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Structure of yeast regulatory gene *LEU3* and evidence that *LEU3* itself is under general amino acid control

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ABSTRACT

Determination of the nucleotide sequence of a DNA region from *Saccharomyces cerevisiae* previously shown to contain the *LEU3* gene revealed one long open reading frame (ORF) whose 887 codons predict the existence of a protein with a molecular mass of 100,162 daltons. The codon bias index of 0.02 suggests that *LEU3* encodes a low-abundance protein. The predicted amino acid sequence contains a stretch of 31 residues near the N-terminus that is rich in cysteines and basic amino acids and shows strong homology to similar regions in five other regulatory proteins of lower eukaryotes. Additional regions with a predominance of basic amino acids are present adjacent to the cysteine-rich region. A stretch of 20 residues, 19 of which are glu or asp, is found in the carboxy terminal quarter of the protein. The 5' flanking region of *LEU3* contains a TATA box 111 bp upstream from the beginning of the long ORF and two transcription initiation elements (5'TCAA3') 58 and 48 bp upstream from the ORF. The 3' flanking region shows a tripartite potential termination-polyadenylation signal. The predicted 5' and 3' ends of the transcript are in very good agreement with the previously determined size of the *LEU3* message. Analysis of a *LEU3'-'lacZ* translational fusion suggests that the *LEU3* gene, whose product is involved in the specific regulation of the leucine and possibly the isoleucine-valine pathways, is itself under general amino acid control. Consistent with this observation is the finding that the 5' flanking region of *LEU3* contains two perfect copies of the general control target sequence 5'TGACTC3'.

INTRODUCTION

At present, *LEU3* is the only specific genetic element in leucine biosynthesis in yeast known to exert a regulatory function. Strains carrying a spontaneous mutation or partial deletion in *LEU3* grow very sluggishly in the absence of leucine because of greatly diminished expression of the *LEU1* and *LEU2* genes which encode isopropylmalate (IPM) isomerase and β-IPM dehydrogenase, respectively (1,2). The fact that *leu3* mutations are uninducible and are recessive in diploids suggests that the *LEU3* product acts in a positive fashion. The 5' flanking region of *LEU2* has been shown to contain a G+C-rich, palindromic sequence important for the expression of that

gene (3). Similar "leucine-specific" upstream activating sites (UAS_L) with the consensus sequence:

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    T (G)          A                G (C)
5'  G  C  C  G  G  G  A  C  C  G  G  C  T  3'
    A              T                T
```

are also present in the 5' flanking regions of *LEU1* (4), *LEU4* (5), *ILV2* (6), and *ILV5* (7). It is possible that these sites represent target sequences for the *LEU3* product, although other possibilities cannot be ruled out at this time.

The *LEU3* gene has recently been cloned and shown to specify an mRNA of 2.9-3.0 kilonucleotides (2). Here we report that *LEU3* is capable of elaborating a 100 kDa protein with features typical of DNA binding proteins. Analysis of an in-frame *LEU3'-'lacZ* fusion shows that *LEU3* expression increases when general amino acid control signals are given.

MATERIALS AND METHODS

Strains, Plasmids, and Special Materials

The following strains were used as hosts in transformations: *S. cerevisiae* CG219 (*MATα ura3*-52) (2), *E. coli* MC1000 (Δ[*lacIPOZYA*] X74, *galU*, *galK*, *rpsL*, Δ[*ara,leu*] (8), and *E. coli* JM101 and JM103 (9). Plasmids pSEY101 and pSEY102 (10) (gifts from S. Emr of CalTech) contain the yeast *URA3* gene, a polylinker region, and either yeast 2 μm DNA (pSEY101) or yeast *ARS1-CEN4* DNA (pSEY102). DNA to be sequenced was derived from plasmids pGB4 and pTSC36 (2). Cloning vectors M13mp18 and M13mp19, universal single-stranded M13 primer (17 bases), and mung bean nuclease were obtained from P-L Biochemicals. DNA polymerase I (Klenow enzyme), T4 DNA ligase, and exonuclease III from *E. coli* B were from Boehringer Mannheim. All other biochemicals, including restriction endonucleases, were purchased from various national suppliers. [α^{32}P]dCTP (800 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from Amersham Corp.

Growth Conditions

The growth conditions and media were as described elsewhere (2), except that yeast cells were harvested at an OD_{580} of about 0.8. 3-Amino-1,2,4-triazole was added to exponentially growing cells for the final doubling prior to harvest.

DNA Preparation and Treatment

Plasmid DNA isolation, restriction enzyme digestions and fragment isolation procedures were described previously (2). Mung bean nuclease and Klenow enzyme treatments were performed as described (5). Ligations were performed

at 22°C for >12 hours using 1-2 units of T4 DNA ligase per μg DNA. Ligation mixtures for blunt end ligations also contained T4 RNA ligase at a concentration of 1 μg per μg DNA. Synthetic linkers were phosphorylated essentially as described by Maniatis, Fritsch and Sambrook (11) using unlabeled ATP.

Transformation Procedures

Yeast transformations (12) and bacterial transformations (11) were performed essentially as described.

Nucleotide Sequencing

All sequencing was performed by the dideoxy chain termination method (13). Fragments of plasmids pGB4 or pTSC36, generated by restriction enzyme digestion, were inserted into the replicative form of two M13 vectors (9). When fragments were too long, exonuclease III was used to create a series of controlled unidirectional deletions (14). In addition, synthetic oligonucleotides corresponding to known *LEU3* sequences were sometimes used in place of the universal primer.

Assay For β-Galactosidase

Yeast cell-free extracts were prepared as described by Baichwal *et al.* (1), except that the extraction buffer contained 0.1 M TRIS·HCl, pH 8.0, 20% (v/v) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.05% Triton X-100. β-Galactosidase activity was measured by the procedure of Miller (15). Protein was determined by the method of Bradford (16) using bovine serum albumin as a standard. For plate assays, bacteria were put on minimal plates containing ampicillin (50 μg/ml) and X-Gal (5-bromo-4-chloro-3-

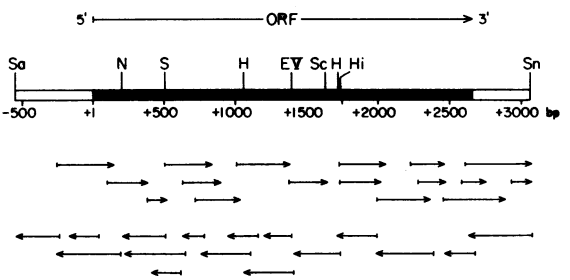
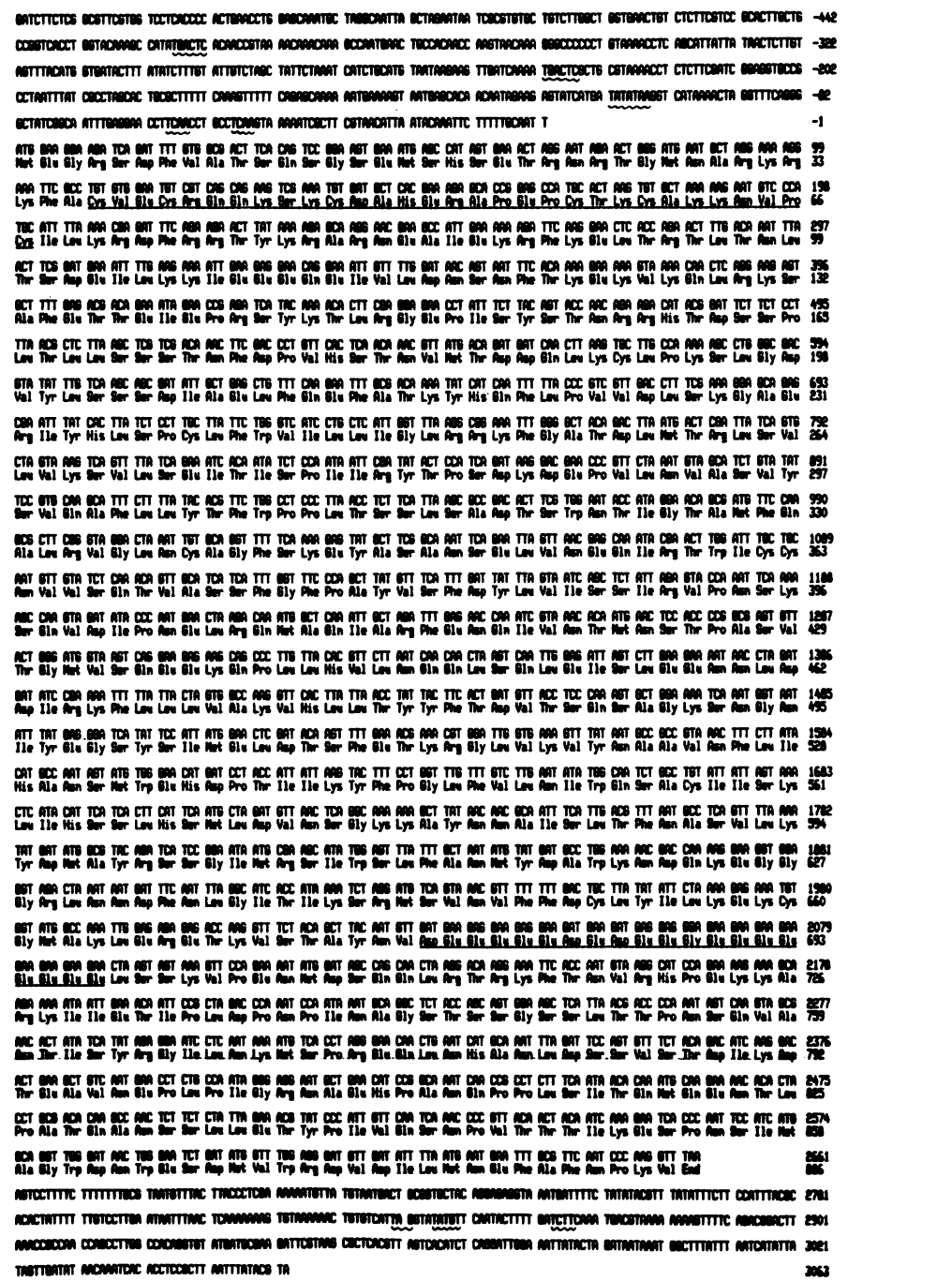


Figure 1. Sequencing strategy and position of long open reading frame. The long open reading frame is indicated by the top arrow. The short arrows indicate the direction and the extent of sequencing. Arrows pointing to the right, noncoding strand; arrows pointing to the left, coding strand. For more than 95% of the DNA between positions -250 and +3060, sequence was obtained from both strands. The restriction sites shown are those whose presence was confirmed by digestion with the appropriate enzymes. EV, *Eco*RV; H, *Hpa*I; Hi, *Hind*III; S, *Sal*I; Sa, *Sau*3A (only the most upstream site is shown); Sc, *Sca*I; Sn, *Sna*BI.



indolyl-β-D-galactoside; Research Organics Inc., Cleveland, OH) (40 μg/ml). Yeast cells were spotted onto minimal (SD) plates containing X-Gal (17).

RESULTS AND DISCUSSION

Nucleotide and Deduced Amino Acid Sequence of LEU3

Figure 1 shows a partial restriction map of the *LEU3* region and the sequencing strategy. The nucleotide sequence between restriction sites *Sau*3A (left end of clone) and *SnaB*I is shown in Figure 2. A computer-aided search of all six reading frames revealed only one extended open reading frame (ORF). Its 886 amino acid codons are indicated by the shaded region in Figure 1. The next-longest ORF contains 90 codons. Identification of the long ORF as that of *LEU3* is based on the following arguments: (i) Earlier subcloning experiments showed that of three DNA fragments, all of which extended beyond the *SnaB*I site, only the one that included the *Sau*3A site at position -561 was able to complement *leu3* mutations; two others, starting at the *Nsi*I and the *Sal*I site, respectively, were unable to do so (2). (ii) Deletion of the 0.66 kb *Hpa*I-*Hpa*I fragment from the *LEU3* gene destroyed *LEU3* function (2). (iii) The size of the long ORF (2.658 kb) agrees well with the size of the *LEU3* message, previously determined to be 2.9-3.0 kilonucleotides (2).

The deduced amino acid sequence of the *LEU3* protein yields a calculated molecular weight of 100,162. Codon usage is shown in Table 1. The codon bias index is 0.02, implying that the *LEU3* product is a low-abundance protein (18).

Special Features of the Predicted LEU3 Protein

Inspection of the deduced amino acid sequence reveals several prominent features. One of these extends from residue 37 to residue 67. This segment of 31 amino acids contains six cysteines, is rich in basic residues, and shows a remarkable homology to five other lower-eukaryotic regulatory proteins (Figure 3). In addition to the six cysteines, one arginine, one lysine, and one proline residue are conserved in all six proteins; two more lysine residues are present in comparable positions in five of the six proteins; and

Figure 2. Nucleotide sequence of the LEU3 gene and flanking regions (noncoding strand).

The sequence covers the region shown in Fig. 1. Nucleotides upstream from the proposed translation start at +1 carry negative numbers, those downstream carry positive numbers. The deduced amino acid sequence of the long ORF is shown below the nucleotide sequence. The following features have been highlighted by wavy lines: Two general amino acid control boxes, a potential Goldberg-Hogness box, two sequences potentially signalling transcription starts (all in the 5′ noncoding region), and a potential tripartite transcription termination signal (3′ noncoding region). A potential DNA binding motif and an acidic amino acid cluster have been underlined (coding region). See text for further details.

Table 1. Codon usage in *LEU3*.

TTT	Phe	21	(2.4)	TCT	Ser	17	(1.9)	TAT	Tyr	19	(2.1)	TGT	Cys	7	(0.8)
TTC	Phe	13	(1.5)	TCC	Ser	8	(0.9)	TAC	Tyr	7	(0.8)	TGC	Cys	7	(0.8)
TTA	Leu	29	(3.3)	TCA	Ser	32	(3.6)	TAA	End	1	(0.1)	TGA	End	0	(0.0)
TTG	Leu	12	(1.4)	TCG	Ser	7	(0.8)	TAG	End	0	(0.0)	TGG	Trp	11	(1.2)
CTT	Leu	10	(1.1)	CCT	Pro	11	(1.2)	CAT	His	10	(1.1)	CGT	Arg	2	(0.2)
CTC	Leu	7	(0.8)	CCC	Pro	9	(1.0)	CAC	His	5	(0.6)	CGC	Arg	0	(0.0)
CTA	Leu	15	(1.7)	CCA	Pro	13	(1.5)	CAA	Gln	26	(2.9)	CGA	Arg	8	(0.9)
CTG	Leu	5	(0.6)	CCG	Pro	6	(0.7)	CAG	Gln	7	(0.8)	CGG	Arg	2	(0.2)
ATT	Ile	26	(2.9)	ACT	Thr	16	(1.8)	AAT	Asn	39	(4.4)	AGT	Ser	19	(2.1)
ATC	Ile	10	(1.1)	ACC	Thr	13	(1.5)	AAC	Asn	21	(2.4)	AGC	Ser	12	(1.4)
ATA	Ile	17	(1.9)	ACA	Thr	23	(2.6)	AAA	Lys	37	(4.2)	AGA	Arg	19	(2.1)
ATG	Met	23	(2.6)	ACG	Thr	8	(0.9)	AAG	Lys	17	(1.9)	AGG	Arg	13	(1.5)
GTT	Val	27	(3.0)	GCT	Ala	16	(1.8)	GAT	Asp	30	(3.4)	GGT	Gly	9	(1.0)
GTC	Val	5	(0.6)	GCC	Ala	12	(1.4)	GAC	Asp	11	(1.2)	GGC	Gly	4	(0.5)
GTA	Val	16	(1.8)	GCA	Ala	14	(1.6)	GAA	Glu	52	(5.9)	GGA	Gly	14	(1.6)
GTG	Val	6	(0.7)	GCG	Ala	9	(1.0)	GAG	Glu	18	(2.0)	GGG	Gly	4	(0.5)

The numbers in parentheses are percentages.

one aspartate and one asparagine residue each is conserved in four of the six proteins. The cysteine/basic amino acid-rich regions of *GAL4* and *qa-1F* have been shown to be part of the DNA binding domain (19,20); it is likely that the corresponding regions of the other four proteins have the same function.

It has been pointed out that the cysteine-containing region of the *GAL4* and *PPR1* products resembles the putative DNA binding regions in TFIIIA of *Xenopus* as well as proteins encoded by the *Krüppel* and *Serendipity* genes of *Drosophila*, and the yeast regulatory protein encoded by *ADR1*, and might conform to the zinc-binding "finger" motif found in those proteins (21). How-

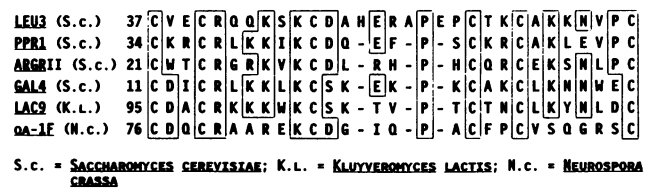


Figure 3. Comparison of potential DNA binding regions in six lower-eukaryotic regulatory proteins.

The numbers preceding the sequences indicate the distance (in residues) from the N-terminus. The hyphens designate spaces introduced for maximum homology. Sequence data are from the following references: *PPR1* (40), *ARGRII* (41), *GAL4* (19), *LAC9* (42), *qa-1F* (20).

ever, this interpretation ignores the potential significance of the highly conserved third and sixth cysteines of the region shown in Figure 3, as well as other differences. It is more likely that the sequences listed represent a variation of the finger motif. Metal binding has yet to be demonstrated for any of these proteins. The sequence present in *LEU3* is unique even among the lower eukaryotic examples, since its middle region contains three additional amino acids, including one histidine. Participation of this histidine in the motif would create a very regular Cys-X_2-Cys-X_6-Cys-X_2-His-X_6-Cys-X_2-Cys-X_6-Cys structure. The importance of cysteine/histidine-rich domains in nucleic acid binding proteins is emphasized by the fact that at least six classes of such proteins are now known, including *gag* encoded proteins of retroviruses, amino acyl tRNA synthetases, and steroid hormone and vitamin D receptors (22,23).

Another notable feature of the *LEU3* protein is the uneven distribution of charged amino acids. Thus, an accumulation of lysine and arginine residues is found between amino acid positions 31 and 94 and again between positions 123 and 148. In both of these regions, the basic amino acids constitute about one third of the total number of amino acids present, as opposed to 8.4% for the remainder of the protein. An extraordinary accumulation of acidic amino acids occurs between positions 678 and 697, in the carboxy terminal quarter of the protein. In this stretch of 20 amino acid residues, one glycine is surrounded by 16 glutamates and 3 aspartates. While similar sequences exist in a number of other proteins, there appears to be no common denominator with respect to their function. Among the proteins containing a cluster of acidic amino acids are yeast ubiquinol-cytochrome c reductase (24), a pig neurofilament protein (25), the major capsid protein of adenovirus 2 (26), homeotic proteins (27), bovine non-histone nucleosomal proteins HMG1 and HMG2 (28), and frog nucleoplasmin, a histone binding protein (29). The last three examples are of obvious interest with respect to potential functions of the *LEU3* product.

Features of the 5' and 3' Flanking Regions

The promoter region of *LEU3* contains both near-upstream "selector" and potential far-upstream "modulator" elements. A good example of a Goldberg-Hogness (TATA) box is present at position -111. This is followed by two 5'TCAA3' sequences approximately 50 and 60 bp further downstream (at positions -58 and -48, respectively). The TCAA element was recently recognized as one of two preferred sequences in yeast that usually signal transcription initiation when present 50-120 bp downstream from the TATA box (30). The first ATG downstream from the putative transcription initiation sites is located at position +1. This is followed by two additional in-frame ATG's at

positions +49 and +82. A decision as to whether any of the latter two ATG's could be utilized as a translation start will have to await experimental determination of the transcription start point(s).

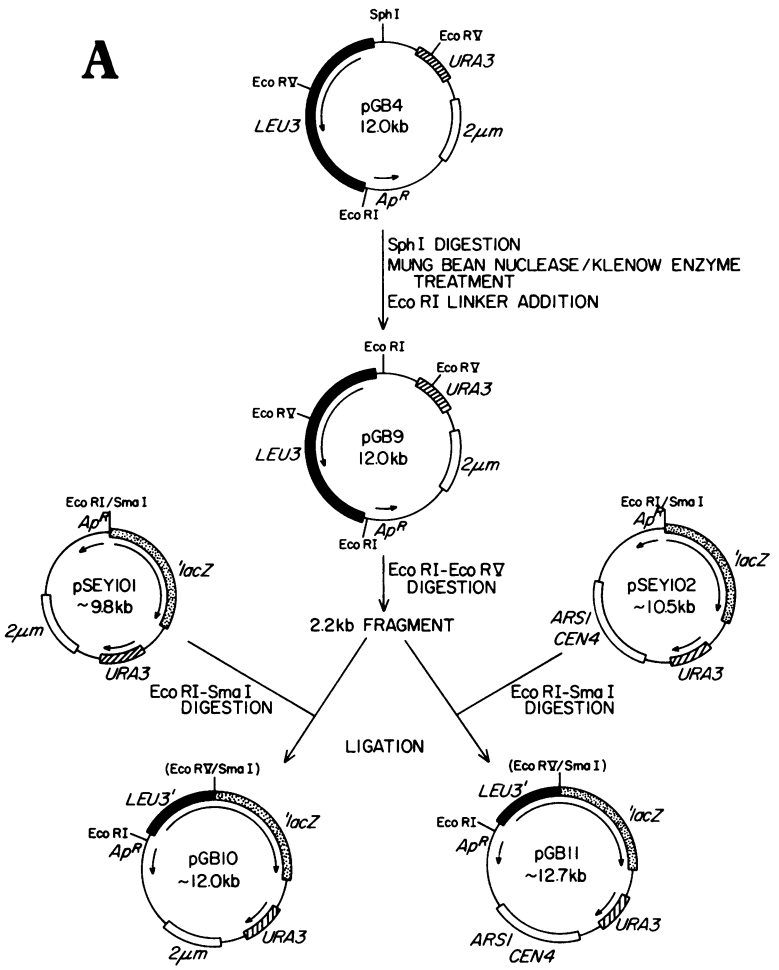
Two hexanucleotide sequences with a perfect homology to the general amino acid control box 5'TGACTC3' are present at positions -417 and -241. The sequence TGACTC appears in at least one copy in the 5' flanking region of all genes that are subject to the cross-pathway regulation termed general control of amino acid biosynthesis (reviewed in 31). It is part of the target sequence for positive control by the *GCN4*-encoded protein. *GCN4* itself is the last, most direct-acting element in a hierarchy of regulatory genes that respond to amino acid starvation. It has been shown that binding of the *GCN4* protein extends to bases on either side of TGACTC (32,33). The environment of the *LEU3* general control boxes would predict intermediate affinity for the *GCN4* protein (32).

A sequence somewhat akin to the UAS_L consensus sequence (see INTRODUCTION) is present between positions -442 and -429. However, this sequence contains two additional bases in a region (5'ACCGG3') that is perfectly conserved in the five known examples of UAS_L sequences, and its significance is therefore unclear.

The likely translational stop codon of the *LEU3* message (UAA at position 2659) is followed within the next 90 nucleotides by five additional stop codons in all three reading frames. A tripartite termination-polyadenylation signal of the kind proposed by Zaret and Sherman (34) is found between positions 2840 and 2867 (TAG...TATGT..[A+T rich]..TCTT). The proposed positions of transcription start and transcription termination agree very well with the approximate length of the *LEU3* encoded message (2.9-3.0 kilonucleotides) determined earlier (2). In combination with the absence of the sequence 5'TACTAAC3' (35,36), these results also suggest that introns are not present within the *LEU3* gene.

Construction of LEU3'-'lacZ Fusions

Utilizing the unique *EcoRV* restriction site between nucleotide positions 1387 and 1392, a translational fusion to the *E. coli lacZ* gene was constructed for the dual purpose of proving the existence of a long ORF and of being able to study regulation of *LEU3* expression at the protein level. Plasmid pGB4 (2) which contains the *LEU3* region (including the *LEU3* promoter) served as starting material (Figure 4A). Its unique *Sph*I site was changed to an *EcoR*I site by *Sph*I digestion, mung bean nuclease and Klenow enzyme treatment, and the addition of *EcoR*I linkers. The plasmid was subsequently digested with



B

LEU3		from polylinker			lacZ	
... GAT	GAT	GGG	GAT	CCC	GTC	GTT ...
Asp	Asp	Gly	Asp	Pro	Val	Val
462	463				9	10

Figure 4. Construction of LEU3'-'lacZ translational fusion plasmids. A, details of construction of in-frame fusion that contains 561 bp of the *LEU3* 5' flanking region, the first 463 *LEU3* codons, and a truncated *lacZ* gene lacking its promoter and the first eight codons. Blocks represent yeast DNA, thin lines, bacterial DNA. Arrows within the plasmid circles indicate direction of transcription. See text for additional information. B, details of the fusion region.

Table 2. β-Galactosidase activities of a *LEU3'-'lacZ* fusion protein in cell-free extracts.

Strain/plasmid[1]	Additions to minimal media	Specific activity (nmoles/min x mg)	-fold change[4]
CG219	Uracil, 0.2 mM	N.D.[2]	
CG219/pSEY101	None	N.D.	
CG219/pSEY102	None	N.D.	
CG219/pGB10	None	3.03 ± 0.06[3]	30.3
CG219/pGB11	None	0.10 ± 0.02	1.0
	3-AT[2], 10 mM	0.31 ± 0.02	3.1
	Leucine, 2 mM	0.21 ± 0.03	2.1
	Leucine, 2 mM, plus valine, isoleucine, 1 mM	0.08 ± 0.02	0.8

[1] See MATERIALS AND METHODS for description.
[2] N.D. = not detectable; 3-AT = 3-amino-1,2,4-triazole.
[3] Experimental error is given as standard deviation ($n \geq 6$).
[4] Relative to the β-galactosidase level in CG219/pGB11 cells grown with no addition.

*EcoR*I and *EcoR*V. Among the fragments thus generated was one, 2.2 kb in length, that contained the *LEU3* promoter and *LEU3* ORF sequence up to the *EcoR*V site. This fragment was ligated to plasmids pSEY101 and pSEY102 (described in MATERIALS AND METHODS), both of which had been digested with *EcoR*I and *Sma*I. This strategy forced the insertion of the 2.2 kb fragment to occur in the proper orientation. The resulting new plasmids were designated pGB10 and pGB11. That the fusion had occurred at the desired point (Figure 4B) was confirmed by diagnostic digestion with restriction enzymes *Hpa*I and *Sal*I (one *Hpa*I and one *Sal*I site each are carried in by the *LEU3* fragment), and by nucleotide sequencing.

LEU3'-'lacZ Expression is Under General Control of Amino Acid Biosynthesis

β-Galactosidase activity was absent from yeast strains containing the parent plasmids pSEY101 or pSEY102, but was present in strains containing the *LEU3'-'lacZ* fusions (Table 2). This result demonstrates that a long ORF indeed exists in *LEU3*, at least to the *EcoR*V site. The strain carrying the 2 μm DNA-containing plasmid pGB10 showed about 30 times as much β-galactosidase activity as the strain carrying the *CEN4*-containing plasmid pGB11. This probably reflects copy number differences. For the study of *LEU3* regulation, we concentrated on a strain carrying plasmid pGB11. Centromere-containing

plasmids are known to be stably maintained in a copy number that is very close to one (37, and S. Emr, personal communication). Extracts from strain CG219/pGB11 grown in minimal medium showed low but statistically significant fusion enzyme levels (Table 2). When this strain was grown under two conditions known to elicit a general control derepression signal, i.e., in the presence of 3-amino-1,2,4-triazole (3-AT) or in the presence of excess leucine, the β-galactosidase levels increased 3.1-fold and 2.1-fold, respectively. Growing strain CG219/pGB11 in the presence of leucine, isoleucine and valine resulted in essentially unchanged β-galactosidase levels, compared to cells grown with no additions. The histidine analog 3-AT acts by causing a histidine deficiency (38). Leucine is one of several amino acids which, when present by themselves at elevated concentrations, cause amino acid imbalance, resulting in "derepression factors" of 1.2-2.0 (39). The leucine effect disappears when isoleucine and valine are also present (39). It is evident from the results shown in Table 2 that expression of the *LEU3'-'lacZ* fusion is increased by general control stimuli to an extent typical for this system. Qualitatively similar results were seen with a strain harboring the multicopy plasmid pGB10 (data not shown). The conclusion that *LEU3* is under general amino acid control is corroborated by the presence of two 5'TGACTC3' boxes in the promoter region of this gene. To our knowledge, this is the first known example of general control being exerted on a regulatory, as opposed to a structural gene, and thus of an intertwining of general and specific controls. Stimulation of the production of a positive regulator by the general control system might reinforce the overall upward trend of amino acid biosynthesis in situations of amino acid imbalance. However, it is not clear at present exactly where and how an increase in *LEU3* expression would manifest itself. It was observed previously that cells harboring multicopy *LEU3*-containing plasmids exhibited elevated levels of *LEU3* message, but did not show significant changes in the levels of IPM isomerase or β-IPM dehydrogenase (2). It is possible that other potential targets of *LEU3* action (*LEU4, ILV2, ILV5*) are more sensitive to changes in *LEU3* expression. Also, the existence of additional controls of *LEU3* expression cannot be excluded at this point. Experiments to study these questions are underway.

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