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LEU3 of *Saccharomyces cerevisiae* Encodes a Factor for Control of RNA Levels of a Group of Leucine-Specific Genes

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Although the majority of genes for amino acid biosynthesis which have been examined are under general amino acid control, *LEU1* and *LEU2* of *Saccharomyces cerevisiae* respond specifically to leucine. We report here an analysis of *LEU3*, a putative leucine-specific regulatory locus. We show that *LEU3* is necessary for expression of wild-type levels of *LEU1*- and *LEU2*-specific RNAs and, further, that the levels of *LEU4*-specific transcripts are also affected by *LEU3*. We cloned *LEU3* and showed by DNA sequence analysis that it contained an open reading frame of 886 amino acids. A striking feature of the predicted *LEU3* protein was a cluster of acidic amino acids (19 of 20) located in the C-terminal half of the coding region. The protein also had a repeated cysteine motif which was conserved in a number of other yeast proteins implicated in gene regulation. We show that whole-cell extracts contained a *LEU3*-dependent DNA-binding activity that interacted with the 5′ region of *LEU2*. Subdivision of the *LEU2* 5′ region established that the *LEU3*-dependent DNA-binding activity interacted with the segment which had the previously reported homology with *LEU1*.

In bacteria, operons for amino acid biosynthetic enzymes are controlled specifically by their respective end product amino acids (reviewed in reference 54). In *Saccharomyces cerevisiae*, however, many of the genes encoding amino acid biosynthetic enzymes are subject to regulation by the general amino acid control system (reviewed in reference 26). This regulatory network consists of a hierarchy of proteins whose function is to modulate the levels of a number of amino acids in the cell. Starvation for any one of these amino acids leads to derepression of all of the genes subject to general control.

Previous studies of enzymes involved in the biosynthesis of leucine in *S. cerevisiae* revealed a combination of both general and specific amino acid regulation. *LEU4*, which encodes the first enzyme in the pathway (α-isopropyl malate [α-IPM] synthase), is subject to regulation by the general amino acid control system (23). In addition, α-IPM synthase is specifically controlled by leucine through feedback inhibition (47, 53). *LEU1* and *LEU2*, which encode the second and third enzymes in the pathway, respectively, are under specific amino acid control. Expression of both genes is repressed by elevated levels of leucine (1, 10, 24, 30). This sensitivity to a specific amino acid seems to distinguish these genes from most others that are associated with amino acid biosynthesis in yeast.

The leucine-specific control of *LEU1* and *LEU2* is thought to be indirect. Expression of these two genes appears to be a function of the level of α-IPM, the product of the first enzyme in the pathway. This is based on the following results: (i) the levels of the *LEU1* and *LEU2* gene products are sharply decreased in a strain that lacks a functional synthase; (ii) *LEU1* and *LEU2* mutants, which are expected to accumulate intermediates in the pathway (such as α-IPM), exhibit increased levels of β-IPM dehydrogenase and α-IPM isomerase, the products of the *LEU2* and *LEU1* genes, respectively; (iii) a strain that contains a feedback-resistant α-IPM synthase, which produces high levels of α-IPM, also has increased levels of the *LEU1* and *LEU2* gene products (3). Because α-IPM synthase is feedback-inhibited by leucine, the levels of α-IPM are directly related to the levels of leucine in the cell. Thus, were α-IPM to function as an inducer, it could mediate leucine-specific control of *LEU1* and *LEU2*.

Analysis of mutants defective in leucine biosynthesis has uncovered a single genetic locus which has the potential for being a factor in the regulation of *LEU1* and *LEU2*. A strain containing this mutant allele, designated *leu3*, is a leaky leucine auxotroph which produces low levels of both the *LEU1* and *LEU2* gene products (30). One hypothesis is that, in conjunction with α-IPM as inducer, the *LEU3* gene product functions as a positive activator of *LEU1* and *LEU2* (3). Based on genetic studies, a similar regulatory pathway has been proposed for the corresponding genes of *Neurospora crassa* (39, 43).

In previous work, the nucleotide sequences of *LEU1* and *LEU2* were established and it was shown that levels of the respective mRNAs are sensitive to intracellular leucine concentrations (1, 2, 24). This work also established a section of partial nucleotide sequence homology between the 5′ regions of the two genes. Brisco et al. (9) recently reported the cloning of *LEU3* along with preliminary genetic studies.

To understand more fully the role of *LEU3* in the leucine-specific regulation of these genes, we have undertaken further characterization of this locus. Through complementation of the mutant allele, a genomic clone of the *LEU3* gene was isolated and its nucleotide sequence was determined, revealing a single large open reading frame. The results of Northern (RNA blot) hybridization experiments show that *LEU3* is required to obtain wild-type levels of *LEU1*- and *LEU2*-specific transcripts and that this effect extends somewhat to *LEU4*. DNA-binding studies suggest that there is a *LEU3*-dependent DNA-binding activity which interacts specifically with *LEU2* upstream sequences.

MATERIALS AND METHODS

Strains and genetic methods. The following yeast strains were used in these studies: PDY139-11B (α *LEU4-103* [Tfl^r; see Results] *leu3-781 ura3-52*) (Peter Drain, Massachusetts Institute of Technology [MIT]); PDY102-1A (**a** *LEU4-103 ura3-52*) (Peter Drain, MIT); F23 (**a** *his5 ura3-52*) (Peter

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Drain, MIT) (16); and PFY400-2C (α *his5 ura3-52 leu3-781*), a segregant derived from the cross F23 × PDY139-11B. Yeast growth media were prepared and general yeast methods performed as described (50). Yeast were transformed by the method of Hinnen et al. (21) or Ito et al. (25).

Escherichia coli HB101 and JM101 were used for maintaining pBR322- and M13-derived plasmids, respectively. Bacterial methods were as described (15).

Hybridization procedures. Yeast RNA for Northern hybridization was prepared as described (12). RNAs were resolved on agarose-formaldehyde gels as described (35) except that the gels were run in 10 mM $NaPO_4$ buffer (pH 7.0) and blotted directly to nitrocellulose after washing in sterile H_2O.

Yeast DNA for chromosome blots was prepared as described by Schwartz and Cantor (48). Orthogonal-field-alternation gels were run and blotted to nitrocellulose as described by Carle and Olson (11).

Prehybridization and hybridization solutions contained 50% formamide, 5× SSPE (sodium chloride, sodium phosphate, EDTA [35]), 5× Denhardt solution, and 0.5% sodium dodecyl sulfate; hybridization was at 37°C. DNA probes (10^7 cpm) labeled by nick translation (35) were incubated with the filter for 12 h. The final wash for all blots contained 0.1× SSPE and 0.1% sodium dodecyl sulfate and was done at 37°C (55°C for chromosome blots).

Plasmid constructions. The details of the plasmid constructions are as follows: plasmid pPF711, deletion of a 5.5-kilobase-pair (kbp) *Bst*EII fragment from plasmid pPF701; plasmid pPF712, deletion of a *Sal*I fragment from plasmid pPF702 which extends from the insert *Sal*I site through the *Bst*EII site to a *Sal*I site in the adjacent vector sequences; plasmid pPF715, deletion of a *Sal*I fragment from plasmid pPF701 which extends from the insert *Sal*I site through the *Avr*II site to a *Sal*I site in the adjacent vector sequences; plasmid pPF741, the 4.6-kbp *Hin*dIII fragment of plasmid pPF701 was inserted into the *Hin*dIII site of plasmid YIp5 (52), followed by the insertion of the *Eco*RI fragment of YEp24 (8) (containing the 2μm replication functions) into the unique vector *Eco*RI site; plasmid pPF750, deletion of an *Nhe*I fragment from plasmid pPF702 which extends from the insert *Nhe*I site to an *Nhe*I site in the adjacent vector sequences; plasmid pPF751, plasmid pPF750 was digested with both *Nhe*I and *Avr*II (which have complementary overhangs) and religated to delete the region between the two sites.

DNA sequence analysis. Large fragments of plasmids pPF701 and pPF702 were cloned into the M13 vectors mp18 and mp19. The DNA inserts were sequenced by the chain termination method (46) as modified for [α-^{35}S]dATP by Biggin et al. (7). DNA sequence information was initially obtained by commercially available sequencing primers (New England Biolabs). Extended stretches of DNA were sequenced by synthesizing additional sequencing primers with a Microsyn-1450A automated DNA synthesizer (Systec, Inc.).

S1 mapping. The synthetic oligonucleotide 5′-CCTTTT CCTAGCATTCATCC-3′, which is complementary to nucleotides +99 to +80 of the *LEU3* coding region, was used to make a single-stranded probe for S1 nuclease protection analysis. The oligonucleotide was labeled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP (35). The labeled primer was annealed to single-stranded DNA from an M13mp19 clone containing the *Sal*I-*Hin*dIII fragment which spans the *LEU3* promoter region, extended using the Klenow enzyme and deoxynucleotide triphosphates and digested with *Bst*EII to give a defined 3′ end. The single-stranded end-labeled probe was isolated from an alkaline low-melting-point agarose gel (35). The S1 reactions were carried out essentially as described (24) except that the probe (10^5 cpm) and RNA were denatured at 65°C and annealed overnight at 30°C. Polyadenylated [poly(A)$^+$]RNA was prepared by two passages over oligo(dT)-cellulose as described (35).

Gel retardation assays. Yeast extracts were prepared and assays performed as described (42). DNA-binding reaction mixtures contained a 2,000-fold excess of sonicated salmon sperm DNA to eliminate nonspecific complexes. Complexes were separated on 4% polyacrylamide gels that were run with 0.5× TBE buffer. The DNA fragments used as substrates were purified from 4% polyacrylamide gels by electroelution and labeled with ^{32}P by using polynucleotide kinase or Klenow enzyme (large fragment, DNA polymerase I) (35).

RESULTS

Isolation and initial characterization of *LEU3*-containing plasmids. To select a genomic clone of *LEU3* by plasmid transformation, strain PDY139-11B was constructed. The relevant genotype of this strain is *leu3-781 ura3-52 LEU4-103*. The product of the *LEU4-103* allele, which is no longer subject to feedback inhibition by leucine, confers dominant resistance to the toxic analog trifluoroleucine (Tfl^r) (3). Because of the low levels of the *LEU1* and *LEU2* gene products in a *leu3* strain, PDY139-11B has a leucine auxotrophy which renders the cells sensitive to trifluoroleucine. The rationale is that the introduction of *LEU3*-containing plasmids into PDY139-11B, by raising the levels of the *LEU1* and *LEU2* gene products, will impart both leucine prototrophy and trifluoroleucine resistance.

PDY139-11B was transformed with DNA from a yeast genomic library. This library was constructed by ligating yeast chromosomal DNA, which had been partially digested with *Sau*3A, into the *Bam*HI site of the multicopy shuttle vector YEp24 (12). The vector also carries the *URA3* gene as a selectable marker. Approximately 9,000 Ura$^+$ transformants were screened for leucine prototrophy and resistance to trifluoroleucine. From this screen, 10 independent Leu$^+$ transformants were obtained. All of these were also Tfl^r.

Four of the transformants were arbitrarily chosen for further study. Each was grown under nonselective conditions and monitored for loss of the plasmid-borne *URA3* marker and of the Leu$^+$ and Tfl^r phenotypes. Each of these transformants cosegregated the Ura$^+$ and Leu$^+$ Tfl^r phenotypes. As an additional test that the Leu$^+$ and Tfl^r phenotypes were plasmid dependent, total DNA was prepared from each transformant, and the plasmids were isolated by transformation of *E. coli* and then reassayed by transforming strain PDY139-11B. All of the resulting Ura$^+$ transformants were also Leu$^+$ and Tfl^r.

Dissection and further analysis of the *LEU3* locus. Restriction mapping of plasmid isolates identified two distinct types, designated pPF701 and pPF702. These plasmids, which each conferred Leu$^+$ and Tfl^r to PDY139-11B, had in common a 3.8-kbp yeast genomic DNA segment. Based on this observation, we constructed a number of deletion plasmids to further define the *LEU3* locus (Fig.1). This analysis established that the smallest region common to the plasmids which fully complemented the *leu3* allele was a 3.4-kbp segment that extended from the *Bst*EII site to the *Nhe*I site.

We tested whether cloned DNA which complemented *leu3* could direct integration to the *LEU3* locus in the yeast

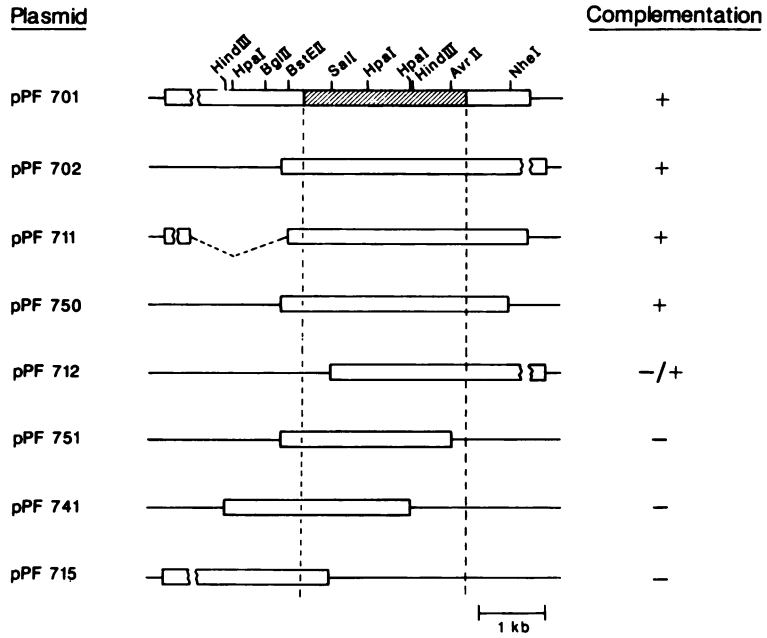

FIG. 1. Deletion analysis of the *LEU3* locus. Various fragments of the *LEU3*-complementing plasmids pPF701 and pPF702 were cloned into yeast shuttle vectors containing 2μm DNA and the *URA3* gene. The insert sizes of pPF701 and pPF702 are approximately 10.7 and 11.2 kbp, respectively. Plasmid pPF711 was constructed by deleting a 5.5-kbp *Bst*EII fragment from pPF702, the result of which is to fuse a distal 1.5-kbp insert fragment to the remainder of the insert. The open rectangles represent insert DNA; vector sequences are represented by the thin lines. The hatched area corresponds to the *LEU3* open reading frame. Complementation was measured in strain PDY139-11B as the ability of transformants to grow on minimal medium without leucine or with 0.6 mM trifluoroleucine. The significance of the weak complementation observed with plasmid pPF712 is unclear due to the possibility of interaction with an altered *leu3* protein being synthesized from the mutant chromosomal copy of *leu3*.

chromosome. This was achieved by cloning a 4.6-kbp *Hin*dIII fragment (the same *LEU3* segment that is in plasmid pPF741 [Fig. 1]) into the yeast integrating vector YIp5 (52). This plasmid contains *URA3* as a selectable marker but has no means for autonomous DNA replication in *S. cerevisiae*. To direct integration to the *LEU3* locus, the constructed plasmid was linearized by cleavage at the unique *Bst*EII site within the insert. This DNA was then transformed into a *ura3-52 his5* strain designated F23. Stable Ura$^+$ transformants were crossed to PDY139-11B, and the resulting diploid was sporulated and subjected to tetrad analysis. The integrated *URA3* gene was found to be tightly linked to the wild-type *LEU3* allele (Table 1). However, the heterozygous *HIS5* marker in the diploid segregated randomly with respect to *LEU3* and *URA3*. These observations suggest that the 4.6-kbp *Hin*dIII fragment contains sequences that direct integration to the *LEU3* locus.

We mapped the *LEU3* locus to chromosome XII. This was accomplished by hybridizing *LEU3*-specific probes to a blot of intact yeast chromosomes that were separated by orthogonal-field-alternation gel electrophoresis (11) (data not shown). Our result is in agreement with the recently reported map position of *LEU3* (9). In addition, the restriction map of the *LEU3* region reported by Brisco et al. (9) is in agreement with our results.

TABLE 1. Cloned DNA directs integration to the *LEU3* locus[a]

Segregation of Leu and Ura phenotypes		Segregation of Leu and His phenotypes	
Spore phenotype	No. of spores displaying phenotype	Spore phenotype	No. of spores displaying phenotype
Leu$^+$ Ura$^+$	37	Leu$^+$ His$^+$	19
Leu$^+$ Ura$^-$	0	Leu$^+$ His$^-$	18
Leu$^-$ Ura$^+$	0	Leu$^-$ His$^+$	21
Leu$^-$ Ura$^-$	41	Leu$^-$ His$^-$	20

[a] Results are for three- and four-spore tetrads. All four-spore tetrads displayed 2:2 segregation of the markers examined.

Effect of *LEU3* on the levels of *LEU1*- and *LEU2*-specific RNAs. We used the Northern blot hybridization method to examine *LEU1*- and *LEU2*-specific RNAs in isogenic strains that differed only at the *LEU3* locus. These strains (PDY102-1A [*LEU3*] and PDY139-11B [*leu3*]) contained the *LEU4* Tfl^r allele. As a result, the product of the *LEU4* gene in these strains was no longer subject to feedback inhibition by leucine and therefore was unimpeded in the synthesis of α-IPM. The latter is proposed to be a cofactor in the induction of synthesis of the *LEU1* and *LEU2* gene products (see above).

The denatured RNA was resolved on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with the appropriate ^{32}P-labeled fragment. The level of *LEU2*-specific RNA was found to be dependent on *LEU3* (Fig. 2A). Introduction of the *LEU3* plasmid pPF711 into a *leu3* strain returned the *LEU2*-specific RNA to levels observed in the *LEU3* strain. It is of interest that *LEU3* on a multicopy plasmid did not increase the level of *LEU2* RNA substantially above that observed in PDY102-1A, which contains a single chromosomal copy of *LEU3*.

Similar hybridization experiments showed that the level of *LEU1* RNA was also dependent on *LEU3* (Fig. 2B). In addition, an RNA blot was probed with DNA from the *HIS3* and *LEU2* genes. *HIS3* is known to be controlled by the general amino acid control system (26). *HIS3* RNA levels

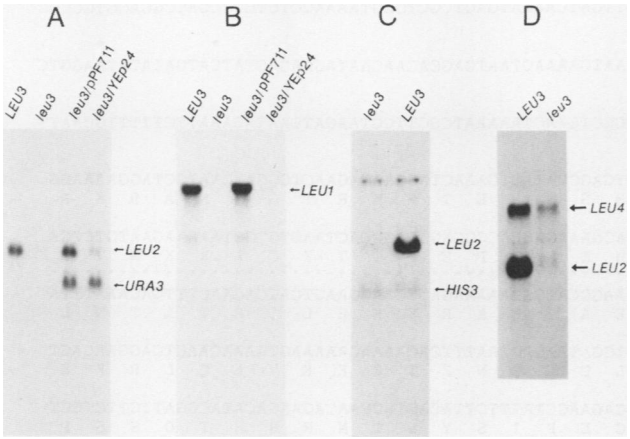

FIG. 2. Northern blot analysis of *LEU1*-, *LEU2*-, and *LEU4*-specific RNAs from wild-type and *leu3* cells. Equivalent amounts of RNA from strains PDY102-1A (*LEU3*), PDY139-11B (*leu3*), and PDY139-11B transformed with the indicated plasmids were resolved on 1.5% agarose–formaldehyde gels and transferred to nitrocellulose. The ^{32}P-labeled probes were (A) a 1,415-bp *Sal*I-*Bst*EII fragment of the *LEU2* gene and a 1,166-bp *Hin*dIII fragment containing the *URA3* gene, (B) an 860-bp *Eco*RI fragment of the *LEU1* gene, (C) the same *LEU2* fragment as in A in addition to a 1.7-kbp *Bam*HI fragment of the *HIS3* locus, (D) a 960-bp *Bam*HI fragment of the *LEU4* gene and the same *LEU2* fragment as in A. The amount of RNA per lane is 75 μg for blots A and B and 60 μg for blots C and D. RNA for blots A and B was prepared from cells grown in supplemented minimal medium. RNA for blots C and D was prepared from cells grown in YPD medium.

were not sensitive to the *LEU3* allele (Fig. 2C). This demonstrates that the effects of *LEU3* are distinct from those of the general amino acid control system.

Effect of *LEU3* on level of *LEU4*-specific RNA. We also examined the effect of *LEU3* on the transcription of *LEU4*. Regulation of *LEU4* expression by leucine (as opposed to feedback regulation acting on the gene product) has been suggested but not observed (4). The recently determined sequence of *LEU4* contains a few regions of partial homology to those 5′ regions of *LEU1* and *LEU2* that are thought to be important for regulation by leucine (see below). The level of *LEU4* RNA was sensitive to the presence or absence of the *LEU3* gene, but not as sensitive as the levels of *LEU1* and *LEU2* RNA were (Fig. 2D).

Cloned *LEU3* gene restores leucine sensitivity to *LEU1* and *LEU2*. *LEU1* and *LEU2* gene product levels are believed to be controlled through leucine-dependent alterations in the amount of α-IPM (see above). Synthesis of this intermediate is catalyzed by the *LEU4* gene product. To determine whether the cloned *LEU3* allele conferred leucine-sensitive synthesis of *LEU1* and *LEU2* RNA to the *leu3* strain, we constructed a *leu3 LEU4* strain. Strains containing the wild-type *LEU4* allele are subject to feedback inhibition and leucine-dependent modulation of the levels of α-IPM. This strain (designated PFY400-2C) was then transformed with a *LEU3* plasmid (plasmid pPF711; Fig. 1). RNA was extracted from this transformed strain after it was grown in the absence or presence of 2 mM leucine. RNA blots were prepared and hybridized to *LEU1*-, *LEU2*-, and *URA3*-specific probes (Fig. 3).

In RNA isolated from cells grown in the presence of 2 mM leucine, the level of *LEU2*-specific RNA was decreased significantly. An identical pattern of regulation by leucine was seen with *LEU1*-specific transcripts. (The differences in the level of *URA3* RNA may be due to small variations in plasmid copy number resulting from the different growth conditions.) These results indicate that the cloned *LEU3* gene confers leucine sensitivity to the expression of both *LEU1* and *LEU2* RNA.

Sequence of the *LEU3* locus. A 3.5-kbp region between the *Bst*EII and *Nhe*I sites of the *LEU3* locus was sequenced by the dideoxy method of Sanger et al. (46). Both strands of the DNA were sequenced independently. A single large open reading frame of 886 codons was found (Fig. 4). Initiation of translation at the proposed AUG would result in the synthesis of a protein with a calculated molecular weight of 100,127.

The 5′ ends of the *LEU3* transcripts were determined by the S1 mapping technique (6). Four major transcription initiation sites were located between base pairs (bp) −116 and −94 (Fig. 5). In addition, a few minor sites were located between bp −94 and −45. In all cases, the first AUG downstream from the 5′ end of the mRNA was the one we have proposed as the start of translation (it is unlikely that the AUG immediately adjacent to the most distal initiation site would be utilized). The sequence TTTAT at position −196 was homologous to the TATA sequence thought to be important in eucaryotic promoters. Sequences that correspond to one of the proposed yeast transcription termination sites (56) were located 50 bp downstream from the UAA stop codon.

The most striking feature of the *LEU3* coding region was a run of 19 of 20 acidic amino acids, spanning codons 678 to 697, which consisted primarily of glutamic acid residues. The *LEU3* coding region also contained sequences homologous to two proposed DNA-binding domains. A basic region with a repeated cysteine motif, which is highly conserved in

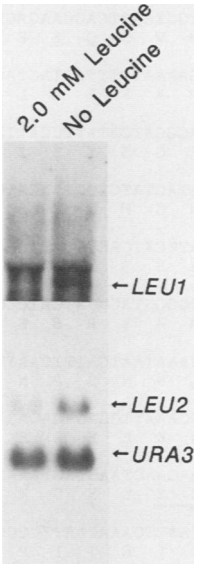

FIG. 3. Leucine-dependent regulation of *LEU1* and *LEU2* by the cloned *LEU3* gene. A Northern blot containing RNA (35 μg per lane) prepared from PFY400-2C(pPF711) grown in minimal medium with and without 2.0 mM leucine was hybridized to ^{32}P-labeled probes to the *LEU1*, *LEU2*, and *URA3* genes (same fragments as in Fig. 2). Regulation of *LEU1* and *LEU2* RNA levels by leucine in *LEU3* strains has been shown previously (1, 24). This figure is a composite; the top half of the blot containing the *LEU1* band is from a longer exposure. The low intensity of the *LEU1* band is due to the low specific activity of the *LEU1* probe. The nature of the band immediately above the *LEU1* band is unknown.

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(-300)  TATCTTTGTATTGTCTAGCTATTCTAAATCATCTGCATGTAATAAGAAGTTGATCAAAATGACTCGCTGCGTAAAACCTCTCTTCGATCGGAGGTGCCGC

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(-200)  CTAATTTATCGCCTAGCACTGCGCTTTTTCAAAGTTTTTCAGAGCAAAAAATGAAAAGTAATGAGCACAACAATAGAAGAGTATCATGATATATAAGGTC

          *   *
(-100)  ATAAAACTAGGTTTCAGGGGCTATCGGCAATTTGAGGAACCTTCAACCTGCCTCAAGTAAAAATCGCTTCGTAACATTAATACAAATTCTTTTTGCAATT

        ATGGAAGGAAGATCAGATTTTGTGGCGACTTCACAGTCCGGAAGTGAAATGAGCCATAGTGAAACTAGGAATAGAACTGGGATGAATGCTAGGAAAAGG
   1     M  E  G  R  S  D  F  V  A  T  S  Q  S  G  S  E  M  S  H  S  E  T  R  N  R  T  G  M  N  A  R  K  R

        AAATTCGCCTGTGTGGAATGTCGTCAGCAGAAGTCGAAATGTGATGCTCACGAAAGAGCACCGGAGCCATGCACTAAGTGTGCTAAAAAGAATGTCCCA
  34     K  F  A  C  V  E  C  R  Q  Q  K  S  K  C  D  A  H  E  R  A  P  E  P  C  T  K  C  A  K  K  N  V  P
                 ..........................................................................................

        TGCATTTTAAAACGAGATTTCAGAAGAACTTATAAAAGAGCAAGGAACGAAGCCATTGAAAAAAGATTCAAGGAACTCACCAGAACTTTGACAAATTTA
  67     C  I  L  K  R  D  F  R  R  T  Y  K  R  A  R  N  E  A  I  E  K  R  F  K  E  L  T  R  T  L  T  N  L
        ..

        ACTTCGGATGAAATTTTGAAGAAAATTGAAGAGGAACAGGAAATTGTTTTGGATAACAGTAATTTCACAAAAGAAAAAGTAAAACAACTCAGGAAGAGT
 100     T  S  D  E  I  L  K  K  I  E  E  E  Q  E  I  V  L  D  N  S  N  F  T  K  E  K  V  K  Q  L  R  K  S

        GCTTTTGAGACGACAGAAATAGAACCGAGATCATACAAAACACTTCGAGGAGAACCTATTTCTTACAGTACCAACAGAAGACATACGGATTCTTCTCCT
 133     A  F  E  T  T  E  I  E  P  R  S  Y  K  T  L  R  G  E  P  I  S  Y  S  T  N  R  R  H  T  D  S  S  P

        TTAACGCTCTTAAGCTCGTCGACAAACTTCGACCCTGTTCACTCAACAAACGTTATGACAGATGATCAACTTAAGTGCTTGCCAAAAAGCCTGGGCGAC
 166     L  T  L  L  S  S  S  T  N  F  D  P  V  H  S  T  N  V  M  T  D  D  Q  L  K  C  L  P  K  S  L  G  D

        GTATATTTGTCAAGCAGCGATATTGCTGAGCTGTTTCAAGAATTTGCGACAAAATATCATCAATTTTTACCCGTCGTTGACCTTTCGAAAGGAGCAGAG
 199     V  Y  L  S  S  S  D  I  A  E  L  F  Q  E  F  A  T  K  Y  H  Q  F  L  P  V  V  D  L  S  K  G  A  E

        CGAATTTATCACTTATCTCCTTGCTTATTCTGGGTCATCCTGCTCATTGGTTTAAGGCGGAAATTTGGGGCTACAGACTTAATGACTCGATTATCAGTG
 232     R  I  Y  H  L  S  P  C  L  F  W  V  I  L  L  I  G  L  R  R  K  F  G  A  T  D  L  M  T  R  L  S  V

        CTAGTAAAGTCAGTTTTATCAGAAATCACAATATCTCCAATAATTCGATATACTCCATCAGATAAGGACGAACCCGTTCTAAATGTAGCATCTGTATAT
 265     L  V  K  S  V  L  S  E  I  T  I  S  P  I  I  R  Y  T  P  S  D  K  D  E  P  V  L  N  V  A  S  V  Y

        TCCGTGCAAGCATTTCTTTTATACACGTTCTGGCCTCCCTTAACCTCTTCATTAAGCGCCGACACTTCGTGGAATACCATAGGAACAGCGATGTTCCAA
 298     S  V  Q  A  F  L  L  Y  T  F  W  P  P  L  T  S  S  L  S  A  D  T  S  W  N  T  I  G  T  A  M  F  Q

        GCGCTTCGGGTAGGACTAAATTGTGCAGGTTTTTCAAAAGAGTATGCTTCGGCAAATTCAGAATTAGTTAACGAGCAAATACGAACTTGGATTTGCTGC
 331     A  L  R  V  G  L  N  C  A  G  F  S  K  E  Y  A  S  A  N  S  E  L  V  N  E  Q  I  R  T  W  I  C  C

        AATGTTGTATCTCAAACAGTTGCATCATCATTTGGTTTCCCAGCTTATGTTTCATTTGATTATTTAGTAATCAGCTCTATTAGAGTACCAAATTCAAAA
 364     N  V  V  S  Q  T  V  A  S  S  F  G  F  P  A  Y  V  S  F  D  Y  L  V  I  S  S  I  R  V  P  N  S  K

        AGCCAAGTAGATATACCCAATGAACTAAGACAAATGGCTCAAATTGCTAGATTTGAGAACCAAATCGTAAACACAATGAACTCCACCCCGGCGAGTGTT
 397     S  Q  V  D  I  P  N  E  L  R  Q  M  A  Q  I  A  R  F  E  N  Q  I  V  N  T  M  N  S  T  P  A  S  V

        ACTGGGATGGTAAGTCAGGAAGAGAAGCAGCCCTTGTTACACGTTCTTAATCAACAACTAAGTCAATTGGAGATTAGTCTTGAAGAAAATAACCTAGAT
 430     T  G  M  V  S  Q  E  E  K  Q  P  L  L  H  V  L  N  Q  Q  L  S  Q  L  E  I  S  L  E  E  N  N  L  D

        GATATCCGAAAATTTTTATTACTAGTGGCCAAAGTTCACTTATTAACCTATTACTTCACTGATGTTACCTCCCAAAGTGCTGGAAAATCAAATGGTAAT
 463     D  I  R  K  F  L  L  L  V  A  K  V  H  L  L  T  Y  Y  F  T  D  V  T  S  Q  S  A  G  K  S  N  G  N

        ATTTATGAGGGATCATATTCCATTATCGAACTCGATACAAGTTTTGAAACGAAACGTGGATTGGTGAAAGTTTATAATGCCGCCGTAAACTTTCTTATA
 496     I  Y  E  G  S  Y  S  I  I  E  L  D  T  S  F  E  T  K  R  G  L  V  K  V  Y  N  A  A  V  N  F  L  I

        CATGCCAATAGTATGTGGGAACATGATCCTACCATTATTAAGTACTTTCCTGGTTTGTTTGTCTTGAATATATGGCAATCTGCCTGTATTATTAGTAAA
 529     H  A  N  S  M  W  E  H  D  P  T  I  I  K  Y  F  P  G  L  F  V  L  N  I  W  Q  S  A  C  I  I  S  K

        CTCATACATTCATCACTTCATTCAATGCTAGATGTTAACTCAGGCAAAAAAGCTTATAACAACGCAATTTCATTGACGTTTAATGCCTCAGTTTTAAAA
 562     L  I  H  S  S  L  H  S  M  L  D  V  N  S  G  K  K  A  Y  N  N  A  I  S  L  T  F  N  A  S  V  L  K

        TATGATATGGCGTACAGATCATCCGGAATAATGCGAAGCATATGGAGTTTATTTGCTAATATGTATGATGCCTGGAAAAACGACCAAAAGGAAGGTGGA
 595     Y  D  M  A  Y  R  S  S  G  I  M  R  S  I  W  S  L  F  A  N  M  Y  D  A  W  K  N  D  Q  K  E  G  G

        GGTAGACTAAATAATGATTTCAATTTAGGCATCACCATAAAATCTAGGATGTCAGTAAACGTTTTTTTTGACTGCTTATATATTCTAAAAGAGAAATGT
 628     G  R  L  N  N  D  F  N  L  G  I  T  I  K  S  R  M  S  V  N  V  F  F  D  C  L  Y  I  L  K  E  K  C

        GGTATGGCCAAATTGGAGAGAGAGACCAAGGTTTCTACAGCTTACAATGTTGATGAAGAGGAAGAGGAAGATGAAGATGAGGAGGGAGAAGAAGAAGAA
 661     G  M  A  K  L  E  R  E  T  K  V  S  T  A  Y  N  V  D  E  E  E  E  E  D  E  D  E  E  G  E  E  E  E

        GAAGAAGAAGAACTAAGTAGTAAAGTTCCAGAAAATATGGATAGCCAGCAACTAAGGACAAGGAAATTCACCAATGTAAGGCATCCAGAAAAGAAAGCA
 694     E  E  E  E  L  S  S  K  V  P  E  N  M  D  S  Q  Q  L  R  T  R  K  F  T  N  V  R  H  P  E  K  K  A

        AGAAAAATAATTGAAACAATTCCGCTAGACCCAAATCCAATAAATGCAGGCTCTACCAGCAGTGGAAGCTCATTAACGACCCCAAATAGTCAAGTAGCG
 727     R  K  I  I  E  T  I  P  L  D  P  N  P  I  N  A  G  S  T  S  S  G  S  S  L  T  T  P  N  S  Q  V  A

        AACACTATATCATATAGAGGAATCCTCAATAAAATGTCACCTAGGGAACAACTGAATCATGCAAATTTAGATTCCAGTGTTTCTACAGACATCAAGGAC
 760     N  T  I  S  Y  R  G  I  L  N  K  M  S  P  R  E  Q  L  N  H  A  N  L  D  S  S  V  S  T  D  I  K  D

        ACTGAAGCTGTCAATGAACCTCTGCCAATAGGGAGGAATGCTGAACATCCGGCAAATCAACCGCCTCTTTCAATAACACAAATGCAAGAAAACACACTA
 793     T  E  A  V  N  E  P  L  P  I  G  R  N  A  E  H  P  A  N  Q  P  P  L  S  I  T  Q  M  Q  E  N  T  L

        CCTGCGACACAAGCCAACTCTTCTCTATTAGAAACGTATCCCATTGTTCAATCAAACCCCGTTACAACTACAATCAAAGAATCACCCAATTCCATCATG
 826     P  A  T  Q  A  N  S  S  L  L  E  T  Y  P  I  V  Q  S  N  P  V  T  T  T  I  K  E  S  P  N  S  I  M

        GCAGGTTGGGATAACTGGGAATCTGATATGGTTTGGAGGGATGTTGATATTTTAATGAATGAATTTGCGTTCAATCCCAAGGTTTAAAGTCCTTTTCTT
 859     A  G  W  D  N  W  E  S  D  M  V  W  R  D  V  D  I  L  M  N  E  F  A  F  N  P  K  V  -

(2674)  TTTTTGCGTAATGTTTACTTACCCTCGAAAAAATGTTATGTAATGACTGCGGTGCTACAGGAGAGGTAAATGATTTTCTATATACGTTTATATTTCTTC

(2773)  CATTTACGCACACTATTTTTTGTCCTTGAATAATTTAACTCAAAAAAAGTGTAAAAAACTGTGTCATTAGGTATATGTTCAATACTTTTGATCTTCAAA
```

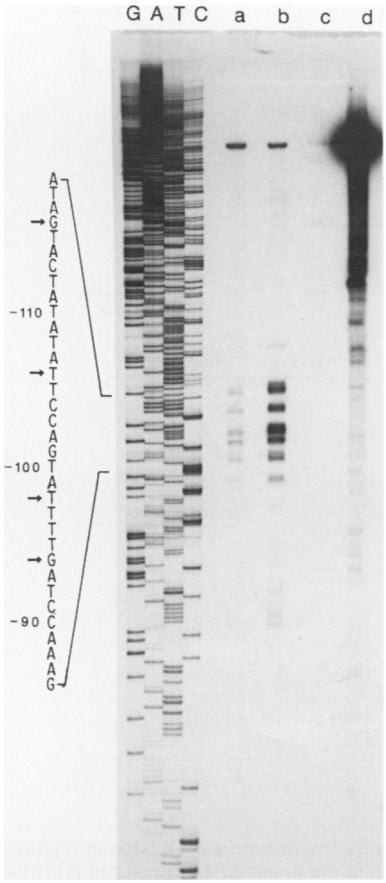

FIG. 5. S1 mapping of *LEU3* transcripts. An approximately 530-bp single-stranded 5′-labeled probe (extending from bp +99 to the *Bst*EII site) was hybridized to RNA and digested with nuclease S1, and the resulting protected fragments were separated on a 6% polyacrylamide sequencing gel. In addition, a sequencing ladder which was generated using the same oligonucleotide primer as that used to synthesize the S1 probe was run on the same gel. The 5′ ends of the *LEU3* transcripts can be directly determined from the sequencing ladder because all of the labeled DNA fragments have identical 5′ ends. Lanes: a, probe plus 12 μg of poly(A)-selected RNA; b, probe plus 70 μg of total RNA from strain PDY139-11B containing plasmid pPF711; c, probe plus 50 μg of wheat germ tRNA; d, undigested probe. Note that the DNA sequence is that of the noncoding strand. The locations of the major mRNA start sites are indicated by arrows.

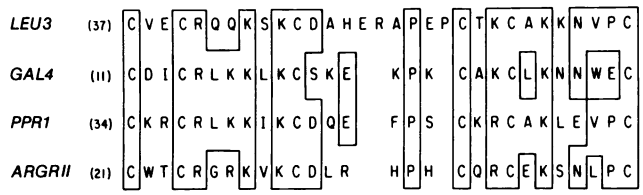

FIG. 6. Sequence motif found in several yeast proteins implicated in gene regulation. Conserved amino acids are boxed. Gaps were introduced to account for the three extra amino acids in *LEU3*. Numbers in parentheses indicate the amino acid position of the first cysteine. Amino acid sequences are from Laughon and Gesteland (31), Kammerer et al. (27), and Messenguy et al. (37).

a few other yeast proteins thought to be involved in gene regulation, was located in the amino-terminal region of *LEU3* (Fig. 6). In addition, there was a short stretch of amino acids (349 to 361; Fig. 4) with partial homology to a portion of the homeo box domain which is conserved in the *MATα2* and *MATa1* genes (49).

***LEU3*-dependent DNA-binding activity.** We explored the possibility that a *LEU3*-dependent product was a regulatory protein by virtue of interactions with the 5′ region of *LEU2*. Extracts from a *LEU3* strain were compared with those from *leu3* cells to test for a *LEU3*-dependent DNA-binding activity that recognized the upstream region of *LEU2*. We used the gel retardation technique, which can detect protein-DNA complexes because the mobility of a DNA fragment in a polyacrylamide gel is shifted due to protein binding (18, 19). The extent of the shift depends on the number of bound proteins and their molecular mass.

Cell extracts for binding experiments were prepared from strains PDY102-1A (*LEU3*) and PDY139-11B (*leu3*). To examine binding to the *LEU2* upstream region, we used a *Hin*cII fragment of 279 bp that extended from −405 to −126 (Fig. 7A, fragment A). (The fragments are numbered with respect to +1 as the start of the coding region.) This fragment contained the region of homology with *LEU1*. After combining the end-labeled DNA with the extracts, the resulting complexes were separated on a 4% nondenaturing polyacrylamide gel. The experiments were done in the presence of a 2,000-fold excess of sonicated salmon sperm DNA to eliminate nonspecific complexes. Both extracts gave rise to two distinct complexes, although the gel migration patterns of these complexes were different (Fig. 7B). These results suggest that a protein which binds to the *LEU2* upstream region was altered or not present in the *leu3* extracts. The intensity of all the bands observed was dependent on the amount of protein extract added to the binding reaction.

Figure 7C shows the results of experiments which defined more precisely the location of the 5′ region of *LEU2* which interacted with the *LEU3*-dependent DNA-binding activity. The 279-bp *Hin*cII fragment was cleaved with *Hga*I to generate two fragments that were designated B (170 bp) and C (114 bp) (Fig. 7A). Fragment C contained the previously reported region of partial homology with the 5′ region of *LEU1*. Only fragment C formed complexes that were dependent on *LEU3*. The intensity of the bands observed with fragment C was decreased by the addition of unlabeled fragment A (data not shown).

DISCUSSION

Until these studies, very little was known about the role of *LEU3* in the regulation of the genes encoding the leucine biosynthetic enzymes in *S. cerevisiae*. In a screen for Leu$^-$ auxotrophs, a minor class, designated *leu3*, was found which

FIG. 4. Nucleotide and deduced amino acid sequence of the *LEU3* gene. The numbering of the DNA sequence, in parentheses, is from the start of translation. The region from +20 to +2790 was sequenced on both strands. DNA 5′ to bp +20 was sequenced at least twice using different templates. The amino acids are numbered from the proposed initial methionine. The region of acidic amino acids is underlined. The region containing the repeated cysteine residues is underscored with dots. The dashed lines underscore the region of partial homology with *MATα2* and the homeo box domain. The major transcription initiation sites, as determined by S1 mapping (Fig. 5), are indicated by asterisks.

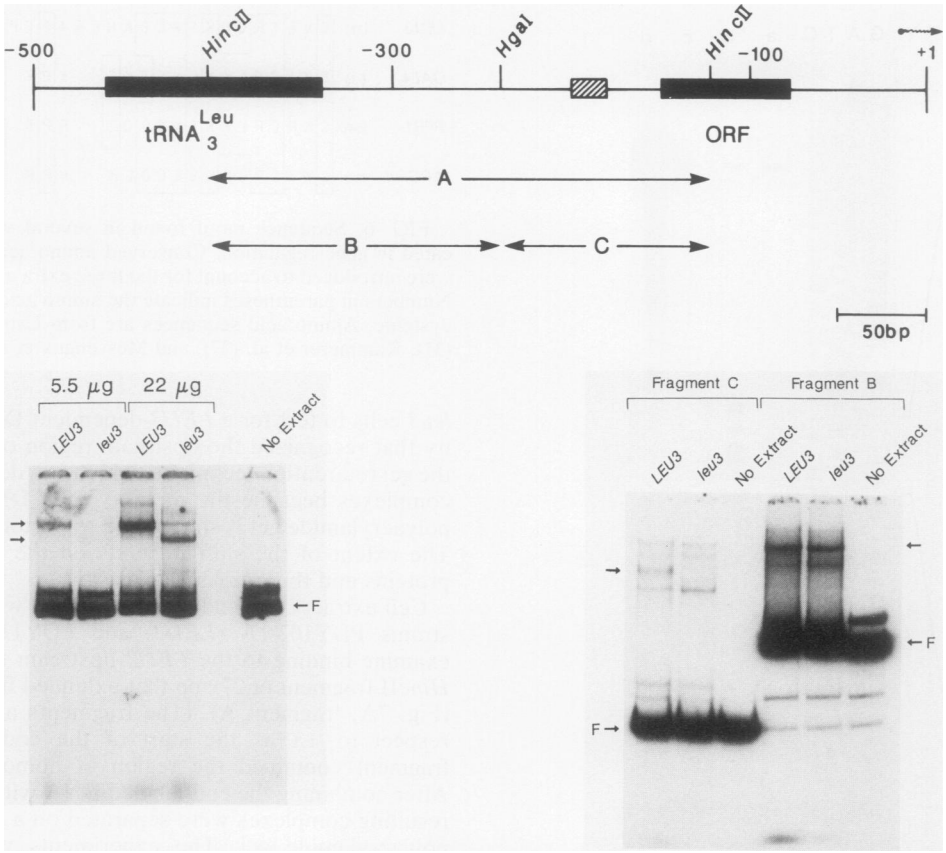

FIG. 7. *LEU3*-dependent protein binding to the upstream region of *LEU2*. (A) Organization of the *LEU2* upstream region. Numbering is relative to the start of translation. ORF, Leucine-rich open reading frame (2). The 5′ end of the major *LEU2* transcript (1) is indicated by the wavy arrow. The putative leucine-specific regulatory site is indicated by the hatched rectangle. The sizes of fragments B (170 bp) and C (114 bp) are inconsistent with the size of fragment A (279 bp) due to the in vitro synthesis (35) of DNA complementary to the 5-base overhangs that were generated in the cleavage of fragment A to yield fragments B and C. (B) Analysis of *LEU3*-dependent protein binding to the *LEU2* upstream region by the gel retardation assay. Extracts were prepared from strains PDY102-1A (*LEU3*) and PDY139-11B (*leu3*). ^{32}P-labeled fragment A (see above) was used as the substrate. F indicates free DNA. Major complexes are indicated by arrows. (C) As in panel B except for the replacement of fragment A with fragment B or C as indicated. The amount of extract per reaction was 40 μg. The origin of the minor contaminating bands in some of the fragment preparations is unknown.

exhibited low levels of both the *LEU1* and *LEU2* gene products (30). Based on analysis of mutations affecting leucine biosynthesis, it has been proposed that the *LEU3* gene product, in conjunction with α-IPM as inducer, regulates *LEU1* and *LEU2* in response to leucine (3).

By examining RNA from wild-type and *leu3* cells, we have shown that the levels of *LEU1*- and *LEU2*-specific transcripts are almost completely dependent on *LEU3*. In addition, a plasmid that contained the *LEU3* gene, isolated by its complementation of the *leu3* mutation, restored *LEU1* and *LEU2* transcripts to wild-type levels when introduced into a *leu3* strain.

An interesting observation from these studies is that *LEU3* on a multicopy plasmid did not result in levels of *LEU1*- and *LEU2*-specific transcripts above those seen with a single chromosomal copy of the gene. Unless an upper limit has been reached in the amount of transcripts that can be synthesized from *LEU1* and *LEU2*, this finding suggests that a component necessary for *LEU3* function (other than *LEU3* itself) is limiting. Brisco et al. (9) drew a similar conclusion in their studies of the levels of the *LEU1*- and *LEU2*-encoded enzymes in strains transformed with plasmids containing *LEU3*. These results are consistent with the hypothesis that *LEU3* alone is not able to activate transcription, but it is functional only when complexed with α-IPM.

The sequences found in the *LEU1* and *LEU2* 5′ regions which are thought to be important for leucine-specific regulation are shown in Fig. 8. Sequences from the upstream region of *LEU4* (4) which are similar to those from *LEU1* and *LEU2* are also shown. It is possible that these sequences in *LEU4* are involved in leucine-specific regulation, as suggested by our observation that the levels of *LEU4*-specific transcripts appeared to be responsive to regulation by *LEU3*. The significance, if any, of this regulation remains to be determined. It may be that regulation of *LEU3* is coordinated with the feedback inhibition of α-IPM synthase by leucine. It is of little value for the cell to continue to produce the synthase in the presence of leucine, only to have the enzyme inactivated. Regulation of *LEU4* by *LEU3* would result in lower levels of *LEU4*-specific RNA in the presence of excess leucine due to diminished *LEU3*-dependent transcription activation.

The nucleotide sequence of the *LEU3* locus was determined so that the primary sequence of the predicted *LEU3* protein could be examined. We found a single long open reading frame of 886 amino acids contained within the

LEU1	(−218)	C C G G G A C C G G	(−209)
LEU1	(−188)	T C G T A A C C G G	(−179)
LEU2	(−196)	C C G G A A C C G G	(−187)
LEU4	(−455)	C C G G A G C G G G	(−446)
LEU4	(−450)	G C G G G A C C G G	(−441)
ILV2	(−466)	C C G G T A C C G G	(−457)
ILV2	(−476)	C C G G A G C C T G	(−467)
ILV5	(−378)	C C G G T A C C G G	(−369)

FIG. 8. Comparison of potential leucine-specific regulatory sequences. Numbering for all sequences is from the start of translation. Sequences are from Hsu and Schimmel (24), Beltzer et al. (4), Falco et al. (17), and Petersen and Holmberg (41).

smallest fragment which could fully complement the *leu3* mutation (the *Bst*EII-*Nhe*I fragment, Fig. 1). The size of the putative *LEU3* protein is not unusual for a yeast regulatory protein; the *GAL4* protein is approximately the same size at 881 amino acids (31), and the *ADR1* protein is even larger, 1,323 amino acids (20). An examination of the codon usage for the *LEU3* coding region revealed a pattern similar to that found in low-abundance yeast proteins. The codon bias index (5) for *LEU3* was 0.02, indicating essentially no bias towards the preferred codons utilized in the highly expressed yeast genes. This finding is not unexpected, as regulatory proteins are usually present in the cell at extremely low levels. Similar codon usage profiles have been found for other yeast regulatory proteins (i.e., *PPR1*, *PHO4*, *ADR1*, and *GAL4* [20, 27, 33]).

The most striking feature of the *LEU3* coding region was a stretch of 19 of 20 acidic amino acids in the C-terminal half of the protein. Similar clusters of acidic amino acids have been found in a number of other proteins, including the *RAD6* gene of yeast (45), the N1/N2 protein of *Xenopus laevis* (29), and the HMG chromatin-associated proteins, specifically the HMG1 and HMG2 proteins isolated from calf thymus (55). It has been proposed that proteins with regions of high-density negative charge could serve as nucleosome assembly and disassembly factors (51). In the case of the N1/N2 protein, the acidic regions appear to be required for the in vitro binding of this protein to histones (29). It is possible that an acidic region on a DNA-binding protein could aid in the removal of histones from chromatin, leading to the activation of adjacent genes.

Recent work on *GCN4* in yeast supports the idea that a region of acidic amino acids is important in the activation of transcription by a yeast regulatory protein. Functional dissection of the *GCN4* protein, which is a DNA-binding protein involved in general amino acid control, has identified a 19-amino-acid segment containing 6 acidic residues which is critical for transcription activation (22). The sequences surrounding this critical segment also have a high proportion of acidic amino acids. Similar studies on *GAL4* have shown that the two domains responsible for transcription activation coincide with the two most acidic regions of the protein (34). It will be of interest to target mutations to the acidic region of *LEU3* to determine its functional significance.

Examination of DNA-binding proteins has revealed two structural motifs which appear to be important for protein-DNA interactions. In the *X. laevis* transcription factor TFIIIA (38), and possibly *ADR1* in *S. cerevisiae* (20), a structure termed metal-binding fingers has been identified which consists of repeated cysteine and histidine residues and appears to be involved in the DNA-binding activity of these proteins. An analogous sequence motif was present in the amino-terminal portion of the *LEU3* coding region. This portion of *LEU3* is similar to sequences located in the amino terminus of several proteins which have been implicated in gene regulation in yeast (*GAL4*, *PPR1*, and *ARGRII* [27, 31, 37]). In the case of *GAL4*, the DNA-binding activity was localized to that portion of the protein containing these sequences (28). However, it is unclear whether these sequences, despite their similarity to those in TFIIIA, could form a metal-chelating structure or function as a DNA-binding domain.

The helix-turn-helix structure, which was originally identified as a DNA-binding domain in a number of phage repressors (40), appears to be functionally important in the *MAT*α*2* gene of *S. cerevisiae* (44). A portion of the *LEU3* protein (amino acids 349 to 361) is somewhat similar to the region of conserved amino acids found in the homeo box, *MAT***a***1*, and *MAT*α*2* (32, 49). While the similarity is neither extensive nor compelling, the conserved amino acids include those found to be essential for diploid functions in *MAT*α*2* (44). The amino acid sequence of this region of *LEU3* is also similar to the helix-turn-helix domain of procaryotic DNA-binding proteins and, based on Chou-Fasman analysis (13), could potentially form such a structure. The identification of sequences in *LEU3* which are similar to those of two proposed DNA-binding domains suggests a mechanism by which *LEU3* could control the levels of *LEU1*-, *LEU2*-, and *LEU4*-specific transcripts. Future mutagenic studies will determine what role, if any, these sequences play in the function of *LEU3*.

The similarities noted above between *LEU3* and other yeast regulatory proteins suggest that the *LEU3* protein may bind to DNA and thereby activate transcription. This hypothesis is strengthened by the finding of homologous DNA sequences upstream of the genes controlled by *LEU3* which could serve as leucine-specific regulatory sites (4, 24). Previous work has shown that deletions extending into this region of *LEU2* have significant effects on promoter function (36). Using DNA fragments of the *LEU2* upstream region as substrates, we have identified a *LEU3*-dependent DNA-binding activity which specifically interacts with fragments containing the proposed regulatory site. Because no leucine-specific regulatory loci other than *LEU3* were found in a screen for such mutants, we propose that this DNA-binding activity is encoded by the *LEU3* gene.

Taken together, these results can be interpreted to suggest that the *LEU3* gene encodes a protein which binds to the 5′ region of leucine-specific genes and, when complexed with α-IPM, activates transcription of these genes. This would be the first case in which transcription in *S. cerevisiae* has been shown to be regulated by an activator responding to a specific amino acid. In the arginine system, which is also subject to specific amino acid control, regulation appears to be largely posttranscriptional by an as yet undetermined mechanism (14, 37).

It is also possible that the *LEU3* regulatory network extends to genes outside the leucine biosynthetic pathway. Analysis of *N. crassa* mutants indicates that genes encoding enzymes in the isoleucine and valine biosynthetic pathways are also controlled by the product of the *LEU3* gene in that organism (39). In this regard, sequences with a strong homology to the proposed leucine-specific regulatory site

have been found in the upstream regions of the *S. cerevisiae* *ILV2* and *ILV5* genes, which encode the first and second common enzymes in the isoleucine/valine pathway, respectively (17, 41) (Fig. 8). It is therefore possible that the product of the *LEU3* gene, in addition to regulating enzymes in the leucine biosynthetic pathway, serves to coordinate the synthesis of all three branched-chain amino acids in *S. cerevisiae*.

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