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LEU3 of *Saccharomyces cerevisiae* Activates Multiple Genes for Branched-Chain Amino Acid Biosynthesis by Binding to a Common Decanucleotide Core Sequence

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***LEU3* of *Saccharomyces cerevisiae* encodes an 886-amino-acid polypeptide that regulates transcription of a group of genes involved in leucine biosynthesis and has been shown to bind specifically to a 114-base-pair DNA fragment of the *LEU2* upstream region (P. Friden and P. Schimmel, Mol. Cell. Biol. 7:2707–2717, 1987). We show here that, in addition to *LEU2*, LEU3 binds in vitro to sequences in the promoter regions of *LEU1*, *LEU4*, *ILV2*, and, by inference, *ILV5*. The largely conserved decanucleotide core sequence shared by the binding sites in these genes is CCGGNNCCGG. Methylation interference footprinting experiments show that LEU3 makes symmetrical contacts with the conserved bases that lie in the major groove. Synthetic oligonucleotides (19 to 29 base pairs) which contain the core decanucleotide and flanking sequences of *LEU1*, *LEU2*, *LEU4*, and *ILV2* have individually been placed upstream of a *LEU3*-insensitive test promoter. The expression of each construction is activated by *LEU3*, although the degree of activation varies considerably according to the specific oligonucleotide which is introduced. A promoter construction with substitutions in the core sequence remains *LEU3* insensitive, however. One of the oligonucleotides (based on a *LEU2* sequence) was also tested and shown to confer leucine-sensitive expression on the test promoter. The results demonstrate that only a short sequence element is necessary for *LEU3*-dependent promoter binding and activation and provide direct evidence for an expanded repertoire of genes that are activated by LEU3.**

Regulation of protein synthesis in both procaryotes and eucaryotes is primarily at the level of transcription (20, 21, 32, 36). Activator and repressor proteins, which bind to specific DNA sequences located in the promoter regions of regulated genes, serve to modulate mRNA synthesis. The activity of these regulatory molecules is in turn controlled at the level of synthesis or by the requirement for a limiting cofactor (either a small molecule or an additional protein) or a protein modification. A single regulatory protein can coordinately control a number of unlinked genes if each promoter contains a site with which the protein interacts.

In the yeast *Saccharomyces cerevisiae*, the general amino acid control system is an example of such a regulatory network (16). The majority of genes encoding amino acid biosynthetic enzymes are coordinately controlled. When cells are starved for any one of a number of amino acids, transcription of all of the genes subject to general control is derepressed. This transcriptional control is mediated by the *GCN4* protein, the synthesis of which increases under conditions of amino acid starvation. GCN4 activates transcription by binding to a specific DNA sequence that is present (usually in multiple copies) upstream of genes subject to general control (3, 12). Other yeast regulatory genes, such as *GAL4* and *HAP1*, operate in a similar manner by binding to specific DNA sequences located in the promoters of genes subject to their control (8, 27).

Regulation of leucine biosynthesis in *S. cerevisiae* is somewhat unique in that it contains elements of both general control and pathway-specific control. *LEU4*, which encodes the first enzyme in the pathway (α-isopropylmalate [α-IPM] synthase), is subject to regulation by the general control system (14). In addition, *LEU1* and *LEU2* are coordinately controlled by leucine at the level of transcription (1, 5, 15, 17). Based on genetic studies, this pathway-specific control was thought to be mediated by the product of the *LEU3* gene and to require α-IPM as an inducer (4, 17). 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.

Recent work revealed that mutations in *LEU3* resulted in decreased levels of *LEU1*- and *LEU2*-specific transcripts (5). This provided direct evidence for a role for *LEU3* in the pathway-specific control of these genes. Moreover, evidence was also obtained for *LEU3*-dependent regulation of *LEU4* expression. This was the first demonstration that *LEU3* exerts control over a gene other than *LEU1* or *LEU2* and raised the possibility that additional genes are also regulated by *LEU3*.

The *LEU3* gene product (886 amino acids) has near its amino terminus a sequence that resembles the "zinc finger" motif of the *Xenopus laevis* transcription factor IIIA and of other transcriptional regulatory proteins (5, 18, 22, 23, 37). The possibility that LEU3 interacts with DNA was supported by the detection of a *LEU3*-dependent DNA-binding activity whose target site was localized to within a 114-base-pair (bp) region of the *LEU2* promoter (5). We show here that LEU3 binds to a specific decanucleotide sequence element that is found in the promoter regions of at least five genes and that this element is sufficient to confer *LEU3*-dependent and leucine-sensitive expression to a downstream promoter. The results suggest an expanded role for *LEU3* such that it regulates the synthesis of all three branched-chain amino acids through a sequence-specific interaction with a set of promoters that are dissimilar in sequence except for the LEU3-binding site.

MATERIALS AND METHODS

Strains and genetic methods. The following yeast strains were used in these studies: PDY102-1A (a *LEU4-103 ura3-*

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52) (5); F23 (a *his5 ura3-52*) (5); DBY746 (α *his3Δ1 ura3-52 leu2-3,112 trp1-289* (Peter Drain, Massachusetts Institute of Technology); PFY415-1C (a *ura3-52 LEU4-103 his3Δ1*), a segregant from the cross PDY102-1A × DBY746. Yeast growth media were prepared and general methods were used as described previously (30).

Escherichia coli HB101 was used to maintain pBR322-derived plasmids. Bacterial methods were as described previously (19).

Gel retardation assays. Yeast extracts were prepared and assays were performed as described previously (5). Oligonucleotides used as substrates were synthesized on a Systec Microsyn-1450A automated DNA synthesizer. Complementary oligonucleotides were radioactively labeled in a single reaction with T4 polynucleotide kinase and [γ-^{32}P]ATP (19). The reaction was diluted with water to give a final concentration of 1 ng of double-stranded oligonucleotide per μl, heated to 80°C for 4 min, and allowed to cool slowly to anneal the complementary strands. All binding reactions contained 1 μl (1 ng) of labeled oligonucleotide.

***LEU3* deletion-disruption construction.** The *LEU3*-encoding plasmid pPF701 was linearized by digestion with *Avr*II, which cleaves at a site that is 338 bp upstream of the *LEU3* translation-termination site. The resulting ends were made flush with deoxynucleoside triphosphates and the Klenow enzyme (19). *Bam*HI linkers (New England Biolabs) were ligated onto the blunt-ended DNA, which was then digested with *Bam*HI (which also cleaves the plasmid at a unique site ~1 kbp upstream of the *LEU3* coding region). The DNA was then ligated under dilute conditions and a plasmid containing a deletion of the *LEU3* locus was isolated. This deletion removes ~1 kbp of the *LEU3* 5′-noncoding region and almost 90% of the coding region. A 1.7-kbp *Bam*HI fragment containing the yeast *HIS3* gene was cloned into this plasmid at the unique *Bam*HI site. The resulting plasmid was used to replace the *LEU3* locus by transforming strain PFY415-1C with plasmid DNA digested with *Sph*I, which cleaves the plasmid on either side of the *HIS3* insertion, and selecting for histidine prototrophs (29).

DNA was isolated (34) from a His$^+$ transformant which also displayed a *leu3* phenotype (PFY420), as well as from the parent strain PFY415-1C, for DNA blot analysis. The digested DNA was resolved on a 1% agarose–Tris–borate–EDTA gel and blotted to nitrocellulose (5, 19). A LEU3-specific radioactive probe was synthesized by extending a duplex which had been formed with a primer annealed to single-stranded DNA derived from an M13 clone containing a fragment of the *LEU3* locus (a *Sal*I fragment from plasmid pPF701 [5] which contains the C-terminal portion of *LEU3* plus flanking sequences) by using the Klenow enzyme with α-^{32}P-labeled deoxynucleoside triphosphates. Hybridization conditions were as described previously (5). The results of the Southern blot hybridization experiment were consistent with the construction diagrammed in Fig. 3 (data not shown). In addition, tetrad analysis indicated that the gene replacement occurred as predicted.

Methylation interference footprinting. The procedure for methylation interference footprinting described by Pfeifer et al. (26) was followed. The samples were analyzed by electrophoresis on 20% polyacrylamide sequencing gels.

***CYC1-lacz* fusion constructions.** Double-stranded oligonucleotides were cloned into plasmid pLGΔ-312-178 (11) at the unique *Xho*I site that is 178 bp upstream of the most proximal RNA start site. Each oligonucleotide encodes a *Pvu*I site (5′ CGATCG 3′), so that plasmids containing inserts could be easily identified. In addition, the sequence of the oligonucleotides is such that an *Xho*I site would be regenerated at only one of the two oligonucleotide-vector junctions, so that the orientation of the oligonucleotides could be easily determined. β-Galactosidase activity was assayed essentially as described previously (9); cultures were grown overnight in YPD medium, diluted into SD minimal medium containing the appropriate supplements, and grown to an optical density at 600 nm of ~1.0. Activities represent the results of at least two experiments, each performed in duplicate. In most cases, several different transformants were analyzed. Standard errors were less than 25%.

RESULTS

Identification of sequences within the *LEU2* promoter necessary for LEU3 binding. Previous results localized the sequences which interact with a *LEU3*-dependent DNA-binding activity to a 114-bp fragment of the *LEU2* upstream region which extends from bp −110 to −224 (numbering is relative to the 5′ end of the major transcript) (5). This DNA fragment contains a sequence beginning at position −176 (5′ CCGGNNCCGG 3′) which is also present upstream of the coding regions of *LEU1*, *LEU4*, *ILV2*, and *ILV5*. This is the only significant sequence similarity among the 5′-noncoding regions of these five genes. Transcript levels from three of these genes (*LEU1*, *LEU2*, and *LEU4*) have been shown to be dependent on *LEU3*. An involvement of *LEU3* in the regulation of *ILV2* and *ILV5* is suggested by the observation that, in a *leu3* strain grown in minimal medium supplemented with leucine, there is a derepression of genes that are under general amino acid control; this derepression is alleviated by the addition of isoleucine and valine (Friden and Schimmel, unpublished observation). These correlations suggested that the *LEU3*-dependent DNA-binding activity might interact with the conserved decanucleotide sequence.

To investigate this possibility, a short DNA oligomer was synthesized for use in gel mobility shift assays (6, 7). The sequence of this oligonucleotide (designated LEU2-26) was derived from that of the *LEU2* promoter and consists of 26 bp of double-stranded DNA which includes the decanucleotide core sequence (Fig. 1). Each strand of the oligonucleotide was radioactively labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and the two strands were annealed. Protein-DNA complexes were formed by incubating a whole-cell yeast extract prepared from a wild-type *LEU3* strain with the labeled oligomer. Complexes were separated from free DNA by electrophoresis on nondenaturing polyacrylamide gels.

A single major protein-DNA complex was observed in assays with the LEU2-26 oligonucleotide as the substrate (Fig. 2). The formation of this complex is diminished specifically by the addition of oligonucleotide competitors which contain the decanucleotide core sequence (see below). These results indicate that the sequences within the LEU2-26 oligonucleotide are sufficient for the formation of a protein-DNA complex.

Complex formation is dependent on *LEU3*. To verify that the complex detected in the above gel shift assay is *LEU3* dependent, a deletion-disruption of the *LEU3* locus was constructed (Fig. 3). This construction deleted the *LEU3* promoter region and almost 90% of the coding sequences. The deletion-disruption strain (designated PFY420) behaved similarly to the original *leu3* mutant strain in that it displayed a leaky leucine auxotrophy (5, 17).

In gel mobility shift assays, the major protein-DNA complex observed with extracts prepared from a *LEU3* strain

LEU2-26	TCGTGAGAGGCCGGAACCGGCTTTTC
LEU2-19	TCGGCCGGAACCGGCTTTC
LEU2M-19	TCGGCCAGAACTGGCTTTC
LEU2M-19T	TCGTCCGGAACCGGCTTTC
LEU2M-19A	TCGACCGGAACCGGCTTTC
LEU1-27	T CGCAAAATTCCGGGACCGGGCGAAAC
LEU4-28	TCGCTACCGGAGCGGGACCGGCTCTGAC
ILV2-29	TCGCCGGAGCCTGCCGGTACCGGCTTGGC

FIG. 1. Sequences of synthetic oligonucleotides that were used to determine the sequence requirements for LEU3 site-specific DNA binding and gene activation. The sequence of the coding strand from the double-stranded region of each oligomer is shown. In addition, each oligonucleotide has at its 5′ terminus the sequence 5′ TCGA 3′, which is complementary to the cohesive ends generated by the restriction enzyme *Xho*I. This facilitated the cloning of the oligonucleotides into the *CYC1-lacZ* fusion vector pLGΔ-312-178 (see Fig. 7).

was not detected when extracts prepared from PFY420 were used (Fig. 2). This result confirms that *LEU3* is required for the formation of a protein-DNA complex with the LEU2-26 oligonucleotide. A second, slower-migrating, *LEU3*-dependent complex (B′) was also detected in some experiments. Preliminary studies suggest that this is a higher-order complex containing multiple LEU3 molecules. That LEU3 itself was binding directly to DNA was suggested by the observation that with the 279- or 114-bp *LEU2* promoter fragment or the LEU2-26 oligonucleotide as substrate, extracts prepared from the *leu3-781* strain gave rise to a complex whose migration was different from that of the wild type (5; data not shown).

Binding of LEU3 to other oligonucleotides. Oligonucleotides which contained the conserved decanucleotide and flanking sequences from the *LEU1*, *LEU4*, and *ILV2* promoter regions were synthesized. The *LEU4* upstream region contained two overlapping copies of the decanucleotide, each of which had a one-base deviation from a perfect match to the consensus sequence. The *ILV2* upstream region contained two tandem copies of the decanucleotide; one had a perfect match, and the other had a single mismatch with the consensus sequence. A shorter version of the LEU2-26 oligonucleotide, which consisted of a double-stranded region only 19 bp in length, was also synthesized. The sequences of these oligonucleotides, designated LEU1-27, LEU4-28, ILV2-29 and LEU2-19, respectively, are shown in Fig. 1. The only sequences these oligonucleotides had in common were within the conserved decanucleotide.

Each of these oligonucleotides gave rise to a single major *LEU3*-dependent complex in gel mobility shift assays (Fig. 4). The complexes observed with each of these oligonucleotides migrated at the same position in the gel, suggesting that all of the complexes had identical compositions. In addition, the amount of each complex formed was diminished by the addition of unlabeled LEU2-26 as a competitor (Fig. 4).

Each oligonucleotide was also tested as a competitor against the labeled LEU2-26 oligonucleotide. There was little observable difference among these oligonucleotides in

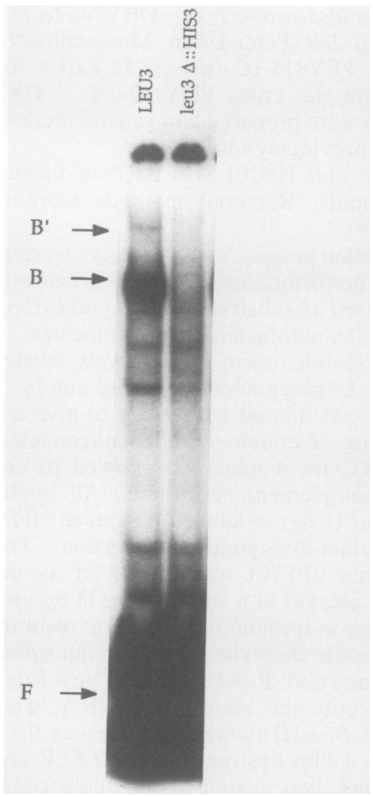

FIG. 2. *LEU3*-dependent protein binding to the LEU2-26 oligonucleotide. Extracts were prepared from PDY102-1A (*LEU3*) and PFY420 (*leu3*Δ::*HIS3*). B and B′ indicate the major and minor *LEU3*-dependent complexes, respectively. F indicates the free DNA. The amount of extract per reaction was 40 μg.

their ability to compete against the LEU2-26 oligonucleotide and therefore in their ability to bind LEU3 (Fig. 5). Note that, although the *LEU4* oligonucleotide appeared to bind LEU3 as well as the other oligonucleotides, it contained an imperfect decanucleotide core sequence (Fig. 1).

Substitutions within the decanucleotide can eliminate LEU3 binding in vitro. The above results suggest that the sequence CCGGNNCCGG is critical for LEU3 binding because sequences that flank the decanucleotide core are not conserved among the *LEU1*, *LEU2*, *LEU4*, and *ILV2* oligonucleotides. However, the *LEU4* oligonucleotide, which contains an imperfect decanucleotide core sequence, did not appear to have a lower affinity for LEU3 (Fig. 4 and 5). To test whether some positions within the decanucleotide sequence form essential contacts with LEU3, a mutant version of the

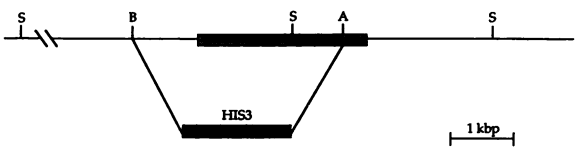

FIG. 3. Schematic diagram of the construction of the *LEU3* deletion-disruption. A fragment of the *LEU3* locus, extending from a *Bam*HI site (B) ~1 kbp upstream of the *LEU3*-coding region to an *Avr*II site (A) 338 bp upstream of the *LEU3* translation-termination codon, was deleted and replaced with a 1.7-kbp DNA fragment containing the yeast *HIS3* gene. The stippled region indicates the *LEU3* open reading frame. S denotes an *Ssp*I restriction site.

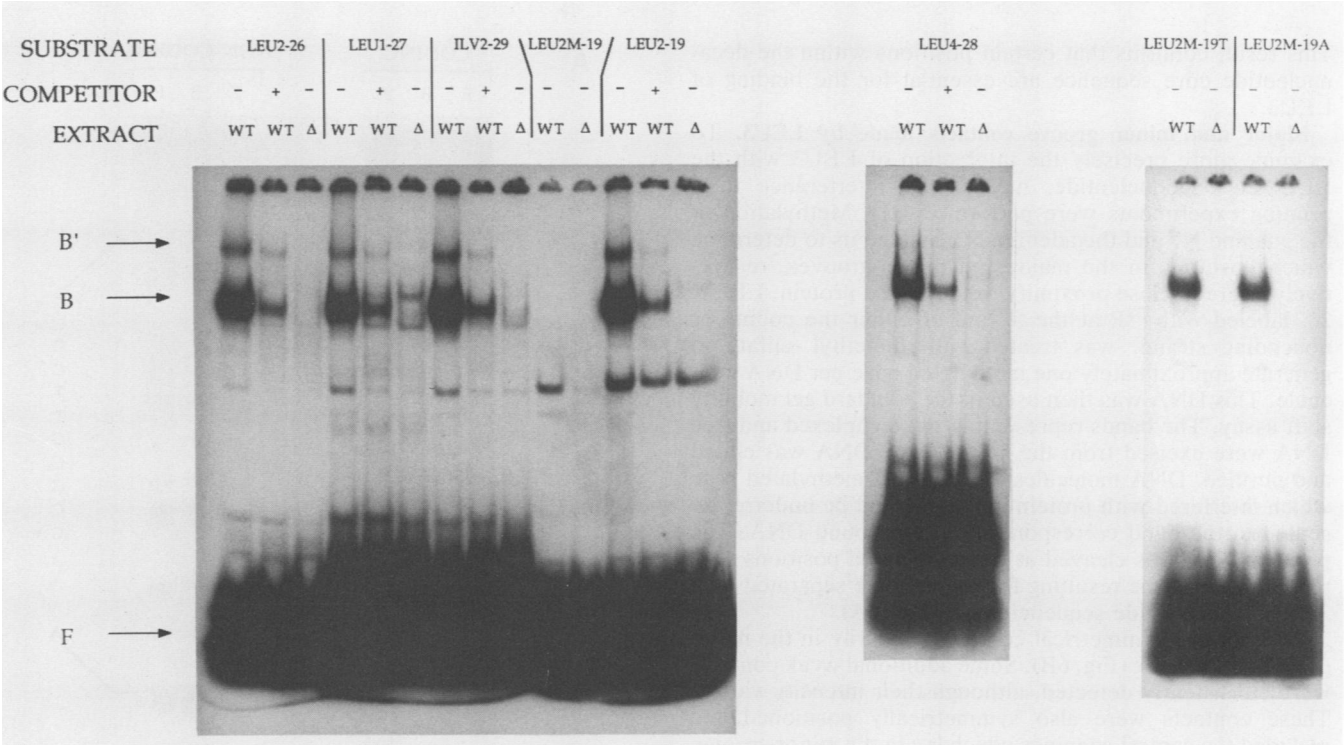

FIG. 4. LEU3 binding to oligonucleotides derived from *LEU1*, *LEU2*, *LEU4*, and *ILV2*. Various oligonucleotides (see Fig. 1 for sequences) were radioactively labeled and used as substrates in gel mobility shift assays. Extracts were prepared from strain PFY420 untransformed (Δ) or transformed with the *LEU3*-encoding plasmid pPF750 (WT). Each reaction contained 20 μg of protein extract. Where indicated, the LEU2-26 oligonucleotide was added as an unlabeled competitor at a 100-fold molar excess. B and B′ indicate the major and minor *LEU3*-dependent protein-DNA complexes, respectively. F denotes uncomplexed DNA.

LEU2-19 oligonucleotide was synthesized. The decanucleotide sequence of this oligonucleotide, designated LEU2M-19, has been changed to 5′ CCAGAACTGG 3′ (the altered positions are in boldface type; see also Fig. 1). These changes do not affect the dyad symmetry of the sequence. This oligonucleotide did not bind LEU3 nor did it serve as an effective competitor against LEU3 binding to the LEU2-26 oligonucleotide in gel mobility shift assays (Fig. 4 and 5).

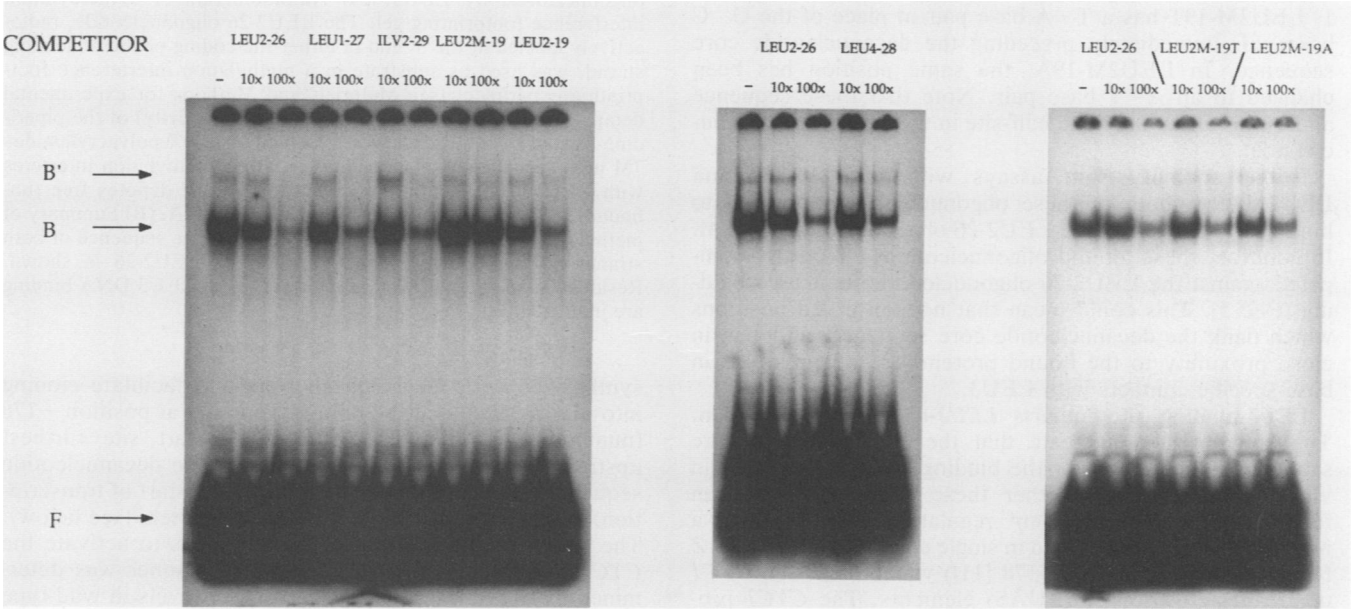

FIG. 5. Competition for LEU3 binding between the LEU2-26 oligonucleotide and oligonucleotides derived from *LEU1*, *LEU2*, *LEU4*, and *ILV2*. Each of the oligonucleotides used as labeled substrates in Fig. 4 was tested for its ability to compete with the radioactively labeled LEU2-26 oligonucleotide for LEU3 binding. The unlabeled oligonucleotides were added at either a 10- or 100-fold molar excess, as indicated. Competition of the LEU2-26 oligonucleotide with itself was included in each of the three experiments for standardization. Extracts were prepared from strain PFY420 transformed with the *LEU3*-encoding plasmid pPF750. Each reaction contained 20 μg of protein extract.

This result confirms that certain positions within the decanucleotide core sequence are essential for the binding of LEU3.

Major and minor groove contacts made by LEU3. To examine more precisely the interaction of LEU3 with the LEU2-26 oligonucleotide, methylation interference footprinting experiments were performed (31). Methylation of the guanine N7 and the adenine N3 enabled us to determine which positions in the major and minor grooves, respectively, were in close proximity to the bound protein. LEU2-26, labeled with ^{32}P at the 5′ end of either the coding or noncoding strand, was treated with dimethyl sulfate to generate approximately one methylated base per DNA molecule. This DNA was then used in the standard gel mobility shift assay. The bands representing the complexed and free DNA were excised from the gel, and the DNA was eluted and purified. DNA molecules containing a methylated base which interfered with protein binding would be underrepresented in the band corresponding to the bound DNA. The purified DNA was cleaved at the methylated positions with piperidine, and the resulting fragments were separated on a 20% polyacrylamide sequencing gel (Fig. 6A).

LEU3 made symmetrical contacts primarily in the major groove of the helix (Fig. 6B). Some additional weak contacts were consistently detected, although their intensity varied. These contacts were also symmetrically positioned and included the central adenines which lay in the minor groove. All of the contacts were centered around the conserved decanucleotide sequence CCGGNNCCGG.

Substitution of a single flanking base pair does not prevent LEU3 binding. Nonconserved positions flanking the decanucleotide core sequence appeared to be in close proximity to the bound protein, as indicated by the significant inhibition of binding when these positions were methylated (Fig. 6). To examine whether LEU3 makes specific contacts with these positions, two variations of the LEU2-19 oligonucleotide were synthesized. The sequences of these oligonucleotides, designated LEU2M-19T and LEU2M-19A, are shown in Fig. 1. LEU2M-19T has a T · A base pair in place of the G · C base pair immediately preceding the decanucleotide core sequence. In LEU2M-19A, the same position has been changed to an A · T base pair. Note that these sequence alterations affect only one half-site in the conserved decanucleotide.

In gel mobility shift assays with LEU2M-19T and LEU2M-19A, both of these oligonucleotides appeared to bind LEU3 as well as the LEU2-26 oligonucleotide (Fig. 4). In addition, these altered oligonucleotides effectively competed against the LEU2-26 oligonucleotide for LEU3 binding (Fig. 5). This could mean that nonconserved positions which flank the decanucleotide core sequence, although in close proximity to the bound protein, are not involved in base-specific contacts with LEU3.

LEU3-binding site imparts *LEU3*-dependent expression. The above results indicate that the decanucleotide core sequence is necessary for the binding of LEU3 to DNA in vitro. To determine whether these oligonucleotides can function as *LEU3*-dependent regulatory sites in vivo, a number of them were cloned in single copy into a *CYC1-lacZ* fusion plasmid (pLGΔ-312-178 [11]) which lacks the *CYC1* upstream activation site (UAS) elements. The *CYC1* promoter in this plasmid contains the TATA sequences and RNA start sites which are normally utilized. This fusion gene is not expressed because of the deletion of the UAS1 and UAS2 transcriptional activating elements. All of the oligonucleotides used in the above DNA-binding studies were

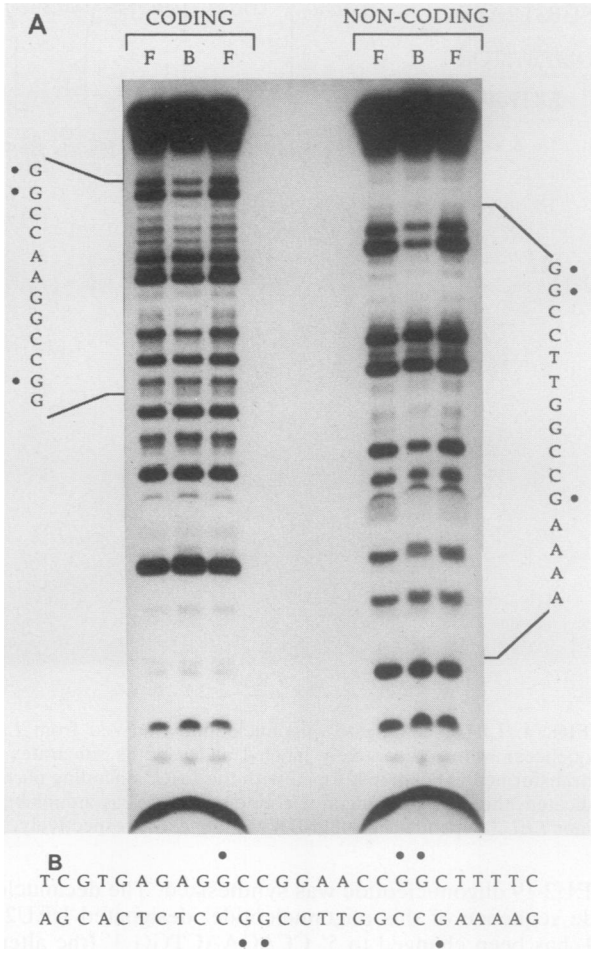

FIG. 6. Methylation interference footprinting of the LEU2-26 oligonucleotide by LEU3. (A) Autoradiogram of a methylation interference footprinting gel. The LEU2-26 oligonucleotide, radioactively labeled at the 5′ end of either the coding or the noncoding strand, was used as substrate in a methylation interference footprinting experiment (see Materials and Methods for experimental details). Equivalent amounts (based on radioactivity) of the piperidine-treated DNA samples were resolved on a 20% polyacrylamide–7M urea sequencing gel. Residues in which methylation interferes with LEU3 DNA binding are indicated (●). F denotes free (unbound) DNA; B denotes bound (complexed) DNA. (B) Summary of methylation interference footprinting results. The sequence of both strands of the double-stranded portion of LEU2-26 is shown. Residues in which methylation interferes with LEU3 DNA binding are indicated (●).

synthesized with *Xho*I cohesive ends to facilitate cloning into pLGΔ-312-178 at the unique *Xho*I site at position −178 (numbering is relative to the RNA start site furthest upstream). Note that the positioning of the decanucleotide sequences in this promoter (relative to the start of transcription) differs from that in their native promoters (see below). The ability of the inserted oligonucleotides to activate the *CYC1* promoter in a *LEU3*-dependent manner was determined by measuring the β-galactosidase levels in wild-type and *LEU3* deletion-disruption strains which were transformed with the various plasmids. These strains contain the *LEU4* Tfl^r allele, which eliminates the feedback inhibition by leucine of α-IPM synthase, the product of the *LEU4* gene (4). The growth of both of these strains in media containing

leucine (which is necessary in the case of PFY420) should not alter the levels of α-IPM, the putative inducer of LEU3.

Each oligonucleotide which bound LEU3 in vitro also imparted *LEU3*-dependent expression when positioned upstream of the *CYC1-lacZ* fusion gene (Fig. 7A). This activation appeared to be independent of the orientation of the binding site. However, unlike the results of in vitro DNA-binding studies, there was considerable variation among the oligomers in their ability to activate transcription. The mutant oligonucleotide, LEU2M-26, which did not bind LEU3 in vitro, failed to act as a *LEU3*-dependent activation site in vivo. Evidently, the presence of a functional LEU3-binding site upstream of a yeast promoter is sufficient for *LEU3*-dependent transcriptional activation in vivo.

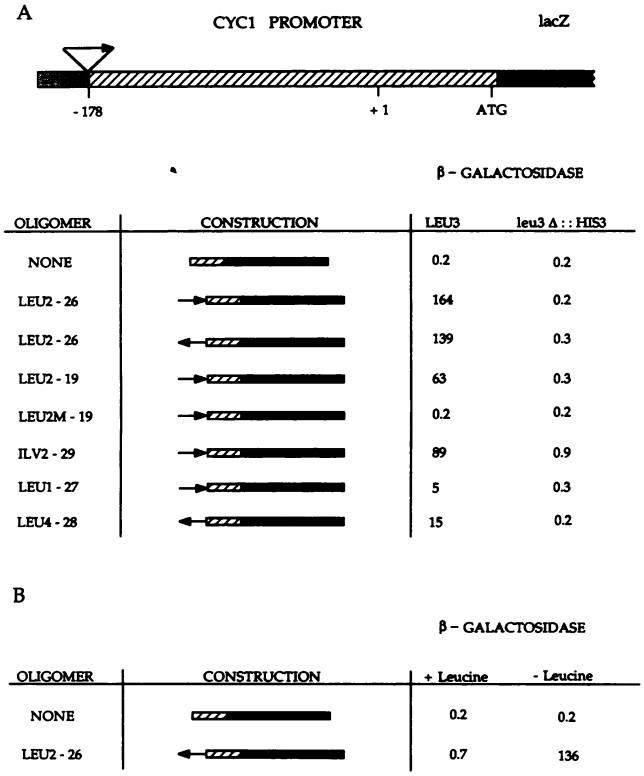

FIG. 7. Oligonucleotides which bind LEU3 in vitro confer *LEU3*-dependent and leucine-sensitive expression on a *CYC1-lacZ* fusion gene. (A) Analysis of *LEU3*-dependent expression of a *CYC1-lacZ* fusion gene containing various oligonucleotides. The top portion of the figure contains a schematic diagram of the *CYC1-lacZ* fusion gene promoter region. Sequences derived from *CYC1* are indicated by the striped area. *lacZ* and vector sequences are indicated by the checkered and stippled areas, respectively. ATG indicates the start of translation of the fusion gene. All oligonucleotides were inserted in single copy at the *Xho*I site at bp −178, where the most proximal RNA start site is labeled +1. The oligonucleotides (see Fig. 1 for sequences) and their orientation are indicated by arrows. Sizes are not to scale. The results of β-galactosidase assays are given for each construction in *LEU3* (PDY102-1A) and *leu3Δ::HIS3* (PFY420) backgrounds. See Materials and Methods for details of the β-galactosidase assays. (B) Analysis of leucine-sensitivity of a *CYC1-lacZ* fusion gene containing a LEU3-binding site. The β-galactosidase activity of a *CYC1-lacZ* fusion gene containing the LEU2-26 oligonucleotide in the indicated orientation was assayed in strain F22 (*LEU4*, Tfl^s) which was grown in the presence or absence of 2.0 mM leucine.

LEU3-binding site defines a leucine-sensitive upstream activation site. We investigated whether these oligonucleotides, when cloned into the *CYC1* promoter, impart leucine-sensitive as well as LEU3-dependent gene expression. This was tested by using the LEU2-26 oligonucleotide. β-Galactosidase levels were measured in the presence and absence of 2 mM leucine in a wild-type *LEU4* strain that had been transformed with *CYC1-lacZ* plasmids containing the LEU2-26 oligonucleotide. The wild-type *LEU4* allele rendered this strain sensitive to leucine-specific modulation of the levels of α-IPM.

Expression from a heterologous yeast promoter became sensitive to leucine levels when a DNA oligomer containing the LEU3-binding site was inserted into its upstream region (Fig. 7B). These oligonucleotides therefore appear to confer all of the properties previously shown to be associated with the leucine-sensitive expression of *LEU1* and *LEU2*.

DISCUSSION

In this report we have shown that the decanucleotide CCGGNNCCGG (where N is predominantly a purine residue) is important for in vitro LEU3 DNA binding. This was determined by examining the in vitro binding activity of a number of oligonucleotides derived from promoter regions (of *LEU1*, *LEU2*, *LEU4*, and *ILV2*) which had only the decanucleotide sequence in common. Each of the oligonucleotides bound LEU3 in gel mobility shift assays, indicating that all of the information required for LEU3 recognition is contained within the conserved sequence. Consistent with these observations, LEU3 was shown to make contacts with the DNA at positions within and immediately adjacent to the decanucleotide sequence. When placed upstream of a heterologous promoter, this sequence imparted *LEU3*-dependent and leucine-sensitive expression in a manner that was independent of orientation and, at least to some degree, position. Substitutions within the decanucleotide which abolished DNA binding also abolished in vivo transcriptional activation. This observation provides a direct link between LEU3 DNA binding and leucine-sensitive transcriptional regulation. Taken together, these results indicate that the decanucleotide element confers all of the characteristics associated with leucine-sensitive gene regulation in *S. cerevisiae*.

Although no qualitative differences were detected in the ability of these oligomers to bind LEU3 in vitro, some differences were apparent in their ability to activate transcription in vivo. The LEU2-26 oligonucleotide is clearly the most responsive element tested, giving rise to an approximately 450-fold activation in the presence of LEU3 (Fig. 7A). This activation is comparable to that observed for a bifunctional *LEU2-lacZ* fusion which has been integrated into the chromosome (data not shown). When a subsequence of the LEU2-26 oligomer (LEU2-19) was tested in the fusion gene construct, the transcriptional activation was about half of that of the larger oligomer. The oligomer derived from the *LEU4* promoter (LEU4-28) activates the fusion gene approximately 10-fold less well than does the LEU2-26 oligomer. This result is consistent with previous in vivo studies, which showed that transcription of *LEU4* is not as strongly regulated as *LEU2* by *LEU3* (5).

An unexpected result was obtained when the *LEU1*-derived oligomer (LEU1-27) was tested in the fusion gene construct. The in vivo regulation of *LEU1* and *LEU2* is very similar, with both genes being repressed by leucine and almost completely dependent on *LEU3* for expression (5, 17). However, the LEU1-27 oligomer is approximately 30-

fold less active in the test promoter than is the LEU2-26 oligomer. These results suggest that, although the minimum sequence requirement for in vitro LEU3 DNA binding and in vivo *LEU3*-dependent gene activation is the CC GGNNCCGG element, nucleotides that flank the sequence CCGG can have a dramatic effect on in vivo transcriptional activation. This effect is apparently not due to major differences in protein-DNA binding if the in vitro DNA binding results accurately reflect the ability of LEU3 to bind to the various oligomers in vivo. This influence of flanking nucleotides may extend beyond the sequence of the oligonucleotides used here and may reflect subtle effects on the ability of DNA-bound LEU3 to form an active protein-DNA complex. This phenomenon could provide a mechanism for fine tuning the degree of regulation derived from the DNA element. As noted above, there is a difference in vivo in the degree to which the *LEU1*, *LEU2*, and *LEU4* genes are regulated by LEU3. It is also quite likely that the *ILV2* and *ILV5* genes are not tightly regulated by LEU3 because, although *leu3* strains appear to have decreased levels of isoleucine and valine (which results in a derepression of the general amino acid control system), they are not obvious isoleucine-valine auxotrophs (17; data not shown).

In addition to conferring LEU3-dependent gene expression on the test promoter, oligomers which contain the decanucleotide sequence also impart leucine-sensitive expression. The difference in β-galactosidase levels that we observed for cells grown in the presence or absence of leucine (~200-fold; Fig. 7B) is considerably greater than the 5- to 10-fold effect that has been observed for *LEU1* and *LEU2* in vivo (1, 5, 15, 17). This might be attributed to the lack of other promoter elements in the test promoter which are present in *LEU1* and *LEU2* and modulate the leucine effect or to the high copy number of the test plasmid (or a combination of both). Results obtained with an integrated *LEU2-lacZ* fusion gene which contains an intact *LEU2* promoter suggest that this discrepancy is not due to an elevated, *LEU3*-independent, basal level of transcription from *LEU2* (data not shown).

The decanucleotide sequence appears to behave like other yeast upstream activating sites (10) in that it is not functionally dependent on orientation. The palindromic nature of the LEU3-binding site is consistent with this observation. This may also suggest that LEU3 binds DNA as a dimer (or tetramer), as does GCN4 (of *S. cerevisiae*) (13) and most procaryotic regulatory proteins which bind to palindromic sites (25, 33). Another characteristic of yeast upstream activating sites is that they are position independent. The position of the decanucleotide sequence in the *LEU1*, *LEU2*, and *LEU4* promoter regions indicates that the element is functional within the range of 130 to 376 nucleotides upstream from the most proximal start of transcription. The location of the decanucleotide sequence in the *CYC1-lacZ* fusion construction is within this range at position −178. Although this is not an extensive analysis, it appears that the decanucleotide sequence element can be moved relative to the start of transcription and remain active. By these criteria, the decanucleotide element defines a leucine-sensitive upstream activation sequence.

As is the case in enteric bacteria (35) and, apparently, *Neurospora crassa* (24, 28), the yeast *S. cerevisiae* appears to have a specialized mechanism for coordinately controlling the biosynthesis of the branched-chain amino acids leucine, isoleucine, and valine. Cells starved for leucine require sufficient amounts of the precursor α-ketoisovalerate feeding into the leucine biosynthetic pathway from that for valine. The *LEU3* regulatory pathway would assure that this occurs. However, it is unclear why this regulation could not be achieved through the general amino acid control network alone. Possibly the levels of leucine in the cell are quite critical, necessitating the additional regulatory system. Genetic evidence suggests that rRNA synthesis may also be subject to regulation by the *LEU3* equivalent in *N. crassa* (2, 17). We have shown here that at least five genes in *S. cerevisiae* are subject to the *LEU3* control system. Whether rRNA or other genes are also part of the *LEU3* regulatory system in *S. cerevisiae* remains to be determined.

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