The upstream activating sequence for L-leucine gene regulation in *Saccharomyces cerevisiae*

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ABSTRACT

The upstream activating sequence (UAS) conferring leucine-specific regulation of transcription in *Saccharomyces cerevisiae* was identified by analysis of the *LEU2* promoter and by comparison to other genes regulated by leucine. The UAS was localized with deletions and cloned synthetic DNA. Point mutations and sequence rearrangements were used to identify important basepairs and to construct an improved UAS with increased regulation and expression. The improved UAS contains a core ten basepair, GC-rich, palindromic sequence, which is sufficient to confer minimal levels of activation and regulation, within a 36 basepair palindromic sequence which confers maximal activation and regulation. Deletions downstream of the UAS indicated that the UAS must act in conjunction with at least one other site, perhaps a TATAA region, in order to confer high levels of activation. Tandem copies of the UAS in front of *LEU2* increased expression and regulation. Tandem UAS elements in *trans* on a multi-copy 2μ-based plasmid decreased expression and regulation. These results are consistent with a model that the UAS serves as the DNA-binding site for diffusible activation factor(s), possibly the *LEU3* gene product.

INTRODUCTION

In *Saccharomyces cerevisiae*, leucine coordinately regulates the production of a number of enzymes involved in leucine, isoleucine, and valine biosynthesis. Induction of these enzymes requires a-isopropyl malate (α-IPM), a leucine precursor (1), the *LEU3* gene product (2, 3, 4, 5) and an upstream activating sequence (UAS) specific for leucine regulation (3, 4, 6). α-IPM synthesis is reduced by leucine feedback repression of the *LEU4* enzyme α-isopropyl malate synthase (1, 5, 7), which catalyzes the first committed step in leucine biosynthesis and is responsible for the synthesis of over 80% of the cell's α-IPM (1).

Genes regulated by leucine include the leucine biosynthetic genes *LEU1* for α-isopropyl malate isomerase, *LEU2* for β-isopropyl malate dehydrogenase, *LEU4*, and at least three branched-chain amino acid genes, *ILV2*, *ILV3*, and *ILV5* (1, 3, 4, 6, 8, 9). For these genes, high levels of leucine in the growth media represses the genes enzymatic activities. In particular, the *LEU2* enzymatic activity and mRNA levels are repressed 10-fold and approximately 5-fold (5, 10, 11, 12), respectively.

In the *LEU2* promoter, a region responsible for leucine regulation was mapped to a region containing an imperfect GC-rich palindrome (6), A. Martinez-Arias and M. Casadaban, unpublished). This regulatory region was identified as an upstream activating sequence (UAS), based on its ability to promote transcription and mediate gene-specific regulation (6, 13, 14). The region functions in a distance- and orientation-independent fashion with respect to its downstream promoter elements (6) and confers leucine-specific regulation when it is substituted for the UAS of the gene *CYC1* (6).

The GC-rich palindromic sequence within the UAS region in *LEU2* is homologous to sequences in other genes responsible for the biosynthesis of branched-chain amino acids, including *LEU1* (15), *LEU4* (8), *ILV2* (16), and *ILV5* (9, 17) of *Saccharomyces cerevisiae*, and *LEU2* of *Candida maltosa* (18) and *Yarrowia lipolytica* (19). All of these genes, except for the last one which has not been tested, are regulated by leucine in *S. cerevisiae* (2, 8, 9, 18).

Here we report the characterization and optimization of the UAS for leucine regulation using deletions, point mutations, sequence rearrangements, and cloned synthetic DNA of the *LEU2* gene.

MATERIALS AND METHODS

Strains, plasmids, phages, and media

Relevant strains, phages, and plasmids are listed in Table 1. The *S. cerevisiae* strain M12b was used to test all *LEU2* promoter constructs. *E. coli* strains JM103, MC1065, and MC1066TR were used to construct the various vectors and promoters. M13mp phages and the *E. coli* strain BW313 were used to construct the oligonucleotide-directed base changes and also for sequencing. Phage pMH14 is a recombinant containing the M13 phage sequences of M13mp19 (20), the polylinker sequences of M13mp9 (21), and the *LEU2* sequences from −221 to +39 flanked by EcoRI and BamHI sites. The plasmids not listed in Table 1 are described elsewhere.

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Table 1. Strains and Plasmids[a]

Name	Description of Genotypes	Reference
Strains:		
Escherichia coli:		
BW313	*dut⁻ ung⁻*	(26)
JM103	F'Δ(*lacZ*)*M15 proAB traD36 / hsp*⁺ Δ(*lacI-A proAB*)*5 rpsL supE44*	(20)
MC1065	*galE15 galK16 hsdR leuB6* Δ(*lacI-A*)*74 rpsL trpC9830*	(22)
MC1066TR	*galE15 galK16 hsdR leuB6* Δ(*lacI-A*)*74 pyrF74*::Tn*5 recA56 rpsL srl*::Tn*10 trpC9830*	C. André & M. Casadaban
Saccharomyces cerevisiae :		
M12b	α *trp1-289 ura3-52 gal2*	K. Struhl
Plasmids:		
pAMA18	pMC1790: EcoRI, BamHI: *LEU2* bases −88 to +39	A. Martinez-Arias
pAMA19	Same as pAMA18 but with *LEU2* bases −75 +/− 5 to +39	A. Martinez-Arias
pAMA20	Same as pAMA18 but with *LEU2* bases −40 +/− 5 to +39	A. Martinez-Arias
pAMA21	Same as pAMA18 but with *LEU2* bases −32 to +39	A. Martinez-Arias
pBR322	*Ap Tc* rep_{pMB1}	(44)
pMC1790	*Ap lac'ZY'* rep_{pMB1} *TRP1-ARS1*	(22)
pSKS101	*Ap lac'IPOZ'* PstI::*Km* rep_{pMB1}	(22)
pUC19	*Ap lac'IPOZ'* rep_{pMB1}	(20)
pYe(*CEN11*)12	*Ap CEN11* rep_{pMB1} *TRP1-ARS1*	(45)
YEp24	*Ap Tc* rep_{pMB1} *URA3 2μ*	(46)

[a]*E. coli* and yeast gene designations are listed according to Bachmann (47) and Jones and Fink (43), respectively. *LEU2* base numbers are listed with respect to the ATG translational start.

The bacterial and yeast media were described previously (6, 22). Yeast minimal media (1× yeast nitrogen base without amino acids, 2% glucose, and appropriate amino acids or nucleotide supplements) were buffered with M63 salts (23) when the colorimetric indicator 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) was utilized or when the yeast cells were to be assayed for β-galactosidase expression. In order to repress the *LEU2* promoter the L-amino acids leucine, threonine, isoleucine, and valine were added at a concentration of 2mM each to M63 liquid media, and leucine and threonine alone were added at concentrations ranging from 2−10mM to solid media. Repressed media was supplemented by isoleucine and valine to prevent the drop in growth caused by the addition of high concentrations of leucine and threonine; at the concentrations used isoleucine and valine do not affect leucine-specific regulation (H. Tu and M. Casadaban, unpublished).

Genetic methods

Cells were transformed and transfected with DNA by standard methods. *E. coli* cells were transformed by the $CaCl_2$ method (24) or according to Morrison (25). Bacterial strains were transfected with phage DNA as described by Messing (20) or Kunkel (26). Yeast cells were transformed by the spheroplast method (27).

LEU2 gene induction and β-galactosidase measurement

The *LEU2* promoters of the various *LEU2-lacZ* gene fusions were induced by transferring M12b transformants from repressed M63-minimal media into derepressed and repressed M63-minimal media. The cells were washed three times with water prior to transferring, and inoculated to a final OD_{600} of 0.050. The cultures were grown at 30°C to an OD_{600} of 0.400 to 0.500 and then spun down, resuspended and frozen at −70°C as described by Rose and Botstein (28). β-galactosidase liquid assays were carried out as outlined by Martinez-Arias et al. (6). β-galactosidase-specific activities are given as nanomoles of *o*-nitrophenyl-β-D-galactoside cleaved per minute per milligram protein (23). Leucine-specific regulation was determined as the ratio (D/R) of β-galactosidase values from cells grown under derepressive (D) versus repressive (R) conditions. Values represent an average of at least three different transformants each assayed in duplicate (individual values differed from the average by <20%).

DNA manipulations

Standard techniques were used for all constructs (29). Constructs were verified by DNA sequencing, restriction endonuclease analysis, or both. DNA was sequenced by the chemical base cleavage method (30), and by either the single stranded M13 (31) or the double stranded dideoxy primer extension method (32). Sequencing from plasmids was facilitated by SEQUENASE (USA Biochemicals). Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer.

Vector constructions

Vectors pHT102 (Fig. 1B) and pHT214 are centromeric versions of the *lacZ* expression vector pMC1790 (22) and were constructed to reduce the effect of copy number instability of *ARS1*-containing vectors on β-galactosidase measurements (33). The vector pHT102 was constructed from a pMC1790 plasmid derivative, pHT92, and pYE(*CEN11*)12. The plasmid pHT92 contains the XhoI linker (GCTCGAGC) inserted into the center of the PvuII site downstream of the *lac'ZY'* sequence in pMC1790. This XhoI site was cleaved and the SalI, *CEN11* fragment of pYE(*CEN11*)12 inserted to form pHT102. The vector pHT214 was obtained by cleaving pHT102 with SmaI and ligating its termini with the XhoI linker.

Table 2. Effect of Single Base changes on UAS Activity[a]

Plasmid	Description of the Mutation	β-Galactosidase Specific Activity		
		D	R	D/R
pHT104	–	270	47	5.7
pHT301	(−191T) BamHI	58	20	2.9
pHT259	(−192T) Asp718	400	67	6.0
pHT141	(−178C) XbaI	190	35	5.4

[a]All plasmids except plasmid pHT104 contain a single base change in the *LEU2* promoter. The plasmid pHT104 contains the wildtype *LEU2* promoter sequence and the first 39 basepairs of its coding sequence as shown in Figure 1A. Each of the above plasmids were transformed into M12b cells and assayed for their β-galactosidase activities as described in Figure 2A. The base changes, their positions, and the restriction sites they form are as indicated in Figure 1A.

Deletions into the UAS region

Deletions from upstream of the UAS utilized the EcoRI site upstream of base −221 and were generated with BAL31 exonuclease (Fig. 2A). The deletions were constructed from the plasmid pHT141 (Table 2); pHT141 contains the *LEU2* promoter from bases −221 to +39, flanked by an EcoRI and a BamHI site, respectively. This promoter contains the XbaI-created mutation (Fig. 1A, Table 2) centered at base −176. pHT141 was linearized with EcoRI, and treated sequentially with BAL31 exonuclease and Klenow. XhoI linkers were ligated to the deletion endpoints. The deletions were subcloned as XhoI, BamHI fragments into the vector pHT214.

Deletions into the UAS from downstream utilized the XbaI or Asp718 mutations present in pHT141 and pHT259 (Fig. 1A, Table 2), respectively, and the HincII site centered at −125. The XbaI- or Asp718-created sites were cleaved and treated with either Klenow or Mung Bean Exonuclease to generate the upstream deletion endpoints in pHT470, pHT468, pHT459, pHT423, and pHT182 (Fig. 2A). SalI linkers (GGTCGACC) were ligated to the treated endpoints. To connect the deleted sequence with the sequences downstream of base −129, the HincII site was converted into a SalI site. The SalI site was then used to ligate the two *LEU2* regions together while the EcoRI, SalI upstream deletion fragment was ligated to the SalI, BamHI downstream fragment. The ligated fragments were then subcloned into pHT102.

Deletions downstream of the UAS

Deletions downstream of the UAS (Fig. 2B), from base −173 to bases −89, −75 +/− 5, −40 +/− 5, and −33, were constructed from plasmids pAMA18, pAMA19, pAMA20, and pAMA21 (Table 1), respectively, and pHT141. To construct the downstream deletions an XbaI linker (CTCTAGAG) was inserted at the EcoRI sites of the pAMA plasmids; the pAMA plasmids were cleaved with EcoRI, treated with Klenow, and circularized with T4 DNA ligase and XbaI linkers (CTCTAGAG). The XbaI linker sites were used to ligate the downstream promoter regions with the −221 to −174 upstream region of pHT141; the downstream regions were subcloned as XbaI, BamHI fragments and the upstream regions as EcoRI, XbaI fragments into the EcoRI and BamHI sites of the vector pHT102.

The deletion downstream of the UAS from base −173 to base −110 was constructed utilizing the AvaII site (Fig. 1A) centered at base −111 in the *LEU2* promoter of pHT104. pHT104 contains the wildtype promoter from base −221 to +39 flanked by the EcoRI and BamHI sites, respectively, in the vector pHT102. The plasmid was cleaved with AvaII, treated with Klenow, and cleaved with BamHI. In parallel, the plasmid pHT182 (Fig. 2A) was sequentially linearized with SalI, treated with Klenow, and cleaved with EcoRI. The appropriate fragments were ligated together into the EcoRI and BamHI sites of vector pHT102 to form pHT463.

The deleted region in the *LEU2* promoter of pHT333 was replaced with spacer DNA (Fig. 2B). The XbaI site that marks the deletions endpoints in pHT333 between bases −174 and −88 was cut, filled in and ligated to a 33 basepair XmnI, PvuII fragment from pBR322 and a 39 basepair EcoRV fragment from lambda in two orientations. The pBR322 insertion formed pHT450; the lambda insertions formed pHT415 and pHT418.

Oligonucleotide-directed point changes

Single base changes were introduced into the UAS region at bases −192, −191, and −178, using synthetic DNA (Fig. 1A, Table 2) and the recombinant phage pMH14 following the method described by Kunkel (26).

2RIGHTS and 2LEFTS palindromes

The UAS was replaced by two palindromes with homology to either the downstream (RIGHT) or upstream (LEFT) 'halves' of the UAS palindrome and flanking region (Fig. 3). The 2LEFTS palindrome was obtained from pHT233 (Fig. 2A) and the 2RIGHTS palindrome from pHT141 (Table 2). The 2RIGHTS and 2LEFTS palindromes were isolated as XbaI and EcoRI fragments, respectively, and inserted into their respective sites upstream of the *LEU2* base −178 in pHT254.

Oligonucleotide UASs

Palindromic sequences that contained various amounts of the UAS were inserted into UAS-deleted *LEU2* promoters (Fig. 4). The resulting oligonucleotides were synthesized with the following features: a SalI or XbaI restriction site adjacent to the 5′ end, an Asp718 site at the 3′ end, and interior bases matching the *LEU2* region from base −189 to −188, −187, −185, −179, and −174. The oligonucleotides were converted into double-stranded molecules by the methods of Oliphant, Nussbaum, and Struhl (34) and Hill et. al. (35). These molecules were cleaved with SalI or XbaI and ligated into the SalI or XbaI sites of pHT515, between the XhoI and SalI sites of pHT486, or into the XbaI site of pHT486 (Fig. 4).

Multiple UASs in *cis* and in *trans*

Multiple UASs were constructed from a 45 basepair UAS cassette (Table 3). The cassette, constructed from pHT233 (Fig. 2A), contains the *LEU2* region from base −210 to −174 flanked by an XhoI and a SalI site, respectively. The XbaI created site (Fig. 1A) in the *LEU2* promoter of pHT233 was cleaved, and the ends were filled in and ligated together with SalI linkers. The result was pHT293 containing an XhoI, SalI UAS cassette. The cassette was inserted into the XhoI linker site of pHT237 (Fig. 2A) to produce the various multiples in *cis*. To construct the multiples in *trans*, the cassette was modified and inserted between the SalI sites of pSKS101. This resulted in the UAS cassette being flanked by BamHI linker sites. This cassette was then inserted into the BamHI site of YEp24.

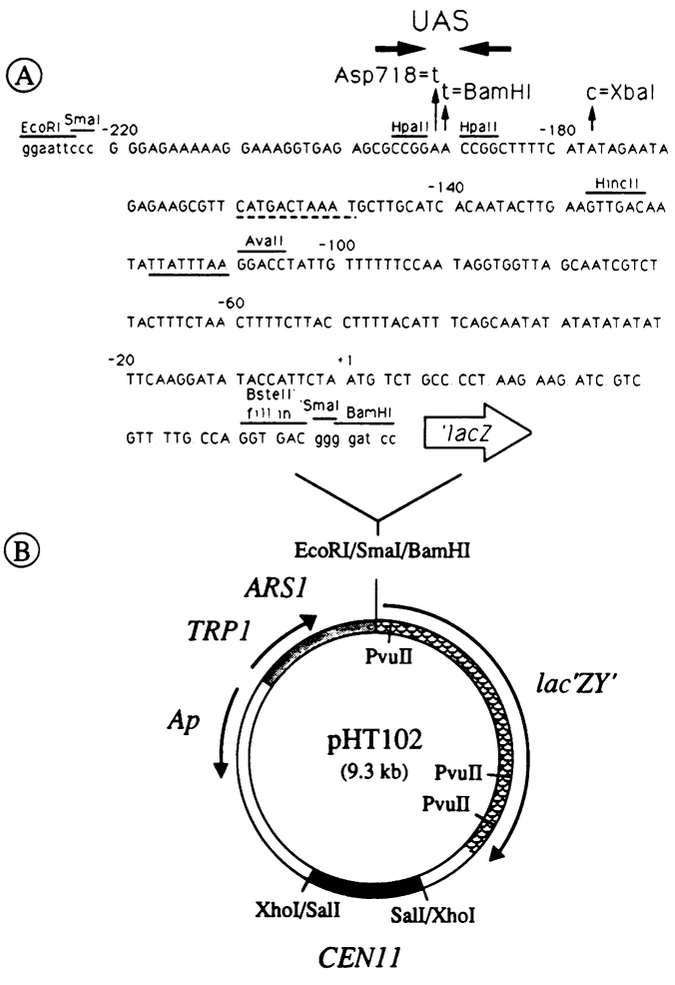

Fig. 1. *LEU2* sequence and β-galactosidase gene fusion vector pHT102. *A.* The *LEU2* gene from base −221 to +39 was inserted into the SmaI site of the *lacZ* expression vector pHT102 as shown to form a *LEU2-lacZ* gene fusion. *LEU2* bases are numbered with respect to the first base of the start codon. The *LEU2* sequence of the wildtype fusion construct and its relevant features are indicated: the GC-rich palindrome in the UAS (→ ←), the degenerate *GCN4* protein binding site (--) (36), the downstream sequence that is also present in the *LEU1* promoter (_) (15), the ATG start site and the reading frame into the *lacZ* gene. Indicated above the *LEU2* sequence are the three transversion changes that were introduced by site-directed mutagenesis, and the resulting restriction sites that were formed. The sequence used was obtained from Andreadis et al. (48) and Heusterspreute et al. (49). These sequences differ in two places between the bases −199 and −180. Our sequencing indicates that these differences are due to one error in each paper. *B.* Vector pHT102 is a yeast-*E. coli* shuttle plasmid. It contains the following functions: a truncated *lacZ* gene deleted for its promoter and first seven codons, a yeast *TRP1* episomal marker, an *E. coli* Ap^R episomal marker, a *colE1* replicon, a yeast *ARS1* replicon and a *CEN11* centromere function. An EcoRI-SmaI-BamHI polylinker region precedes the *lac'Z* sequence. The polylinker sequence with the *lac'Z* reading frame included is 5'...GA.ATT.CCC.GGG.GAT.CC...

RESULTS

β-galactosidase fusion

To facilitate measuring *LEU2* gene expression, the complete *LEU2* promoter region from positions −221 to +39, as measured from the ATG initiation codon, was fused to the *lacZ* gene in vector pHT102 to form the *LEU2-lacZ* gene fusion of pHT104 (Fig. 1A, B). This region contains all the sequences necessary for *LEU2* activation and regulation (6) A. Martinez-Arias and M. Casadaban, unpublished). Levels of activation and expression from this and subsequent *LEU2-lacZ* gene fusions were measured by assaying for β-galactosidase activity in the absence (derepressed) versus presence (repressed) of 2mM leucine and 2mM threonine for maximum leucine control.

Deletion analysis of the UAS

The upstream endpoint of the UAS was mapped with deletions made on the plasmid pHT141 (Fig. 2A). Plasmid pHT141 is identical with pHT104 except for a base change at position −178, which creates an XbaI site (Fig. 1A, see below). The deletions were made with BAL31 exonuclease from the EcoRI site upstream of the −221 position of the promoter. Deletions to position −202 (pHT229, pHT233, pHT234, and pHT240) (Fig. 2A) retained full levels of regulation. (These deletions, however, displayed an increase in both repressed and derepressed levels, an observation which we cannot explain.) In contrast, those deletions mapping to base −196 and further downstream to position −126 (pHT232, pHT231, pHT254, pHT230, pHT237, and pHT486) resulted in low levels of derepressed and repressed expression and therefore low regulation levels. Defining the loss of leucine-specific regulation as a regulation ratio of less than 1.0, these results indicate that deletions extending past base −202 to base −196 eliminate regulation.

The downstream endpoint of the UAS was mapped with deletions from the HincII site at position −125, as described in methods and shown in Fig. 2A. Deletion of the promoter region to base −173 (pHT182) had no effect on regulation. In contrast, deletions extending to base −186 (pHT423, pHT459, and pHT468) decreased the regulation ratio from the wildtype levels to intermediate levels that ranged from a ratio of 2.2 to 2.8. Regulation was lost with the deletion into the GC-rich palindrome to base −189 (pHT470).

The two sets of deletions map a minimal and maximal UAS sequence necessary for activation and regulation. The endpoints of the sequence required for minimal UAS function are between bases −201 and −196 and between bases −189 and −187, inclusive (Fig. 2C). The endpoints of the sequence required for maximal UAS function are between bases −201 and −196 and between bases −178 and −174, inclusive (Fig. 2C). Both sequences contain the GC-rich palindrome important to UAS function, assymetrically positioned within the maximal sequence. Furthermore, a six basepair deletion within this GC-rich palindromeabolishes regulation whether the deletion is in a *LEU2* promoter piece of 640 (31) or 221 (pHT123) basepairs. These results demonstrate the necessity of the GC-rich palindrome for even minimal UAS function.

Deletions downstream of the UAS

Deletions were constructed downstream of the UAS from base −173 to determine if any other promoter sequences were required for activation (Fig. 2B). Deletions moving the UAS to between bases −110 and −40 +/−5, inclusive, drastically reduced expression levels to between 0.7 and 3.0 percent of the respective values of the control pHT182, while causing only a moderate drop of 20 to 33% in regulation. Deletions that moved the UAS further downstream to base −33 further reduced expression and abolished regulation.

To demonstrate that the region between bases −129 and −88 was important for expression, the deleted region in pHT333 (Fig.

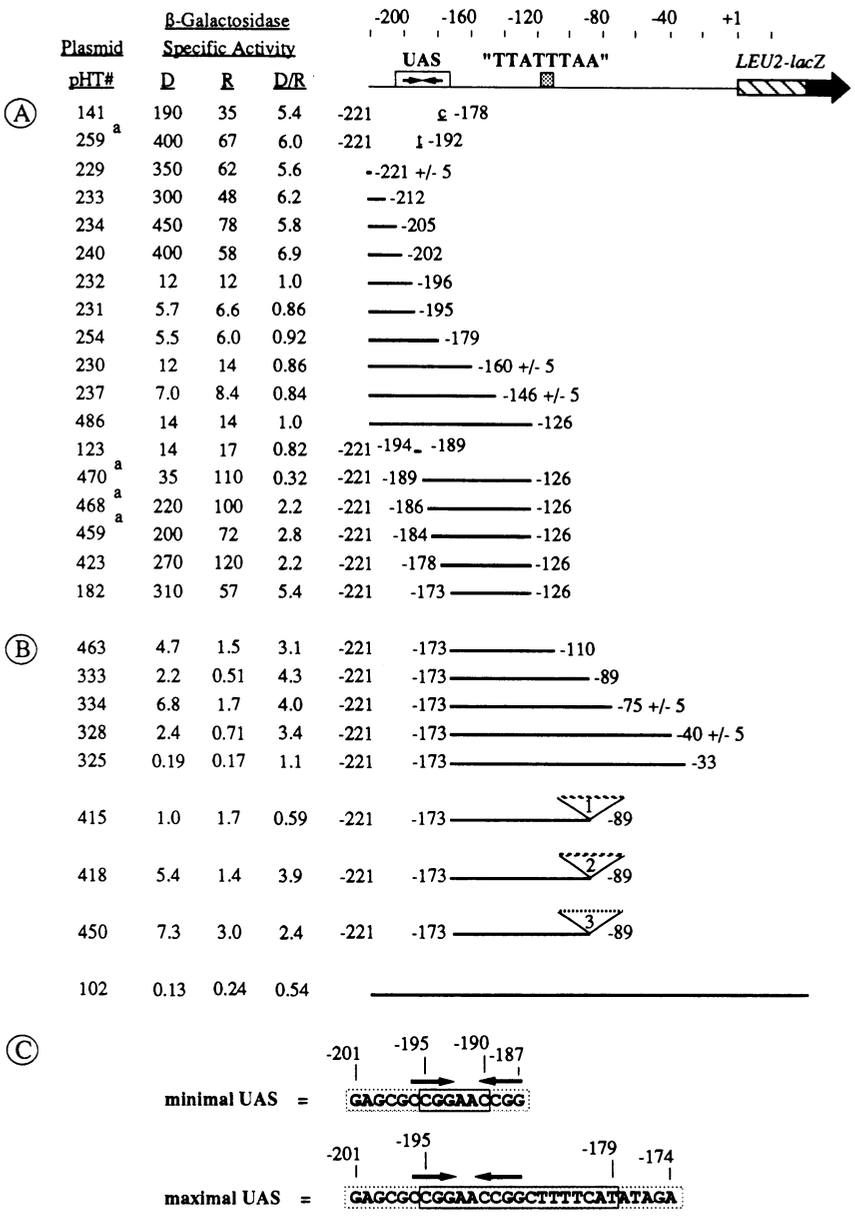

Fig. 2. Deletion analysis of the *LEU2* promoter. The *LEU2-lacZ* fusion indicated in Figure 1 is shown schematically. Deletions are indicated by a solid bar, and the inclusive endpoints of the deletions are listed. *A*. Deletions into the UAS were constructed from the *LEU2* base −221 or base −126. *B*. Deletions downstream of the UAS were constructed from base −173 to the positions indicated. Spacer DNA from lambda and pBR322 was inserted at the junction of the deletion shown for the pHT333 promoter construct. The spacer DNA from lambda was inserted in both orientations, represented by pHT415 and pHT418; the spacer DNA from pBR322 was inserted in one orientation, represented by pHT450. *C*. The sequence necessary for minimal and maximal UAS function, as defined by deletion analysis, is shown. The dashed and solid enclosed regions refer to the deletion endpoints that were used to define the two sequences. All deletions were derived from the pHT141 plasmid, which contains the XbaI change at base −178, except for those plasmids marked with an 'a'; the latter were made starting with the pHT259 plasmid, which contains the Asp718 change at base −192.

2A) was restored with spacer DNA (Fig. 2B). A 39 basepair lambda sequence and a 35 basepair pBR322 sequence were used for this purpose; neither sequence was homologous with the deleted *LEU2* region or with the other sequences in the orientation tested. The 39 basepair lambda sequence was inserted in two orientations (pHT415 and pHT418), whereas the 35 basepair pBR322 sequence was inserted in only one (pHT450). Although expression was significantly higher for one lambda insert than the other, none of the inserts restored expression to wildtype levels.

Point mutants

To test the effect of mutations on the UAS, single base changes were introduced into the maximal UAS by site-directed mutagenesis (Table 2, Fig. 1A). Three transversion mutants were constructed, each of which resulted in the formation of a unique restriction site within the *LEU2* promoter. Two changes, the Asp718 and BamHI mutations, mapped to the center of the imperfect GC-rich palindrome, at bases −192 (pHT259) and −191 (pHT301), respectively. The third change, the XbaI

mutation, mapped to the distal end of the maximal UAS, at base −178 (pHT141).

Each of the three mutations affected UAS function differently. The Asp718 and BamHI mutations equally increased the symmetry within the GC-rich palindrome, but whereas the BamHI mutation reduced expression and regulation compared to the value of the control pHT104, the Asp718 mutation marginally increased expression and regulation . In contrast, the XbaI mutation reduced expression levels almost proportionately to the control values, with the net effect that regulation was not substantially altered.

Palindromic nature of the UAS

The importance of the flanking regions to UAS function was addressed by extending the GC-rich palindrome to include either of its flanking sequences (Fig. 3). The ability of the two extended palindromes to confer leucine-specific expression and regulation was measured by β-galactosidase assays and the values werecompared to each other and to the appropriate controls. The 2RIGHTS construct increased derepressed and repressed expression by 60% and 2%, respectively, whereas the 2LEFTS construct decreased the levels 57% and 15%, respectively, relative to the values of their positive control pHT233. The net effect was that regulation was increased by 60% for the 2RIGHTS construct and decreased by 48% for the 2LEFTS construct compared to control values.

Oligonucleotide UASs

The minimum sequence necessary for UAS function was determined by using palindromic oligonucleotides (Fig. 4). Each palindrome had a center coincident with the GC-rich palindrome of the Asp718 mutation (Fig. 1A). Each palindrome was inserted upstream of base −125 of the UAS-deleted *LEU2* promoters pHT515 and pHT486, using the polylinker sites upstream of the −125 position, and each was assayed for its ability to confer specific activation and regulation.

Palindromes that contained less than 10 basepairs of the region from −191 to −187 (pHT516 and pHT520) were unable to confer activation or regulation greater than the 0.49 value of their respective negative control (pHT515). In contrast, palindromes that contained 10 or more basepairs of the region from −191 to −174 were able to confer substantially higher levels. For the latter constructs, the regulation ratio ranged from 2.1 to 9.5, with the largest palindrome, pHT553, conferring the highest regulation value.

To determine whether both halves of the decanucleotide core were equally important to UAS function, two bases were changed in the 36-mer of pHT553 to form the two mutant constructs pHT496 and pHT499 (Fig. 4). The mutations mapped to the bases equivalent to positions −190 and −193: in pHT496 a C was changed to an A and in pHT499 a G to a T, yielding complementary changes. Compared to wildtype levels, a drop in regulation of 16% and 22% were measured for the two mutants. The six percent difference between these values themselves is less than the approximate error of the measurements; thus, in the context of the 36 basepair UAS and the bases tested, the two halves of the UAS appear to be functionally equivalent.

Multiple UAS elements in *cis* and *trans*

To test the effect of multiple copies of the UAS element on regulation of the *LEU2* gene, additional copies of it were placed

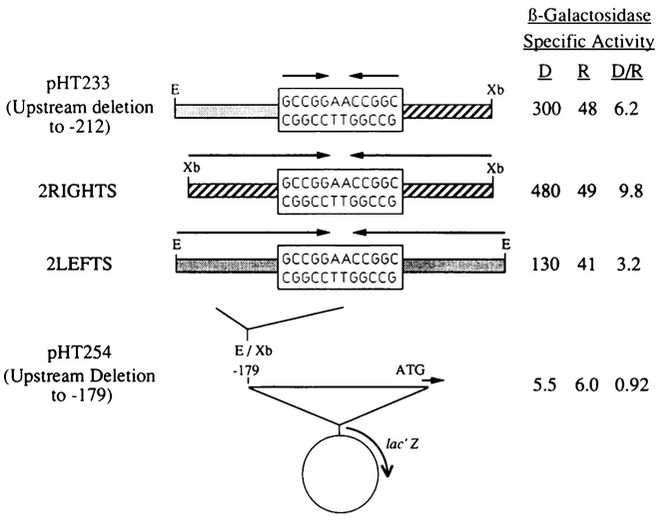

Fig. 3. 2RIGHTS and 2LEFTS palindromes. Three different UAS palindromes (→ ←) were compared to each other and to the UAS-deleted promoter fusion pHT254. All three promoter constructs contain the GC-rich palindrome of the wildtype UAS at their centers. The 2RIGHTS and 2LEFTS palindromes were constructed from the *LEU2* promoter sequences of pHT141 and pHT233, respectively. The 2RIGHTS palindrome is symmetric for the bases −191 to −179 inclusive, in pHT141, and the 2LEFTS for the bases −212 to −192 inclusive, in pHT233. The center bases of these palindrome are asymmetric as in the *LEU2* promoter. E, EcoRI; Xb, XbaI.

in *cis* and in *trans* to the *LEU2-lacZ* gene fusion on test plasmids (Table 3). One to five elements were inserted tandemly in various orientations in *cis* and four multiples in uniform orientation in *trans*. The levels of activation and regulation conferred by each of the multiples in *cis* were determined directly from the construct, and those in *trans* from the recorder *LEU2-lacZ* fusion pHT259. This fusion contains a functional UAS that confers relatively high levels of expression, which should facilitate the detection of any *trans* effect.

The levels of expression and regulation conferred by each of the multiples in *cis* were determined by β-galactosidase assays. Increased levels of expression over those of a single wildtype element (pHT141, pHT182, and pHT346) were obtained in all cases as elements were added sequentially. However, derepressed and repressed expression levels did not increase linearly with the addition of each element. Thus, two (pHT249) and four (pHT421, pHT348) elements had higher levels of expression than either three (pHT345 and pHT354) or five (pHT351) elements. Also, the three inverted (pHT354) elements had the highest regulation ratio of all of the multiples. This mutant had a ratio of 9.4, while the others had ratios ranging from 6.7 to 7.8.

The levels of activation and regulation in the presence or absence of the multiples in *trans* were tested by assaying cells transformed with the recorder fusion pHT259 and either the test plasmid pHT458 or its isogenic control plasmid YEp24. Decreased levels of expression and regulation were observed for pHT259 when multiple copies of the UAS were present. Relative to the control, cells cotransformed with pHT259 and pHT458 had derepressed and repressed expression levels that were reduced by 56% and 43%, respectively, and regulation reduced by 24%.

DISCUSSION

The upstream activating sequence of the *LEU2* gene was mapped with deletions (Fig. 2) and clones of synthetic DNA (Fig. 4).

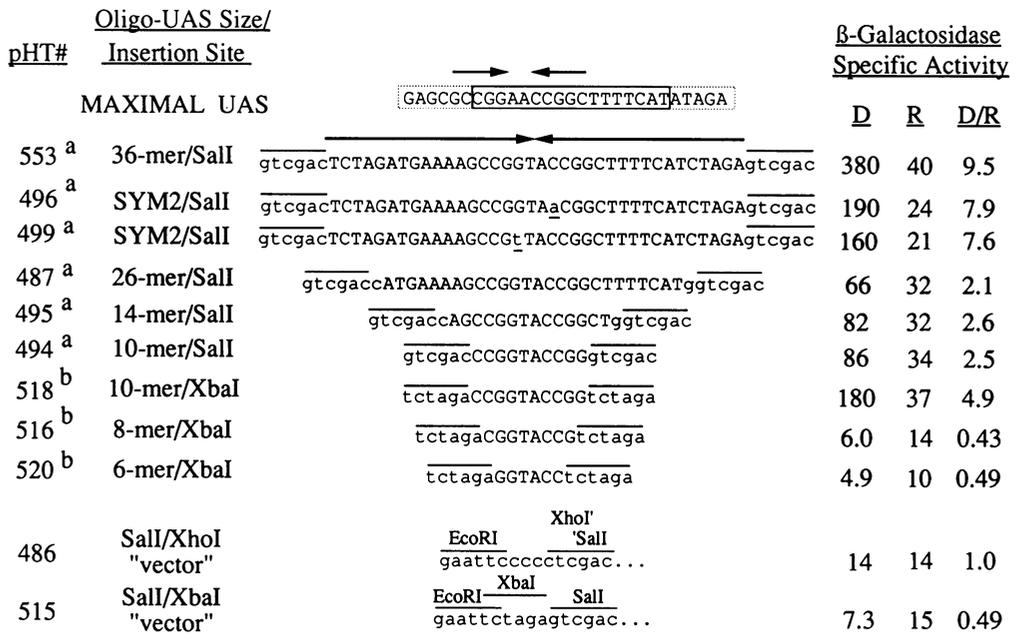

Fig. 4. Oligonucleotide UASs. Symmetric UASs were constructed by use of oligonucleotides. The oligonucleotides varied in size and were used to generate a series of double-stranded palindromes that were symmetric for the wildtype UAS from bases −191 to bases between −189 and −174, inclusive. The sequences of the palindromes are entirely symmetric, with the exception of the SYM2 constructs of pHT496 and pHT499; the mismatched bases in these two constructs are underlined (__). As a result of the symmetry, each of the constructs contains the Asp718 mutation found in the point mutant of pHT259 (Fig. 1A, Table 2). The palindromes were flanked with either SalI or XbaI linkers (‾), and inserted either into the XhoI or SalI site of pHT486 or into either the SalI or the XbaI sites of pHT515. The deletion-defined UAS element is listed at the top of the diagram for reference. Linker sites are overlined (‾). Superscript 'a' indicates inserts into plasmid pHT486; 'b' indicates inserts into plasmid pHT515.

This sequence contained a core ten base-pair, GC-rich palindromic sequence, CCGGAACCGG, which is sufficient to confer minimal levels of activation and regulation. The core ten basepair region was made into two perfect palindromes by changing the middle AA sequence to TA (Asp718 mutation) and AT (BamHI mutation) (Table 2). The TA change did not significantly change regulation, whereas the AT change reduced it by fifty percent. Also, the insertion of four, twelve and fourty-three basepairs between the center bases of the palindrome abolished activity of the UAS (data not shown).

An additional non-palindromic 13 basepair, AT rich, sequence on the right (downstream) side was necessary for full levels of activation and regulation, as seen both with deletions (Fig. 2) and with synthetic DNA clones (Fig. 4). Inclusion of part of the 13 basepair sequence resulted in intermediate levels of regulation. The 13 basepair right flanking sequence was used to make an improved UAS by placing a second copy of it onto the left side, in the opposite orientation, of the 10 basepair core to create a 36 basepair palindromic sequence (Figs. 3 and 4)

The *LEU2* UAS region is homologous to sequences upstream of other leucine-regulated genes for branched chain amino acid synthesis, including the *LEU1* (4, 15), *LEU4* (4, 8), *ILV2* (4, 16), and *ILV5* (17) genes of *Saccharomyces cerevisiae* (Fig. 5). (At least two additional genes that are leucine regulated have not been sequenced: *ILV3* and branched chain amino acid transaminase). The sequences from the *LEU1*, *LEU4*, and *ILV2* genes have been tested and shown to have leucine-regulated UAS activity (4), while the *ILV5* sequence has not yet been tested. The *LEU2* UAS region is also homologous to sequences upstream of the *LEU2* genes from *Yarrowia lypolytica* (19) and *Candida maltosa* (18) and to another fortuitous sequence from near the bacterial *Cm* gene of Tn*9* (6) (Fig. 5). The latter two of these sequences have been tested and shown to have leucine-regulated UAS activity in *Saccharomyces cerevisiae*.

The homology between these sequences is limited to the 10 basepair core region and not to the 13 basepair flanking region. It is possible that the flanking region is not used for its sequence but for its structure, such as in Z or bent DNA. Alternatively, the different flanking sequences may interact with other proteins, any of which may function with the UAS.

Interestingly, two regions with homology to the *LEU2* UAS are present in several of the leucine-regulated genes, including the *LEU1* (4, 15), *LEU4* (4, 8), *ILV2* (4, 16) (Fig. 5). We have tested the effect of additional copies of the *LEU2* UAS element placed in tandem upstream of the *LEU2* promoter and have found that they result in increased activation and regulation. This effect has also been observed with other UAS elements, including those which bind *GCN4* for general amino acid control (36) and *GAL4* for galactose induction (37), and with the UAS1 of the *CYC1* gene (38). Larger increases were found for additions to even numbers than to odd numbers of the element in our constructs (Table 3).

Multiple copies of the *LEU2* UAS element were also tested in *trans* (Table 3) by placing four copies of the UAS on a high copy 2μ-plasmid (39). A slight 24% reduction in the regulation level was observed. This reduction in regulation is consistent with a model that the UAS is used as a DNA-binding site for a positive control factor such as the product of *LEU3*. The limited ability of the *trans* UAS elements to titrate leucine-specific regulation may be due to a high concentration of the control factor in the cell, to a high number of sites in the cell to which the control factor has affinity for, or to a weak ability of the UAS on the 2μ plasmid to effectively compete for binding of the control factor.

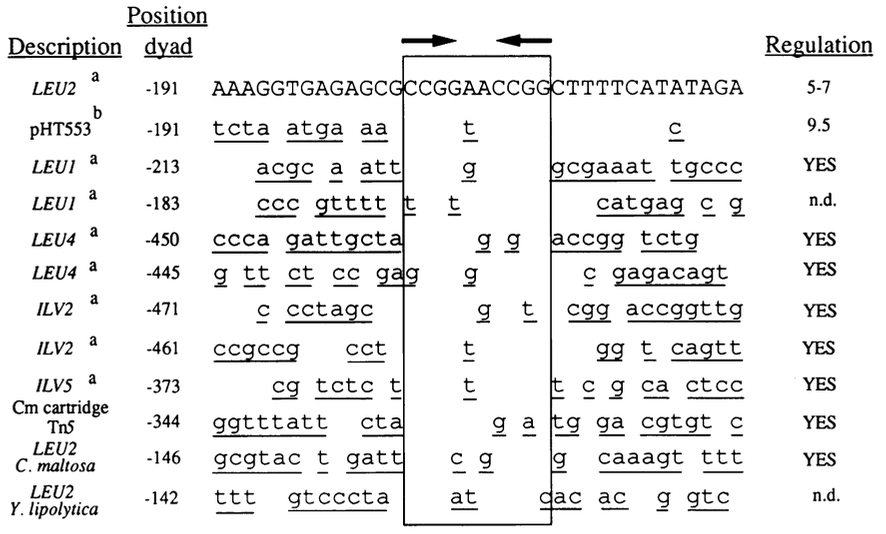

Fig. 5. Sequences homologous to the UAS. The various sequences were compiled based on their homology to the core sequence of the *LEU2* UAS and UAS constructs such as the improved UAS of pHT553 (Fig. 4). The sequences are aligned with respect to the 10 basepair core of the *LEU2* UAS. Bases not homologous to the *LEU2* UAS bases are indicated in lower case and underlined. The position of the dyad cores refer to the position of the nearest center base of the core to the translation start site. The ability of the various genes or homologous sequences to confer leucine-specific regulation is indicated as 'yes' or 'no'. The actual levels of regulation are listed only for the *LEU2-lacZ* fusions of this study, which were measured under identical conditions. *Cm*, chloramphenicol gene; n.d., not determined; a, wildtype sequence; b, sequence construct.

Genetic analysis of leucine-specific regulation indicates that regulation is dependent on the *LEU3* product (2, 3, 4, 5). Band shift assays show that fragments containing the *LEU2* UAS are specifically retarded by factors present in a *LEU3* crude cell extract but not in a *leu3* null extract (3, 4). This model has been further supported by Friden and Schimmel's (4) methylation-interference footprinting of the *LEU2* element between bases −207 and −180, which includes the ten basepair core and six of the 13 basepair flanking sequence. In a *LEU3* extract, strong protection was observed for the distal guanines in the decanucleotide and the guanines adjacent to the decanucleotide on both strands of the element, while weak protection was observed for the center two adenines and none observed for the remaining six bases of the UAS_L flank.

Deletion analysis and sequence substitution (Fig. 2B) of the region downstream of the UAS indicates that high levels of activation require at least one other sequence region, which begins between basepairs −126 and −110, inclusive. Deletions of this region (pHT463, Fig. 2B) drastically reduced expression but retained much of the regulation. The reduction was sequence-specific, as spacer DNAs used to replace a larger deleted area (−125 to base −89 in pHT333 to make pHT415, pHT418, and pHT450) were unable to restore wild type function. Within this region, mapping between bases −118 and −111, inclusive, is a TATA-like AT-rich sequence (TATTTAA, Fig. 1A) which was shown to bind with high affinity ($K_d \sim 10^{-9}$M) to the yeast TATA-binding factor TFIID (40). This sequence is identical to the proposed TATA element sequence of the *LEU1* gene (15). These TATA sequences fit the known criteria of a yeast TATA: they are downstream of the UAS (13, 14) and are between 50 to 120 basepairs upstream of the transcription initiation start sites (41, 42).

In addition to leucine-specific control, many of the genes for branched-chain amino acid biosynthesis are also regulated by the *GCN4* general amino acid control system (36), including *LEU4*, *ILV2*, *ILV3*, and *ILV5*. Some genes, including *LEU1* and *LEU2*, are regulated only by leucine, and others, including *ILV1*, only by general amino acid control. These two systems thereby interact to control the important branched-chain amino acid biosynthesis pathway, which converts pyruvate, a precursor to the tricarboxylic acid cycle, into.isoleucine, leucine, and valine (43).

Table 3. Multiple UASs in *Cis* and *Trans*

Plasmids[a]		Tandem UASs[b]	β-Galactosidase Specific Activity[c]		
TRP1 *ARS1, CEN11*	*URA3* *2μ*		D	R	D/R
UASs in cis:					
pHT346	–	←	170	41	4.1
pHT249	–	→→	680	94	7.2
pHT345	–	→→→	620	93	6.7
pHT354	–	←←←	660	70	9.4
pHT421	–	→→→→	900	130	6.9
pHT348	–	←→→→	870	120	7.2
pHT351	–	←→→→→	760	97	7.8
UASs in trans:					
pHT259	YEp24	–	480	96	5.0
pHT259	pHT458	→→→→	210	55	3.8

[a]M12b cells were transformed with the various *TRP1* plasmids containing tandem UASs in *cis* configuration in its *LEU2* promoter or cotransformed with the *TRP1* plasmid pHT259 (Fig. 2A, Table 2) and either isogenic *URA3* plasmid, YEp24 or pHT458. The plasmid pHT259 contains a single UAS in its *LEU2* promoter and in the wildtype orientation (→), whereas the plasmid pHT458 contains four UASs inserted into the *Tc* gene of YEp24. All plasmids are listed according to their yeast replicon system and selectable marker.

[b]The number of UASs in tandem and the orientation of each UAS with respect to the *LEU2* gene are shown; → and ← indicates the wildtype and inverted orientation, respectively. All UASs in the *cis* configuration were derived by insertion of a 45 basepair UAS cassette at position −145 +/− 5 of the UAS-deleted *LEU2* promoter of pHT237 (Fig. 2B).

[c]Assays for the cotransformants were performed with cells grown in media lacking tryptophan and uracil.

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