Leucine Biosynthesis in Fungi: Entering Metabolism through the Back Door

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INTRODUCTION

There has in recent years been a renewed interest in the intricacies of branched-chain amino acid biosynthesis, particularly leucine biosynthesis, sparked by a number of new and sometimes surprising observations in the yeast *Saccharomyces cerevisiae*, the organism of choice for many investigators in this field. These observations include the following. (i) First is the unusual partitioning of the pathway enzymes between the mitochondrial matrix and the cytoplasm and its consequences (Fig. 1). While the general aspects of this distribution pattern have been known for a long time, there have been recent refinements, especially with respect to the α-isopropylmalate synthase isoenzymes. In addition, hitherto unknown transport systems have come to light that are essential for efficient branched-chain amino acid biosynthesis, as well as for other processes. They include a transporter required for the accumulation of coenzyme A (CoA) inside the mitochondria; a

system for the export of Fe-S clusters from the mitochondrial matrix to the cytoplasm, involving proteins whose primary function is to serve as branched-chain amino acid aminotransferases; and a hypothetical transporter that facilitates the exit of α-isopropylmalate from the mitochondria. (ii) Second is completion of the *Saccharomyces* genome sequencing project and the resulting definitive information on the number and structural relatedness of isoenzymes, questions of particular importance for the first specific step in leucine biosynthesis. It is now evident that there are two very closely related genes that encode α-isopropylmalate synthase. Together, they are capable of producing three isoforms of the enzyme, two located in the mitochondrial matrix and one that remains cytoplasmic. (iii) Third are new findings regarding metabolic regulation, which emphasize the signal character of the leucine pathway intermediate α-isopropylmalate and also assign a role to the transcriptional regulator Leu3p that reaches beyond branched-chain amino acid biosynthesis. Great strides have been made toward understanding the mechanism of action of Leu3p; it appears to be a "self-masking" regulator that represses transcription in the absence of α-isopropylmalate and becomes a strong transcriptional activator in its presence. Remarkably,

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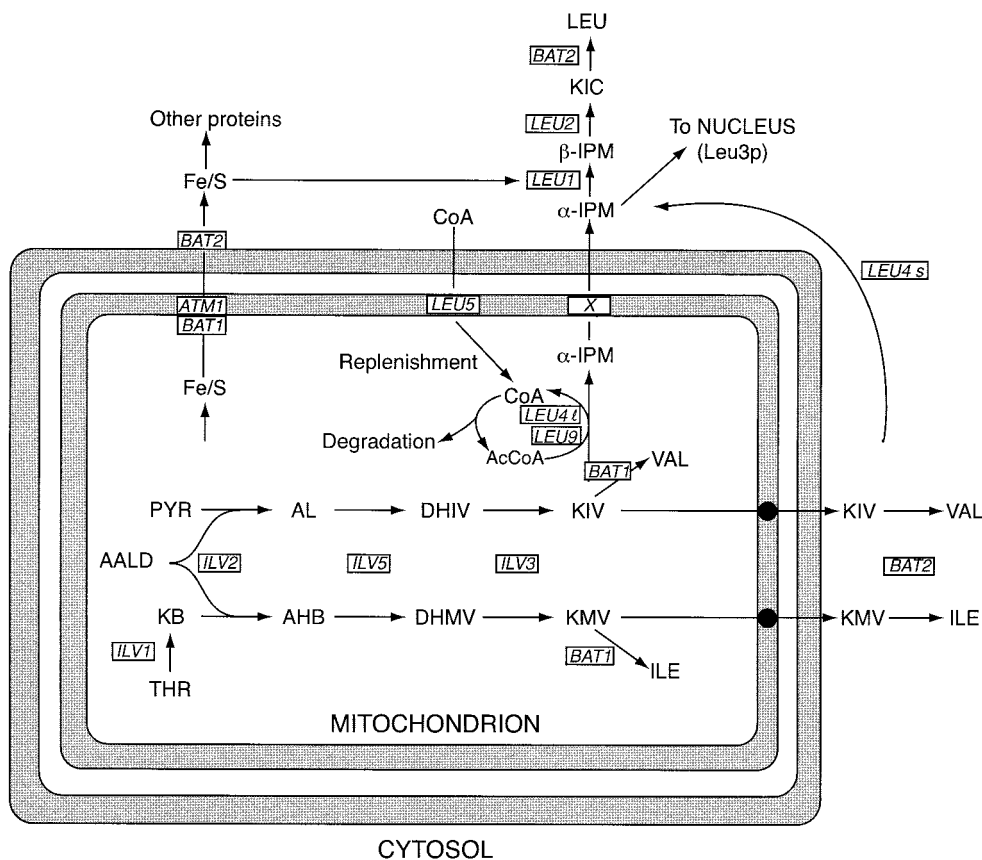
FIG. 1. Compartmentation of the branched-chain amino acid biosynthetic pathways of *S. cerevisiae*. The boxed characters refer to the genes involved directly or indirectly in the pathways. Their protein products are as follows: *ILV1*, threonine deaminase; *ILV2*, acetohydroxy acid synthase catalytic subunit; *ILV5*, acetohydroxy acid reductoisomerase; *ILV3*, dihydroxy acid dehydratase; *BAT1*, mitochondrial branched-chain amino acid aminotransferase; *BAT2*, cytosolic branched-chain amino acid aminotransferase; *LEU4*l, α-isopropylmalate synthase I, long form; *LEU4*s, α-isopropylmalate synthase I, short form; *LEU9*, α-isopropylmalate synthase II; X, hypothetical α-isopropylmalate transporter; *LEU1*, isopropylmalate isomerase; *LEU2*, β-isopropylmalate dehydrogenase; *LEU5*, protein necessary for the accumulation of CoA within the mitochondria; presumably an importer of CoA or precursor thereof; *ATM1*, ABC transporter involved in exporting Fe-S clusters to the cytosol; the implied interaction between Bat1p and Atmp is hypothetical (58, 66). *Abbreviations*: KB, α-ketobutyrate; AALD, active acetaldehyde; PYR, pyruvate; AL, acetolactate; AHB, α-aceto-α-hydroxybutyrate; DHIV, α,β-dihydroxyisovalerate; DHMV, α,β-dihydroxy-β-methylvalerate; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; α-IPM, α-isopropylmalate; β-IPM, β-isopropylmalate; KIC, α-ketoisocaproate; Fe/S, iron-sulfur cluster. Not shown for reasons of clarity: transport of leucine and/or KIC back into the mitochondria.

the Leu3p–α-isopropylmalate system works as well when expressed in mammalian cells as it does in yeast. Since mammals do not have the capacity to produce branched-chain amino acids, the yeast Leu3p–α-isopropylmalate system may be useful as a novel hormone- and antibiotic-independent regulator of therapeutic genes.

In this review, an attempt is made to critically evaluate earlier results and use them together with recent developments to give the reader a comprehensive picture of the extended leucine pathway and its metabolic ramifications in *S. cerevisiae*. In addition, to gain a wider perspective, information available on branched-chain amino acid biosynthesis in other fungi is summarized.

EVOLUTIONARY ASPECTS

As depicted in Fig. 1 (using the nomenclature for *S. cerevisiae*), the biosynthesis of the branched-chain amino acids consists of a common pathway that leads from pyruvate and α-ke-tobutyrate to valine and isoleucine and a branch that leads from the immediate precursor of valine, α-ketoisovalerate, to leucine. These pathways operate in eubacteria (106), archaebacteria (112), fungi (41, 59), and green plants (43, 62, 79). They are not found in mammals, a fact that was important in the development of herbicides that specifically inhibit branched-chain amino acid biosynthesis (24). Whenever nucleotide or amino acid sequences of genes or enzymes involved in the branched-chain amino acid pathways were compared in different species, significant similarities were found (an example is shown in Fig. 2). There are also strong functional similarities among enzymes from different species that relate to such properties as pH optima, kinetics, and cation requirements. It has been concluded, based on these observations, that the branched-chain amino acid pathways are very ancient and may have been derived from a common ancestor of the three major lineages (112). However, while the basic catalytic functions of the enzymes that make up these pathways have remained largely unchanged throughout evolution, differences

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                    514                                               552
ScI     DIEGT-GNGPISSLVDALSNLLNVRFAVANYTEHSLGSGSSTQAASYIHLS
ScII    NIEGY-GNGPISSLVDALSNLLNVKLSVQNYSEHSLGSGSATQAASFINLS
Cg      TVDGR-GNGPLAAYANALEKL-GIDVEIQEYNQHARTSGDDAEAAAYV---
Ba      IESATTSNGPIDAIYQALNRITHFPIILQKYQLIAKGKGKDALGQVDILVE
Hi      STSSIGGNGPVDAVYNAILNLTGLEIKMSHYNLTAKGEGAEALGQVDIVVE
Mj      KKSSAIGVGPVDAAVKAIQKAIGEKIKLKEYHINAITGGTDALAEVIVTLE
St      KAEAANGNGPVDAIYQAINRITGYDVELVKYDLNAKGRARRA-GSGRYRRE
Ll      YVSQGEGSGSVDAIFKAIDKVFNHQLKLISYSVDAVTDGIDAQATTLVSVE
Ta      RLATHTGDGPVDAVYKAIQEAIGLRPELELYRVEAITGSTEALGQVTVRL-
        *   * *  *      *           *     *   *   *  *
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R-region

FIG. 2. Alignment of the R-regions (boxed) of known α-isopropylmalate synthase sequences. Amino acid sequence identities are indicated by bold letters, similarities are indicated by asterisks. Abbreviations and references: ScI, *S. cerevisiae* α-isopropylmalate synthase I (10; the amino acid positions shown at the top refer to this sequence); Sc II, *S. cerevisiae* α-isopropylmalate synthase II (107); Cg, *Corynebacterium glutamicum* (82); Ba, *Buchnera aphidicola* (16); Hi, *Haemophilus influenzae* (34); Mj, *Methanococcus jannaschii* (18); St, *Salmonella enterica* serovar Typhimurium (90); Ll, *Lactococcus lactis* (38); Ta, *Thermus aquaticus* (GenBank accession number U52907). Reprinted from reference 23 with permission of the copyright owner.

with respect to other properties such as gene organization, gene and enzyme regulation, and pathway compartmentation clearly exist. For example, the genes involved in branched-chain amino acid biosynthesis in *Escherichia coli* are organized in operons whereas those of *S. cerevisiae* or *Neurospora crassa* are found throughout the genome, often on different chromosomes. The *ilvGMEDA*, *ilvBN*, and *leuABCD* operons of *E. coli* are regulated by attenuation of transcription in response to the availability of the cognate aminoacyl-tRNAs (106, 113); the *ilvIH* operon is under the control of Lrp, the leucine-responsive regulatory protein (19). By contrast, the transcription of the *ILV* and *LEU* genes of *S. cerevisiae* and *N. crassa* is individually controlled by a regulatory protein (Leu3p) in conjunction with the leucine precursor α-isopropylmalate and is also subject to other, more general controls (see below). Comparing compartmentation of the branched-chain amino acid biosynthetic enzymes in different eukaryotic species again reveals significant differences that demonstrate how difficult it is to foretell the way(s) in which problems of adaptation to new environments have been solved. For example, all of the enzymes of the common pathway plus most of the α-isopropylmalate synthase activity of *S. cerevisiae* are found in the mitochondrial matrix while the two remaining enzymes specific for leucine synthesis are cytoplasmic (Fig. 1; also see below). By contrast, the enzymes for leucine biosynthesis in spinach are located entirely to the chloroplasts (43); subfractionation showed that spinach α-isopropylmalate synthase is tightly associated with the thylakoid membranes, site of the light reactions.

As an aside, the leucine pathway may have played a more important role in evolution than is evident from its present "peripheral" function. Miller and coworkers (54) have proposed that it may have served as the forerunner of the Krebs cycle (which, in its ancient form, is thought to have arisen as a means of supplying the cell with glutamate, aspartate, and related amino acids). This scenario assumes that the leucine pathway-specific genes were duplicated and mutated so that their protein products would accommodate the respective substrates of the Krebs cycle. Assuming that an α-isopropylmalate synthase homologue would act as citrate synthase, that isopropylmalate isomerase homologues would function as aconitase

and fumarase and that β-isopropylmalate dehydrogenase homologues would act as isocitrate dehydrogenase and malate dehydrogenase, and also that a fatty acid reductive carboxylase was available to catalyze the interconversion of succinate and α-ketoglutarate, the only additional enzyme required to complete the cycle would then have been succinate dehydrogenase. It was also assumed that the Krebs cycle-related amino acids, because of their apparent great abundance under prebiotic conditions, would have been depleted later than the less abundant branched-chain amino acids, implying that branched-chain amino acid biosynthesis preceded that of glutamate and aspartate.

α-ISOPROPYLMALATE SYNTHASE OF *S. CEREVISIAE*: ISOFORMS, COMPARTMENTATION, AND REGULATION

The Leu4p and Leu9p Isoforms and Their Intracellular Localization

It had long been surmised on genetic grounds that yeast cells elaborate two independent α-isopropylmalate synthases (7). With the completion of the sequencing of the *S. cerevisiae* genome, it became clear that there are indeed two, and only two, genes, designated *LEU4* and *LEU9*, that encode α-isopropylmalate synthases (21, 107). *LEU4* is located on chromosome XIV (27), and *LEU9* is located on chromosome XV (107). The primary structures of the two isoenzymes Leu4p (α-isopropylmalate synthase I) and Leu9p (α-isopropylmalate synthase II) are 83% identical. In wild-type cells, Leu4p accounts for about 80% of the total synthase activity. It is also the better understood of the two isoenzymes.

An interesting aspect of the *LEU4* gene is that it itself can generate two forms of α-isopropylmalate synthase. This conclusion was first arrived at when it was observed that two of four major transcription start sites utilized in vivo are located downstream from the ATG at the beginning of the open reading frame (ORF) of *LEU4* (10), which suggested that *LEU4* mRNAs might be translated into full-length and shortened versions of the isoenzyme. This was subsequently shown to be true. As it turns out, the full-length form of α-isopropylmalate

synthase I contains at its N terminus a signal sequence that directs the enzyme to the mitochondrial matrix; the short form, starting at what would be the second in-frame AUG of the long mRNA, lacks the 30 N-terminal residues of the long form and hence the mitochondrial import sequence. It remains in the cytoplasm (11). These observations provided an early example of how differential transcription can be used to generate mitochondrial and nonmitochondrial forms of the same enzyme.

Why should α-isopropylmalate synthase be localized in two cellular compartments? Its presence in the mitochondrial matrix can be rationalized by arguing that both of the enzyme's substrates, acetyl-CoA and α-ketoisovalerate, are prevalent in that compartment. The reason that α-ketoisovalerate accumulates in mitochondria stems from the fact that all of the enzymes required to convert pyruvate and active acetaldehyde to α-ketoisovalerate are also located in this organelle (93) (Fig. 1). Given this logic, it is not immediately obvious why α-isopropylmalate synthase should also be available in the cytoplasm. It has been speculated that the reason for this may have to do with the ability of *S. cerevisiae* to grow both aerobically and anaerobically, i.e., both with and essentially without functional mitochondria. Under anaerobic conditions, the mitochondrial form of α-isopropylmalate synthase might be unstable or nonfunctional for other reasons and the cytosolic form might take over. A similar situation might arise under glucose repression, a condition in which cells also elaborate only very few mitochondria with poorly developed cristae (68). These questions of compartmentation seem to be limited to Leu4p, since Leu9p is apparently found only in the mitochondria (86).

While the synthesis of α-isopropylmalate occurs largely within the mitochondria, its conversion to leucine takes place in the cytoplasm (94), as discussed in more detail below and shown in Fig. 1.

The Unexpected Role of Leu5p

An early tetrad analysis (7) had indicated that α-isopropylmalate synthase activity in yeast depended on two separate genes, which were designated *LEU4* and *LEU5*. (A *leu4 leu5* strain is a leucine auxotroph.) While *LEU4* was identified as a structural gene, *LEU5* was thought to either directly encode the second synthase or provide some function needed for the expression of a second structural gene. Subsequent experiments showed that strains deficient in *LEU5* behaved as *petite* strains, i.e., did not grow well on most nonfermentable carbon sources, and that *LEU5* was probably not the second structural gene (32). The conundrum of *LEU5* was solved by the recent surprising demonstration that the gene encodes a carrier protein located in the mitochondrial inner membrane that is required for the buildup of CoA inside the mitochondria (86). It presumably serves to transport CoA, or a precursor thereof, from the cytosol, its likely site of synthesis, to the mitochondrial matrix to replenish the CoA pool and allow efficient synthesis of acetyl-CoA (Fig. 1). The fact that a Δ*leu5* strain exhibits a 15-fold drop in the mitochondrial CoA level, accompanied by a slight increase in the cytoplasmic CoA level, is consistent with the carrier role of Leu5p and might also explain the *petite*-like nature of *leu5* cells. Additional support for a carrier role of Leu5p comes from the observation that intact mitochondria from *leu5* cells are essentially incapable of synthesizing α-isopropylmalate, even though they contain α-isopropylmalate-synthesizing activity that can easily be detected after lysis of the mitochondria (86).

LEU5 was later joined by several additional genes whose absence in a *leu4* background substantially decreases the capacity to synthesize α-isopropylmalate (33). The additional genes (complementation groups) were designated *LEU6*, *LEU7*, and *LEU8*. They have not been analyzed, and we do not know the functions of their protein products. It is entirely possible, though, that either *LEU7* or *LEU8* (neither of which shows *petite*-like deficiencies when mutated) is identical to *LEU9*.

Regulation of α-Isopropylmalate Synthases

Regulation of *LEU4* expression. The regulation of *LEU4* and its major gene product is more complex than might be expected if the synthesis of α-isopropylmalate were to serve the leucine pathway only. Gene expression control, studied with the aid of a *LEU4-lacZ* fusion, was found to depend on three distal elements and one proximal element of the *LEU4* promoter (52). Of these, the most distal element corresponded to a binding site for the transcriptional regulator Leu3p (UAS_{LEU}). Mutation of UAS_{LEU} resulted in the loss of control by Leu3p. The other two elements in this region were identified as likely Gen4p binding sites; their inactivation by mutation eliminated general amino acid control of *LEU4*. Transcriptional activation from all three distal elements was additive, implying that the regulatory proteins bound to these elements do not compete with one another or act synergistically. The fourth, proximal regulatory element showed similarity to the Bas2p response element of the *HIS4* promoter (102). Bas2p (Phos2p) is a "global," homeobox-containing regulatory protein that is required, together with Bas1p, for incremental *HIS4* transcriptional activation and also activates transcription of the secreted acid phosphatases. *LEU4* gene expression decreased by as much as 45% when the inorganic phosphate concentration in the growth medium was increased from a depleted level to 5 mM. This response was absent when the presumed BRE of the *LEU4* promoter was made nonfunctional by mutation (52). Elimination of the BRE also caused a drop in *LEU4* expression under phosphate sufficiency.

Regulation of synthase activity. The enzymatic activity of α-isopropylmalate synthase is subject to two major controls by small molecules. The first is feedback (end product) inhibition by leucine. The apparent inhibitor constants for L-leucine were determined to be about 0.1 mM for the long form of α-isopropylmalate synthase I (26), about 0.4 mM for the short form of synthase I (11), and about 1 mM for α-isopropylmalate synthase II (27), all at pH 7.2. The second type of activity regulation of α-isopropylmalate synthase is Zn^{2+}-dependent reversible inactivation by CoA (44, 103, 104, 105). While CoA is a product of the reaction catalyzed by α-isopropylmalate synthase, the inactivation by CoA clearly is not simple product inhibition. Rather, it requires a second binding site for CoA that, in contrast to the substrate/product site, opens up only in the presence of zinc ions (104). In the absence of zinc and other divalent metal ions, CoA (bound at the product site) is a competitive inhibitor with respect to acetyl-CoA, with an apparent inhibitor constant of 65 to 70 μM; binding to the second

CoA site in the presence of 50 μM Zn^{2+} occurs with an apparent dissociation constant of about 35 μM and causes a rapid, albeit noninstantaneous inactivation of the enzyme. Acetyl-CoA neither prevents nor reverses this inactivation. However, experiments performed in situ, i.e., in permeabilized cells, demonstrated that reactivation is possible with physiological concentrations of ATP or ADP. ATP is also capable of protecting α-isopropylmalate synthase against CoA inactivation. As might be expected from these results, protection was found to increase with increasing adenylate energy charge values (44).

Further support for the physiological importance of the CoA inactivation and its occurrence in vivo comes from a recent demonstration that strains in which α-isopropylmalate synthase I is no longer regulated by CoA are resistant to 5′,5′,5′-trifluoroleucine, owing to overproduction and secretion of leucine (23). Two such strains were identified within a group of seven spontaneous, trifluoroleucine-resistant mutants; the other five were shown to contain an α-isopropylmalate synthase I that had largely or completely lost its sensitivity to leucine. Remarkably, all seven mutations (six point mutations and one codon deletion) map to a short, 39-residue region near the C terminus of the enzyme, designated the R region by the authors. The R region is also present in α-isopropylmalate synthase II, with only six conservative changes, and is found largely conserved (69 to 56% similarity) in the synthases from seven bacterial species (Fig. 2). It has not been established whether the R region contains part or all of the leucine and CoA-Zn^{2+} binding sites or whether it is involved in the signal propagation caused by these molecules. The fact that leucine can partially protect yeast α-isopropylmalate synthase against CoA inactivation (105) is consistent with both interpretations. Whatever the mechanism, the observations by Cavalieri et al. (23) clearly show that the R region of α-isopropylmalate synthase contains subregions that respond to either leucine or CoA-Zn^{2+} and that both types of control must function to prevent overproduction of leucine.

Yet another hint at the metabolic importance of the CoA-Zn^{2+} inactivation of yeast α-isopropylmalate synthase comes from the work of Pronk et al. (87). These authors observed that a null mutant lacking *PDA1*, the gene encoding the E1α subunit of the pyruvate dehydrogenase complex, exhibited a partial leucine requirement. Since this requirement could not be satisfied by valine, the basis for this phenotype was thought to lie within the leucine pathway itself. Knowing that deletion of the *PDA1* gene eliminates pyruvate dehydrogenase activity and sharply elevates the CoA/acetyl-CoA ratio in the mitochondria, the authors suggested that the Leu⁻ phenotype of the *pda1* null mutant might stem from increased CoA inactivation of α-isopropylmalate synthase. It has not been ruled out, however, that the diminished availability of acetyl-CoA itself might contribute to the partial leucine auxotrophy.

In a wider metabolic context, it is noteworthy that CoA inactivation is not limited to α-isopropylmalate synthase but is also observed with homocitrate synthase, the enzyme catalyzing the first committed step in lysine biosynthesis, and with β-hydroxy-β-methylglutaryl-CoA reductase, a key enzyme in the biosynthesis of sterols (101, 103). (In the latter case, the actual inactivating species is probably CoA disulfide [37].) By contrast, citrate synthase is entirely resistant to inactivation by

CoA or derivatives thereof. This scenario was interpreted to mean that under conditions where the intramitochondrial CoA/acetyl-CoA ratio is high due to a low acetyl-CoA level, the remaining acetyl-CoA would be channeled into the citric acid cycle by limiting its consumption in biosynthetic pathways (44).

Why is the production of α-isopropylmalate subject to such elaborate controls? This question can be answered at least in part by pointing to the likely involvement of α-isopropylmalate, when complexed with Leu3p, in metabolic processes that go distinctly beyond the biosynthesis of the branched-chain amino acids (see below). The CoA-Zn^{2+} inactivation of α-isopropylmalate synthase may then be looked on not just as an acetyl-CoA saving device but also as an important link between those metabolic processes and central energy metabolism.

ISOPROPYLMALATE ISOMERASE (LEU1P) AND β-ISOPROPYLMALATE DEHYDROGENASE (LEU2P) OF *S. CEREVISIAE*

Intracellular Localization and the Question of an Isomerase-Dehydrogenase Complex

Of several reasons to lump together the two yeast enzymes needed to convert α-isopropylmalate to α-ketoisocaproate, their intracellular localization is perhaps the most compelling. In contrast to the enzymes responsible for the conversion of α-ketobutyrate and pyruvate to the α-ketoacid precursors of isoleucine and valine and to α-isopropylmalate, which are largely mitochondrial, isopropylmalate isomerase and β-isopropylmalate are entirely cytoplasmic (94). A recent independent confirmation of the cytoplasmic localization of the isomerase came from work done by Lill and coworkers on the maturation of Fe-S proteins (57, 58, 67, 75, 85). These authors observed that lesions in the *ATM1* gene lead to leucine auxotrophy. They explained this behavior by pointing out that Atm1p, a mitochondrial ATP binding cassette (ABC) transporter, is required for the efficient generation of extramitochondrial (but not intramitochondrial) Fe-S clusters and that, in the absence of functional Atm1p, isopropylmalate isomerase, an Fe-S protein, would not be able to function if it were cytoplasmic.

The spatial separation of (most of) the α-isopropylmalate synthases from the isomerase and the dehydrogenase means that α-isopropylmalate would have to be transported from the mitochondrial matrix to the cytoplasm. The molecule also would have to enter the nucleus in order to form an active complex with Leu3p. The possibility that α-isopropylmalate binds to Leu3p in the cytoplasm cannot be dismissed but is considered unlikely since Leu3p localizes to the nucleus and interacts with its target promoters irrespective of whether α-isopropylmalate is present (17, 56). So far, no α-isopropylmalate transporter has been identified.

It has long been known that both isopropylmalate isomerase and β-isopropylmalate dehydrogenase are unusually labile when removed from their natural environment (13, 14, 48). For example, isomerase precipitated from crude extract by ammonium sulfate fractionation has a half-life of only 2 to 3 h. The enzyme can be stabilized by a combination of cryoprotectants
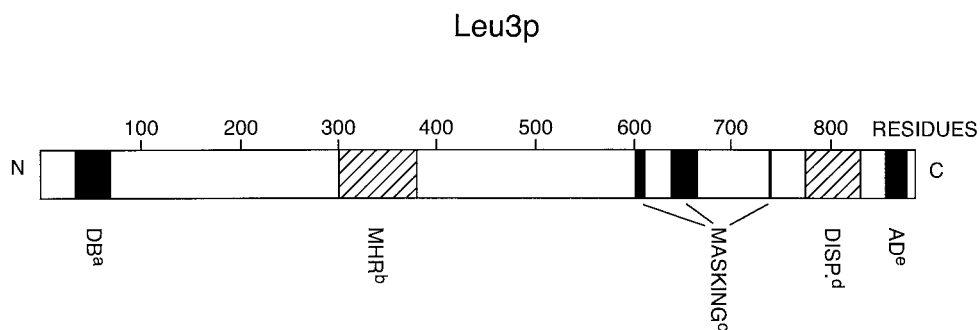
Leu3p

FIG. 3. Regions of interest along the primary structure of Leu3p of *S. cerevisiae.* [a]DNA binding region, residues 37 to 67, containing the cysteine motif for $Zn(II)_2Cys_6$ cluster formation; [b]MHR, residues 300 to 380; [c]three clusters of residues (604 to 611, 643 to 664, and 738 to 741) potentially involved in activation domain masking; [d]region dispensable for all known functions, residues 774 to 854; [e]transcriptional activation domain, residues 857 to 875; N, N terminus; C, C terminus. Note that the 11 C-terminal residues (residues 876 to 886), while not needed for the activation function, are essential for the masking process. See the text for further details.

and, to some extent, by the presence of β-isopropylmalate. The dehydrogenase is exceptionally sensitive to low temperatures (and to dilution), but this occurs only when it is outside the cells; intact yeast cells can be stored frozen for months without loss of dehydrogenase activity. Strikingly, when *LEU2* is expressed in *E. coli*, the yeast dehydrogenase is cold sensitive even in intact cells (60). This behavior of the two enzymes has led to the speculation that they might exist as a mutually stabilizing complex in vivo (59). It should be pointed out in this context that when a polyfunctional complex of isomerase and dehydrogenase was sought in *N. crassa* because of the coordinate expression of the corresponding genes, none was found (41).

Regulation of *LEU1* and *LEU2* Expression

Regulation of the synthesis of isopropylmalate isomerase and β-isopropylmalate dehydrogenase is achieved by controlling the rate of transcription of *LEU1* and *LEU2*, mainly through Leu3p–α-isopropylmalate. The levels of the encoded enzymes and the cognate mRNAs respond similarly to genetic or physiological changes that affect *LEU3* or alter the intracellular concentration of α-isopropylmalate (3, 7, 50). In addition to being controlled by Leu3p–α-isopropylmalate, *LEU1* appears to be mildly up-regulated by Gcn4p, although the effect may be indirect (76). The response of *LEU2* to Gen4p is atypical (17). There is no up-regulation by Gcn4p, but Gcn4p is needed to maintain a low but significant level of expression (basal level I) when Leu3p is absent. By an unknown mechanism, UAS$_{LEU}$-bound, activation-incompetent Leu3p abolishes this Gcn4p effect and reduces *LEU2* expression to basal level II, a very low level of expression (see also the next section).

LEU3P: A DUAL-FUNCTION TRANSCRIPTION FACTOR

Leu3p as Transcriptional Activator and Repressor

The *LEU3* gene of *S. cerevisiae* was originally identified as a positive regulator of the expression of *LEU1* and *LEU2*, the genes encoding isopropylmalate isomerase and β-isopropylmalate dehydrogenase, respectively (7). The activation of these genes was found to depend not only on the presence of an intact *LEU3* gene product but also on the rate at which α-isopropylmalate is produced. In the absence of α-isopropylmalate

synthase activity, the activities of the *LEU1* and *LEU2* gene products were less than 10% of those found in wild-type cells. On the other hand, when α-isopropylmalate was synthesized in largely uncontrolled fashion (in a *LEU4*[fbr] strain that produces feedback-resistant α-isopropylmalate synthase), the *LEU1* and *LEU2* gene product activities rose three- to fivefold over those seen in wild-type cells. When, in addition, α-isopropylmalate was made to accumulate to even higher concentrations by preventing it from being metabolized (using *LEU4*[fbr] *leu1* and *LEU4*[fbr] *leu2* strains), the activities of the *LEU2* or the *LEU1* gene product increased about 15- and 10-fold, respectively. It was concluded that, by analogy to the pattern seen previously with *N. crassa* (41), the expression of *LEU1* and *LEU2* is subject to induction by a Leu3p–α-isopropylmalate complex. Subsequent refinement of the analysis of *LEU2* expression using a *LEU2-lacZ* fusion confirmed this conclusion and demonstrated, furthermore, that Leu3p is a dual regulator (17): in the presence of α-isopropylmalate, it is a transcriptional activator; in its absence, it acts as a repressor. To understand this behavior, one has to realize that in the total absence of Leu3p, *LEU2* gene expression is not zero but still proceeds at about 8% of that of the wild-type control, rendering Δ*leu3* strains leaky (basal level I expression). Leu3p molecules that are activation-incompetent but retain an intact DNA binding function decrease basal level I expression of *LEU2* by another fourfold, to basal level II, a very low level of expression also seen in cells that are wild type with respect to *LEU3* but are essentially unable to produce α-isopropylmalate (e.g., in a *leu4 leu5* strain). The switch from basal level I to basal level II defines the repressive effect of Leu3p. It follows from these observations that Leu3p should bind to its cognate DNA irrespective of the presence or absence of α-isopropylmalate. That was indeed shown to be the case (17, 99). It was shown independently that Leu3p localizes to the nucleus (and binds to target sites) under a variety of growth conditions which included the presence and the absence of α-isopropylmalate (56).

Structure-Function Relationships: the Self-Masking Model

Structural aspects of Leu3p. Important regions along the primary structure of Leu3p and their significance are shown schematically in Fig. 3.

Leu3p is a homodimeric DNA binding protein with 886 residues per monomer and a deduced monomer molecular weight of close to 100,000 (89, 118). It binds to cognate promoters at upstream activating sequences (UAS$_{LEU}$) with the structure 5′-CCGN$_4$CGG-3′ (45). This structure is not entirely invariable. For example, the element found in the promoter of the *LEU4* gene fits the above palindrome only when one mismatch is allowed (10, 45). Also, the sequence of the spacing nucleotides is not random but provides additional information for binding specificity (78). The DNA binding region of Leu3p is of the Zn(II)$_2$Cys$_6$ binuclear cluster type and is located near the N terminus. The DNA binding motif is Cys-Xxx$_2$-Cys-Xxx$_6$-Cys-Xxx$_n$-Cys-Xxx$_2$-Cys-Xxx$_6$-Cys, where Xxx is any amino acid. This motif is found in at least 79 fungal proteins, including at least a dozen transactivators (95). Apparent dissociation constants of Leu3p-DNA complexes using purified Leu3p and a *LEU2* promoter-derived 30-mer containing the UAS$_{LEU}$ were determined to be in the low nanomolar range (97), values very close to those seen with other yeast transcriptional factors and with mammalian hormone receptors. Circular permutation and cyclization experiments showed that binding of Leu3p to UAS$_{LEU}$ causes DNA bending (H. Guo and G. B. Kohlhaw, unpublished results). The apparent flexure angle, calculated from circular permutation assays, was found to be 47° with full-length Leu3p and 25° with a Leu3p fragment containing residues 17 to 147, i.e., little more than the DNA binding region. This result suggests that interaction between Leu3p and its cognate DNA not only depends on the DNA binding motif but also is influenced by other regions of the protein.

The transcriptional activation domain of Leu3p is located near the C terminus. This region is promiscuous: it is functional across species and when attached to other DNA binding domains (42, 98, 110). There is very little sequence homology among the activation domains of this class of transactivators. Rather, what seems to be important is the presence (and probably the spatial arrangement) of both acidic and hydrophobic residues. The activation domain of Leu3p is surprisingly small. The 30-C-terminal-amino-acid residue region originally identified as encompassing the activation function was subsequently whittled down to only 19 residues; even a Leu3p molecule lacking 17 of the 30 C-terminal residues retains about 25% of the activation potential of the wild-type protein (109). Reassuringly, its activation domain still contains three acidic and four hydrophobic residues.

In agreement with the notion that the regions of Leu3p that are responsible for DNA binding, dimerization, and transcription activation operate largely autonomously, a large internal segment of Leu3p that encompasses more than two-thirds of the entire protein can be deleted without infringing on the above functions. However, the shortened protein now behaves as a constitutive activator that maximally stimulates the expression of genes under its control irrespective of the presence or absence of α-isopropylmalate (35, 117). These results suggest that some aspects of the internal region (consisting of residues 174 to 773) are essential for the modulation of Leu3p by α-isopropylmalate. Additional deletion analysis showed that an 81-residue region on the N-terminal side of the activation domain is dispensable for all known functions of Leu3p (119). Since deletions often led to instability, other strategies were used to elucidate the mechanism by which the activation domain of Leu3p is masked in the absence of α-isopropylmalate and unmasked in its presence. One important question was whether auxiliary factors were needed for the masking-unmasking process, as is the case with some other modulated transactivators, e.g., Gal4p (63, 64) and possibly Hap1p (115, 116).

Are auxiliary factors needed to render the activation domain of Leu3p ineffective? In a first approach to answer this question, the *LEU3* gene was expressed in mammalian cells (42). To this end, mouse preadipocytes were transfected with two plasmids, one containing the *LEU3* gene behind the human cytomegalovirus major intermediate-early promoter and the other carrying a luciferase reporter gene with four consecutive UAS$_{LEU}$ sequences in its promoter. Cultured cells strongly expressed Leu3p, irrespective of whether α-isopropylmalate was added to the cell suspension. A basal level of reporter gene expression was seen in the absence of *LEU3*. This level decreased by about fourfold when *LEU3* was present. When both *LEU3* and α-isopropylmalate were present, reporter gene expression increased about 17-fold compared to the "repressed" level. Similar results were obtained in in vitro transcription assays using nuclear extract from mouse preadipocytes and highly purified Leu3p. These assays showed in addition that induction by α-isopropylmalate was specific since it was not observed when β-isopropylmalate was used instead. Several conclusions may be drawn from these experiments. First, Leu3p assumes its masked (and repressive) configuration when expressed in the absence of the coactivator. This is in striking contrast to what was observed when Lac9p, a Gal4p-related activator from *Kluyveromyces lactis*, was expressed in mammalian cells (96). In that case, it was necessary to coexpress the inhibitory yeast Gal80 protein in order to prevent transcription activation of a reporter gene. Second, both repression and activation by Leu3p occur in mammalian cells very much like they do in yeast. Third, since *LEU3* was the only yeast gene present in the mammalian cell experiment and since mammalian cells do not elaborate branched-chain amino acid pathways, masking and unmasking of the activation domain of Leu3p very probably occur without the participation of additional Leu-specific factors.

More direct support for the idea that masking and unmasking of the activation domain are intra-Leu3p events comes from extensive mutant analyses and physical interaction studies (109, 110). Permutation of the activation domain showed that the modulation function was much more sensitive to amino acid changes and deletions than was the activation function. The effects seen on modulation could be classified as either permanent unmasking of the activation domain (i.e., generation of constitutive activators), diminished masking, or intensified masking. Of particular interest was a doubly mutated Leu3p (Leu3p[D872N/D874N], designated Leu3-dd) that is essentially unresponsive to α-isopropylmalate, even though it is able to interact with the coactivator and possesses strong intrinsic activation capability. The latter was demonstrated by creating a Leu3-dd molecule from which the large internal segment recognized earlier as important for modulation had been deleted; it was fully active. To further investigate whether the "permanent mask" for the activation domain of Leu3-dd was provided by the Leu3 protein itself, a modified two-hybrid experiment was designed in which a Leu3p wild-

type molecule minus its activation domain served as "bait" and separate activation domains served as potential "prey." The results were consistent with a self-masking model. It was found that (i) both wild-type and Leu3-dd activation domains interacted with the remainder of the Leu3p molecule; (ii) the interaction between the Leu3-dd activation domain and the bulk of Leu3p was much stronger than the interaction between the wild-type activation domain and the remainder of Leu3p, which, nevertheless, was clearly visible; (iii) most importantly, the interaction in both cases depended on the α-isopropylmalate concentration, i.e., took place only when little or no coactivator was present, demonstrating that the interactions were specific and physiologically meaningful. It should be noted that interaction between the activation domain and an internal region of Leu3p might well involve both subunits of the protein; i.e., activation domain masking could be an intradimer rather than an intramonomer event.

As expected, the Leu3-dd protein caused repression, and cells containing Leu3-dd grew only very slowly on minimal medium, opening the door to select for faster-growing suppressors. Methods were developed to select for intragenic suppressors of the Leu3-dd phenotype that were limited to an internal region of *LEU3* covering residues 172 to 772 of Leu3p (109, 110). Interestingly, all nine missense mutations thus identified mapped to three very short regions located within the C-terminal one-third of Leu3p and extending from residues 604 to 611, 643 to 664, and 738 to 741, respectively (Fig. 3). Every one of the nine mutations restored substantial activation potential and various degrees of modulation, i.e., response to α-isopropylmalate, to the Leu3-dd molecule. When transferred to wild-type Leu3p, each of the nine mutations gave rise to constitutive, highly active activators, apparently abolishing masking altogether. It has been argued that at least some of the nine residues, in their wild-type form, might be directly involved in trapping the activation domain. However, more structural knowledge is needed to answer that question.

Cha4p is a serine/threonine-responsive transcriptional activator in yeast with an arrangement of functional and structural regions along the primary structure that is not unlike that of Leu3p (47). Domain swapping between Leu3p and Cha4p demonstrated that each activation domain requires its cognate internal segment in order to be eclipsed (110). This requirement does not extend to the DNA binding domains since normal regulatory behavior was seen in both cases when only these regions were swapped.

It is not known exactly where and how α-isopropylmalate interacts with Leu3p. It has, however, been possible to determine apparent binding constants for the coactivator, using in vitro transcription assays. The values obtained were 0.2 mM using an *S. cerevisiae* system (99) and 0.25 mM using a nuclear extract from mammalian cells (42). No interaction was detected with β-isopropylmalate.

Masking versus repression. The masked form of Leu3p is also the form that represses gene expression below the basal level. However, masking and repression are two entirely different processes. While it is now clear that masking of the activation domain requires (part of) the central region of the protein and does not require any part of the extended DNA binding domain (defined as residues 1 to 173), it has been shown that repression is as efficient with a peptide encompassing residues 17 to 147 of Leu3p as it is with the full-length protein (98). The region of Leu3p from residues 17 to 147 contains the cysteine residues that give rise to the $Zn(II)_2Cys_6$ cluster and a heptad repeat that is similar to the one observed in Gal4p (72) and is therefore probably involved in dimerization. The fragment from residues 17 to 147 is identical to the one that has been shown to bind to and bend UAS_{LEU}-containing DNA (Guo and Kohlhaw, unpublished), and repression could be a (direct or indirect) consequence of this change in DNA structure. It is also possible, however, that the region of Leu3p from residues 17 to 147 recruits another protein(s) that in turn exerts a negative effect on transcription. It has been shown that repression by Leu3p requires the presence of Mot1p (108). Mot1p is an evolutionarily conserved repressor of transcription by RNA polymerase II that acts by displacing the TATA box binding protein from its sites, perhaps through ATP-dependent conformational changes (1, 6). However, since it is unclear how Leu3p and Mot1p communicate, the exact pathway by which Leu3p exerts its repressive effect remains unresolved.

The 'middle homology region' of Leu3p. Many of the $Zn(II)_2Cys_6$ cluster-forming proteins, Leu3p included, contain an internal region known as the middle homology region (MHR). This homology was first identified by Chasman and Kornberg (28) and was thereafter reported to be present in as many as 50 such proteins but not in other protein classes (95). The MHR of Leu3p is located between residues 300 and 380. No conclusive function has been established for the MHR, but it has been speculated that it might be involved in decreasing the affinity of $Zn(II)_2Cys_6$ cluster proteins for "wrong" genomic binding sites, based mainly on the observation that binding site specificity of full-length Gal4p in vivo is stricter than that of the Gal4p binding domain in vitro (72, 73). As far as Leu3p is concerned, it is possible that the much stronger bending of cognate DNA by the full-length molecule than by the Leu3p [17–147] fragment is due to the presence of the MHR, but direct evidence to support this idea is not available.

HOW LARGE IS THE LEU3P REGULON?

Genetic and biochemical analyses have demonstrated that the expression of seven *S. cerevisiae* genes is, at least in part, under the control of Leu3p (Table 1, first category). Five of these genes belong to the branched-chain amino acid pathways. The product of the sixth gene (*BAP2*) belongs to a family of permeases concerned primarily with the uptake of branched-chain amino acids (reference 77 and references therein). The seventh gene (*GDH1*) is responsible in a major way for the assimilation of ammonia in *S. cerevisiae*. To date, no systematic DNA microarray study relating to Leu3p has been carried out. There are, however, two recent items that bear on the potential range of Leu3p-mediated regulation. The first of these is a genome-wide analysis of regions located upstream of ORF's with regard to Leu3p binding and occupancy (69). These authors determined the equilibrium dissociation constants of 43 variants of the consensus Leu3p binding site which made it possible to predict an equilibrium dissociation constant for all potential binding sites in the genome and to calculate the fractional occupancy of these sites at an assumed intranuclear concentration of Leu3p. A total of 6,276

TABLE 1. Targets for regulation by Leu3p–α-isopropylmalate in *S. cerevisiae*

Gene	Function	Reference(s)
First category: established targets		
LEU1/YGL009c	Isopropylmalate isomerase	7, 36
LEU2/YCL018w	β-Isopropylmalate dehydrogenase	7, 17, 36
LEU4/YNL104c	α-Isopropylmalate synthase I	36, 52, 83
ILV2/YMR108c	Acetohydroxyacid synthase	36
ILV5/YLR355c	Acetohydroxyacid reductoisomerase	36
BAP2/YBR068c	Branched-chain amino acid permease	77
GDH1/YOR375c	Glutamate dehydrogenase (NADP$^+$)	51
Second category: potential targets[a]		
BAT1/YHR208w	Branched-chain amino acid aminotransferase	
GAP1/YKR039w	General amino acid permease	
OAC1/YKL120w	Mitochondrial oxaloacetate, sulfate carrier	
MAE1/YKL029c	Mitochondrial malic enzyme	
LEU9/YOR108w	α-Isopropylmalate synthase II	

[a] The first four of these genes meet two criteria: first, they rank very high in a genome-wide Leu3p binding-occupancy analysis (see text); second, they show diminished expression, as determined by a DNA microarray experiment, in cells whose genetic background allows only very little accumulation of α-isopropylmalate compared to cells whose genetic background allows strong accumulation of the coactivator (see text). The fifth gene (*LEU9*) meets only the first criterion.

ORFs were analyzed in this way and ranked according to the number and quality of Leu3p binding sites present in their potential 5′-"regulatory" regions (defined as the 600 bp 5′ to an ORF). Over a range of free Leu3p concentrations between 1 and 100 nM, all seven genes known to be regulated by Leu3p were found among the top 5% of all yeast genes (with four of them being among the top 0.2%), demonstrating the predictive power of the method. The second item concerns results from a limited DNA microarray experiment (L. Breeden, personal communication). In this experiment, which was a by-product of an unrelated project, the following genotypes were compared: *leu2 HAA1* with *LEU2 haa1* and *LEU2 HAA1* with *LEU2 haa1*. Cells with an intact *LEU2* gene accumulate much less α-isopropylmalate than do cells with a deficient *LEU2* gene and would therefore be expected to show lesser induction of genes controlled by the Leu3p–α-isopropylmalate complex. Genes whose expression was specifically depressed when *LEU2* was intact included not only *LEU1*, *ILV3*, *ILV5*, and *GDH1* but also about two dozen other genes and hypothetical ORFs. Four of the additional genes (*BAT1*, *GAP1*, *OAC1*, and *MAE1*) were also found to rank within the top 5% of all genes in the above occupancy calculation (three of them ranked within the top 0.5%). Since the likelihood that these four genes are regulated by Leu3p is based on the results of two very different approaches, they are included as a second category ("potential Leu3p targets") in Table 1. Also added to this category was *LEU9*, a close homologue of *LEU4* that likewise ranked very high in the occupancy analysis. The 12 genes listed in Table 1 can be assigned to three classes. The first and largest class encompasses nine genes involved in branched-chain amino acid biosynthesis and amino acid uptake into the cell (*LEU1*, *LEU2*, *LEU4*, *LEU9*, *ILV2*, *ILV5*, *BAT1*, *BAP2*, and *GAP1*). The second class includes two genes that function in central nitrogen and carbohydrate metabolism (*GDH1* and *MAE1*). Finally, the one gene of the third class (*OAC1*) produces an inner mitochondrial membrane-based carrier protein for oxaloacetate and sulfate. It is relatively easy to see connections between these classes. For example, the well-established activation of *GDH1* by Leu3p–α-isopropylmalate may be looked on as a signal transduction from the periphery of nitrogen metabolism (represented by the leucine pathway) to an essential early step in nitrogen metabolism (the assimilation of ammonia). The product of the *MAE1* gene, mitochondrial malic enzyme, functions in the intramitochondrial synthesis of pyruvate and may therefore be considered a supplier of an important precursor in the biosynthesis of valine, leucine, and isolecine within the very compartment where most of these biosynthetic reactions occur (15). Also, given the sensitivity of α-isopropylmalate synthase I (and probably II as well) to changes in the mitochondrial CoA/acetyl-CoA ratio and the dependence of α-isopropylmalate synthase II on functioning CoA import into the mitochondria, it is perhaps not surprising to find that the Leu3p–α-isopropylmalate complex might regulate a gene like *OAC1*. In terms of the number of genes controlled, the presumptive extended Leu3p regulon is comparable to several other yeast regulons. For example, the Zap1p zinc-responsive regulon encompasses some 40 genes (70), Mac1p activates seven genes of a copper regulon (39), and a multidrug resistance regulon consists of 26 targets controlled by Pdr1p-Pdr3p (31).

MULTIPLE LAYERS OF CONTROL

As illustrated in Fig. 4, the most direct control of the genes that define the extended leucine pathway is exerted by the Leu3p–α-isopropylmalate complex, which functions at six of the seven steps shown (*ILV2*, *ILV5*, *LEU4*, *LEU1*, *LEU2*, and *BAT1*), with the caveat that Leu3p control of *BAT1* has not yet been directly demonstrated. Superimposed on this leucine-specific regulation is general amino acid control, mediated by Gcn4p. The effect of Gcn4p is twofold. First, it increases the Leu3p level, as inferred from experiments showing an increase in the rate of production of a Leu3p–β-galactosidase fusion protein in a manner typical for the general amino acid control system (118). This probably has physiological consequences since increased production of Leu3p can lead to increased target gene expression (110). Second, it acts directly on at least four genes (*ILV3*, *LEU4*, and *BAT1-BAT2*) of the extended leucine pathway; the effect on three more genes (*ILV2*, *ILV5*, and *LEU1*) may be indirect, through Leu3p (49, 52, 76). The

FIG. 4. Major regulatory mechanisms impacting the extended leucine pathway of *S. cerevisiae*. For abbreviations of intermediates and the protein products of the genes shown, see the legend to Fig. 1. See the text for further explanation.

Leu3p regulon thus becomes part of a remarkable regulatory network that encompasses at least 539 bona fide targets, among them a total of 26 genes that encode DNA binding transcription factors (76). By stimulating *LEU3* expression, Gcn4p would amplify its impact in cascade-type fashion. Its simultaneous stimulation of *LEU3* and *LEU4* ensures that both components of the Leu3p–α-isopropylmalate complex will be made at increased rates. It can be imagined that, in a typical sequence of events, starvation for amino acids (or purines or glucose) would elicit an overriding response in a multitude of pathways, through the Gcn4p general amino acid control system. The Leu3p regulon would obey these starvation signals and augment them. In addition, it would respond independently to other signals, e.g. changes in the CoA/acetyl-CoA ratio, the cell's adenylate energy charge, or the leucine pool, that have an effect on the intracellular α-isopropylmalate level. This would give the yeast cell even more flexibility in its adjustment to environmental changes and demands.

Yet another layer of control that acts on genes of the extended leucine pathway is represented by Tpk1p (Fig. 4). This protein is one of three catalytic subunits of yeast protein kinase A. Like Tpk2p and Tpk3p, Tpk1p is released from an inactive complex under conditions that raise the intracellular cyclic AMP level, for example when cells leave stationary phase after gaining access to glucose or other fermentable carbon sources.

The functions of the three subunits were recently studied by genome-wide transcriptional profiling, and it was established by comparing a *TPK1* mutant strain with the wild type that there was a significant reduction in the expression of *BAT1* and *ILV5* in the *TPK1* mutant in YPD medium (91). Why should *BAT1* and *ILV5* be regulated by Tpk1p? It turns out that the enzymes encoded by these two genes play other important roles besides their catalytic functions in the branched-chain amino acid pathways, and it is likely that the stimulatory effect of Tpk1p is aimed at these other functions, which have to do with mitochondrial integrity.

The pathway function of Bat1p is to serve as mitochondrial branched-chain amino acid aminotransferase; Bat2p is an isoenzyme located in the cytosol (58, 85). Cells deficient in both proteins do not grow on minimal media unless supplied with all three branched-chain amino acids. However, even in the presence of these amino acids, growth remains sluggish. A possible explanation for this behavior comes from the observation that the Bat proteins, in particular Bat1p, perform an essential function in iron homeostasis by being involved in the efficient transfer of Fe-S clusters from the mitochondria, where the clusters are synthesized, to the cytosol, a process that also involves the mitochondrial ABC transporter Atm1p. In fact, the *BAT1* gene was isolated as a suppressor of a temperature-sensitive *ATM1* mutant, and elevated levels of Bat1p were able

to stabilize mutant Atm1p at the nonpermissive temperature. This suggested direct interaction between Bat1p and Atm1p, but other possibilities have not been ruled out (57, 58, 66) (Fig. 1). An important point is that a disturbance in the process of maintaining iron homeostasis can lead to a severe accumulation of iron in the mitochondria with subsequent damage and loss of mitochondrial DNA (mtDNA).

Ilv5p, which normally functions as acetohydroxy acid reductoisomerase, is also involved in enhancing the stability of mtDNA (114). This feature of Ilv5p was first discovered when *ILV5* was identified as a suppressor of the mtDNA instability phenotype of Δ*abf2* cells grown on rich glucose medium (Abf2p is required for maintenance of mtDNA under these conditions). Suppression was achieved with a relatively small increase in *ILV5* copy number or increased expression of the *ILV5* gene in a strain with a constitutively active *GCN4* allele. The fact that an *ILV5* null mutant showed a strong tendency to produce ρ^- *petite* strains further supported the notion that Ilv5p stabilizes mtDNA. More recently, it was shown that Gcn4p-mediated activation of *ILV5* expression results in increased formation of nucleoids (mtDNA-protein complexes considered to be the basic unit of mtDNA segregation) and that such an increase in nucleoid number also increases the transmission of mtDNA to daughter cells (71). It was proposed that the simultaneous activation of amino acid biosynthesis and nucleoid formation by Gcn4p might enhance the chance of progeny survival.

By increasing the expression of *BAT1* and *ILV5*, Gcn4p and Tpk1p are likely to achieve similar results with respect to preservation of mtDNA, parsing of mtDNA into nucleoids, and respiratory competence. Apparently, the action of Tpk1p is reinforced by Gcn4p since the *TPK1* gene is controlled, at least in part, by Gcn4p (76). Also, given the possibility that Gcn4p regulation of *ILV5* actually works through the Leu3p–α-isopropylmalate complex, the latter might likewise contribute to mtDNA maintenance.

BRANCHED-CHAIN AMINO ACID BIOSYNTHESIS IN OTHER FUNGI

Neurospora crassa

Our knowledge of leucine as well as isoleucine-valine biosynthesis and its regulation in *N. crassa* stems to a large extent from the pioneering biochemical and genetic work of S. Gross and coworkers. The three leucine pathway-specific enzymes of *N. crassa* are encoded by three genes: *leu-4* (α-isopropylmalate synthase), *leu-2* (isopropylmalate isomerase), and *leu-1* (β-isopropylmalate dehydrogenase). These genes are not only unlinked but actually located on different linkage groups. It was observed early on (40, 41) (i) that the synthesis of the *leu-2* and *leu-1* gene products was proportional to the amount of *leu-4* product generated; (ii) that *leu-4* mutants unable to make synthase would also produce very little isomerase and dehydrogenase; (iii) that *leu-2* mutants, which accumulate α-isopropylmalate, produce relatively large amounts of the *leu-1* and *leu-4* gene products (and that *leu-1* mutants overproduce *leu-2* and *leu-4* gene products); and (iv) that the expression of *leu-2* and *leu-1* was reduced to very low levels in *leu-3* mutants. It was suggested on the basis of these and other results that α-iso-

propylmalate, in conjunction with the *leu-3* gene product, induces the expression of *leu-2* and *leu-1*. Variations in the intracellular concentration of α-isopropylmalate were initially achieved by indirect means. Later, isolation of an α-isopropylmalate-permeable strain allowed direct demonstration of the induction of isopropylmalate isomerase by the leucine precursor (88). Additional work showed that *leu-4* as well as four genes involved in isoleucine-valine biosynthesis and encoding threonine deaminase, acetohydroxy acid synthase, acetohydroxy acid isomeroreductase, and dihydroxy acid dehydratase require the *leu-3* gene and α-isopropylmalate for full expression (80, 84).

Of considerable interest in terms of defining a "regulon" for *leu-3* of *N. crassa* was the discovery of a connection between the leucine and histidine pathways (55). A connection was suspected after it was observed that the degree of resistance to 3-amino-1,2,4-triazole exactly paralleled the ability of the cell to generate *leu-3* product and α-isopropylmalate. It was then established (again by using the α-isopropylmalate-permeable mutations mentioned above) that the target for regulation was *his-1*, which encodes imidazoleglycerol-phosphate dehydratase, a key enzyme in histidine biosynthesis. There thus exists a second layer of control for histidine biosynthesis in addition to what is kown as "cross-pathway control," a *cpc-1*–governed mechanism that affects the histidine pathway and several other amino acid biosynthetic pathways by responding to amino acid starvation and that is analogous to the *GCN4* system of *S. cerevisiae* (20, 81). The physiological importance of the *N. crassa leu-3* product–α-isopropylmalate complex was underscored even further by the observation that the complex is able to cause transient repression of stable RNA and overall protein synthesis, an effect reminiscent of the "stringent response" of bacteria (5). While the mechanism of the negative effect of the complex on macromolecular synthesis is not known, it was established that the onset of repression is very rapid compared to the *Neurospora* doubling time and that one of the consequences is a significant efflux of Ca^{2+} from the cells, which is also transient.

Viewing the information gathered for both *S. cerevisiae* and *N. crassa* with regard to the metabolic effects of the Leu3p–α-isopropylmalate complex, one would seem justified in concluding that the fungal regulator known as Leu3p plays a rather more comprehensive role than its name suggests.

As far as the intracellular localization of branched-chain amino acid biosynthetic enzymes in *N. crassa* is concerned, it appears that only the enzymes of the isoleucine-valine pathway have been studied in this respect. Of these, threonine deaminase is apparently cytosolic while most, if not all, of a cell's acetohydroxy acid synthase, acetohydroxy acid isomeroreductase, and dihydroxy acid dehydratase is localized to the mitochondrial matrix (22, 80).

Other Species

There are very few studies with fungi other than *S. cerevisiae* and *N. crassa* that deal with the full set of genes and gene products concerned with the biosynthesis of all three branched-chain amino acids or even just the leucine branch. One exception is a report on leucine auxotrophs of the basidiomycete *Phanerochaete chryosporium*, an organism that is

potentially useful in schemes for the bioprocessing of ligno-cellulose (74). The authors of this report found three complementation groups corresponding to three genes encoding α-isopropylmalate synthase, isopropylmalate isomerase, and β-isopropylmalate dehydrogenase. Mutations in any one of these genes caused the loss of the encoded enzyme only. In auxotrophs that lack synthase, the activities of the isomerase and the dehydrogenase were found to be 3- to 4-fold higher than in wild-type cells; auxotrophs that lack isomerase had 2-fold-elevated synthase and unchanged dehydrogenase levels; and auxotrophs deficient in dehydrogenase had 20% more synthase than did wild-type cells and up to 10-fold elevated activities of isomerase. The authors concluded that these results are indicative of leucine pathway regulation but did not elaborate further. However, the regulation seems to differ in at least some respects from that seen in *S. cerevisiae* and *N. crassa*, where very little formation of isomerase and dehydrogenase occurs in synthase-minus strains (see above). Another study of leucine biosynthesis was carried out with *Candida maltosa*, an imperfect yeast that is able to assimilate liquid hydrocarbons for biomass production (9). In this case, 13 leucine auxotrophs were found to constitute five complementation groups (determined by protoplast fusion since *C. maltosa* lacks a sexual cycle), one affecting α-isopropylmalate synthase, three affecting isopropylmalate isomerase, and one affecting β-isopropylmalate dehydrogenase. Again, a unique pattern of apparent regulation was observed, in that in a synthase-minus strain, isomerase activity was decreased only marginally and dehydrogenase still proceeded at about 25% of wild-type activity. When isomerase was missing, causing significant excretion of α-isopropylmalate, synthase activity increased four- to sixfold while dehydrogenase activity remained essentially unchanged. When dehydrogenase was missing, synthase activity again increased 4- to 6-fold while isomerase activity rose 12- to 20-fold. These data are not easily interpreted in terms of known regulatory mechanisms and appear to be at odds with an earlier report that a cloned gene from *C. maltosa* that showed 76% homology (at the protein sequence level) to *LEU2* from *S. cerevisiae* (4), and was therefore named *C-LEU2*, was regulated just like *LEU2* from *S. cerevisiae* when transferred into that organism (100). The interpretation of genetic data from *C. maltosa* is complicated by evidence of a highly aneuploid genome and the spontaneous occurrence of auxotrophy-prototrophy-auxotrophy changes due to the presence of silent gene copies (8).

Ever since the first successful transformation of yeast, which used cloned *LEU2* from *S. cerevisiae* to complement a *leu2* double mutant (46), the *LEU2* gene or its homologues have been the selectable marker of choice in developing new host-vector systems. This gene is therefore one of the best-studied genes of the branched-chain amino acid pathway in fungi. A partial list of organisms from which a *LEU2* homologue has been cloned, sequenced, or otherwise characterized includes (in addition to the *C. maltosa* example mentioned above) the yeast *Yarrowia lipolytica* (29), the methylotrophic yeasts *Hansenula polymorpha* (2) and *Pichia anomala* (30), and the industrially used *Kluyveromyces marxianus* (12) and *Aspergillus niger* (111). *A. niger* elaborates two isoenzymes of β-isopropylmalate dehydrogenase (Leu2A and Leu2B) that are only 52% identical at the amino acid level. In fact, Leu2A is more closely related to its *S. cerevisiae* and *N. crassa* homologues than to Leu2B. Since *A. niger* has been shown previously to incorporate DNA from other organisms in its environment, the authors speculate that the gene encoding Leu2B was introduced from another species. In all of the above cases, with the exception of *A. niger*, where this question was not studied, sequences were found in the 5′-noncoding regions that strongly resemble the consensus binding site for Leu3p, suggesting that Leu3p–α-isopropylmalate regulation of the kind seen in *S. cerevisiae* and *N. crassa* may be important in these other yeasts as well.

LEUCINE AS METABOLIC SIGNAL

The use of leucine or a precursor of leucine to coordinate the status of peripheral nitrogen metabolism with other cellular processes is not limited to the fungi. Another prominent example of this type of signal transduction is the leucine-responsive regulatory protein (Lrp) of *E. coli*. This DNA binding protein controls its own regulon of more than 20 operons that are involved principally in amino acid biosynthesis, the assimilation of ammonia, amino acid degradation, nutrient transport, and the formation of pili (19). (Interestingly enough, Lrp does not directly affect the *leuABCD* operon which, as pointed out above [see "Evolutionary Aspects"] is controlled by transcription attenuation [61].) Lrp functions as a positive regulator for some operons and as a negative regulator for others. Leucine either potentiates or diminishes the action of Lrp or has no effect in some cases. It has been proposed that the Lrp regulon may serve to facilitate metabolic changes in cells subjected to shifts from feast to famine and back (19). Evidence has been presented suggesting that leucine affects the equilibrium among different oligomeric states of Lrp, thus influencing Lrp occupancy at its target sites (92).

There appears to be no obvious structural similarity between the 165-residue Lrp and the 886-residue Leu3 protein and no similarity in the molecular mechanism of action of the two regulators (for example, the effect of leucine is only to modulate the efficiency of Lrp whereas α-isopropylmalate alters the regulatory properties of Leu3p by changing it from a repressor to an activator). However, there clearly are physiological similarities between the two regulons, in terms of what types of genes (operons) are being regulated. One is led to conclude that leucine was selected at least twice in evolution—once in (mostly enteric) bacteria and once in fungi—to function, directly or indirectly and by very different mechanisms, as a signal molecule in a wider metabolic context.

OUTLOOK

One of the more interesting lessons learned from studying the branched-chain amino acid pathways in fungi has been the realization that it is somewhat artificial to look on them in isolation since components of these pathways fulfill functions that go much beyond the task of supplying the cell with valine, leucine, and isoleucine. A prominent example of the involvement of branched-chain amino acid biosynthetic intermediates or proteins in other cellular processes is the Leu3p–α-isopropylmalate complex. In *S. cerevisiae*, this complex is involved in

the regulation of ammonia assimilation by Gdh1p and is likely to up-regulate carrier functions (both at the plasma membrane and at the inner mitochondrial membrane) as well as mitochondrial malic enzyme. There is a good chance that the current list of genes affected by yeast Leu3p is incomplete. While it is possible that some of even the top-ranking candidates for Leu3p regulation identified by the promoter occupancy calculation of Liu and Clarke (69) will turn out to be statistical noise, as was recently suggested for the occurrence of Gal4p binding sites in the *S. cerevisiae* genome (65), it is nevertheless reasonable to assume that others will be recognized as true targets for Leu3p–α-isopropylmalate. It is intriguing that among the genes that rank within the upper 1% on the basis of the occupancy calculation (beyond those listed in Table 1), there are three that encode proteins with a mitochondrial function and eight that specify proteins with transport functions. In any event, a systematic search for genes targeted by Leu3p is clearly called for. It would be of interest to do a corresponding study with respect to the Leu3p regulon of *N. crassa*, where a solid base of information already exists, and to learn whether, and if so, how the use of Leu3p as a "higher-order" metabolic regulator has changed in the course of fungal evolution.

Next, one might ask how widespread among fungi is the surprisingly close relationship between branched-chain amino acid biosynthesis and the mitochondria, with regard to both the subcellular distribution of enzymes and the role of IIv5p and Bat1p or their homologues in maintaining mitochondrial (DNA) integrity.

There are several other loose ends that need to be tied up. One of these concerns the repressor function of Leu3p of *S. cerevisiae*. How does Mot1p come into play? Are other factors involved? The fact that Leu3p-caused repression of target gene transcription below basal level is seen not only in yeast but also in mammalian cells suggests that a more general mechanism may be at work. It is therefore important to find what factors of the transcription machinery are in direct contact with Leu3p. How do these interactions compare with those seen with other transactivators of the zinc cluster class, such as Gal4p? A recent study using an in vivo protein interaction assay has identified seven proteins as candidates for recognizing the activation domain of Gal4p (25). In addition, the activation domain of Gal4p was shown to contact Gal11p and possibly another component of the mediator complex (53). One also needs to remember that Leu3p exists in phosphorylated and nonphosphorylated states (97). The question whether the degree of phosphorylation of Leu3p has any functional significance has not yet been answered.

Finally, there is the question of how and why α-isopropylmalate synthases and other enzymes are regulated by CoA or a derivative thereof. The identification by Cavalieri et al. (23) of an R region that is involved in, if not responsible for, the CoA inactivation of α-isopropylmalate synthase of *S. cerevisiae* and is found in the synthases of a variety of bacteria puts this question on a new footing and should be an impetus to further explore the connections between the activity of the leucine pathway and metabolic energy supply as reflected by the CoA/acetyl-CoA ratio.

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