Biotin Sensing in *Saccharomyces cerevisiae* Is Mediated by a Conserved DNA Element and Requires the Activity of Biotin-Protein Ligase*

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Biotin is a water-soluble vitamin that functions as a prosthetic group in carboxylation reactions. In addition to its role as a cofactor, biotin has multiple roles in gene regulation. We analyzed biotin effects on gene expression in the yeast *Saccharomyces cerevisiae* and demonstrated by microarray, Northern, and Western analyses that all yeast genes encoding proteins involved in biotin metabolism are up-regulated following biotin depletion. Many of these genes contain a palindromic promoter element that is necessary and sufficient for mediating the biotin response and functions as an upstream-activating sequence. Mutants lacking the plasma membrane biotin transporter Vht1p display constitutively high expression of biotin-responsive genes. However, they react normally to biotin precursors that do not require Vht1p for uptake. The biotin-like effect of precursors with regard to gene expression requires their intracellular conversion to biotin. This demonstrates that Vht1p does not act as a sensor for biotin and that intracellular biotin is crucial for gene expression. Mutants with defects in biotin-protein ligase, similar to vht1Δ mutants, also display aberrantly high expression of biotin-responsive genes. Like vht1Δ cells, they have reduced levels of protein biotinylation, but unlike vht1Δ mutants, they possess normal levels of free intracellular biotin. This indicates that free intracellular biotin is irrelevant for gene regulation and identifies biotin-protein ligase as an important element of the biotin-sensing pathway in yeast.

*Saccharomyces cerevisiae* responds to the presence of a variety of environmental nutrients via sensing and signaling pathways capable of identifying the nutrients, determining their concentration, and using this information to regulate gene expression, metabolism, and cell growth. The nutrient sensors identified to date are either typical plasma membrane receptors, plasma membrane proteins with transporter-like structures, or cytoplasmic nutrient-binding proteins (for recent reviews see Refs. 1 and 2). In addition, some plasma membrane transporters have been claimed to have additional functions as sensors for their transported substrate (3, 4).

Biotin, a water-soluble vitamin (vitamin H) that functions as a prosthetic group of carboxylases, is an essential nutrient for most yeast species (5). In *S. cerevisiae*, six biotin-containing proteins are known. These include two isoforms of acetyl-CoA carboxylase, cytosolic Acc1p (6), and mitochondrial Hs1p (7), the two cytosolic isoforms of pyruvate carboxylase (Pyc1p and Pyc2p (8) and urea amidohydrolase (Dur1p,2p (9)). Although these proteins use biotin in carboxylation reactions, the biotin group linked to Arc1p is currently not known to fulfill an enzymatic function (10). The establishment of a covalent linkage of biotin to these proteins is catalyzed by biotin-protein ligase, which is encoded by the essential *BPL1* gene (11). All known biotin ligases operate via a two-step mechanism. First, a biotinyl-AMP intermediate is formed from biotin and ATP that is then transferred to a selected lysine residue in the biotin domain of an acceptor protein (12). Because Acc1p is the only essential substrate of Bpl1p, the essential function of Bpl1p lies in the biotinylation of Acc1p that is necessary for the production of malonyl-CoA for fatty acid biosynthesis and elongation.

Most strains of *S. cerevisiae* are not able to synthesize *de novo* biotin. However, they can perform the last three steps of biotin biosynthesis and generate biotin from the precursors KAPA2, DAPA, or DTB (13) by making use of the enzymes encoded by *BIO3* (DAPA aminotransferase), *BIO4* (DTB synthase), and *BIO2* (biotin synthase) (14, 15). Uptake of DTB is mediated by the biotin permease Vht1p (16), and uptake of KAPA and DAPA requires the high affinity plasma membrane permease Bio6p (14). An interesting feature of the *BIO3*, *BIO4*, and *BIO5* genes is their presence in a closely packed gene cluster on chromosome XIV (14). Recently, sake-producing strains of *S. cerevisiae*, as well as a few laboratory strains, have been found to produce biotin *de novo* (17). Prototrophic strains contain Bio6p, a protein required for an early step of biotin biosynthesis, which is absent from most laboratory strains (17).

Pioneering studies by Rogers and Lichstein (18) demonstrated that biotin uptake in yeast is a high affinity process that leads to biotin accumulation. Moreover, biotin uptake appeared to be regulated and to correlate inversely with the biotin concentration present in the medium (19). Once the biotin transporters of *S. cerevisiae* and *Schizosaccharomyces pombe* had been identified, these results were confirmed and demonstrated to be due to the increased expression of the transporter genes (*VHT1* in *S. cerevisiae* and *vht1* in *S. pombe*) in low biotin medium (16, 20). Together, this demonstrates that biotin uptake in yeast is regulated at the level of transcription and raises the question of which mechanism allows the cells to measure the abundance of biotin.

Regulation of gene expression by biotin is not unique to yeast cells. This field was pioneered by Dakshinamurti and co-workers (21, 22), and many biotin-controlled genes are now known from both biotin-utilizing (plants, most bacteria) and biotin-auxotrophic organisms.

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Dedicated to the memory of Nathalie Vallon.

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1 The abbreviations used are: KAPA, 7-keto-8-aminopelargonic acid; BRE, biotin-responsive element; DAPA, 7,8-diaminopelargonic acid; DTB, desthiobiotin; strip-PO, streptavidin-conjugated peroxidase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ONPG, ortho-nitrophenyl-

nyl-β-D-galactoside; UAS, upstream-activating sequence.
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including mammals (23–25). Mammalian biotin-regulated genes include the biotin transporter, biotin-dependent carboxylases, cytokines, and oncogenes (reviewed in Ref. 24). The sensor for biotin or the precise signaling pathway that mediates the effect of biotin on gene expression has not been unveiled in any eukaryotic system. This is different from the situation in some eubacteria and archaea, where biotin-protein ligase contains an N-terminal DNA-binding domain and acts as a repressor of the biotin operon (25). DNA binding requires that BirA contains bound biotinyl-AMP, which is an intermediate in the biotinylation reaction that accumulates when the biotin acceptor proteins are completely present in the holo-form (26). This regulatory circuit ensures that biotin is not produced in higher amounts than required for protein biotinylation (26). Many prokaryotic and all eukaryotic biotin-protein ligases, in contrast, lack DNA-binding domains, indicating that these organisms use different mechanisms to monitor biotin availability (27, 28).

Here we address the question of where the biotin signal is generated and which target genes are affected by biotin deficiency in S. cerevisiae cells. We demonstrate that yeast mutants with defects in protein biotinylation display typical features of low biotin grown cells, which identifies biotin-protein ligase as a critical element in controlling biotin-responsive genes. In addition, we identify a conserved DNA sequence that confers biotin-dependent regulation. Pathways that may link the low biotin signal to gene expression are discussed.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Culture Conditions—W303-1A (MATα ura3-1 ade2-1 trpl-1 his3-11,15 leu2-3,112 (29)) was used as a wild-type strain for most experiments. The vht1Δ::HIS3 allele was amplified with specific primers from JSYΔvht1 (20) and transformed into W303-1A. Correct transformants were identified by PCR. To replace the promoter (nucleotides −167 to −5) of BPL1 with the galactose-regulated GAL1 promoter, we fused a HindIII-SpeI fragment from pYES2 (Stratagene) containing the GAL1 promoter to the kanMX4 marker gene (obtained as a SpeI/Smal fragment from pFA6a-kanMX (30) in a HindIII/SmaI cut of the pUC19 plasmid. The kanMX4-GAL1 fusion was amplified with oligonucleotides that contained terminal regions of homology derived from BPL1 and mediated homologous recombination after transformation of the PCR product. The same strategy was used to replace the promoter (nucleotides −213 to −5) of ACC1 with that of GAL1 to create strain GAL-ACC1. Correct transformants, as verified by PCR, displayed slow growth on glucose-containing plates. Epitope-tagged versions of Bio5p or Vht1p were generated by PCR-mediated homologous recombination using S. pombe his5+ (VHT1-3HA) or kanMX4 (BIOS-9myc) as a marker (30, 31). Correct integration and C-terminal fusion were verified by Western analysis that detected Vht1p-3HA and Bio5p-9myc at the expected molecular weight. The W303-1A pyc1Δ::LEU2 deletion strain was described before (8). The pyc2Δ::kanMX4 deletion in W303-1A was generated by transformation with a PCR fragment from W303-1A that was transformed into an EUROSCARF strain (Frankfurt/Main, Germany). BY4742 (MATa his3Δ1 leu2Δ0lys2Δ0 ura3Δ0) and isogenic bio2Δ::kanMX4, arc1Δ::kanMX4, his1Δ::kanMX4, and dur1Δ::kanMX4 strains were also received from EUROSCARF. Identical growth conditions produced lower β-galactosidase activities in BY strains when compared with W303, but the induction of VHT1 in low biotin medium was similar in both strain backgrounds. SC medium contained 2% glucose or 2% galactose and 0.67% yeast nitrogen base without amino acids and without vitamins (Bio 101, Inc.). Amino acids and nucleobases were added as required. SC was supplemented with all vitamins except biotin for standard concentrations (32), and biotin, DTB, or KAPA was added as indicated. Difco bacto-agar was used for solidification. Growth assays on Fig. 7A made use of bpl1Δ-3826 (34, 35). Medium for bpl1Δ-3826 additionally contained 0.03% hydrolyzed butter and 1% Tween 40. Medium for both pyc mutants contained 40 mM L-aspartate and 15 mM potassium hydrogen phthalate and was adjusted to pH 5.0 with KOH.

β-Galactosidase Assays—Most reporter assays made use of a construct that fused the VHT1 promoter (starting from the natural HindIII site at nucleotide −760) to the Escherichia coli lacZ gene in the S. cerevisiae centromeric plasmid YCplac33 (36). Alternatively, 1000 bp of the BIO2 promoter were amplified by PCR and fused to lacZ in YCplac33. Binding sites for Gcn4p, Aft1p, or the BRE were replaced by unrelated sequences of identical length using PCR-mediated site-directed mutagenesis. Alternatively, the fragments of the VHT1 promoter depicted in Fig. 3 were cloned as PCR products with terminal XhoI or Sall sites into the unique XhoI site of pMEL-β2, a centromeric yeast vector that contains the UAS-less MEL1 promoter fused to lacZ (37). Reporter plasmids were transformed into yeast cells, and β-galactosidase activity was assayed according to published protocols (38) with minor modifications. Standard assays were performed by growing cells overnight in SC containing 2 μg/liter biotin. The cells were centrifuged, washed, and used to inoculate three cultures in SC with 2 μg/liter biotin and three cultures with 0.02 μg/liter biotin for A600 = 0.2. Six hours later, when the cultures had reached A600 ~1.0, about 10 A600 units of cells were broken with glass beads in 250 μl of 50 mM sodium phosphate buffer, pH 7.0, containing 4 mM phenylmethylsulfonyl fluoride using a Systems FastPrep instrument (Bio 101, Inc.). After addition of 250 μl of 50 mM sodium phosphate buffer, pH 7.0, the extract was centrifuged for 5 min, and the protein concentration of the supernatant was determined in Bradford assays (39). In parallel, the β-galactosidase activity of the supernatant was determined with ortho-nitrophenyl-β-d-galactoside (ONPG) at 37 °C as described (38). One unit of β-galactosidase corresponds to 1 μmol of ONPG hydrolyzed per mg of protein per min at 37 °C. The data reported are mean values obtained from the three individual cultures ± S.D.

Immunological Techniques—A serum against Vht1p was produced by immunization of rabbits with a protein containing amino acids 4–126 of Vht1p fused to the C terminus of maltose-binding protein. The fusion protein was produced from pMAL-c2X (New England Biolabs) as a soluble protein in E. coli and purified using amylose columns as described by the manufacturer (New England Biolabs). Polyclonal sera to detect the 9myc (sc-789) or 3HA (sc-805) epitope tags were obtained from Santa Cruz Biotechnology. Antisera against Bio2p and porin were a gift from U. Mühlenhoff and R. Lill (Marburg, Germany). Secondary antibodies coupled to peroxidase and ECL reagents (Amersham Biosciences) were used for detection. Alternatively, proteins harboring covalently bound biotin were detected with streptavidin-conjugated peroxidase (streP-PO) (catalog number 21126; Pierce).

Determination of Intracellular Free Biotin—To quantify cellular free biotin, 30–100 A600 units of cells were washed, resuspended in PBS containing 0.1% Tween 20 (PBST), boiled for 10 min at 95 °C, and centrifuged to remove cell fragments and denatured proteins. Free biotin present in the supernatant was determined with a competition ELISA (40) with the following modifications. Biotinylated BSA for coating of ELISA plates was prepared by incubation of 25 μg of BSA (catalog number A0846; AppliChem) with 5 μg of biotinamidocaproate N-hydroxysuccinimide ester (catalog number B2643; Sigma) in 0.1 M borate buffer, pH 8.8, for 6 h at room temperature, followed by extensive dialysis against the same buffer. Plates were coated with a solution containing
3 μg/ml biotinyl-BSA in PBS overnight, followed by blocking with 10 mg/ml unlabeled BSA in PBS. Strept-PO (Pierce) was used in a dilution of 1:10,000 in PBST and either incubated with biotin standard solutions or with cell extracts for 60 min at 25 °C before incubation with the plates for 60 min at 25 °C. After washing with PBST, peroxidase activity was determined as described (40). The biotin concentration in the cell extracts was determined as described (40). Briefly, a curve was generated with biotin solutions in PBS to determine the minimal amount of biotin required to saturate strep-PO and thus prevent strep-PO binding to the biotinyl-BSA-coated plates. Next, different volumes of cell extracts were used as competitors of strep-PO binding, and the smallest volume of extract, which already prevented binding of strep-PO, was determined. The biotin concentration of the extract was calculated by division of the above-determined amount of biotin with the extract volume. Cellular concentrations are based on an assumed intracellular volume of 11.25 μl/10 A600 units of cells.

DNA Microarrays — A yeast culture was grown to saturation in SC medium containing 2 μg/liter biotin and used to inoculate parallel cultures containing 2 or 0.02 μg/liter biotin. The cells were harvested 6 h later (A600 = 1.0–1.1), washed with diethyl pyrocarbonate-treated water, and frozen. RNA was extracted with acid phenol according to standard protocols (41) and further purified using RNeasy maxi columns (Qiagen). RNA (15 μg) was converted to double-stranded cDNA (Invitrogen), followed by synthesis of biotin-labeled cRNA using the BioArray HighYield RNA transcript labeling kit (ENZO Diagnostics). Two Affymetrix yeast genome S98 GeneChips were hybridized for each condition as described by the manufacturer. Affymetrix microarray suite 5.0 was used for single array and base-line comparison analysis, using a global scaling strategy and setting the average signal intensity of all arrays to a target value of 100.

Northern Analysis — To confirm the results of the microarray experiment, we performed Northern analysis using an independent preparation of RNA prepared by the procedure described above. RNA (20 μg/lane) was separated on formaldehyde gels and blotted to nitrocellulose filters. Probes were obtained by random labeling of PCR products derived from the coding regions of VHT1, BPL1, BIO2, BIO5, or ACT1 (encoding actin) with [α-³²P]dCTP.

RESULTS

A Conserved DNA Element in the Promoters of Genes Involved in Biotin Metabolism — We have shown previously that S. cerevisiae and S. pombe possess plasma membrane biotin permeases (named Vht1p for vitamin H transporter 1 in both yeasts) that are transcriptionally regulated by the amount of biotin present in the growth medium (16, 20). To investigate this regulation, we aligned the promoter of VHT1 from S. cerevisiae and other Saccharomyces species (42, 43) and searched for the presence of conserved DNA elements. This approach identified a DNA element, which we name BRE, that was present in all species (Fig. 1A). The promoters of biotin metabolic genes. The BRE was identified in the genes of biotin permease VHT1 (6), biotin synthase BIO2 (8), and biotin-protein ligase BPL1 (9) of the related yeast species S. cerevisiae, Saccharomyces paradoxus, Saccharomyces mikatae, Saccharomyces kudriavzevii, Saccharomyces bayanus, and Saccharomyces castelli. The numbering indicates the position relative to the start ATG (where A is +1) of the gene. Positions that are identical in at least 16 of the 17 sequences are printed in bold. Weblogo (available online) was used with default settings to create a graphic representation of the sequences displayed in A–C.

Another study demonstrated that the transcription factor Gcn4p, a central player in the response to amino acid starvation, targets VHT1 (11). In the case of Gcn4p, a canonical binding site (UA5GCRE (47)) is present in the VHT1 promoter (−110AGACTTCCTTT−100) and conserved in all sequenced Saccharomyces species. To address the question whether Gcn4p or Aft1p

**FIGURE 1.** A conserved sequence is present in the promoters of biotin genes. A conserved 20-bp DNA element (termed BRE) was identified by promoter alignments of biotin metabolic genes. The BRE was identified in the genes of biotin permease VHT1 (A), biotin synthase BIO2 (B), and biotin-protein ligase BPL1 (C) of the related yeast species S. cerevisiae, Saccharomyces paradoxus, Saccharomyces mikatae, Saccharomyces kudriavzevii, Saccharomyces bayanus, and Saccharomyces castelli. The numbering indicates the position relative to the start ATG (where A is +1) of the gene. Positions that are identical in at least 16 of the 17 sequences are printed in bold. D, Weblogo (available online) was used with default settings to create a graphic representation of the sequences displayed in A–C.

![Image](http://www.jbc.org/content/281/18/12383/F1.large.jpg)
mediate the low biotin response, we individually deleted their binding sites from the VHT1 promoter. Deletion of the Gcn4p site caused an overall reduction of β-galactosidase activity, which was apparent at all biotin concentrations tested (Fig. 2). The promoter lacking the UAS-Gcn4p, however, still responded to low biotin concentrations, which caused a 7.8-fold increase in activity (Fig. 2). Deletion of the Aft1p site caused no gross alterations of the promoter activity and did not influence the response to low biotin concentrations (Fig. 2). Another promoter element, which is present in S. cerevisiae VHT1 but not conserved in all Saccharomyces species, is a binding site for the heterodimeric Ino2p/Ino4p transcription factors (46). Although expression of VHT1 was reduced 3.9-fold in a microarray experiment in inositol choline-containing medium and the BIO genes were affected to a similar extent (47), a more careful transcriptional analysis of inositol-responsive genes could not confirm these findings (50). The lack of regulation of VHT1 by inositol is also consistent with reporter assays performed by Hoppen et al. (51) and with our own data that failed to demonstrate gross differences in the expression of VHT1 in medium containing none or 50 μM inositol (data not shown). This makes it unlikely that the Ino2/Ino4 proteins participate in the biotin response.

We next deleted the BRE identified above in the VHT1 promoter and repeated the reporter assays. This construct possessed wild-type activity in normal biotin medium, but the increased activity in low biotin medium was almost completely nullified. However, some activation (1.7-fold) by biotin limitation was still noticeable. We have shown above that deletion of the Gcn4p site from the VHT1 promoter did not influence the biotin response. However, we noticed that the BRE in itself had similarities to a canonical Gcn4p-binding motif (Fig. 1D). To exclude that the BRE acted as a Gcn4p-binding site, we exposed cells to histidine deficiency, a condition that activates Gcn4p. In these experiments, both the wild-type and the VHT1 promoter lacking the BRE were activated, but this activation was lost upon deletion of the Gcn4p site (data not shown). This confirms earlier findings that VHT1 is regulated by Gcn4p (46), confirms that the Gcn4p-binding site identified above is functional, and excludes that the low biotin response of VHT1 is mediated via Gcn4p.

To gain independent proof that the BRE mediates the low biotin response, we used pMEL-β2, a centromeric yeast vector that contains the UAS-less MEL1 (melibiose) promoter and the lacZ reporter gene. This plasmid shows no β-galactosidase activity unless a binding site for a transcriptional activator is introduced (37). Fragments of the VHT1 promoter not including the regulatory sites for Ino2p/Ino4p, Gcn4p, and Aft1p were amplified by PCR and introduced into pMEL-β2, and yeast cells transformed with these constructs were assayed for reporter activity (Fig. 3). Although a fragment containing 134 bp of VHT1 promoter, including the BRE (fragment A), caused a 14.6-fold increase in β-galactosidase activity in low biotin medium, this increase was abolished when the BRE was removed (fragment B). Next, we further truncated the BRE-containing fragment to 80 (fragment C) and 63 bp (fragment D). Both constructs mediated large increases in reporter activity in low biotin medium (48.3-fold for fragment C and 22.3-fold for fragment D), and this response was nullified by removal of the BRE (fragment E). Additionally, a 90-bp fragment that was similar to fragment A but did not extend into the BRE (fragment F) did not cause expression of lacZ. In summary, the BRE acts as a typical upstream-activating sequence that mediates the low biotin response (UAS_BIO). The BRE is the dominant site of biotin regulation in the VHT1 promoter and is sufficient to confer the biotin response.

The BRE in BIO2 Is Necessary for the Biotin Response—Although all genes listed in Fig. 1 contained similar promoter elements, the BREs present in the control regions of BIO2 and BPL1 appeared to be more similar to each other than to the element present in VHT1. To check if BIO2 also responds to biotin, we fused the BIO2 promoter fragment to lacZ in a yeast centromeric plasmid. Cells carrying this construct displayed a 4.2-fold increase in β-galactosidase activity when grown in low biotin medium (Fig. 4). An identical plasmid harboring no BRE did not show this response but rather displayed reduced activities at both low and high biotin concentrations (Fig. 4). This confirms that the BRE functions as a general UAS_BIO for genes involved in biotin metabolism and mediates the low biotin response.

A Genome-wide Search for Genes Induced by Low Biotin Concentrations—To increase our knowledge about genes whose expression is affected by biotin, we performed a DNA array experiment in which the transcriptome of cells grown in normal and low biotin medium was compared. Interestingly, the transcription of all yeast genes involved in biotin metabolism was increased by biotin limitation.
FIGURE 4. The BRE is necessary for the biotin-responsive expression of BIO2. β-Galactosidase assays were performed with W303-1A wild-type (wt) yeast cells carrying the unmodified BIO2-lacZ reporter plasmid (wt) or a reporter plasmid from which the BRE sequence had been deleted (w/o BRE). The cells were grown in medium containing 2 or 0.02 μg/liter biotin for 6 h as indicated and used for the determination. Units are defined as in Fig. 2.

(Fig. 5A). BIO5 showed the greatest increase (12.9×), followed by BIO2 (6.6×) and BIO4 (3.6×). The expression of BPL1 (2.7×), BIO3 (2.4×), and VHT1 (1.7×) was also increased in low biotin medium. A detailed analysis of the genome-wide response to biotin limitation will be presented elsewhere. To confirm this analysis, we used independent RNA preparations of cells grown in various biotin concentrations in Northern blots (Fig. 5B). We analyzed VHT1, BIO2, BIO5, and BPL1 expression, and we found all these genes to be more strongly expressed in low biotin medium. Their expression changes were similar to the changes found in the microarray with BIO5 showing the biggest and VHT1 the smallest increase (Fig. 5B). Together this analysis adds the BRE-containing gene BPL1 as well as BIO3, BIO4, and BIO5 to the list of biotin-regulated genes. The genes BIO3, BIO4, and BIO5 lie in a closely spaced cluster on chromosome XIV, and we also find this cluster to contain BRE-related sequences. These putative BREs, however, lie within the BIO4 open reading frame, which prevented their identification in our initial analysis of intergenic regions (Fig. 1). One element (5′-GAGT-CAGATCAAGGTGACTCC-3′, numbering relative to the BIO4 start ATG) is present in all sequenced Saccharomyces species, whereas a related but shorter sequence upstream of the BIO4 stop codon (5′-GAGTCAATGACCAGGCTCC-3′) is conserved in all species except Saccharomyces kluwyeri. The high degree of similarity to the BRE sequences in the promoters of VHT1, BIO2, and BPL1 makes it possible that these elements mediate the biotin-responsive expression of BIO3, BIO4, and BIO5.

Western blots were used to prove that the differences in gene expression revealed by the Northern blot, microarray, and reporter gene analyses indeed resulted in changes of the abundance of the proteins. To this end, we used cells carrying tagged versions of BIO5 and VHT1 in Western blots. Bio2p, for which a specific antibody was available, was also included in the analysis. The Western blots (Fig. 5C) demonstrated that Bio2p and Vht1p were more abundant in low biotin-grown cells. This is consistent with the fact that growth in low biotin medium increases the activity in biotin uptake (19, 20). Bio5p, whose gene was most strongly regulated by biotin, was not detectable in cells from normal biotin medium but gave a strong band in cells from low biotin medium (Fig. 5C). Together, this confirms that all tested genes are regulated by biotin.

Identical blots were decorated with strep-PO to detect biotin proteins (Fig. 5D). Growth in low biotin caused a fainter appearance of all biotin proteins. Because yeast cells lack biotinidase, a human enzyme necessary for removing biotin from proteins (52), this phenomenon unlikely reflects biotin removal. Because the expression of the genes of the biotin proteins was unaffected by biotin depletion (see legend to Fig. 5A), we rather speculate that growth in low biotin medium lowers the intracellular biotin concentration to levels that do not support normal protein biotinylation. This hypothesis is supported by the free intracellular biotin concentrations, which were determined by ELISAs. Wild-type cells grown in 2 μg/liter biotin contained 0.5–0.55 ng of biotin/μl of cell volume, whereas growth in low biotin medium (0.02 μg/liter) for 6 h lowered their intracellular biotin concentration to 0.03–0.05 ng/μl (Table 1). Thus, although we cannot rule out an effect of biotin on the translation of the RNAs coding for the biotin proteins, the simplest interpretation of our findings is that low intracellular biotin concentrations cause an overall reduction in biotinylation.

Although growth in low biotin caused a fainter appearance of all biotin proteins in Western blots (Fig. 5), some biotin acceptors were more affected by biotin deficiency than others. Although biotinyl-Arc1p, a protein with a noncanonical biotin domain (10), was undetectable at low biotin concentrations, the band corresponding to the two isoforms of pyruvate carboxylase (Pyc1p and Pyc2p) was the least affected. This finding may be explained by differences in RNA translation, RNA or protein stability, or different affinities of the apoproteins toward biotin-protein ligase.

In summary, growth in biotin-restricted medium results in low intracellular biotin concentrations and causes a biotinylation defect, although this condition leads to overproduction of the biotin transporter, the enzymes involved in biotin biosynthesis, and biotin-protein ligase.
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### TABLE 1

**Intracellular biotin concentrations**

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>High extracellular biotin</th>
<th>Normal extracellular biotin</th>
<th>Low extracellular biotin</th>
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<tr>
<td>W303-1A</td>
<td>12.8 ± 3.4</td>
<td>0.50 ± 0.08</td>
<td>0.053 ± 0.004</td>
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<tr>
<td>W303-1A</td>
<td>ND</td>
<td>0.56 ± 0.16</td>
<td>ND</td>
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<td>W303-1A vht1Δ</td>
<td>0.053 ± 0.010</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>GAL-BPL1</td>
<td>ND</td>
<td>0.91 ± 0.18</td>
<td>ND</td>
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<tr>
<td>GAL-BPL1</td>
<td>ND</td>
<td>1.09 ± 0.21</td>
<td>ND</td>
</tr>
<tr>
<td>BY4742</td>
<td>ND</td>
<td>0.55 ± 0.22</td>
<td>0.03 ± 0.008</td>
</tr>
<tr>
<td>BY4742 arc1Δ</td>
<td>ND</td>
<td>1.12 ± 0.17</td>
<td>0.029 ± 0.009</td>
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</table>

* a Grown in medium containing 2% glucose.
* b Grown in medium containing 2% galactose.
* c ND indicates not determined.

### Biotin Sensing Is Independent of the Extracellular Biotin Concentration—What is the signal that mediates the expression of the biotin-responsive genes in biotin-deficient medium? To check if extracellular or intracellular biotin is relevant for biotin signaling, we experimentally altered the cytoplasmic biotin concentrations by using mutants lacking the plasma membrane biotin permease Vht1p. vht1Δ cells are viable only when supplied with high outside concentrations of biotin (200 μg/liter) (20). Consistent with the findings of Fig. 2, wild-type cells grown in high biotin concentrations displayed much weaker reporter activities (Fig. 6A). vht1Δ mutants from identical high biotin medium, in contrast, possessed drastically increased (18×) reporter activities (Fig. 6A). The intracellular free biotin concentrations were 12.8 ng of biotin/μl cell volume for wild-type cells grown in 20 μg/liter biotin and 0.053 ng of biotin/μl cell volume for vht1Δ cells grown in 200 μg/liter biotin (Table 1). Thus, vht1Δ cells grown in high biotin medium and wild-type cells grown in low biotin medium both have low levels of intracellular free biotin, and this correlates with high reporter gene activities. Similar results were obtained with vht1Δ cells harboring the minimal promoter construct D (Fig. 3) as a reporter (data not shown).

Moreover, we observed that the conditions that created high reporter gene activities did not allow full growth of the strains (Fig. 6B). This growth defect likely resulted from the reduced biotinylation of the biotin-dependent enzymes that is demonstrated by the Western analysis shown in Fig. 6C. This finding is consistent with our earlier observation (20) and with the finding that wild-type cells grown in low biotin medium display much weaker signals for all biotin proteins (Fig. 5D). In summary, low concentrations of intracellular free biotin cause reduced protein biotinylation and slow growth, and this correlates with high levels of VHT1 promoter activity. This demonstrates that extracellular biotin does not provide the signal for biotin-mediated gene expression.

### Biotin Precursors Mediate Biotin-dependent Gene Regulation—To resolve if the Vht1 protein itself is involved in biotin sensing, or if the absence of Vht1p had only reduced the intracellular availability of biotin, we performed reporter assays with vht1Δ cells grown in medium in which biotin was replaced by KAPA. KAPA can be converted to biotin by the activities of Bio3p, Bio4p, and Bio2p but is structurally unrelated to biotin and is taken up by Bio5p (14) (Fig. 5A). This allowed us to check if KAPA influences the expression of biotin-responsive genes and if this requires Vht1p.

To deplete intracellular biotin, we first cultured wild-type and vht1Δ cells for several days in medium containing 2 μg/liter KAPA and overnight in 20 μg/liter KAPA. On the day of the analysis, the cultures were split and grown in medium containing 20 or 0.2 μg/liter KAPA. lacZ reporter gene activities were determined 6 h later. This demonstrated that wild-type cells from low KAPA medium possessed 2.4× increased β-galactosidase activities (Fig. 6D) and that vht1Δ cells produced a similar response (2.9×). This shows that low KAPA concentrations result in the activation of a biotin-responsive promoter, irrespective of the presence or absence of Vht1p. We also noted that the reporter gene is well expressed in medium with high KAPA concentrations. This likely reflects that the BIO genes must be expressed to allow biotin synthesis even when KAPA is present in excess. Control experiments demonstrated that KAPA-adapted wild-type and vht1Δ cells displayed similar growth rates with KAPA and that the growth of both strains was reduced at the lower concentration used in the assay (Fig. 6E). Protein
extracts from the cells used in the reporter assays were analyzed by Western blotting (Fig. 6F). Both wild-type and vht1Δ cells showed reduced levels of protein-bound biotin and increased abundance of Bio2p when grown in low KAPA conditions (Fig. 6F).

We have also addressed if DTB has biotin-like effects with regard to gene expression. Wild-type cells showed similar growth rates on biotin or DTB, but bio2Δ mutants lacking biotin synthase failed to utilize DTB as a biotin source. Growth in low concentrations of either biotin or DTB caused an increase in the expression of the reporter gene in wild-type cells (Fig. 6G). Although bio2Δ cells responded normally to low biotin conditions, they possessed high reporter activities in high DTB medium, which were only marginally increased following DTB depletion (Fig. 6G). Additionally, high KAPA concentrations failed to repress the activity of the reporter when its conversion to biotin was blocked (data not shown). Taken together, the biotin precursors KAPA and DTB both cause an increase in the expression of the reporter gene in wild-type cells grown in low biotin conditions (Fig. 6F).

A reporter gene assay was performed with wild-type (W303-1A) or bpl1-3826 cells carrying the VHT1 promoter-lacZ construct. Cells were passaged for 24–40 h in medium containing fatty acids and 2 or 0.2 μg/liter biotin as indicated. β-Galactosidase activity was assayed with cells from logarithmic phase (A600 = 0.4–1.2). Units are defined as in Fig. 2. A reporter gene assay was performed with the VHT1 promoter-lacZ construct in a wild-type strain or in the GAL-BPL1 strain in 2 μg/liter biotin. The cells were grown for 48 h in repressing (Glc) or inducing (Gal) conditions prior to the assay. C, Western blots were performed on protein extracts from GAL-BPL1 cells or wild-type cells as indicated in B. The vht1Δ strain was grown in minimal medium containing 200 μg/liter biotin and glucose. The extracts were probed with strep-PO to reveal biotin proteins or sera directed against Bio2p or Vht1p as indicated. Because of its low reactivity, the Vht1p serum does not produce a signal unless VHT1 is overexpressed, such as in GAL-BPL1 cells grown in glucose. The faint band marked with an asterisk is a cross-reacting protein also present in vht1Δ mutants. A serum against the mitochondrial porin Port1p was used as a loading control.

The Role of Biotin-Protein Ligase in Biotin Sensing—Intracellular biotin could be monitored at various levels, such as the amount of free biotin, the degree of protein biotinylation, or the activity of the biotin-dependent enzymes. To alter the degree of protein biotinylation, we made use of mutants affected in the activity of biotin-protein ligase. BPL1 is an essential gene, but viable bpl1 mutants that have retained some enzymatic activity have been isolated (34). These mutants require a supplement of fatty acids because they have reduced activity of acetyl-CoA carboxylase and are deficient in fatty acid biosynthesis and elongation (34). We transformed the VHT1-lacZ reporter plasmid into bpl1−3826 and performed reporter assays as above (Fig. 7A). Although the wild-type strain showed a typical response to low biotin concentrations, the bpl1 mutant had a high reporter activity in high biotin medium, which was not further increased at low biotin concentrations (Fig. 7A).

To confirm the role of Bpl1p, we replaced the promoter of BPL1 with the galactose-inducible GAL1 promoter in the genome of a wild-type strain. GAL-BPL1 cells were grown in normal biotin medium under either repressing (glucose) or inducing (galactose) conditions and were used for the determination of β-galactosidase activities (Fig. 7B). Although lacZ expression was similar in wild-type cells grown in glucose or galactose, GAL-BPL1 cells showed increased β-galactosidase activities following depletion of Bpl1p (Fig. 7B), and similar to bpl1−3826 (Fig. 7A), their activity was not further increased by growth in low biotin medium (data not shown). These results were confirmed using GAL-BPL1 cells containing the minimal promoter construct D (see Fig. 3) as a reporter plasmid (data not shown). Protein extracts from GAL-BPL1 cells grown in glucose demonstrated low levels of protein biotinylation, whereas the normal pattern of biotin proteins was apparent after growth in galactose (Fig. 7C). The amount of Bio2p in GAL-BPL1 cells in galactose was similar to wild-type cells grown with either carbon source, but it was increased in GAL-BPL1 cells grown in glucose (Fig. 7C). Similar to these findings, Vht1p could only be detected when BPL1 was repressed (Fig. 7C). These results were corroborated by Northern blots demonstrating increased expression of BIO2 and BIO5 in bpl1−3826 mutants (data not shown). Moreover, the free intracellular biotin concentrations in GAL-BPL1 cells were similar in cells from glucose- and galactose-containing medium (Table 1). In summary, this shows that a normal activity of Bpl1p is necessary for normal biotin sensing. Cells with defects in biotin-protein ligase initiate a low biotin response even though the cells contain a normal concentration of free biotin.

Are Biotin Proteins Involved in Biotin Sensing?—Because biotin-protein ligase affected biotin sensing, we next asked if this effect is caused by any of the proteins modified by Bpl1p. To this end, we analyzed reporter gene activities in strains lacking individual biotin proteins. For Arc1p, Dur1,2p, Hfa1p, Pyc1p, and Pyc2p, knock-out strains were used in the analysis. For the essential ACC1 gene, we replaced its promoter with the galactose-regulated GAL1 promoter, which resulted in undereexpression of ACC1 after growth in glucose-containing medium. Standard reporter assays were performed after growth in normal or low biotin medium for 6 h (Table 2). In every case, removal of a biotin protein resulted in the reduced ability of the cells to express the reporter gene in low biotin medium. This effect was most pronounced for Pyc2p and Arc1p, but even loss of the mitochondrial acetyl-CoA ligase Hfa1p or the low abundance protein Dur1,2 diminished the low biotin response (Table 2).

We speculated that the lack of a single biotin protein might lead to an increase in the free biotin present in the cells, and this might cause a later onset of the low biotin response. In support of this hypothesis, we found that arc1Δ cells grown in standard medium contained twice the amount of free biotin when compared with wild-type cells (Table 1). Moreover, we repeated reporter assays with arc1Δ cells but kept the cells for a longer time in low biotin medium. These assays demonstrated that arc1Δ cells were competent to produce a normal low biotin response (data not shown). Thus, although the biotin proteins affect biotin sensing, it is possible that they merely have an indirect role.

FIGURE 7. Biotin sensing requires the activity of biotin-protein ligase. A, reporter gene assays were performed with wild-type (W303-1A) or bpl1-3826 cells carrying the VHT1 promoter-lacZ construct. Cells were passaged for 24–40 h in medium containing fatty acids and 2 or 0.2 μg/liter biotin as indicated. β-Galactosidase activity was assayed with cells from logarithmic phase (A600 = 0.4–1.2). Units are defined as in Fig. 2. B, reporter gene assays were performed with the VHT1 promoter-lacZ construct in a wild-type strain or in the GAL-BPL1 strain in 2 μg/liter biotin. The cells were grown for 48 h in repressing (Glc) or inducing (Gal) conditions prior to the assay. C, Western blots were performed on protein extracts from GAL-BPL1 cells or wild-type cells as indicated in B. The vht1Δ strain was grown in minimal medium containing 200 μg/liter biotin and glucose. The extracts were probed with strep-PO to reveal biotin proteins or sera directed against Bio2p or Vht1p as indicated. Because of its low reactivity, the Vht1p serum does not produce a signal unless VHT1 is overexpressed, such as in GAL-BPL1 cells grown in glucose. The faint band marked with an asterisk is a cross-reacting protein also present in vht1Δ mutants. A serum against the mitochondrial porin Port1p was used as a loading control.

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Biotin Sensing and Signaling in *S. cerevisiae*

**TABLE 2**

**Reporter gene activities of yeast strains lacking biotin proteins**

Deletion strains lacking the biotin proteins Arc1p, Dur12p, Hfa1p, Pyc1p, and Pyc2p, or carrying a galactose-controlled version of ACC1 (GAL-ACC1), were transformed with the VHT1 promoter-lacZ reporter plasmid. The cultures were grown as indicated, washed, inoculated into medium containing either 2 or 0.02 μg/liter biotin, and assayed 6 h later for β-galactosidase activity. One unit corresponds to 1 μmol of ONPG hydrolyzed per mg of protein per min at 37 °C. Activities represent means ± S.D. of three separate determinations.

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>2 μg/liter biotin</th>
<th>0.02 μg/liter biotin</th>
<th>-Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742 (wild type)</td>
<td>0.295 ± 0.016</td>
<td>1.978 ± 0.171</td>
<td>6.7x</td>
</tr>
<tr>
<td>arclΔ</td>
<td>0.218 ± 0.014</td>
<td>0.400 ± 0.059</td>
<td>1.8x</td>
</tr>
<tr>
<td>dur12Δ</td>
<td>0.410 ± 0.027</td>
<td>1.077 ± 0.122</td>
<td>2.6x</td>
</tr>
<tr>
<td>ifa1Δ</td>
<td>0.281 ± 0.014</td>
<td>1.254 ± 0.080</td>
<td>4.5x</td>
</tr>
<tr>
<td>W303-1A (wild type)*</td>
<td>0.734 ± 0.083</td>
<td>3.525 ± 0.048</td>
<td>4.8x</td>
</tr>
<tr>
<td>W303-1A (wild type)*</td>
<td>0.727 ± 0.040</td>
<td>4.237 ± 0.275</td>
<td>5.8x</td>
</tr>
<tr>
<td>GAL-ACC1*</td>
<td>1.187 ± 0.109</td>
<td>4.681 ± 0.513</td>
<td>3.9x</td>
</tr>
<tr>
<td>GAL-ACC1*</td>
<td>0.640 ± 0.063</td>
<td>1.407 ± 0.144</td>
<td>2.2x</td>
</tr>
<tr>
<td>W303-1A (wild-type)*</td>
<td>0.733 ± 0.041</td>
<td>2.407 ± 0.268</td>
<td>3.3x</td>
</tr>
<tr>
<td>pyc1Δ*</td>
<td>0.478 ± 0.037</td>
<td>1.830 ± 0.107</td>
<td>3.8x</td>
</tr>
<tr>
<td>pyc2Δ*</td>
<td>0.628 ± 0.065</td>
<td>0.931 ± 0.025</td>
<td>1.5x</td>
</tr>
</tbody>
</table>

* A grown in medium containing 2% galactose.
* A grown in medium containing 2% glucose.
* A grown for 22 h in medium containing 2% galactose.
* A grown in medium containing 2% glucose, 40 μM L-aspartate, 15 mM potassium hydrogen phthalate, pH 5.0.

**DISCUSSION**

In this study we have analyzed the biotin-sensing pathway of yeast. We provide evidence by microarray, Northern, Western, and reporter gene analyses that the *S. cerevisiae* genes involved in biotin metabolism are regulated by biotin. The control regions of these genes contain a novel DNA element that functions as an UAS for biotin (UASBIO) and mediates this response. We postulate that this palindromic sequence, which has no similarity to the 40-bp biotin operon of the *E. coli* biotin operon (53), serves as a binding platform for a transcription factor. The UASBIO has been missed in earlier genome-wide analyses performed using cells from complete medium (54), possibly indicating that it is only occupied at low biotin concentrations. A typical CRE is also present in the promoter of *Bio6*, a biotin-regulated gene required for biotin biosynthesis in recently discovered biotin-prototrophic strains of *S. cerevisiae* (17).

In addition to the UASBIO, the promoter VHT1 contains a regulatory site for Gcn4p (UASGCRE), a transcription factor that regulates many biotin-using proteins and increases the activity of the biotin-dependent pyruvate carboxylases that generate oxaloacetate. This anaplerotic reaction keeps the Krebs cycle functional when intermediates are withdrawn for amino acid biosynthesis. Interestingly, both *Pyc1* and *Pyc2* genes are also under control of Gcn4p (46). Thus, Gcn4p increases the expression of the *PYC* genes and increases the availability of biotin, both of which are necessary to increase pyruvate carboxylase activity. The UASBIO appears to be similar to the Gcn4p-binding site. However, only removal of the UASGCRE but not of the UASBIO affects the expression of *VHT1* following histidine starvation. This strongly indicates that biotin deficiency and starvation for amino acids initiate distinct physiological responses.

The promoter of VHT1 also contains a control site for the Aft1p transcription factor that mediates a low iron response (45). Low iron conditions are known to reduce the expression of many iron-using proteins, including biotin synthase (Bio2p) that contains two iron-sulfur cofactors (44). Thus, the Aft1p-dependent increase of biotin import mediates biotin homeostasis in low iron conditions where biotin cannot be synthesized. Another discernible promoter element is a binding site for the Iho2p/Iho4p transcription factors. However, these proteins do not seem to largely influence the expression of VHT1 (50, 51).

Another possibility consistent with our data is that the biotin sensor is modified by Bipl1p but cannot be detected in Western blots because of low protein abundance. An ideal sensor would have low affinity to Bipl1p so that biotin signaling is initiated before the activity of the biotin-dependent enzymes is compromised. Arc1p, a protein with a noncanonical biotin domain, is a low affinity substrate for Bipl1p (Figs. 5–7) (10). However, arc1Δ mutants are still able to sense low biotin conditions, which is inconsistent with a function in biotin sensing. The surprising finding that yeast Bipl1p can modify nonclassical biotin proteins such as Arc1p may, however, indicate that more targets of Bipl1p await discovery.

The possibility that biotin deficiency is sensed by a lack of metabolites caused by inactivity of a biotin-dependent enzyme can be ruled out. Lack of Acc1p, for example, is expected to result in low levels of malonyl-CoA and fatty acids synthesized. If any of these metabolites would signal biotin deficiency, down-regulation of ACC1 would also signal biotin deficiency. However, neither underexpression of ACC1 nor deletion of the other biotin protein-encoding genes elicits a low biotin response. Thus, although our experiments demonstrate that reduced levels of protein biotinylation (such as in vht1Δ cells, *bpl1* mutants, and *GAL-BPL1* cells grown in glucose and wild-type cells grown in low biotin medium) are a common feature of cells with high expression levels of biotin-responsive reporters, they fail to demonstrate a direct influence of the known biotin proteins on biotin sensing.

Biotin effects on gene expression were recently analyzed in mammalian cell lines. Here, in contrast to *S. cerevisiae*, the expressions of the genes encoding biotin-protein ligase, the biotin transporter SMVT1, and some biotin-acceptor proteins were decreased following biotin starvation (55–57). Because the mRNA levels of these genes were not affected in the brain, this response is thought to spare biotin consumption in the body to ensure the functioning of the brain when biotin is scarce (56, 57). The recovery of certain mRNAs following biotin supplementation was used to investigate signaling molecules involved in biotin regulation (55). Addition of 8-bromo-cGMP to biotin-starved cells had...
the same effect as biotin, although the effect of biotin was nullified by inhibition of cGMP-dependent protein kinase. This led to the conclusion that the mammalian biotin-sensing cascade requires guanylate cyclase and cGMP-dependent protein kinase (55). Moreover, mRNA recovery was slowed in cells derived from patients with mutations in biotin-protein ligase, leading to the conclusion that biotin-protein ligase is important in mediating the effect of biotin on gene expression (55). Because biotin analogues that do not serve as enzymatic cofactors have biotin-like activities with regard to gene expression, the effect of biotin-protein ligase is independent of the formation of enzymatically active holo-carboxylases (58). This lead to the interesting speculation that biotinyl-AMP, which is produced in the first half-reaction of biotin-protein ligase, could be the intracellular signal that mediates gene regulation in mammalian cells (55). This hypothesis is fully consistent with our findings in yeast where biotin-protein ligase is involved in biotin signaling, but this effect does not appear to involve biotin proteins. It is thus intriguing to speculate that biotinyl-AMP also acts as the primary intracellular biotin signal in yeast. However, yeast cells lack homologues of guanylate cyclase and cGMP-dependent protein kinase. Thus, although biotinyl-AMP might be the common signal in yeast and higher eukaryotes, the pathways downstream of it appear to be different. Nevertheless, we hope that this study will establish S. cerevisiae as a model system to investigate biotin sensing. Further experiments to close the remaining gaps in our understanding of the yeast biotin-signaling pathway can now be devised.

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