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Aromatic Amino Acid Biosynthesis in the Yeast *Saccharomyces cerevisiae*: a Model System for the Regulation of a Eukaryotic Biosynthetic Pathway

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INTRODUCTION

The biosynthesis of the aromatic amino acids tryptophan (Trp), phenylalanine (Phe), and tyrosine (Tyr)—especially the tryptophan branch of this pathway—has become one of the best-studied examples of a biosynthetic pathway. Studies of this pathway have contributed to the understanding of topics such as gene-enzyme relationships, promoters, protein-DNA interactions, translational control, enzyme structure and catalysis, protein-protein interactions, and control of flow through a pathway in a wide range of organisms. Earlier reviews about the aromatic amino acid biosynthesis often concentrate on prokaryotic organisms, mainly on the tryptophan branch, and only a smaller portion also deal with eukaryotic organisms and includes other parts of the pathway (5, 6, 37, 46–48, 66, 97, 98, 206, 207). Since the prokaryotic paradigm of regulatory mechanisms does not completely extend to eukaryotes, this review focuses on the regulation of the pathway in a simple eukaryotic system, the unicellular yeast *Saccharomyces cerevisiae*. This yeast is one of the oldest commercially cultured organisms and is also one of the best-studied genetic systems available. Since the first yeast transformation (91), yeast research has boomed and is giving rise to numerous new insights in the understanding of various aspects of a branched biosynthetic pathway.

Archaebacteria, eubacteria, plants, and fungi are competent to synthesize de novo the three aromatic amino acids phenylalanine, tyrosine, and tryptophan. Animals are generally able to synthesize tyrosine only by hydroxylation of phenylalanine and require the other aromatic amino acids in their diet (79). Specific inhibitors of the aromatic pathway, e.g., glyphosate (*N*-phosphomethylglycine), can therefore

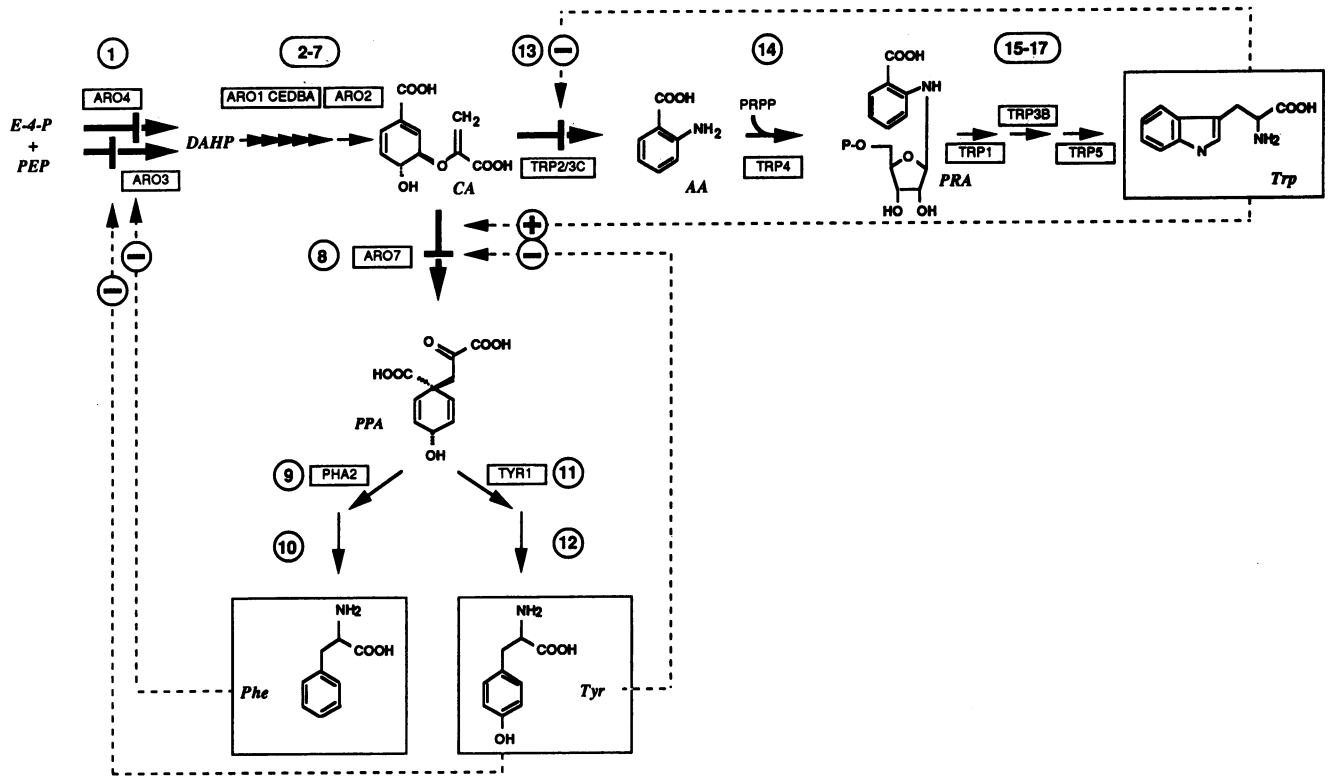

FIG. 1. Biosynthesis of aromatic amino acids and regulation of the enzymes in *S. cerevisiae*. The numbers correspond to the numbering of the enzyme reactions as used in the text. Abbreviations: CA, chorismate; AA, anthranilate; PPA, prephenic acid. Enzymes are indicated by their gene designations: *ARO3/ARO4*, DAHP synthases (EC 4.1.2.15); *ARO1C*, DHQ synthase (EC 4.6.1.3); *ARO1E*, DHQ dehydratase (EC 4.2.1.10); *ARO1D*, DHS dehydrogenase (EC 1.1.1.25); *ARO1B*, shikimate kinase (EC 2.7.1.71); *ARO1A*, EPSP synthase (EC 2.5.1.19); *ARO2*, chorismate synthase (EC 4.6.1.4); *ARO7*, chorismate mutase (EC 5.4.99.5); *PHA2*, prephenate dehydratase (EC 4.2.1.51); *TYR1*, prephenate dehydrogenase (EC 1.3.1.13); *TRP2*, anthranilate synthase (EC 4.1.3.27); *TRP3C*, glutamine amidotransferase; *TRP4*, anthranilate phosphoribosyltransferase (EC 2.4.2.18); *TRP1*, PRA isomerase; *TRP3B*, InGP synthase (EC 4.1.1.48); *TRP5*, tryptophan synthase (EC 4.2.1.20).

be used as herbicides and are also inhibitors of microbial growth (115).

The seven enzyme-catalyzed reactions of the shikimate pathway from erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) to chorismic acid are common for all aromatic amino acids. The series of reactions is invariable in all eukaryotic and prokaryotic organisms studied so far (81). Chorismic acid is the last common intermediate of the three aromatic amino acids and is distributed towards tryptophan, phenylalanine/tyrosine, and derivatives therefrom such as vitamin K, ubiquinone and *p*-aminobenzoate. Whereas the biosynthesis of tryptophan from chorismic acid proceeds in five invariable steps in all organisms so far studied, two separate routes of phenylalanine and tyrosine biosynthesis exist. Thus, phenylalanine may be formed from arogenate or from phenylpyruvate, whereas tyrosine synthesis may proceed from either arogenate or 4-hydroxyphenylpyruvate. In *S. cerevisiae* only the phenylpyruvate and the 4-hydroxyphenylpyruvate pathways have been found (81).

An outline of the biosynthetic pathway in *S. cerevisiae* from E4P and PEP through chorismate to the aromatic amino acids and the other metabolically important compounds is given in Fig. 1.

The aromatic amino acids are energetically the most costly amino acids for the living cell: 78 mol of ATP is required to synthesize 1 mol of tryptophan; the respective values for phenylalanine and tyrosine are 65 and 62 mol. On average this is approximately twice the energy required for any other amino acid (12). Accordingly, the concentration of these amino acids in the cell is among the lowest of all amino acids: in *S. cerevisiae* the total pool of phenylalanine, tyrosine, and tryptophan was determined as 0.6, 0.5 and 0.02 mM, respectively (61, 101).

Although the enzymatic steps involved in aromatic amino acid biosynthesis are very similar in all species studied so far (79), there are striking differences among various species in the genetic organization of the enzyme activities that catalyze the reactions and in their regulation (46, 203). For example, in the enteric bacterium *Escherichia coli*, all genes of the tryptophan branch of the pathway are arranged in the well-studied tryptophan operon that permits simultaneous regulation of gene expression by repression and attenuation (206). In contrast, the tryptophan genes are scattered over the genome in all eukaryotic organisms studied to date, and therefore each of them requires its own regulatory signal sequences (98).

Some of the encoded enzymes appear to be more highly organized in eukaryotic than in prokaryotic microorganisms. Different fusion patterns have produced multifunctional enzymes with different combinations of activity domains (98). In the eukaryotic microorganisms studied, the activity domains are located on fewer polypeptide chains encoded by

TABLE 1. Genes and enzymes for the biosynthesis of chorismate in *S. cerevisiae*

Reaction	Compound	Enzyme designation	Gene	Chromosome	mRNA length (kb)	Polypeptide size (kDa)[a]
	PEP + E4P					
1	↓	DAHP synthase (EC 4.1.2.15)	*ARO3*	IV	1.2	41 (370 aa)
			ARO4	II	1.4	39 (367 aa)
	DAHP					
2	↓	DHQ synthase (EC 4.6.1.3)	*ARO1C*	IV		175 (1,588 aa): aa 1–392
	DHQ					
3	↓	DHQ dehydratase (EC 4.2.1.10)	*ARO1E*	IV		aa 1059–1293
	DHS					
4	↓	DHS dehydrogenase (EC 1.1.1.25)	*ARO1D*	IV		aa 1306–1588
	Shikimate					
5	↓	Shikimate kinase (EC 2.7.1.71)	*ARO1B*	IV		aa 886–1059
	Shikimate 3-phosphate					
6	↓	EPSP synthase (EC 2.5.1.19)	*ARO1A*	IV		aa 404–886
	EPSP					
7	↓	Chorismate synthase (EC 4.6.1.4)	*ARO2*	VII	1.4	
	Chorismate					
	↓					
	Phe, Tyr, Trp, ubiquinone, *p*-aminobenzoate, vitamin K					

[a] aa, amino acid.

fewer genes than in most prokaryotes. An impressive example is the pentafunctional arom enzyme, which is found in numerous lower eukaryotes and which catalyzes reactions 2 to 6 of the shikimate pathway (Fig. 1). In *S. cerevisiae* the arom enzyme is encoded by the *ARO1* gene (58, 114). In contrast, the genes encoding the corresponding activities of *E. coli* are widely scattered about the genome, encoding five separable enzymes (159). The diversity in the patterns of gene and enzyme organization found in different species is a remarkable feature of the arom system (203).

The aromatic amino acid biosynthesis in *S. cerevisiae* is controlled by two principal mechanisms: (i) regulation of enzyme synthesis by the regulation of gene expression, and (ii) regulation of the enzyme activities that control the carbon flow.

(i) At the transcriptional level, most of the structural genes of the aromatic amino acid biosynthesis in *S. cerevisiae* are regulated by the transcriptional activator GCN4 (10, 83, 87, 94, 191). The GCN4 protein is the regulator of a complex regulatory network, known as the general amino acid control, which couples transcriptional derepression of at least 30 structural genes involved in multiple amino acid biosynthetic pathways (87, 144, 172). (ii) At the enzyme level the carbon flow is controlled mainly by modulating the enzyme activities at the first step and at the branch points. In general, the end products of the major terminal pathways, phenylalanine, tyrosine, and tryptophan, serve as sensors to control carbon flow (Fig. 1).

GENE-ENZYME RELATIONSHIPS

In *S. cerevisiae*, 12 genes encoding enzymes for 15 of the 17 reactions in the biosynthesis of the three aromatic amino acids have been described (Fig. 1; Tables 1 to 3). The number of genes encoding the aminotransferases, which catalyze the final steps in the phenylalanine and tyrosine branches, is as yet unknown.

Shikimate Pathway

The seven enzyme-catalyzed reactions of the common shikimate pathway leading to the branch point compound chorismic acid are encoded by four genes. Table 1 summarizes some features of the genes and enzymes involved in the biosynthesis of chorismate in *S. cerevisiae*.

***ARO3* and *ARO4*: DAHP synthase.** 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) carries out the initial step in the shikimate pathway, which is the condensation of PEP and E4P to form DAHP in a reaction closely analogous to an aldol condensation (for a review, see reference 79).

In the yeast *S. cerevisiae*, two isoenzymes of DAHP synthase exist, one of which is feedback inhibitable by L-phenylalanine and the other by L-tyrosine (117). Other organisms such as *E. coli* (36) or the filamentous fungus *Neurospora crassa* (145) possess three DAHP synthases, each one regulated by one of the three aromatic amino acids. Meuris et al. (127) and Teshiba et al. (190) isolated *aro3* and *aro4* mutations bearing deficiencies in the tyrosine- and phenylalanine-sensitive DAHP synthases, respectively. The corresponding genes, *ARO3* and *ARO4*, are located on different chromosomes (*ARO3* on chromosome IV and *ARO4* on chromosome II) (111a). The two genes were cloned, and they encode a 1.2- and a 1.4-kb mRNA, respectively (111a, 154, 155, 190). From the *ARO3* DNA sequence one can predict a protein of 370 amino acids with a calculated molecular mass of 41 kDa (154); the *ARO4* sequence predicts a polypeptide of 367 amino acids with a molecular mass of 39 kDa (111a). The amino acid sequences of the two genes show strong overall similarity (75% according to the method proposed by Gribskov and Burgess [72]) including 225 identical amino acid residues. A high degree of similarity (65 to 71%) is also found with the three DAHP synthases of *E. coli* (52, 174, 214).

The *ARO3*-encoded enzyme was purified to apparent homogeneity and has a molecular mass of 42 kDa, corre-

TABLE 2. Genes and enzymes for the biosynthesis of phenylalanine and tyrosine in *S. cerevisiae*

Reaction	Compound	Enzyme designation	Gene	Chromosome	mRNA length (kb)	Polypeptide size (kDa)[a]
	Chorismate					
8	↓	Chorismate mutase (EC 5.4.99.5)	*ARO7*	XVI	0.95	30 (256 aa)
	Prephenate					
9	↓	Prephenate dehydratase (EC 4.2.1.51)	*PHA2*	XIV		
	Phenylpyruvate					
10	↓	Phenylalanine aminotransferase				
	Phenylalanine					
	Chorismate					
8	↓					
	Prephenate					
11	↓	Prephenate dehydrogenase (EC 1.3.1.13)	*TYR1*	II		52 (441 aa)
	4-Hydroxyphenylpyruvate					
12	↓	Tyrosine aminotransferase				
	Tyrosine					

[a] aa, amino acid.

sponding to the predicted molecular mass deduced from the DNA sequence (156) (see below).

***ARO1*: arom pentafunctional enzyme.** In *S. cerevisiae* the central five steps of the shikimate pathway (reactions 2 to 6 in Fig. 1 and Table 1) are catalyzed by a pentafunctional enzyme, the arom multifunctional enzyme, which is encoded by the *ARO1* gene located on chromosome IV (54, 114, 133). The protein sequence deduced from the DNA sequence corresponds to a polypeptide of 1,588 amino acids with a calculated molecular mass of 175 kDa (58). The yeast *ARO1* DNA fragment also complements the corresponding *aroA*, *aroB*, *aroD*, and *aroE* mutants of *E. coli* (114). Functional regions within the polypeptide chain have been identified by comparison with the sequences of the five separate monofunctional *E. coli* enzymes whose activities correspond to those of the arom multifunctional enzyme (159). Accordingly, the pentafunctional arom enzyme is a mosaic of monofunctional domains connected by some extra amino acid residues as linkers. The arrangement of the domains in the corresponding *ARO1* gene does not correlate with the succession of the corresponding catalyzed reactions in the pathway (58) (Table 1).

The 5-dehydroquinate (DHQ) synthase (EC 4.6.1.3) catalyzes the second reaction in chorismate biosynthesis, which results in cyclic DHQ after removal of a phosphate and an internal oxidation reaction. In an alignment of the DNA-derived protein sequence, the first 392 amino acid residues of the *ARO1* gene are similar to the *E. coli aroB*-encoded DHQ synthase (129). There is 36% identity between the two sequences; including two subdomains of greater similarity (58).

The 3-dehydroquinate (DHS) dehydratase (EC 4.2.1.10) catalyzes the reaction that converts DHQ into DHS and introduces the first double bond of the aromatic ring (reaction 3). Twenty-one percent of amino acids 1059 to 1293 of the arom enzyme are identical to amino acids in the corresponding *E. coli aroD* gene product (57). Confirmation that this region of the *S. cerevisiae* sequence truly encodes the DHQ dehydratase activity is provided by the presence of a pentadecapeptide of the corresponding *N. crassa* enzyme which is part of the active site of the enzyme (58).

Dehydroshikimate is converted to shikimate by the dehydroshikimate (DHS) dehydrogenase (EC 1.1.1.25) catalyzing the fourth step of the pathway. Shikimic acid gave its name to the pathway and was first described as a natural product from the plant *Illicium religiosum*. It was from the Japanese

TABLE 3. Genes and enzymes for the biosynthesis of tryptophan in *S. cerevisiae*

Reaction	Compound	Enzyme designation	Gene	Chromosome	mRNA length (kb)	Polypeptide size (kDa)[a]
	Chorismate + glutamine					
13	↓	Anthranilate synthase (EC 4.1.3.27)	*TRP2*	V	1.8	60 (528 aa)
		(glutamine amidotransferase)	*TRP3C*	XI	1.75	54 (484 aa): aa 1–206
	Anthranilate + PRPP					
14	↓	Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	*TRP4*	IV	1.4	41 (380 aa)
	PRA					
15	↓	PRA isomerase	*TRP1*	IV	0.8–1.0	28 (224 aa)
	CDRP					
16	↓	InGP synthase (EC 4.1.1.48)	*TRP3B*	XI	1.75	54 (484 aa): aa 218–484
	InGP + serine					
17	↓	Tryptophan synthase (EC 4.2.1.20)	*TRP5*	VII		77 (707 aa)
	Tryptophan					

[a] aa, amino acid.

name of this plant, shikimi-no-ki, that the name shikimic acid was derived (79). In the early 1950s, however, it was revealed that shikimic acid was an obligatory intermediate in the pathway from carbohydrate to the aromatic amino acids (53, 177).

The C-terminal domain of the arom polypeptide (residues 1306 to 1588) is similar to the *E. coli aroE* gene product DHS dehydrogenase (9), with a 25% identity of amino acids.

The shikimate kinase (EC 2.7.1.71) catalyzes the fifth reaction, the formation of shikimate-3-phosphate from shikimate and ATP. A similarity of 23% with the *E. coli aroL* gene product (130) extends from residues 886 to 1059 on the arom peptide. There is one well-conserved region between residues 895 and 909 corresponding to the ATP-binding site of various enzymes.

The 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) condenses shikimate-3-phosphate and a second molecule of PEP to produce EPSP (reaction 6). This enzyme is the target of the commercially important herbicide glyphosate (*N*-phosphomethylglycine) (8), which is widely used as a nonselective herbicide and, in addition, is an inhibitor of microbial growth (115). The finding that glyphosate is antagonized by one or more of the aromatic amino acids is true for many organisms including prokaryotes, algae, and plants (71). The yeast EPSP synthase domain is located between residues 404 and 866 and is the best conserved of the five arom domains when compared with the corresponding *E. coli* domains. It shares an overall 38% identity with the *E. coli aroA* gene product (59), with two subdomains of higher similarity separated by a region with no similarity.

Similar arom enzymes to those in *S. cerevisiae* seem to be restricted to the fungi and the euglenoids (6), whereas in plants and bacteria several separable enzymes encoded by genes in different arrangements have been found (203). Besides *S. cerevisiae*, arom-encoding genes have been isolated from other ascomycetes such as the yeast *Schizosaccharomyces pombe* (141) and *Aspergillus nidulans* (40). The *N. crassa* enzyme was shown to be a dimer consisting of two identical pentafunctional polypeptides (45, 68, 113).

***ARO2*: chorismate synthase.** Finally, chorismate is generated in reaction 7 by removal of a phosphate and introduction of a second double bond by chorismate synthase (EC 4.6.1.4). In *S. cerevisiae* the enzyme is encoded by the *ARO2* gene (54), located on chromosome VII (133). The gene was recently cloned and codes for a 1.4-kb mRNA (100a).

Phenylalanine-Tyrosine Branch

Chorismic acid is the last common intermediate of the three aromatic amino acids and is distributed toward the phenylalanine-tyrosine and the tryptophan branches. In addition, the chorismate pool in the cell is necessary for the synthesis of other aromatic compounds such as vitamin K, ubiquinone, or *p*-aminobenzoate. Synthesis of these compounds will not be discussed. In the phenylalanine-tyrosine sine branch of the pathway, chorismate is converted to prephenate, which is the last common intermediate before the pathway branches again toward either phenylalanine or tyrosine (Fig. 1). The phenylalanine-tyrosine branch includes five enzyme reactions. The genes for three of these reactions have been identified. Since no mutants for transamination of tyrosine or phenylalanine were found, the number of aminotransferases catalyzing the final step in these two branches is unknown. Table 2 summarizes some features of the genes and enzymes involved in the biosynthesis of phenylalanine and tyrosine in *S. cerevisiae*.

***ARO7*: chorismate mutase.** The first step in the phenylalanine-tyrosine branch which is still common in all organisms studied so far is the intramolecular rearrangement of the enolpyruvyl side chain of chorismate to yield prephenate (reaction 8). The reaction is formally analogous to a Claisen rearrangement and is catalyzed by chorismate mutase (EC 5.4.99.5).

The *S. cerevisiae ARO7* gene located on chromosome XVI (133) encodes a monofunctional chorismate mutase (110, 170), a situation also found in other yeasts (21), in different plants (67), and in bacteria such as *Bacillus subtilis* Marburg (118) and *Streptomyces aureofaciens* (69, 70). The yeast *ARO7* gene was cloned (13, 170) and was shown to be identical to a gene necessary for growth in hypertonic medium, *OSM2* (13). The reason for this connection between *ARO7* and osmotic stability is unclear.

ARO7 encodes a 0.95-kb mRNA. DNA sequencing determined a 771-bp open reading frame (ORF) capable of encoding a protein of 256 amino acids (170). The protein was purified (168), and the monomer size of 30 kDa corresponds to the predicted size deduced from the DNA sequence.

The yeast chorismate mutase is not only feedback inhibited by tyrosine, one of the two end products of this biosynthetic branch, but also strongly activated by tryptophan (110), the end product of the other branch. The regulation of the enzyme is discussed in more detail below. The monofunctional *B. subtilis* Marburg chorismate mutase is inhibited by prephenate but unaffected by tyrosine, phenylalanine, or tryptophan (119), and the *S. aureofaciens* enzyme activity is unregulated (70). Other investigated organisms, such as *E. coli*, use two bifunctional enzymes: a chorismate mutase-prephenate dehydratase (*pheA*) that is feedback inhibited by phenylalanine and a chorismate mutase-prephenate dehydrogenase (*tyrA*) that is feedback inhibited by tyrosine (50, 51). In both cases the N-terminal part of the bifunctional enzyme carries the chorismate mutase activity (96, 122). In contrast to other enzymes in this pathway, no significant similarity between the monofunctional yeast chorismate mutase and the corresponding domains of the two bifunctional *E. coli* enzymes was found (170).

***PHA2* and *TYR1*: prephenate dehydratase and prephenate dehydrogenase.** For the biosynthesis of phenylalanine and tyrosine, two alternative routes exist in nature, a phenylpyruvate and a 4-hydroxyphenylpyruvate route, respectively, or an L-arogenate route. Virtually every conceivable combination of possible enzyme arrangements has been found: whereas *S. cerevisiae* or *E. coli* use only the phenylpyruvate–4-hydroxyphenylpyruvate routings (116, 117, 159) (Fig. 1), plants utilize arogenate as the sole precursor of both phenylalanine and tyrosine (23). A widespread combination, e.g., in cyanobacteria, is an arogenate-to-tyrosine/phenylpyruvate-to-phenylalanine pathway. In other bacteria, e.g., *Pseudomonas aeruginosa*, the two alternative pathways to phenylalanine and/or tyrosine coexist (81).

Little is known about the final phenylalanine branch in *S. cerevisiae*. Prephenate dehydratase (EC 4.2.1.51) catalyzes the first reaction, the conversion of prephenate to phenylpyruvate (reaction 9). Lingens et al. (116) isolated mutants with mutations in the prephenate dehydratase-encoding gene *PHA2*, which is located on chromosome XIV (133). No mutants have been isolated for the final reaction (reaction 10), the transamination of phenylpyruvate to phenylalanine by an aminotransferase. This might be explained by the finding that in other organisms there are numerous

aminotransferases, often exhibiting a rather broad range of substrate specificities (159).

The yeast *TYR1* gene (116) in the final tyrosine branch encodes prephenate dehydrogenase (EC 1.3.1.13), which catalyzes the oxidative decarboxylation and dehydratation of prephenate (reaction 11) that results in 4-hydroxyphenylpyruvate. The gene is localized on chromosome II (133). The *TYR1* gene was cloned and contains an ORF of 441 codons for a calculated protein of 52 kDa. There is a consensus sequence for an NAD-binding site within the first 45 amino acids that is typical for dehydrogenases (121). As in the phenylalanine branch, the aminotransferase(s) involved in the final transamination of 4-hydroxyphenylpyruvate to tyrosine (reaction 12) has not yet been characterized.

Tryptophan Branch

The tryptophan branch proceeds in five steps from chorismate to tryptophan, using a set of seven enzyme activity domains encoded by five genes in *S. cerevisiae*. The activities are organized into four separable enzyme components. Reactions 13 and 16 are catalyzed by a bifunctional complex composed of two subunits encoded by the genes *TRP2* and *TRP3*. Table 3 summarizes some features of the genes and enzymes involved in the biosynthesis of tryptophan in *S. cerevisiae*.

***TRP2* and *TRP3C*: anthranilate synthase complex.** The first step of the tryptophan branch (reaction 13) is the conversion of chorismate to anthranilate with glutamine as the donor of the amino group. The enzyme that catalyzes this reaction is called anthranilate synthase (EC 4.1.3.27) and is feedback inhibitable by the end product of the branch, tryptophan. Two genes, *TRP2* and *TRP3*, located on chromosome V and XI, respectively, are necessary to encode the enzyme. Both genes were cloned (3) and sequenced (212). *TRP2* and *TRP3* encode 1.8- and 1.75-kb mRNAs (28, 212) with open reading frames of 528 and 454 codons for calculated polypeptides of 60 and 54 kDa, respectively (212). Purification of the enzyme complex confirmed these data and revealed a molecular mass for the subunits of 64 and 58 kDa, respectively (160) (see below).

The *TRP2* gene product encodes an anthranilate synthase activity that is able to form anthranilate, with considerably reduced efficiency, only if provided with ammonia instead of glutamine (Gln) (160). It also contains the tryptophan-binding site for feedback inhibition. An amino acid sequence alignment between the *S. cerevisiae TRP2* gene and corresponding *E. coli trpE* gene (142) exhibits only a limited amino acid sequence similarity: nine short conserved regions can be found, with eight of them located in the C-terminal half of the enzyme. A comparison of 16 amino acid sequences derived from the corresponding nucleotide sequences of different species, namely 12 anthranilate synthases and 4 sequences of a similar enzyme, *p*-aminobenzoate synthase, also shows high variability in the amino-terminal half of the molecule and conserved regions in the distal part of the molecule (48).

The *TRP3* gene encodes two enzyme activities. The first of these, encoded by the N-terminal part of the enzyme, is a glutamine amidotransferase activity, which provides the nitrogen from glutamine for the synthesis of anthranilate (2, 212). An alignment of the first 206 amino acids of the *TRP3* product with 195 amino acids of the *E. coli trpG* product (208) shows 38% identity (212). There is a 60% identity with the corresponding gene product of *N. crassa* (167). Similar values also have been found with four other known fungal sequences, including *A. nidulans*, *A. niger*, *Penicillium chrysogenum*, and *Phycomyces blakesleeanus* (48). A 74% identity exists with the corresponding gene product of the yeast *Hansenula polymorpha* (163). Whereas the *E. coli trpG* gene is fused to the gene encoding anthranilate phosphoribosyltransferase, which catalyzes the next reaction (reaction 15), the yeast and other fungal glutamine amidotransferases are fused to the indole-3-glycerol-phosphate (InGP) synthase catalyzing the fourth step of the tryptophan branch (see the section *TRP3B*: InGP Synthase, below).

***TRP4*: phosphoribosyltransferase.** The second reaction of the tryptophan branch is the transfer of a 5-phosphoribosyl moiety from 5-phosphoribosylpyrophosphate to the amino group of anthranilate, resulting in phosphoribosylanthranilate (PRA). The reaction is catalyzed in *S. cerevisiae* by a monofunctional anthranilate phosphoribosyltransferase (EC 2.4.2.18) encoded by the *TRP4* gene. The *TRP4* gene, which is located on chromosome IV, was cloned and encodes a 1.4-kb mRNA (63, 64) which contains an ORF of 380 codons for a putative protein of 41 kDa (64). Purification of the enzyme revealed a monomer size of 42 kDa on a denaturing polyacrylamide gel (93).

The product of the corresponding *E. coli* gene, *trpD*, the C-terminal part of a fused *trpG-D* gene (208), is only partially similar to the yeast protein, with 15% overall identity; however, 50 and 44% identities exist in two separate domains of about 50 amino acids each (64, 98). No other fungal amino acid sequences are as yet available for comparison.

***TRP1*: PRA isomerase.** PRA isomerase catalyzes a practically irreversible Amadori rearrangement, the third step (reaction 15) in the tryptophan branch of the pathway. The aminoglycoside PRA undergoes an internal redox reaction, which results in the ketone carboxyphenylamino-1-deoxyribulose 5-phosphate (CDRP).

The corresponding gene in *S. cerevisiae* is the *TRP1* gene located on chromosome IV. The yeast *TRP1* gene was one of the early yeast genes that was cloned by complementation of the corresponding *E. coli* mutant (180, 188). The gene attracted special interest, because an *ARS* (autonomous replication sequence) site is located adjacent to the 3′ end of the *TRP1* gene, which allows the use of the yeast *TRP1-ARS* fragment as a selectable marker in many extrachromosomally maintained yeast vectors. The *TRP1* gene encodes a heterogeneous mRNA of 0.8 to 1.0 kb (27, 28) with an ORF of 224 codons for a calculated protein of 28 kDa (194). Purification of the enzyme (28) revealed a molecular mass of 23 kDa for the protein.

Whereas *S. cerevisiae* exhibits a monofunctional PRA isomerase, the situation is different in other ascomycetes: several genes have been cloned that encode a trifunctional polypeptide with the arrangement NH_2-glutamine amidotransferase-InGP synthase-PRA isomerase-COOH. Genes with this arrangement have been cloned from different fungi including *N. crassa* (167), *A. nidulans* (137), *A. niger* (105), *Cochliobolus heterostrophus* (195), *Penicillium chrysogenum* (166), and *Phycomyces blakesleeanus* (164). For the basidiomycete *Schizophyllum commune* a bifunctional NH_2-InGP synthase-PRA isomerase-COOH was proposed (139). In *S. cerevisiae* two genes encode these three enzymatic functions: *TRP3* encodes the bifunctional NH_2-glutamine amidotransferase-InGP synthase-COOH, and *TRP1* encodes the monofunctional PRA isomerase. The same pattern of two separated genes was found only in a series of *Saccharomyces* strains (25), in *Kluyveromyces lactis* (178), and in *Candida maltosa* (20). A single trifunctional gene seems to be present in *Hansenula* spp. and *S. pombe* (18, 27a, 192).

The apparent restriction of this arrangement to a small group of fungi suggests that the *S. cerevisiae TRP1* gene may have been separated from a trifunctional gene found in other ascomycetes by a translocation that occurred relatively recently in the evolution of yeasts. More argument for this hypothesis are summarized in Regulation of the Anthranilate Synthase Complex (below).

In many bacteria including *E. coli*, the PRA isomerase is fused to the InGP synthase. A monofunctional PRA isomerase, as in *S. cerevisiae*, was found in fluorescent pseudomonads. There is a 25% identity between the amino acid sequences of the *E. coli* and *S. cerevisiae* enzymes, a 40 to 45% identity with those of the various filamentous fungi, and a 53% identity between those of *S. cerevisiae* and *K. lactis* (178). The last value is low if one considers that both are similar species of budding yeasts and that other genes analyzed so far (e.g., the two *URA3* genes encoding orotidine-5′-phosphate decarboxylase) share a much higher proportion (80%) of identical amino acids (178).

The crystal structure of the fused *E. coli* protein NH_2-InGP synthase-PRA isomerase-COOH has been solved (162): both domains are eightfold α/β barrels resembling triose phosphate isomerase (TIM barrel). Priestle et al. (162) aligned all known sequences and demonstrated that the predicted α-helices, turns, and β-strands were coincident with the known ones of *E. coli*. Luger et al. (120) demonstrated, by using mutated yeast *TRP1* genes encoding circularly permutated variants, that the yeast PRA isomerase also folds in a TIM barrel.

***TRP3B*: InGP synthase.** The fourth step of the tryptophan pathway (reaction 16) is the decarboxylation of CDRP and the closure of the second ring to yield InGP. The reaction is catalyzed by InGP synthase (EC 4.1.1.48), the second domain of a bifunctional enzyme which is encoded in *S. cerevisiae* by the 3′ half of the *TRP3* gene (*TRP3B*).

Sequence alignment reveals that codons 218 to 484 of the ORF on the 1.75-kb *TRP3* mRNA correspond to this domain. The degree of identity to the *E. coli* domain *trpC* (208) is 32% (212) and is therefore higher in comparison with the PRA isomerase and lower in comparison with the glutamine amidotransferase of *E. coli*. A multisequence alignment reveals that all InGP synthases are more highly conserved than the PRA isomerases and also fold in a TIM barrel. The main areas of conservation are located in the β-strands of the barrel, and some are located in the turns at the carboxyl ends of the strands, whereas the α-helical regions seem generally more variable (48, 162).

***TRP5*: tryptophan synthase.** In the final reaction of the tryptophan branch (reaction 17) the InGP is cleaved and indole is condensed with serine to yield tryptophan. *E. coli* tryptophan synthase (EC 4.2.1.20) is one of the most intensively studied enzymes of the pathway. The enzyme has two active sites, one for the aldol cleavage of InGP to yield indole and glyceraldehyde-3-phosphate, and the other for the synthesis of L-tryptophan from indole and serine (for a review, see reference 128). In most organisms both functions are on two separate polypeptide chains. In *S. cerevisiae* a single gene, *TRP5*, located on chromosome VII (133) encodes the bifunctional tryptophan synthase protein with a deduced amino acid sequence of 707 amino acids and a calculated molecular mass of 77 kDa (213). Purification of the enzyme reveals a size for the monomer of 76 kDa (55).

The N-terminal domain of 239 amino acids of the yeast enzyme is similar to the *E. coli* α-subunit (29% identity); the distal 389 amino acids correspond to the β-subunit (50% identity). This order of segments is the reverse of the chromosomal order characteristic of all prokaryotes that have been examined. A single tryptophan synthase with the same gene domain order as in *S. cerevisiae* was also found in the filamentous fungus *N. crassa* (34). The two fungal enzymes show strong similarity when compared on the deduced amino acid sequence level: the A domains have 54% identity; the B domains have 75% identity (34). An alignment of known DNA sequences suggests that the basic three-dimensional structure is probably the same whether the subunits are fused or not (49). It is known from the crystal structure that the α-subunit is in the form of a TIM barrel, as are the PRA isomerase and the InGP synthase (99). The 45-amino-acid connector region of *S. cerevisiae* has less than 25% identity to the 54 amino acids of *N. crassa*, although secondary-structure analysis predicts that both connectors would be α-helical.

REGULATION OF ENZYME SYNTHESIS

The amount of a certain enzyme in a cell is determined by the rate of protein synthesis and degradation. Protein synthesis is determined by gene expression, which includes various parameters such as the initiation, elongation, and termination of transcription; the capping, processing, and polyadenylation of the transcript; the packaging into ribonucleoprotein particles; the transport of the mRNA from the nucleus into the cytoplasm; and, finally, the initiation, elongation, and termination of translation.

Not much is known about the regulation of protein degradation of the enzymes of the aromatic amino acid biosynthesis. There is no evidence, however, that there are significant differences in the degradation rates of the different enzymes.

The regulation of enzyme synthesis of the aromatic amino acid biosynthetic genes takes place mainly at the level of transcription and specifically at the initiation of transcription. Additional regulatory points are the mRNA half-life and translational control of the level of the main transcriptional regulator, the protein GCN4.

Regulation of Transcription

Transcription of the genes of the aromatic amino acid biosynthesis is regulated mainly at the 5′ end of the genes, where DNA-binding proteins determine the rate of initiation of transcription at the different promoters. As with most of the yeast genes, the aromatic amino acid biosynthetic genes carry no introns and hence cannot be regulated by splicing (62). As in higher eukaryotic cells, the mature yeast mRNAs possess poly(A) tails, which seem to be either the product of processing and polyadenylation or a coupling of termination and polyadenylation (35, 149, 150). Besides the initiation of transcription, the decay rate of the mRNA can influence gene expression (29).

The transcriptional regulation of the amino acid biosynthetic genes in the yeast *S. cerevisiae* includes three important features which are different from those of the corresponding genes in a bacterium such as *E. coli*. (i) The yeast genes involved in amino acid biosynthesis are spread all over the genome (133) and are not organized in operons, as is the case for some biosynthetic pathways of *E. coli*. Therefore, the expression of all genes takes place independently and the initiation of transcription is performed on individual promoters. (ii) Yeast cells maintain a significant level of amino acid biosynthetic gene expression when amino acids are added to the growth medium or when large internal amino acid pools are present (11, 132). This relatively high level of transcrip-

tion—and, as a consequence, of enzyme synthesis—in the presence of amino acid excess has been named the basal-level control (11). Therefore, unlike bacteria, yeasts have relatively large intracellular amino acid pools (61, 101). (iii) Yeast cells respond to starvation for a single amino acid by turning on the transcription of at least 30 genes in unrelated amino acid biosynthetic pathways. For example, starvation for tryptophan leads not only to the derepression of the enzymes of the aromatic amino acid biosynthetic pathway, but also to the biosynthetic enzymes of arginine, histidine, isoleucine, leucine, lysine, serine, and threonine, and to different aminoacyl-tRNA synthetases. Therefore, the pathway of aromatic amino acid biosynthesis in *S. cerevisiae* is part of a complex regulatory network known as general control (87, 172). This cross-pathway regulation also exists in numerous other yeasts (21) and in other fungi such as *N. crassa* and *A. nidulans* (38, 158), enhancing the potential of the organisms to survive in an environment of external amino acid imbalance (144).

In many bacteria, including *E. coli*, transcription of the genes for their amino acid biosynthetic enzymes is repressed when the amino acids are present in sufficient amounts in the growth medium. Starvation for a single amino acid leads mainly to increased transcription of only the genes in the corresponding pathway. An additional control system, termed metabolic regulation, has been described for the aromatic amino acid biosynthesis in *E. coli* and seems to be independent of the presence or absence of these amino acids (159).

In *S. cerevisiae* at least six amino acid biosynthetic pathways, namely those for arginine, lysine, methionine, leucine, isoleucine, and valine, are, independently of or in addition to the general control system, also controlled by specific regulatory mechanisms (125). With the exception of arginine, these specific control mechanisms seem to operate at the transcriptional level. For the aromatic amino acid biosynthetic enzymes, however, no specific regulatory system has been found.

Initiation of transcription. During the last couple of years it has been demonstrated that the mechanisms necessary for the initiation of transcription at RNA polymerase II promoters are in principle conserved between yeasts and humans. Yeast transcriptional activators often have a related counterpart in other organisms, with a high degree of similarity in functionally important domains. In some cases it has been shown that the related proteins of the higher organism are able to complement defects in the corresponding yeast gene. In addition, some of these proteins have been shown to be oncogene products in the higher organism.

One example is the regulator protein GCN4 of *S. cerevisiae*, which is required for the response to amino acid starvation. GCN4 shares homology with the *jun* oncoprotein and the human *trans*-activator protein AP-1. The GCN4 DNA-binding domain can be exchanged for the *jun* DNA-binding domain, and the resulting chimeric protein is still active in *S. cerevisiae* (22, 184). GCN4 contains the leucine zipper structure responsible for dimerization, which is a characteristic feature of a whole class of DNA-binding proteins (4). GCN4 activates transcription in the general control system of the amino acid biosynthesis network of *S. cerevisiae* (89). As a result, derepressed specific enzyme levels of the gene products of the corresponding regulated genes are measured (132).

Typical amino acid biosynthetic promoters are dual promoters and hence can be regulated by two control systems, namely general (GCN4 dependent) and basal (11, 183). Whereas the general control promoter is active under conditions of amino acid starvation, the basal control promoter is not regulated by amino acids and is responsible for the high basal level of transcription of the amino acid biosynthetic genes, even when amino acids are present in the growth medium. There are some genes for which, in the absence of GCN4 protein, the basal promoter is also affected and which therefore depend on GCN4 protein for at least one component of their basal expression (see below).

Transcriptional regulation of a yeast RNA polymerase II promoter requires three kinds of *cis*-acting sequences, namely upstream, TATA, and initiator elements (reviewed in references 185 and 186).

(i) Upstream elements (or upstream activation sequences [UASs]) are target sites for various activator proteins; they work in a distance- and orientation-independent manner approximately 100 to 600 bp upstream from the transcription initiation site (75, 185, 186). In many respects, upstream elements resemble enhancer elements of higher eukaryotes.

Genes subject to a common control mechanism contain upstream elements that are in general similar in the DNA sequence that allows the binding of the same activator protein (for reviews, see references 73 and 74). For the general and basal control promoters in yeast amino acid biosynthetic genes, different upstream elements exist as binding sites for the various regulatory proteins controlling the basal or general control response.

The optimal promoter-binding site for the general control regulator GCN4 is the well-characterized palindrome 5′-ATGA(C/G)TCAT-3′ (10, 56, 83, 90, 94, 95, 148, 182). Such GCN4 recognition elements (GCREs) have been found repeated upstream of every analyzed structural gene subject to general control (reviewed in reference 87). The naturally occurring sites analyzed so far are not identical to the consensus sequence, but differ by 1 to 2 bp (186). The GCN4 protein binds general control promoters at all GCRE sequences (10). Deletion analysis of a number of these promoters has demonstrated that GCRE sequences are both necessary and sufficient for general control-mediated regulation of transcription in vivo (56, 181) and are therefore a class of upstream activation sequences (UAS). Little is known, however, about the interplay of multiple GCREs in a naturally occurring general control promoter in vivo. Some features of the analyzed GCN4-regulated promoters of the aromatic amino acid biosynthetic genes are summarized in Table 4.

A similar sequence, TGACTA, contained in the recognition element for the mammalian transcription factor AP-1, can interact with the yeast AP-1 homolog, yAP-1, a factor of unknown function, and stimulate transcriptional activation independently of GCN4 (78).

For the basal-level control of the histidine biosynthetic gene *HIS4*, the *trans*-acting factors BAS1 and BAS2 have been identified (11). The *BAS2* gene, which is identical to *PHO2* and *GRF10*, is repressed in its expression by phosphate and is autoregulated (146, 210, 211). PHO2-BAS2 binds to upstream elements of *HIS4*, the acid phosphatase gene *PHO5*, and the aromatic amino acid gene *TRP4* promoter (11, 26, 193, 200) (see below).

For the *HIS3* gene it has been shown that upstream elements necessary for basal gene expression are poly(dA-dT) sequences, and it has been proposed that these act by excluding nucleosomes (187). An oligo(dA-dT)-binding protein might be involved in this kind of basal gene expression. Such a protein of 248 residues, named datin, has been purified and requires at least 9 to 11 bp of oligo(dA-dT) for

TABLE 4. Features of analyzed GCN4-regulated promoters of several aromatic amino acid biosynthetic genes of *S. cerevisiae*

Gene	GCRE				Initiation sites of transcription	
	Position and sequence[a]	Orientation	In vitro binding	Demonstrated function(s)	Basal	GCN4 dependent
ARO3	−180 GTGACTAAT −188	⟵	+	UAS_{GC}[b], basal control	−38, −31, −22, −8	As basal
ARO4	−312 ATGACTCAA −304	⟶	+	UAS_{GC}	−136, −88, −72	As basal
TRP2	−162 CTGACTCAT −155	⟶	ND[c]		−95, −59, −52, −49, −33	
TRP3	−162 TTGACTCAT −155	⟶	ND		−88, −80, −79, −77, −49, −23, −21	
TRP4	−246 ATGACTAAT −238 (GCRE1)	⟶	+	UAS_{GC}, PHO2-binding site	−127, −76	−31, −26, −12
	−166 TTGACTCTC −158 (GCRE2)	⟶	+	Together with GCRE3: UAS_{GC}, TATA box analog		
	−151 ATGATTCAT −143 (GCRE3)	⟶	+	Together with GCRE2: UAS_{GC}, TATA box analog		
TRP5	−233 GTGACTGGT −155	⟶	ND	Minor UAS_{GC}	−45, −28, −18	
	−108 ATGACTAAT −100	⟶	ND	Major UAS_{GC}		

[a] Mismatches with the consensus sequence are indicated by bold letters.
[b] UAS_{GC}, UAS sites functional in general control derepression.
[c] ND, not determined.

high-affinity DNA binding (205). The exact function of datin remains to be examined.

The GCN4 protein can, in specific cases, also regulate the basal expression of amino acid biosynthetic genes, as shown for several genes, including *ARO3* (155) (see below), *HIS4* (11), and *LEU2* (30).

(ii) As in mammalian promoters, TATA elements are necessary but not sufficient for accurate initiation of transcription in *S. cerevisiae* (60, 185). TATA elements are located close to mRNA initiation sites and mediate the first step in the pathway of transcription initiation by binding the general transcription factor TFIID (31, 32, 199). The yeast transcription factor TFIID is able to substitute for the corresponding HeLa cell TATA-binding protein (39). In contrast to higher eukaryotes, in *S. cerevisiae* the distance between TATA element and mRNA initiation site can vary between 40 and 120 bp (41). For the *HIS3* promoter there are different TATA elements for the GCN4-dependent promoter ($TATA_R$) and the basal promoter ($TATA_C$). For the regulatory TATA element ($TATA_R$) in the *HIS3* promoter, a saturation mutagenesis experiment has been carried out, and it appears that only the sequences TATAAA and (to a lesser extent) TATGTA or TATATA are functional in vivo (42). Functional TATA elements are located between UAS and mRNA initiation site(s) (187).

Three possible models have been proposed to explain how specific activator proteins could interact with the basic transcription machinery. In one model the specific activator interacts with the TATA-binding factor TFIID to facilitate assembly of a preinitiation complex. The assembled preinitiation complex would then interact with and activate RNA polymerase II. In another model the activator would perform some step after the assembly of the general factors into a preinitiation complex (31), e.g., a direct interaction of the activator with RNA polymerase II (7, 14, 24). In a third model an additional protein, termed an adaptor or mediator, is necessary to interact with the specific activator, TFIID, and with RNA polymerase II (17, 102).

(iii) The transcription initiator element is the primary determinant of the location where transcription begins in *S. cerevisiae* (for a review, see reference 186). Yeast mRNA initiation sites are determined primarily by specific initiator sequences, not by the distance from the TATA element as in many genes of higher eukaryotes (41). An initiator as a transcription control element is also described for the lymphocyte-specific terminal deoxynucleotidyltransferase gene (176). Two types of start site selection patterns have been found in *S. cerevisiae* GCN4-controlled genes when transcription start sites of the basal expression were compared with the start sites of the GCN4-driven transcription (140). Only a single start site of transcription has been found in the *HIS4* promoter region when the 5′ ends of basal controlled transcripts, as well as GCN4-controlled transcripts, were determined (140). The *HIS3* promoter initiates transcription equally from two sites, at +1 and +12, during basal expression. The GCN4-driven transcription of this promoter then preferentially initiates at the basal initiation site at +12 (41).

In the aromatic amino acid pathway of *S. cerevisiae*, four of the five *TRP* genes (132), the isogenes *ARO3* and *ARO4* (190), and the *ARO2* gene (100a) are derepressed under the general control system. The genes *TRP1*, *ARO7*, and *TYR1* are not derepressible by this system (28, 121, 169, 170). The regulation of the other genes in the pathway remains to be investigated. The arrangement of many of the elements described above can be compared, as the promoters of all five *TRP* genes, the *TYR1* gene, and the *ARO3*, *ARO4*, and *ARO7* genes have been cloned and sequenced (2, 64, 121, 154, 170, 194, 212, 213). Although little is known about the basal control aspect of these promoters, more and more data about the GCN4-regulated parts of the promoters are available. The following section summarizes the available data for several of these promoters, with the main focus on the GCN4-mediated regulation (Table 4).

(i) A single GCN4-binding site has different functions in the promoters of the isogenes *ARO3* and *ARO4*. Both isogenes (*ARO3* and *ARO4*) encoding DAHP synthases in *S. cerevisiae* respond equally well to the general control regulatory system. In fact, DAHP synthase activity can be increased sixfold under derepressing conditions, whereas, for example, *TRP*-encoded enzymes can be derepressed only two- to threefold (132, 190). Cells carrying only one intact isogene are phenotypically indistinguishable from a wild-type strain when grown on minimal medium.

In contrast to *ARO4* and to other genes of the pathway (28,

143), a drop in ARO3 enzyme activity is observed in a *gcn4* background. Concomitantly, the growth rate of an *ARO3 aro4 gcn4* strain is reduced by 50%. Growth and enzyme level can be restored by transforming the mutant strain with the *GCN4* gene on a low-copy plasmid, imitating a wild-type situation. The complete functional *ARO3* promoter comprises 231 bp and contains a −180 GTGACTAAT −188 binding site for GCN4 in an inverse orientation (154) (Table 4). This corresponds to a 2-bp mismatch with the optimal palindromic binding site ATGA(C/G)TCAT. Mutating the *ARO3* element to GTTACTAAT inhibits the binding of GCN4 and results in the same phenotype as that of the *ARO3 aro4 gcn4* strain, namely a decreased basal level of *ARO3* gene product and slow growth of a strain defective in its isogene, *ARO4*. In addition, *ARO3* gene expression cannot be increased under conditions of amino acid starvation (155). The amount of GCN4 protein present in repressed wild-type cells therefore seems to contribute to a basal level of *ARO3* gene expression.

As found for *ARO3* and a few other genes, including *ILV2* and *ARG4* (87), only a single GCN4-dependent UAS is found in the *ARO4* promoter (111a) (Table 4). This element, with the sequence 5′-ATGACTCAA-3′ in normal orientation (one mismatch to the consensus sequence), is located at positions −312 to −304. A second identical element was found in inverse orientation downstream of the *ARO4* ORF at positions +1297 to +1289, located only 185 bp downstream of the translational stop codon. The two elements form a perfect 9-bp inverted repeat with the coding sequence of the *ARO4* gene in between.

The upstream GCN4-binding site was shown to be the upstream activation site of the *ARO4* gene, which is necessary for GCN4-mediated transcription activation. Destroying this sequence does not affect the basal level of *ARO4* expression, but *ARO4* gene expression can no longer be increased under amino acid starvation conditions (155). The sequence elements responsible for the basal level of transcription have not yet been identified. The GCN4-binding site located downstream of *ARO4* has no function with respect to the *ARO4* gene, but is a functional UAS of another amino acid biosynthetic gene of histidine biosynthesis, *HIS7*, located immediately downstream. This configuration demonstrates one of the differences between UASs and mammalian enhancers (111a). Mammalian enhancers also function when located downstream of the gene, whereas a GCN4 site which is actually used in vivo is not able to do so.

The general control activator *GCN4* thus has two functions for these isogenes: (i) to maintain a basal level of *ARO3* transcription (basal control) in the presence of amino acids and (ii) to derepress the *ARO3* as well as the *ARO4* gene to a higher transcription rate under amino acid starvation conditions (general control).

Both promoters contain multiple initiation sites of transcription (Table 4). For *ARO3*, four major 5′ ends were mapped between positions −38 and −8 upstream of the ATG start codon (154). For *ARO4* the three major transcription initiation sites were localized further upstream of the translational start sites at positions −136, −88, and −72. Transcripts starting from all initiation sites are equally elevated under conditions of amino acid starvation by the general control system (111a, 154).

The four *ARO4* transcript ends were mapped 12 to 84 nucleotides upstream of the *HIS7* upstream element, suggesting that there is virtually no intergenic space between transcription termination and promoter elements of these two genes (111a).

(ii) *TRP2* and *TRP3* promoters. The products of the two genes *TRP2* and *TRP3* form a heterodimeric enzyme complex which consists of equimolar amounts of both polypeptide chains (160). Therefore, expression of the two genes must be coordinated. In both genes a GCN4 consensus sequence with a single mismatch is located in the promoter: at position −162 CTGACTCAT −155 for *TRP2* and −124 TTGACTCAT −116 for *TRP3* (Table 4). Several transcription start sites were mapped in both promoters (−95, −59, −52, −49, and −33 for *TRP2*; −88, −80, −79, −77, −49, −23, and −21 for *TRP3* (212). A comparison of the strengths of the two promoters has still not been undertaken.

(iii) Three GCN4-responsive elements have different functions in the *TRP4* promoter. The promoter of the *TRP4* gene of *S. cerevisiae*, coding for the enzyme anthranilate phosphoribosyltransferase (64) contains two putative UAS elements for the GCN4 protein. UAS1 comprises a single GCN4-binding site −246 (relative to the translational start site) ATGACTAAT −238, designated as GCRE1 (one mismatch), and UAS2 comprises two adjacent repeats, −166 TTGACTCTC −158 and −151 ATGATTCAT −143, designated as GCRE2 (three mismatches) and GCRE3 (one mismatch), respectively. UAS1 and UAS2 are both able to specifically bind the activator protein GCN4 in vitro (26) (Fig. 2; Table 4).

All three GCREs are required for a normal GCN4-dependent transcription activation but do not affect basal transcription. A promoter containing a mutation of either UAS1 (*gcre1*) or UAS2 (*gcre2-gcre3*) is no longer inducible by the GCN4 protein (134). The use of the *TRP4* promoter by GCN4 is reduced to approximately 30% when either GCRE2 or GCRE3 is mutated (134).

GCN4 has been shown to compete at the UAS1 site with another transcriptional regulator, PHO2/BAS2 (26). PHO2/BAS2 encodes a homeo-box protein (33, 173) homologous to genes involved in developmental regulation in many different species (65). Among other functions, PHO2/BAS2 appears to be closely involved in regulating P_i metabolism.

PHO2/BAS2 binds directly to the *PHO5*, *HIS4*, and *TRP4* promoters. In the *TRP4* promoter the PHO2/BAS2-protected region in vitro comprises approximately 20 nucleotides and completely overlaps the GCN4-protected UAS1 region. GCN4 and PHO2/BAS2 bind to UAS1 in a mutually exclusive manner. PHO2/BAS2 does not affect the basal level of *TRP4* expression, indicating that additional *cis*- or *trans*-acting factors are involved in basal *TRP4* expression. When PHO2/BAS2 competes with GCN4 at the UAS1 site of the *TRP4* promoter, it prevents *TRP4* derepression under conditions of simultaneous P_i and amino acid starvation (26). Whereas GCN4 mediates the response of the transcriptional apparatus to the environmental signal amino acid limitation, PHO2/BAS2 could be the phosphate sensor that adjusts the response to the availability of phosphate precursors for tryptophan biosynthesis. The physiological significance of this is apparent when it is considered that *TRP4* encodes a phosphoribosyltransferase, requiring 5-phosphoribosyl 1-pyrophosphate (PRPP) as a substrate. Therefore, repression of the GCN4-induced *TRP4* expression prevents more enzyme from being produced under conditions where PRPP as one of the substrates is limiting.

The mode of action of PHO2/BAS2 seems to differ depending on the context of the binding site of the corresponding target genes: PHO2/BAS2 and PHO4 are both necessary for *PHO5* and *PHO11* activation under conditions of phosphate starvation (151, 200, 209–211). In addition, PHO2/BAS2 has another function in another amino acid promoter.

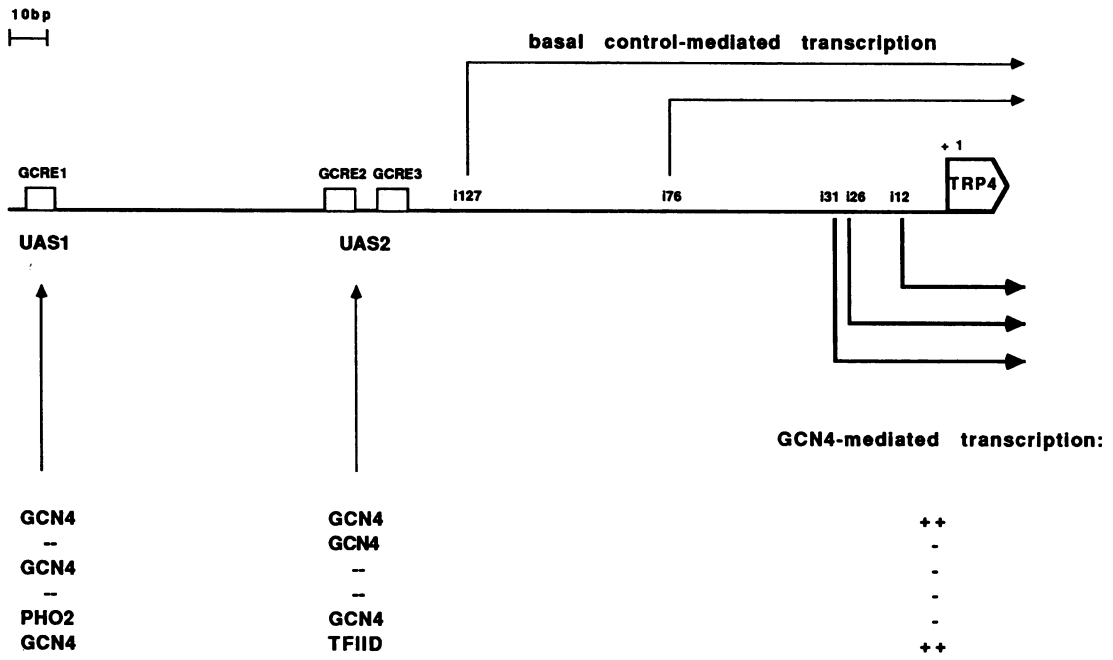

FIG. 2. *TRP4* promoter of *S. cerevisiae*. The effects of the binding or nonbinding of different combinations of the transcription factors GCN4, PHO2, or TFIID to UAS1 and UAS2 on the formation of GCN4-mediated transcripts (starting at initiation sites −31 [i31], −26 [i26], and −12 [i12] relative to the translational start codon) are shown. The expression of the basal control-mediated transcripts (starting at initiation sites −127 [i127] and −76 [i76]) is not affected under these conditions. See the text for details.

Together with BAS1, PHO2/BAS2 is necessary for basal-level expression of the amino acid biosynthetic gene *HIS4* (11). *HIS4* encodes a trifunctional histidine biosynthetic enzyme. The enzyme preceding the *HIS4* gene product is a phosphoribosyltransferase (encoded by the *HIS1* gene) that also requires PRPP as a substrate (101). It is still unclear whether the PHO2/BAS2 protein actually functions as a PRPP sensor in the cell.

Basal transcription and GCN4-mediated transcription initiate at different start sites at the *TRP4* promoter. A basal level of *TRP4* transcription results in transcripts starting at two sites at positions −127 (i127) and −76 (i76) relative to the translational start site. Under conditions of derepression by GCN4, the basal transcripts remain unchanged but three additional signals for mRNA start sites appear at positions −31, −26, and −12 (63, 134); these were named i31, i26, and i12 (Fig. 2). These additional transcripts correspond to the increase in transcription initiation as measured at the mRNA and enzyme levels and therefore represent the product of the GCN4-driven part of the *TRP4* promoter. These GCN4-dependent start sites are lacking when the GCN4 regulator is missing from the cell as well as when the GCN4-driven transcription of the *TRP4* gene is abolished by mutations in UAS1 or UAS2 or both. The use of the initiator elements i31, i26, and i12 by the transcription machinery is therefore solely dependent on the presence of the regulator protein GCN4 and its recognition elements in the *TRP4* promoter. These results show that basal transcription and GCN4-driven transcription of the *TRP4* gene are distinct events, even with respect to their transcription start sites.

Upstream activator proteins such as GCN4 or GAL4 normally stimulate transcription when bound upstream of a TATA element. No functional consensus TATA box (like TATAAA, TATATA or TATCTA [42]) is found in the *TRP4* promoter between UAS2 and the transcription initiation sites of the GCN4-mediated transcription (63). This observation led to the question of whether UAS2 is the analog of a TATA box for the GCN4-dependent *TRP4* promoter. To analyze this question, the *TRP4*-UAS2 element was exchanged for a consensus TATA box, TATAAA, which is identical to the GCN4-dependent TATA element in the *HIS3* promoter (42) and to the *CYC1-52* TATA element that binds to the transcription factor TFIID in vitro (76). Expression studies revealed that the newly introduced TATA box was able to restore the GCN4-driven transcription of a *TRP4* promoter with a mutated UAS2. The basal level of *TRP4* transcription was unaffected; transcription of the mutant *TRP4* promoter started mainly at i127 at repressed levels or in the absence of GCN4 protein, as was found for the wild-type promoter. Transcription initiated again at i31, i26, and i12 at high levels of GCN4 protein in the cell. The regulated initiator elements i31, i26, and i12 can therefore be used in two possible ways: (i) when transcription is driven by GCN4 acting synergistically via UAS1 and UAS2 (wild-type situation) and (ii) when transcription is dependent on GCN4 binding at UAS1 and on a TATA factor (presumably TFIID) binding to a TATA box situated at the position of UAS2. These results show that a consensus TATA box can functionally replace the UAS2 element in the GCN4-dependent *TRP4* promoter, suggesting that the UAS2 element has a function in vivo which is analogous to that of a TATA element in other eukaryotic promoters. A possibility which cannot completely be ruled out is that, in vivo, the three GCN4 sites serve as UASs that activate transcription in combination with a more downstream weak TATA element, which deviates somewhat from a TATAAA sequence. In addition, other factors with binding properties similar to GCN4, e.g., the transcriptional factor yAP-1 (78), might be involved in the function of UAS2. There is, however, additional evidence that the TATA factor function for the

general control transcription in the *TRP4* gene is fulfilled by GCN4. (i) GCN4 is able to interact specifically with RNA polymerase II in vitro (24). The region of GCN4 that contacts polymerase II resides within the DNA-binding domain of the protein and not the short acidic domain, which is required for transcriptional activation in vivo. (ii) GCN4 efficiently activates transcription in an artificial *GAL-HIS3* hybrid promoter in the absence of a TATA element, when bound close to the mRNA initiation site (43). These data demonstrate that there are other factors in *S. cerevisiae*, apart from the general transcription factor TFIID, that recognize sequences unrelated to the consensus TATA box but are nevertheless able to perform the role of TFIID.

Taken together, these results suggest that GCN4 can activate transcription by exhibiting two alternative functions within one natural promoter (Fig. 2).

(iv) Two putative GCN4 elements in the *TRP5* promoter. The *TRP5* promoter contains a putative GCN4-binding site −108 ATGACTAAT −100 relative to the translational start codon, which contains one mismatch in comparison with the consensus sequence (213). Deletion of this sequence abolishes the general control response (135, 136). A second sequence −233 GTGACTGGT −225 contains three mismatches and seems to be necessary for full derepression and also for basal-level expression (Table 4). In addition, other sequences in this region seem to be important for high basal-level expression (136). Three different transcriptional start sites, located at positions −45, −28 and −18, have been identified (213).

(v) The promoters of *ARO7*, *TRP1*, and *TYR1* are not regulated by GCN4. Although the general control system regulates most of the genes of the aromatic amino acid pathway, it does not seem to be necessary to regulate all biosynthetic genes in order to maintain the metabolic flow through the pathway. No GCN4 regulation was found for one gene of the tryptophan branch, *TRP1* (27, 28), and one gene of the tyrosine branch, *TYR1* (121). For the *ARO7* gene, encoding chorismate mutase, neither transcriptional regulation by the general control system nor specific regulation by aromatic amino acids was found (169, 170).

Similar to the promoters of other aromatic amino acid biosynthetic genes, however, a recognition element for the GCN4 transcriptional activator of amino acid biosynthesis in inverse orientation is located 425 bp upstream of the first transcriptional start point in the *ARO7* promoter (−496 ATGACTGAA −504; two mismatches with the consensus sequence). This element binds GCN4 specifically in vitro. Northern (RNA) analysis and determination of the specific enzyme activity reveal that the element is not sufficient to mediate transcriptional regulation by GCN4 in vivo (169). These data suggest that in addition to a consensus sequence capable of binding the GCN4 protein, other DNA-binding proteins or other parameters, such as chromatin structure, determine whether a recognition site for a transcription factor functions as a UAS. For the *ARO7* mRNA, three 5′ ends at positions −36, −56, and −73 relative to the start codon were mapped.

TYR1, the gene which encodes prephenate dehydrogenase, is also not regulated by the GCN4 system, and there is no consensus sequence for a GCN4-binding site located in the promoter. Instead, transcriptional regulation seems to be dependent on the presence or absence of phenylalanine in fusions between the *TYR1* promoter and the *CAT* (chloramphenicol acetyltransferase) reporter gene. Only a single transcriptional start site was found in the *TYR1* promoter, at position −70 relative to the translational start codon (121).

In the *TRP1* promoter the only site similar to a GCN4-binding site (−54 CTGACTATT −46) has three mismatches (194). This sequence is located between the transcription start sites (27) and is unable to bind GCN4 in vitro (94). Transcription from the *TRP1* promoter is initiated only approximately half as frequently as, for example, transcription from the *TRP3* promoter (28). The *TRP1* promoter generates two groups of transcripts (103) corresponding to five transcription initiation sites, organized in two clusters. The two longer transcripts start at positions −209 and −187, and the three shorter transcripts start at positions −36, −26, and −16. A transcriptional terminator element of unknown function located in the 5′ region upstream of the *TRP1* promoter seems to be essential for accurate *TRP1* gene expression. In partial *TRP1* promoters—lacking the terminator—transcription is initiated predominantly in adjacent upstream regions, resulting mainly in large, poorly translated transcripts. The effect can be suppressed by introducing artificial transcription barriers such as transcriptional terminators, centromere sequences, or yeast replicator (*ARS*) sequences in front of the truncated *TRP1* promoter (27). In addition, an A+T-rich region of dyad symmetry was proposed as a promoter element for the shorter transcripts (104). This element consists of two perfect inverted repeats of 12 A+T rich nucleotides separated by a 21-bp spacer and located between positions −81 and −125 upstream of the start codon. Deletions within this element abolished transcription of the shorter transcripts (104).

mRNA decay. mRNA decay is a potential control point of gene expression (for a review, see reference 29). In aromatic amino acid biosynthesis, differences in mRNA stability affect the relative steady-state level of mRNAs in at least one case. For three mRNAs of the aromatic amino acid biosynthetic pathway, the half-life was determined. For the larger *TRP3* and *TRP4* mRNAs the half-lives were determined as 11 and 14 min, respectively (28, 63). For the smaller *TRP1* transcript the half-life was 19 min, indicating a greater stability (28). Therefore, *TRP1* mRNA is approximately twice as stable as *TRP3* mRNA. Since transcription from the *TRP1* promoter initiates only approximately half as frequently as from the *TRP3* promoter, the final steady-state amounts of the two mRNAs without amino acid limitation are similar (28).

Translation

There is no evidence that translation of the structural genes of aromatic amino acid biosynthesis plays a major role in the regulation of gene expression. The expression of the main transcriptional regulator of the pathway, *GCN4*, however, which is the basis of the regulation of gene expression of most of the structural genes of the pathway, is regulated by amino acid availability at the translational level.

The transcriptional regulator of amino acid biosynthesis GCN4 is regulated at the translational level. The translational derepression of *GCN4* mRNA seems to be directly linked to major changes in the protein synthesis machinery of the cell (197). The *GCN4* mRNA has a 600-bp leader sequence containing four short ORFs, each consisting of an AUG codon followed by one or two sense codons and then a termination codon (87, 191). This sequence organization is unusual because in *S. cerevisiae*, like in other eukaryotes, the AUG codon most proximal to the 5′ end of the mRNA in general functions as the translational initiation signal. Additional AUG codons upstream of the normal translation initiation substantially inhibit translation of downstream

coding sequences (106, 107). Under certain circumstances the 40S ribosomal subunit can resume scanning as soon as ribosomes terminate translation of an upstream ORF and can reinitiate to a certain degree at a downstream ORF (107, 108, 157, 198). Removal of the four upstream ORFs from the GCN4 transcript, either by deletion or by point mutations in the four ATG start codons of the corresponding DNA segment, results in high-level unregulated GCN4 expression. In addition, insertion of the four upstream ORFs into the leader sequence of another transcript results in translational regulation of gene expression similar to that of the *GCN4* transcript itself. A combination of the first and the fourth of these upstream ORFS is sufficient for wild-type regulation of GCN4. Whereas the fourth upstream ORF seems to be the strongest translational barrier for GCN4 expression, the first upstream ORF is rather leaky and seems to be a positive element to bypass ORF4 under amino acid starvation conditions (138, 196). In amino acid-starved cells the ribosomal reinitiation at the upstream ORF seems to be suppressed. The 40S ribosomal subunits seem to traverse the upstream ORFs and to ignore their AUG start codons (1). The inhibitory effect of ORF4 on GCN4 expression seems to be determined mainly by sequences surrounding its stop codon (131). Different *trans*-acting regulators are involved in the function of the four upstream ORFs (for reviews, see references 87 and 88).

Two classes of mutations, *gcn* and *gcd*, have been described conferring either a nonderepressible (GCN) or a constitutively derepressed (GCD) phenotype of enzymes regulated under general control. From genetic studies, a cascade model was proposed to explain the interactions between the various genes and gene products (86, 89). In this hierarchy the *GCN4* gene product was identified as the most direct activator of the structural genes subject to general control (87, 191).

According to this model, the *GCD* genes are negative effectors of GCN4 translation. *GCD1* and *GCD2* are essential yeast genes; they have been cloned and sequenced, but their exact function is unknown (84, 152, 153). Among these negative effectors there are α- and β-subunits, respectively, of the eukaryotic initiation factor 2 encoded by the genus *SUI2* and *SUI3* (88, 204). The *GCN* genes *GCN1*, *GCN2*, and *GCN3* are assumed to be positive regulators which antagonize the negative effect of the *GCD*-encoded regulators on *GCN4* expression (77, 152).

The translational derepression of *GCN4* mRNA depends on the small ORFs in the leader region and on positive effectors such as GCN2 and GCN3 (86, 138). One domain of the *GCN2* gene product is a protein kinase that is posttranslationally regulated. Its substrate specificity in vivo is not known (165, 202). There is an additional domain with homology to histidyl-tRNA synthetase (201). Although depletion of an amino acid pool leads to general control derepression, a reduction in the level of tRNA aminoacylation seems to be the more direct signal for derepression (126, 177a). Since aminoacyl-tRNA synthetases bind uncharged tRNA as a substrate and are able to distinguish between charged and uncharged tRNAs, it has been proposed that the putative histidyl-tRNA synthetase domain of GCN2 monitors the concentration of uncharged tRNAs in the cell and activates the adjacent protein kinase domain under starvation conditions when uncharged tRNA accumulates (88, 201). It has been shown that translational activation of GCN4 can be triggered in a cell-free system by uncharged tRNAs (111).

There is evidence that increased levels of the GCN2 protein kinase in the cell increase the ability of 40S ribosomal subunits that have participated in the translation of the first of the four small ORFs (ORF1) to reinitiate at a downstream AUG (198). Whatever the function of the GCN2 protein kinase in this process, it can be implemented only in conjunction with the translation of ORF1 (198). Moreover, the 5′ region of the *GCN2* gene contains a recognition site, which binds the GCN4 protein in vitro (165). It is still an open question whether there exists a transcriptional-translational circuit which involves the GCN4 transcriptional activator and the GCN2 protein kinase (165, 198). The precise mechanism of the mode of action of the GCN2 protein kinase and the other *trans*-acting factors (*GCN* and *GCD* gene products), modulating the expression of *GCN4* directly or indirectly, is a topic of current research.

REGULATION OF ENZYME ACTIVITY

In *S. cerevisiae* the regulation of enzyme activities plays a major role in the establishment of the flow through an amino acid biosynthetic pathway. For the biosynthesis of aromatic amino acids, the main control points are the entrance of the shikimate pathway and the first branch point, where the distribution of chorismate either in the direction of tryptophan or in the direction of phenylalanine-tyrosine is controlled. The effector molecules are the end products of the pathway (Fig. 1).

In *S. cerevisiae* the initial step of the pathway catalyzed by the two DAHP synthases is regulated by feedback inhibition; phenylalanine feedback inhibits the *ARO3*-encoded DAHP synthase, and tyrosine feedback inhibits the *ARO4*-encoded DAHP synthase. A tryptophan-inhibitable DAHP synthase has not been found. This situation leads to tryptophan starvation for yeast cells grown in a medium lacking tryptophan but with an excess of phenylalanine and tyrosine and can be compensated by increasing the rate of enzyme synthesis with the general control system. Mutant yeast strains which are unable to elevate transcription by the GCN4-dependent general control system have a significantly reduced growth rate under these conditions (144). At the first branch point the tryptophan biosynthetic anthranilate synthase complex (encoded by *TRP2-TRP3*) is feedback inhibited by tryptophan and the tyrosine-phenylalanine biosynthetic chorismate mutase (encoded by *ARO7*) is feedback inhibited by tyrosine. In addition to feedback inhibition, the chorismate mutase is also strongly activated by tryptophan, the end product of the other branch. This dual control of enzyme regulation by tyrosine as feedback inhibitor and tryptophan as activator is unique when compared with the corresponding enzymes of other organisms.

The following section focuses mainly on the regulation of the activities of these enzymes.

Phenylalanine-Inhibitable DAHP Synthase

Takahashi and Chan (189) were able to separate and preliminarily characterize the DAHP synthase isoenzymes by affinity chromatography. By using current gene technology, the problem of separating two isoenzymes was circumvented for the phenylalanine-inhibitable DAHP synthase by overexpressing the *ARO3* gene on a high-copy-number plasmid in a yeast strain carrying an *aro4* deletion (156). The phenylalanine-inhibitable DAHP synthase from *S. cerevisiae* has been purified to apparent homogeneity by a 1,250-fold enrichment of the enzyme activity present in wild-type crude extracts, by using the overproducing strain. Hence this DAHP synthase corresponds to approximately 0.1% of the

TABLE 5. Features of branch point enzymes for the biosynthesis of aromatic amino acids in *S. cerevisiae*

Enzyme	Gene	Oligomeric state	Molecular mass of native enzyme (kDa)	$(S)_{0.5}$ or K_m (mM)	k_{cat} (s^{-1})	K_i/K_a (mM)	% of total cellular protein
Phe-inhibitable DAHP synthase (EC 4.1.2.15)	*ARO3*	Monomer	42	For PEP, 0.018; for E4P; 0.130	10	For Phe/E4P, 0.01	0.05–0.1
Chorismate mutase (EC 5.4.99.5)	*ARO7*	Homodimer	60	Without aa[a], 4.0 + Tyr, 8.6 + Trp, 1.2	176 129 264	 For Tyr, 0.05 For Trp, 0.0015	0.01
Gln-dependent anthranilate synthase/InGP synthase (EC 4.1.3.27/EC 4.1.1.48)	*TRP2/3*	Heterodimer	130	For chorismate, 0.05; for CDRP, 0.25	ND	For Trp, 0.056	0.05
PRA isomerase[c]	*TRP1*	Monomer	23	For PRA, 0.005	2 50		0.006

[a] aa, amino acid.
[b] ND, not determined.
[c] PRA isomerase is included for comparison, because in other fungi this enzyme activity is part of a trifunctional enzyme; Gln-dependent anthranilate synthase-InGPsynthase-PRA isomerase.

total cellular protein. This is in agreement with an estimation of the *ARO3* mRNA level in the cell, which, on the basis of the codon usage index of Bennetzen and Hall (15), amounts to roughly 0.05% of the total mRNA.

Gel filtration indicates that the active enzyme is a monomer with a molecular mass of 42 kDa, which corresponds to the calculated molecular mass deduced from the previously determined primary sequence (154). A comparison with the three described DAHP synthases from *E. coli* reveals striking differences in the quaternary structure: the phenylalanine-inhibitable enzyme is a tetramer, whereas the tyrosine-inhibitable enzyme is a dimer (124, 171, 175), although there is 70% sequence similarity between the enzymes (52, 174). The tryptophan-inhibitable DAHP synthase has been described as a tetramer (145). Another monomeric DAHP synthase as in *S. cerevisiae* has as yet not been described.

Atomic absorption spectroscopy suggests that the yeast phenylalanine-inhibitable DAHP synthase is an iron metalloenzyme. The enzyme can be inactivated by EDTA in a reaction which can be reversed by the addition of several bivalent metal ions. Similar results have been found for the phenylalanine-inhibitable *E. coli* enzyme (123, 175, 179).

The kinetic data of the phenylalanine-inhibitable yeast DAHP synthase (summarized in Table 5), with a calculated rate constant of 10 s^{-1} (156), suggest a sequential reaction mechanism similar to that proposed for the tyrosine-inhibitable *E. coli* DAHP synthase (171). The apparent Michaelis constant of the enzyme is 0.018 mM for PEP, which is similar to the values obtained for the two available DAHP synthase isoenzymes from *E. coli* and the tryptophan-sensitive enzyme from *N. crassa* (124, 145, 171, 175). For E4P the Michaelis constant is 0.13 mM, and the reported values for the other described enzymes range between 0.0027 and 0.9 mM. A reason for this finding may be that E4P forms dimers in solution, but the enzyme distinguishes between monomers and dimers (145), so that the concentration of this substrate available to the enzyme could be overestimated.

The reported inhibition constants for all the DAHP synthases are of the same order of magnitude. For the yeast enzyme, inhibition by phenylalanine is competitive with respect to E4P and noncompetitive with respect to PEP, with a K_i of 0.01 mM (156). The *N. crassa* enzyme shows the same pattern of inhibition, but only qualitative results were obtained, as the inhibition by tryptophan was not hyperbolic and the intercept and slope replots curved upwardly (145). The present knowledge suggests that the interplay of DAHP synthase subunits and the regulatory behavior seems to be different in various organisms, although the high degree of sequence homology points to similarities in catalytic behavior.

Once the *ARO4* gene product is purified, it will be interesting to compare the quaternary structure and the kinetic and inhibitory properties of the two enzymes from *S. cerevisiae*.

The Allosteric Chorismate Mutase Can Be Locked in the Activated State

The *ARO7* gene of *S. cerevisiae* encodes a monofunctional chorismate mutase catalyzing the first step in the phenylalanine-tyrosine branch. Interestingly, this Claisen reaction from chorismate to prephenate can also be catalyzed stereospecifically by a monoclonal antibody (85). Whereas the allosteric chorismate mutase activity can be activated up to 10-fold in the presence of the specific effector tryptophan at the enzyme level, tyrosine is able to reduce the chorismate activity up to 10-fold, resulting in a range of regulation of the enzyme of a factor of 100. No effect of phenylalanine, the other end product of this branch of aromatic amino acid biosynthesis, is known (168).

Mutant strains carrying the *ARO7*^{c} (constitutively activated chorismate mutase) alleles show increased sensitivity to the amino acid analog 5-methyltryptophan with respect to growth and exhibit a 10-fold increase in the basal activity of chorismate mutase (110, 168, 170). The mutant enzymes are practically unresponsive to tyrosine and tryptophan. Since anthranilate synthase and chorismate mutase control the distribution of chorismate at the first branch point of aromatic amino acid biosynthesis, a high chorismate mutase activity depletes the chorismate pool, destroys the balance between the two enzymes and the chorismate pool in the cell, and causes tryptophan starvation in the presence of the false anthranilate synthase inhibitor 5-methyltryptophan (132, 160). Overexpression of the cloned mutant enzyme on a high-copy-number vector leads to starvation for tryptophan because of depletion of the chorismate pool even in the absence of the analog.

Recently a tryptophan auxotrophic mutant strain of *Pichia guilliermondii* was isolated that had a sevenfold-increased chorismate mutase activity, leading to a depletion of the

chorismate pool (19). Once the cloned gene and the purified enzyme are available, it will be interesting to compare them with the *S. cerevisiae* chorismate mutases.

The wild-type and the mutant chorismate mutases have been purified approximately 11,000-fold from overproducing strains. On the basis, chorismate mutase represents approximately 0.01% of the total cellular protein, and this figure correlates with the estimated *ARO7* mRNA level in the cell, which, on the basis of the codon usage index of Bennetzen and Hall (15), amounts to roughly 0.01%.

The ARO7^c phenotype is caused by an identical point mutation found in independent mutant alleles in the C-terminal part of the 256-amino-acid protein. This mutation causes a change from threonine in the C-terminal part of the chorismate mutase at amino acid 226. In a Chou and Fasman secondary plot (44), the replacement of the hydrophilic threonine in the wild-type enzyme by the hydrophobic isoleucine in the mutant enzyme interrupts a hydrophilic α-helical conformation from amino acids 220 to 226 (170).

The wild-type and mutant enzymes are dimers consisting of two identical subunits with a molecular mass of 30 kDa, each one capable of binding one substrate and one activator molecule (tryptophan). Each subunit of the wild-type enzyme also binds one inhibitor molecule (tyrosine). The mutant enzyme which is still able to bind tryptophan loses the tyrosine-binding ability, suggesting that there are two conformational states for the wild-type enzyme but only one for the mutant enzyme (168).

A ^{1}H nuclear magnetic resonance spectrum of the enzyme reaction with the pure enzyme shows a direct and irreversible conversion of chorismate to prephenate without the accumulation of any enzyme-free intermediates. Since the other purified chorismate mutases from *E. coli* are bifunctional and do not release prephenate (50, 51), the use of the purified yeast chorismate mutase makes it possible to observe the enzyme reaction for the first time by ^{1}H nuclear magnetic resonance spectroscopy (168).

Table 5 summarizes some features of the wild-type chorismate mutase. The kinetic data for the wild-type chorismate mutase show positive cooperativity toward the substrate with a Hill coefficient of 1.71 and an $(S)_{0.5}$ value of 4.0 mM. In the presence of the activator tryptophan, the cooperativity is lost. The enzyme has an $(S)_{0.5}$ value of 1.2 mM in the presence of 0.01 mM tryptophan and an increased $(S)_{0.5}$ value of 8.6 mM in the presence of 0.3 mM tyrosine (168).

The amino acid substitution in the mutant enzyme destroys the allosteric response toward substrate binding, as well as the ability to bind tyrosine, resulting in a loss of the cooperativity together with a remarkable reduction of the $(S)_{0.5}$. Consequently, a normal Michaelis-Menten substrate saturation was found for the mutant enzyme, with similar parameters to those for the wild-type enzyme measured in the activated state. The curve observed in the presence of tryptophan was the same as that observed in the presence of tyrosine, reflecting the unresponsive character of the mutant enzyme. With the V_{max} values and the known enzyme amount, the turnover numbers [$k_{cat} = V_{max}/(e_0/M_r)$] can be calculated, and they vary from 129 to 264 s^{-1} for the wild-type enzyme and are 219 s^{-1} for the mutant enzyme (168). Compared with other aromatic amino acid biosynthetic enzymes of *S. cerevisiae*, the turnover number (k_{cat}) of the chorismate mutase is clearly higher than that of the PRA isomerase (50 s^{-1} [28]), InGP synthase (2 s^{-1} [28]), phosphoribosyltransferase (2.9 s^{-1} [93]), or the phenylalanine-inhibitable DAHP synthase (10 s^{-1} [156]). However, these in vitro data should be used with caution because the V_{max} was calculated at unphysiologically high substrate concentrations.

The wild-type enzyme can be described according to the common Monod-Wyman-Changeux model as a dimer in which both subunits exist in either an unligated T-state or a ligated R-state. Both states are in equilibrium, but the T-state has a lower affinity for ligands. Tyrosine acts in a mixed inhibition in the presence of 1 to 4 mM substrate with a K_i value of 0.05 mM. The K_a for tryptophan is 0.0015 mM. Increasing substrate concentrations pull the equilibrium toward the R-state, which has a better substrate affinity, resulting in a sigmoidal substrate saturation curve. According to this model, the addition of tryptophan as an activator stabilizes the R-state, which has a lower $(S)_{0.5}$ value, as observed for the wild-type enzyme. Tyrosine has the opposite effect. Different inhibitor and activator concentrations thus modulate a T-R equilibrium. The same effect, namely cooperativity which is destroyed in the presence of an activator, leading to normal Michaelis-Menten substrate saturation, has been observed for other allosteric enzymes such as the isocitrate dehydrogenase of *S. cerevisiae* (80) and the deoxythymidine kinase of *E. coli* (147).

The wild-type chorismate mutase shows a pH optimum at pH 5.5, whereas in the presence of tryptophan the highest activity is found at pH 7.0. The pH optimum is lowered in the presence of tyrosine (pH 5.0). It is interesting that tryptophan acts as an activator only at pHs higher than 4.5, but as an inhibitor at lower pHs. The single-amino-acid substitution in the wild-type enzyme altered the pH optimum to 6.3 for the mutant enzyme. The different pH optima for the wild-type enzyme measured under three different conditions (in the absence of amino acids, in the presence of tryptophan, and in the presence of tyrosine) and for the mutant enzyme can also be explained by using the Monod-Wyman-Changeux model. This suggests that there are two states with different conformations for the wild-type enzyme. Each state has a different pH optimum: the T-state of the enzyme has a pH optimum at 5.0, and the R-state has a pH optimum at 7.0. Thus, the optimum for chorismate mutase is pH 5.0 in the presence of tyrosine but pH 7.0 in the presence of tryptophan. In the absence of amino acids the enzyme exists in a T-R equilibrium and has a pH optimum between pH 5.0 and 7.0. The R-state (modulated by tryptophan) has almost no activity at pH 5.0, which clarifies the result that tryptophan acts as an inhibitor at low pH. The mutant enzyme exists mainly in the R-state and thus has an optimum which is shifted toward pH 7.0.

Point mutations are one basis for evolution. The wild-type and mutant yeast chorismate mutases thus demonstrate the striking effect of a point mutation within a gene on the function of an allosteric enzyme. In an opposing scenario, a point mutation has been found which triggers allostery of the ornithine transcarbamoylase, which is not an allosteric enzyme per se (112). For the *E. coli* phosphofructokinase, for which the crystal structure is known, it was shown that mutations in the active site alter the cooperativity of this allosteric enzyme (16). By using nuclear magnetic resonance and X-ray analysis, the two chorismate mutases may represent an ideal model for studying the R-state of an allosteric enzyme in comparison with the T-R equilibrium.

Regulation of the Anthranilate Synthase Complex

The glutamine-dependent anthranilate synthase-InGP synthase complex from *S. cerevisiae* has been purified to apparent homogeneity by a 2,000-fold enrichment of the

enzyme activity present in wild-type crude extracts, by using a 50-fold-overproducing strain and a further 40-fold purification. The native enzyme complex consists of a heterodimer of two different subunits with a total molecular mass of 130 kDa. The larger subunit (64 kDa) is the anthranilate synthase component (component I, see below), which is active in vitro with ammonia as cosubstrate without the need for complex formation. The smaller polypeptide (58 kDa) carries both glutamine amidotransferase activity and InGP synthase activity, the fourth enzymatic step of the tryptophan branch of the pathway (component II, see below) (160).

This complex corresponds to approximately 0.05% of the total cellular protein. Compared with other aromatic amino acid biosynthetic enzymes, the competing chorismate mutase of the phenylalanine-tyrosine branch makes up 0.01% (168) of total cellular protein and another tryptophan branch enzyme, the PRA isomerase, makes up 0.006% (27) of total cellular protein. Considering the different molecular masses of 130, 30 (for the monomer), and 23 kDa, respectively, all three enzymes are present in roughly the same number of protein molecules per yeast cell. Only the phenylalanine-inhibitable DAHP synthase at the entrance of the whole pathway (0.05 to 0.1%; molecular mass, 42 kDa [156]) is more abundant, with three to six times more molecules per cell.

Anthranilate synthases from a number of microorganisms examined so far are oligomeric proteins containing nonidentical subunits with two different components, called I and II. In all cases, both components are required for the glutamine-dependent anthranilate synthase activity, whereas component I can use ammonia as a cosubstrate without the need for complex formation. The yeast configuration of I_1II_1, with component II associated with the InGP synthase, is so far unique. In different enteric bacteria including *E. coli*, in which component II is associated with the phosphoribosyltransferase catalyzing the second step of the tryptophan branch, a I_2II_2 subunit composition has been found (100). In other bacteria, including *B. subtilis* (92), a I_1II_1 configuration has been found, as in *S. cerevisiae*, but component II is a small polypeptide of less than 20 kDa, carrying only the monofunctional glutamine amidotransferase activity. In other ascomycetes the glutamine amidotransferase is part of a trifunctional polypeptide, NH_2-glutamine amidotransferase-InGP synthase-PRA isomerase-COOH. In *N. crassa* the anthranilate synthase containing the trifunctional component II has a I_2II_2 configuration (167).

As mentioned above, there is some evidence that a trifunctional component II is the original situation in ascomycetes and that the bifunctional *TRP3* and a separate *TRP1* gene coding for the monofunctional PRA isomerase, as found only in different yeast species (25), is actually the result of a rearrangement event. The evidence is summarized as follows: (i) The *TRP1* gene product, PRA isomerase, is a more active enzyme than any other enzyme of the *TRP* branch, providing the cell with a two- to threefold-higher basal enzyme activity (28). (ii) Comparison of the connector regions of known fused and separate InGP synthase and PRA isomerase proteins reveals a remarkable feature: the NH_2 terminus of the yeast PRA isomerase contains additional amino acids, in comparison with prokaryotic monofunctional PRA isomerases, that correspond in length to the natural connector region in the proteins of ascomycetes such as *A. nidulans* and *N. crassa* (28). (iii) The *TRP1* promoter is unusual among *TRP* promoters in being the only *TRP* promoter that is not regulated by the general control system. Transcription from the *TRP1* promoter is weaker than from the regulated *TRP3* promoter. This situation is compensated by a higher stability of *TRP1* mRNA (27, 28).

With the purified yeast anthranilate synthase-InGP synthase complex, apparent K_m values were determined as 0.017 mM for chorismate, 0.74 mM for glutamine, and 0.57 mM for Mg^{2+}. For ammonia a much higher K_m value (22 mM) for the complex, as well as for the dissociated *TRP2* gene product alone, was found compared with 0.74 mM for glutamine (160). It is unlikely that an NH_3-dependent anthranilate synthase has any function in vivo, because (i) mutants defective in the glutamine amidotransferase domain of the *TRP3* gene are auxotrophs and (ii) the K_m value is far beyond the ammonia concentration in the cell, where ammonia is converted to glutamate very readily by the NADP-dependent glutamate dehydrogenase (82). For the other activity of the complex, the InGP synthase, the K_m value was determined as 0.25 mM.

Inhibition of the complex by tryptophan affects both the glutamine-dependent and the NH_3-dependent anthranilate synthase activities. The InGP synthase activity remains unchanged. Inhibition by tryptophan is competitive with respect to chorismate, with a K_i of 0.056 mM (160). A point mutation in the *TRP2* gene, resulting in a single amino acid substitution, causes feedback resistance against tryptophan without changing the catalytic activity of the complex (124a). Therefore, chorismate might bind at a different site from tryptophan, and the competition between tryptophan and chorismate might be due to mutual exclusion.

Anthranilate synthase and chorismate mutase control the distribution of chorismate into the tryptophan and tyrosine-phenylalanine branches. The tryptophan feedback mechanism reduces the flow toward tryptophan in vivo to 10 to 20% of its normal capacity (132). Whereas the anthranilate synthase activity in the cell can be increased only up to 3-fold by the transcriptionally active general control system, the chorismate mutase activity in the cell can be activated up to 10-fold in the presence of the specific effector tryptophan at the enzyme level. A comparison of the in vitro enzyme data of the anthranilate synthase complex and the chorismate mutase (Table 5) suggests that in the absence of aromatic amino acids and at low substrate concentrations, chorismate would preferentially flow into the tryptophan branch of the pathway, whereas the flow into the phenylalanine-tyrosine branch of the pathway proceeds preferentially at higher substrate concentrations. As the chorismate mutase feedback inhibition by tyrosine is canceled by tryptophan, this part of the pathway is enhanced by tryptophan excess, because tryptophan both inhibits the anthranilate synthase and activates the chorismate mutase.

Whereas the anthranilate synthase has the low chorismate Michaelis-Menten constant of 0.017 mM and is inhibited by 50% only at a tryptophan concentration of 0.056 mM, the chorismate mutase has a high $(S)_{0.5}$ value of 8.6 mM in the presence of 0.3 mM tyrosine, which is lowered to 4.0 mM in the absence of amino acids and to 1.2 mM in the presence of 0.01 mM tryptophan (168). It will be interesting to compare the turnover numbers of both enzymes once they are determined for the anthranilate synthase complex.

When there is enough tryptophan in the cell, the anthranilate synthase complex is feedback inhibited by tryptophan. This effect can be counteracted by increasing the chorismate concentration, which makes the anthranilate synthase less sensitive to tryptophan inhibition (160). For instance, an *aro7* mutation, which leads to an increase in the chorismate concentration (109), leads to increased tryptophan accumulation. A similar effect was found for *TRP2* mutations that

result in an anthranilate synthase resistant to feedback inhibition (61). Further increases in tryptophan accumulation are obtained when the mutation *aat2*, which eliminates an inducible aromatic amino transferase-degrading tryptophan, is introduced and when GCN4 overexpression results in a constitutively increased synthesis of the enzymes (109, 161).

The wild-type yeast strain, fed with anthranilate to bypass feedback inhibition of the anthranilate synthase, and the feedback-resistant *TRP2* mutant strain accumulate 0.4 nmol of tryptophan min^{-1} mg of $protein^{-1}$, of which 0.3 nmol is needed for growth on minimal medium for protein biosynthesis, resulting in a free tryptophan accumulation rate of 0.1 nmol min^{-1} mg of $protein^{-1}$ (61, 109, 161). By using a plasmid containing all cloned tryptophan biosynthetic genes, including the feedback-resistant allele of the *TRP2* anthranilate synthase, enzyme levels are elevated about 20-fold and the accumulation rate can be increased up to 10-fold to approximately 4 nmol min^{-1} mg of $protein^{-1}$ (161). This results in an internal tryptophan accumulation of 30 mM, in comparison with 0.02 mM in the wild-type yeast under normal growth conditions (61). The fact that the tryptophan accumulation rate is increased only 10-fold may be due to two reasons: (i) there is not enough chorismate which can be channeled toward tryptophan and (ii) tryptophan is degraded by aromatic amino transferases which have K_m values at least five times lower than this concentration (109). Addition of phenylalanine and tyrosine prevents starvation for these amino acids but lowers the tryptophan accumulation as a result of their feedback inhibition of the DAHP synthases at the entry to the pathway.

CONCLUSIONS

This review focuses on different levels of regulation of the aromatic amino acid biosynthetic pathway of the yeast *S. cerevisiae*. This branched pathway serves as a model system for understanding various aspects of a metabolic pathway in eukaryotic organisms: most genes of the pathway have been cloned, and the DNA sequences have been determined. Some aspects of the regulation of transcription initiation are established. Several enzymes have been purified and to a certain extent characterized. A primary understanding of the flow of carbon through the pathway is now apparent.

Comparison of the DNA sequences and of the deduced amino acid sequences from different organisms clearly points to common ancestor molecules for most genes of the aromatic amino acid biosynthesis. In addition, the three final steps of the tryptophan biosynthetic pathway are encoded by enzymes which all have the same topological characteristics of an eightfold α/β barrel (49, 120, 162). Since one possibility for the way biochemical pathways have evolved is duplication and evolution of new functions in the reverse direction (retrograde evolution) compared with the direction of synthesis, it will be interesting to test the folding motifs of more enzymes. The common enzyme structures and the common pathway suggest that the aromatic amino acid biosynthesis has evolved only once on Earth and connects yeasts and other fungi with the prokaryotic world and with plants. In addition, the knowledge of the various enzyme structures might be an interesting basis from which to design novel enzymes with additional catalytic and regulatory features. A further study of the allosteric chorismate mutase in its wild-type and mutant forms, frozen in the activated state, is a promising project for a better understanding of allosteric enzymes and the effect of conformational change.

Further studies with *S. cerevisiae* are challenging, because gene organization and regulation are different from those in prokaryotes and especially because yeast cells exhibit many features in common with higher eukaryotes. For instance, the proteins GCN4 and PHO2 share similarities to other eukaryotic transcription factors. It seems that many components of the transcriptional machinery are interchangeable, but many basal transcription factors remain to be identified and many aspects of the regulation of the regulators themselves are completely unknown. Obviously, we have just scratched the surface of the transcriptional regulatory network in *S. cerevisiae*, not to mention higher eukaryotic systems. Thus, *S. cerevisiae* can make a substantial contribution to the understanding of basic aspects of any eukaryotic cells, including human cells.

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