Transcriptional Regulation and Differentiation in *Saccharomyces* and *Aspergillus*: *jlbA*, *RPS26*, and *ARO3/4*

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… für Hans-Peter Strittmatter

- Chapter II -

Induction of *jlbA* mRNA synthesis for a putative bZIP protein of *Aspergillus nidulans* by amino acid starvation

- Chapter III -

Functional analysis of the ribosomal RPS26 isogenes in the yeast Saccharomyces cerevisiae

- Chapter IV -

Evolution of DAHP synthase encoding genes in the yeast
Saccharomyces cerevisiae

Abbreviations

3AT	3-amino-1,2,4-triazole
5MT	5-methyl-tryptophan
AA	anthranilic acid
AAS	anthranilate synthase
ADIC	2-amino-2-deoxyisochorismate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CA	chorismic acid
CDRP	carboxy-phenylamino-1-deoxyribulose-5-phosphate
CM	chorismate mutase
CPRE	'cross-pathway' control recognition element
d	days
DAHP	3-Deoxy-D-*arabino*-heptulosonate 7-phosphate
DAHPS	DAHP synthase
DHQ	3-hydroquinate
DHS	3-dehydroshikimic acid
DTT	dithiothreitole
dNTPs	desoxy nucleotide triphosphates
E4P	erythrose-4-phosphate
Ec AroHp	AroH protein from *E. coli*
EDTA	ethylene-diamine-tetraacetate
e.g.	*exempli gratia* (*ejusdem generis*)
eIF	eukaryotic translation initation factor
EPSP	5-enolpyruvyl-shikimate-3-phosphate
et al.	*et alibi*
EtOH	ethanol
GCRE	general control recognition element
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
h	hours
HPP	4-hydroxyphenylpyruvic acid
i.e.	*id est*
IGP	indoleglycerole phosphate
kDa	kilo Dalton
LB	Luria Bertani medium
LiOAc	lithium acetate

MCS	multiple cloning site
MDa	mega Dalton
MV	minimal vitamins
NaOAc	sodium acetate
nt	nucleotide
OD	optical density
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PEP	phosphoenolpyruvic acid
PMSF	phenylmethanesulfonyleflouride
PPY	phenylpyruvic acid
PRA	phosphoribosylanthranilic acid
S	Svendberg Units
S3P	shikimate-3-phosphate
SC	synthetic complete
SDS	sodium dodecylsulfate
TAFs	TATA binding protein associated factors
TBP	TATA binding protein
TEMED	N,N,N',N' tetramethylethylenediamine
U	enzymatic Unit (1 nmole product / 1 min / 1 mg of total protein)
wt	wild type
YEPD	yeast extract / peptone / dextrose
μ	growth rate (h^{-1})

Summary

Increasing data from genomics research revealed that the number of isogenes present within a given organism is significantly higher than anticipated a decade ago. Here we have characterised representatives of isogenes, which are involved in transcriptional regulation and in translation as part of the ribosome. Two different isogenes were characterised involved in the aromatic amino acid biosynthesis. All analysed genes are derived from either of two model fungi, the baker's yeast *Saccharomyces cerevisiae*, and the filamentous fungus *Aspergillus nidulans*.

jlbA from the filamentous fungus *Aspergillus nidulans* encodes a novel putative bZIP-protein of the *JUN* isogene family. Another prominent representative of this family is *cpcA*, which encodes the 'cross pathway control' activator protein, which is required for amino acid biosynthesis under starvation conditions. The transcriptional regulation of *jlbA* was compared under induced and non-induced nutritional and environmental conditions. Under amino acid starvation, expression of the *jlbA* transcript was strongly induced suggesting control by the 'cross pathway' regulator CPCA. In addition, *jlbA* transcription was strongly induced by a second CPCA independent regulation after amino acid starvation.

The two *RPS26* isogenes encode for two proteins of the small ribosomal subunit of *S. cerevisiae*. The deduced amino acid sequence of both proteins differs only in two amino acids. The Rps26Ap was identified as an essential part in normal cell division. Additionally, Rps26Ap functions in the translational regulation of adhesive and pseudohyphal growth that acts on the *FLO11* expression of *S. cerevisiae* Σ1278b wild type strains. In contrast, the Rps26Bp seems to be of minor importance in all these regulation mechanisms, but can partially substitute for Rps26Ap functions.

ARO3 and *ARO4* of *S. cerevisiae* encode two differently regulated DAHP synthase isoenzymes at the initial step of the 'shikimate'-pathway. Aro3p and Aro4p are feedback regulated by phenylalanine and tyrosine, respectively. Both isogenes were substituted for a single gene encoding a tryptophan-regulated enzyme. Comparison of the structural and physiological behaviours of all DAHP isoenzymes resulted in the engineering in regulation of an Aro4p Pro165Gly mutant enzyme. This enzyme is under an efficient dual control. Aro4p Pro165Gly is feedback inhibited by tyrosine and tryptophan. The careful analysis of Aro4p wildtype revealed that this enzyme is also moderately regulated by tryptophan feedback inhibition.

Zusammenfassung

Eine steigende Anzahl von Daten aus Genomuntersuchungen belegt, dass die Anzahl von Isogenen innerhalb eines gegebenen Organismus deutlich höher liegt als noch vor 10 Jahren angenommen. In dieser Arbeit haben wir verschiedene Vertreter von Isogenen charakterisiert, welche an der transkriptionellen Regulation und an der Translation als Teil der Ribosomen beteiligt sind. Zwei Isogene wurden charakterisiert, welche an der aromatischen Aminosäurebiosynthese beteiligt sind. Alle untersuchten Gene stammen aus zwei pilzlichen Modellsystemen, aus der Bäckerhefe *Saccharomyces cerevisiae* und aus dem filamentösen Pilz *Aspergillus nidulans*.

Das *jlbA* Gen aus dem filamentösen Pilz *Aspergillus nidulans* codiert für ein neues, potentielles bZIP-Protein der Familie der *JUN* Isogene. Ein anderer bekannter Vertreter dieser Genfamilie ist das *cpcA* Gen, welches das Regulatorprotein der ‚Cross-Pathway' Kontrolle codiert, welches unter Mangelbedingungen für die Aminosäurebiosynthese benötigt wird. Die transkriptionelle Regulierung von *jlbA* wurde unter induzierten und nicht induzierten Nährstoff- und Umweltbedingungen verglichen. Unter Aminosäuremangel wird die Expression von *jlbA* stark induziert, was auf eine Regulation durch das CPCA der ‚Cross-Pathway' Kontrolle schliessen lässt. Zusätzlich wird die *jlbA* Transkription unter Aminosäuremangelbedingungen aber auch durch eine CPCA unabhängige Regulierung induziert.

Die zwei *RPS26* Isogene von *S. cerevisiae* codieren für zwei Proteine der kleinen ribosomalen Untereinheit. Die abgeleitete Aminosäuresequenz der beiden Proteine unterscheidet sich in nur zwei Aminosäureresten. Das Rps26A Protein wurde als ein essentieller Bestandteil der normalen Zellteilung identifiziert. Zusätzlich nimmt Rps26A an der translationellen Regulation von adhäsivem und pseudohyphalem Wachstum teil, welche auf die *FLO11* Expression von *S. cerevisiae* Σ1278b Wildtyp-Stämmen wirkt. Im Gegensatz dazu scheint das Rps26B Protein bei allen diesen Regulationsmechanismen eine weniger wichtige Rolle zu spielen, es kann aber teilweise die Rps26A Proteinfunktionen übernehmen.

Die *ARO3* und *ARO4* Gene von *S. cerevisiae* codieren für zwei unterschiedlich regulierte DAHP Synthase-Isoenzyme des ersten Schrittes des Shikimat-Synthese-Weges. Die Aro3 und Aro4 Proteine werden durch Phenylalanin bzw. Tyrosin in ihrer Wirkung gehemmt. Beide Isogene wurden durch ein einzelnes Gen ersetzt, welches eine Tryptophan-regulierte DAHP Synthase codiert.

Ein Vergleich des strukturellen und physiologischen Verhaltens aller DAHP Isoenzyme resultierte in einer gezielten Veränderung ('protein engineering') des Regulationsverhalten eines Aro4 Pro165Gly Mutanten-Enzymes. Dieses Enzym steht unter einer effizienten, zweifachen Kontrolle. Eine Aro4 Pro165Gly Mutante wird durch Tyrosin und durch Tryptophan in ihrer Aktivität gehemmt. Eine sorgfältige Analyse des Aro4 Wildtyp-Proteins zeigte, dass dieses Enzym ebenfalls moderat durch Tryptophan gehemmt wird.

Introduction

1.1   Lifecycles of the yeast *Saccharomyces cerevisiae*

In human history, the cultivation of one microorganism seems to play an important role from the early beginning of civilisation. The cultivation of yeast and the brewing of alcoholics started in Egypt more than 2000 years ago. Among the *Sacharomycetaceae*, many organisms play an important role in nutrition of mankind. Whereas the yeast *S. elipsoideus* naturally exists in wild forms on vines and grapes and has an important function in the production of wine, the yeast *S. cerevisiae* was cultivated through the times for brewing and baking of bred. In the last 60 years, *S. cerevisiae* has also become an important organism in the genetic field. In addition, yeast serves as an eukaryotic model organism, since it could be easily handled, modified by genetic approaches and has many genetic features in common with humans.

One main biosynthetic feature of *S. cerevisiae* is the rapid growth, which depends on its normal lifecycle. Haploid (1n) and diploid (2n) yeast strains naturally grow by budding and can easily switch between both genetic phases, either by conjugation (1n $\rightarrow$ 2n) or by sporulation (2n $\rightarrow$ 1n). While haploid yeast cells perform axial budding, diploid cells exhibit a bipolar budding (Mösch, 2000). Beside the fission yeast *S. pombe*, budding is the preferred life form of many naturally occurring yeast strains. Budding yeasts can grow very fast on nutritional surfaces like grapes or fruits and therefore claim living area against antagonists.

Under laboratory conditions, the ability of conjugation and exchange of genetic information is a disadvantage, i.e. haploid yeast strains were selected that are unable to switch their mating types. Additionally, the commonly used S288C or W303 yeast strain backgrounds have lost another genetic feature, the ability to grow invasively. This morphological switch from unicellular adhesive to multicellular invasive growth seems to depend on environmental conditions in both haploid and diploid yeast Σ1278b strains. While haploid yeast wildtype strains exhibit short filaments and grow invasively under glucose limitation on complex media (Cullen & Sprague, 2000), diploid wild type strains (Σ1278b background) exhibit a pseudohyphal development on fermentable carbon sources under nitrogen starvation (reviewed in Mösch 2002). This morphological switch of diploid yeast strains depends on alterations in several

cellular processes. Firstly, the budding pattern of the cells is altered from bipolar to unipolar, resulting in a linear filamentous growth. Secondly, cell morphology changes from the round yeast form towards the thin and elongated pseudohyphal form. Thirdly, cells no longer separate but exhibit an incomplete cell division. Thereby cells stick together and form long chains of multicellular pseudohyphae. Finally, the cell division pattern of diploid pseudohyphal cells changes when compared to yeast-form budding. The entire pseudohyphal growth occurs during G2 phase of the cellcycle, and mitosis is restricted until the bud has reached almost the same size as the mother cell. Subsequently, all cells are budding synchronously without a G1 delay (Mösch, 2000).

1.2   Regulation of yeast pseudohyphal development

Regulation of pseudohyphal development is very complex and requires at least two different conserved signal transduction pathways (Rupp *et al.*, 1999; Pan *et al.*, 2000). The small GTP-binding protein Ras2p can activate a hyper-filamentous growth phenotype under nitrogen starvation conditions and is therefore often designated as Ras-cAMP pathway. Although a direct connection between glucose sensing and nitrogen starvation is not yet known, Ras2p seems to be a transmitter between both regulatory mechanisms. Downstream to Ras2p, a protein kinase is acting in the cAMP-dependent pathway. This kinase is composed of one of three catalytic subunits Tpk1p, Tpk2p and Tpk3p and an inhibitory subunit Bcy1p, respectively (Todd *et al.*, 1997). Tpk2p is the only yet known subunit of the cAMP-dependent protein-kinases that has been demonstrated to activate pseudohyphal development. Downstream to Tpk2p, the Flo8p is an activator of the *FLO11* transcription. Flo8p is required for positive regulation of pseudohyphal growth by Tpk2p (Rupp *et al.*, 1999).

The highly conserved MAPK cascade is the second control pathway for regulation of pseudohyphal growth. Four protein kinases are acting in sequence to activate the expression of *FLO11* transcription via two cooperative transcription factors (Ste12p and Tec1p). A not yet identified signal is activating the MEKK-kinase (Ste20p) via Ras2p and Cdc42p dependent phosphorylation. Ste20p acts on MEKK (MEK kinase; Ste11p), downstream MEK (MAPK kinase; Ste7p), and the mitogen activated protein kinase (MAPK; Kss1p).

Finally, the Kss1p activated Ste12p and the regulatory subunit Tec1p bind to specific filamentation response elements (FRE) in the promoter regions of many target genes and activate their transcription, e.g. transcription of *FLO11* and of other genes (Madhani *et al.*, 1997; Lo & Dranginis, 1998).

1.3   Lifecycles of *Aspergillus nidulans*

It has been assumed that, under natural conditions, the switch from yeast-form growth to pseudohypal growth opens the possibility for *S. cerevisiae* to exploit new nutritional sources that are located in distance. In natural habitats, *Aspergillus nidulans* is one of many competitors of *S. cerevisiae* for nutrients. By default, this mold exhibits filamentous growth and forms real hyphae mycelia, which cannot be found in *Saccharomyces*. A vegetative lifecycle of *A. nidulans* starts with germination of asexual spores. After approximately 20 hours of vegetative filamentous growth, the *Aspergillus* mycelium becomes competent to start an asexual reproduction (Axelrod *et al.*, 1973; Adams *et al.*, 1998). This differentiation process seems to depend on several nutritional influences, such as nitrogen starvation (Skromne *et al.*, 1995) or phosphate availability (Bussink & Osmani, 1998; Bussink & Osmani, 1999). Physical influences like aeration or light were also demonstrated to influence these differentiation processes (Mooney *et al.*, 1990; Mooney & Yager, 1990). The formation of conidiophores starts with the formation of aerial filaments from elder mycelia. This so-called stalks (Adams *et al.*, 1998) further differentiate and bud in a pseudohyphal-like manner. The asexual differentiation and reproduction process ends with the formation of mature conidiophores, that contain many mitotic conidiospores (Adams *et al.*, 1998). When isogenic conidiospores are released from the conidiophores, these spores can germinate and close the asexual cycle.

A. nidulans is also competent to perform a sexual reproduction cycle (Champe *et al.*, 1994; Braus *et al.*, 2002). After about 50 hours of growth, mycelia probably starts the sexual development by fusion of fertile hyphae cells under not yet clear induction conditions. The sexual reproduction cycle continues via the formation of several morphological structures, namely the nests, the micro-cleistothecia, the formation of dikaryotic hyphae cells that are surrounded by a specific cleistothecia wall, and subsequent meiotic and mitotic divisions of diploid zygote cells. The sexual fruit body formation ends with the release of haploid ascospores that germinate and

close the sexual cycle. In contrast to yeast, a unicellular or yeast form-like growth cannot be found in *A. nidulans*.

1.4 Expression control of fungal amino acid biosynthesis

The capability to perform a *de novo* biosynthesis of all amino acids is a widespread common feature among various eukaryotes including plants (Gilchrist & Kosuge, 1974; Huisman & Kosuge, 1974; Gosset *et al.*, 2001) and fungi (Braus, 1991; Sousa *et al.*, 2002). Mammals lack some of the necessary enzymes for the aromatic amino acid biosynthesis. Therefore, mammals rely on their diet to supply aromatic amino acids as an essential nutritional additive. This additive has to be delivered from external sources, e.g. from commensally prokaryotes and eukaryotic or plant foods.

A. *nidulans* and *S. cerevisiae* are two representatives of organisms that can perform a *de novo* biosynthesis of all 20 amino acids including the three aromatic amino acids, *L*-phenylalanine, *L*-tyrosine and *L*-tryptophan, respectively (Braus, 1991). The knowledge of the regulation of the specific biosynthesis systems became detailed the same way as did the general knowledge in molecular bio-science by using both fungi as eukaryotic model systems. The specific genetic control of the enzyme activities of the aromatic amino acid biosynthesis as well as of those of other differential processes (e.g. pseudohyphal growth) are characterised by precise regulation mechanisms on various levels of the entire gene expression.

Firstly, the concentration of a specific mRNA and therefore the transcription rate of the expressed genes has to be adapted. In *S. cerevisiae* the expression of most biosynthetic genes is regulated by the transcriptional activator Gcn4p, which is the regulator of the general amino acid control network (Hinnebusch, 1988; Hinnebusch & Natarajan, 2002). In *A. nidulans*, *A. niger,* and *Neuropora crassa* the same transcriptional regulation system is termed 'cross pathway' control and acts via Gcn4p related proteins (Paluh & Yanofsky, 1991; Wanke *et al.*, 1997; Hoffmann *et al.*, 2001). This transcriptional regulation is genetically balanced in such a fine way that starvation for one single amino acid or imbalances in the amino acid pool are capable to induce the system control, while growth on minimal vitamin medium lacking any amino acid supplementation and the subsequent expression of all 20 amino acids does not result in an induction effect.

Secondly, the translational control and the control of mRNA stability play an important role in the precise regulation of gene expression. In yeast and other fungal organisms, this regulation is mainly achieved by a translational derepession system that acts on expression of *GCN4* mRNA (Hinnebusch, 1985; Hinnebusch, 1997; Hinnebusch & Natarajan, 2002).

Thirdly, functional proteins are controlled by stability effects, modifications, and allosteric feedback regulations. In fungi, this regulation is either achieved by regulation of Gcn4p stability (Kornitzer *et al.*, 1994; Meimoun *et al.*, 2000; Pries *et al.*, 2002) or by feedback regulation of specific enzyme activities of various biosynthetic pathways, such as the aromatic amino acid biosynthesis (Schnappauf *et al.*, 1998a; Krappmann *et al.*, 2000; Helmstaedt *et al.*, 2001).

Finally, degradation of proteins seems to be involved in regulation of cellular enzyme activities. Many proteins are target for degradation by ubiquitination processes and degradation via the 26S proteasome complex (Hershko & Ciechanover, 1992; Hershko & Ciechanover, 1998; Jackson & Eldridge, 2002). Exemplified for the Gcn4p degradation, this process also requires a phosphorylation of specific amino acid residues (Meimoun *et al.*, 2000).

1.5   Transcriptional regulation of biosynthetic genes in fungi

Generation of appropriate amounts of specific mRNAs, is one of the first steps in the control of balanced gene expression activities. One important process for regulated gene expression depends on the rate of transcription initiation (Weber *et al.*, 1977; Struhl, 1995). In eukaryotes, transcription of protein encoding genes is usually performed by the RNA polymerase II complex (RNA pol II) located within the nucleus (Woychik & Young, 1990). Transcription of pre-rRNAs is performed by RNA polymerase I complex (RNA pol I) located in the nucleolus (Paule & White, 2000), while the RNA polymerase III complex (RNA pol III) functions in the transcription of tRNAs, the 5s-rRNAs and some small ribonucleotides (Willis, 1993; Buratowski, 1994).

Although the exact compositions of RNA polymerase holoenzymes I, II, and III, have not yet been entirely established, general structures are conserved among all eukaryotes (Myer & Young, 1998; Asturias & Kornberg, 1999). All three eukaryotic RNA polymerases contain four core units, six regulatory subunits, and about 4 to 7

accessory proteins. The yeast RNA polymerase II has four core units and 10 regulatory subunits (Woychik & Young, 1990; Woychik & Hampsey, 2002).

These four core units of RNA polymerase complexes partially share high sequence homologies, but also contain parts that are specific for their different functions, such as the central terminal domain (CTD) of RNA pol II located at the biggest subunit L' (160-220 kDa) (Howe, 2002). Furthermore, at least five of the RNA pol II regulatory subunits Rpb1 to Rpb12 of *S. pombe* are conserved among eukaryotic RNA polymerases (Sakurai & Ishihama, 2001). RNA polymerase enzyme complexes are competent to *de novo* start the mRNA synthesis. Therefore, these multi-protein complexes have to bind to specific DNA sequences that are located nearby the transcriptional start sides within the upstream regions of the transcribed genes.

1.5.1 Regulation of yeast transcription initiation

One important mechanism of controlling transcription initiation depends on the promoter region of the expressed genes. A typical yeast promoter contains a 6bp TATA binding box at position -90 relatively to the transcriptional start site (Cormack & Struhl, 1992; Schultz *et al.*, 1992) as well as other upstream binding sites that are recognised by specific transcription factors (Fig. 1.1).

The TATA binding protein (TBP) associates with the RNA pol holoenzyme complex and directs the RNA polymerase to the transcriptional start site by interactions with different RNA pol II subunits. Comparison of many promoter regions of protein encoding genes of *S. cerevisiae* revealed a major consensus sequence motif of this TATA binding box (TATA($^A/_T$)A), which is necessary for an efficient transcription initiation (Stewart & Stargell, 2001). Variations of the TATA binding motif within the promoter regulate the efficiency of gene expression by variation of the binding efficiency of the RNA pol II complex (Stewart & Stargell, 2001).

Furthermore, control of transcription initiation can be achieved by activation or repression of the RNA polymerase II complex via variable proteins, termed transcription factors (Struhl, 1995). According to the model of Jacob-Monod (Ullmann *et al.*, 1967), these *trans*-acting proteins direct the RNA pol II to or prevent the RNA pol II from accessing a specific transcriptional start site. Transcription factors bind to specific *cis*-elements within the upstream regions of the controlled

genes, which are termed <u>u</u>pstream <u>a</u>ctivation <u>s</u>ite (UAS) or <u>u</u>pstream <u>r</u>epression <u>s</u>ite (URS). In mammals some additional *cis*-elements are known that share the same functions but are located more than 10 kbp to 50 kbp upstream of the regulated genes. By binding specific transcription factors, these enhancer- and repressor-sites take also part in directing the RNA polymerase II to specific transcription targets within the mammalian genomes (Massari & Murre, 2000; Gill, 2001).
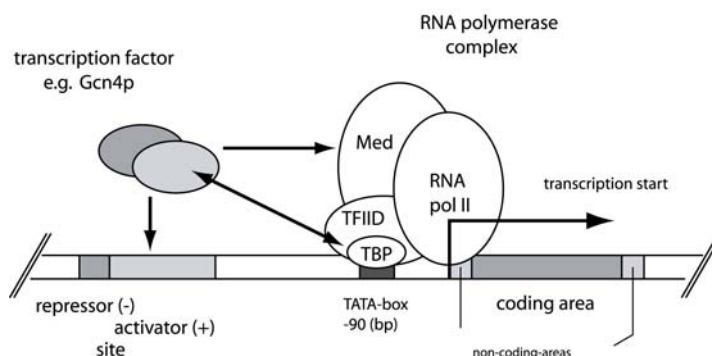
Fig. 1.1: Schematic drawing of *cis*-active sites and transcription initiation of *S. cerevisiae*. A typical yeast promoter is given, containing upstream activation and repressor site, and the TATA-binding element at position -90 relatively to the transcriptional start site. Transcription factors bind to specific upstream elements in the promoter of their targets and direct RNA pol II complex towards the transcriptional start site. RNA pol II complex is simplified by main components referred to in the text. Arrows indicate possible interactions.

1.5.2 Regulation of transcription by specific yeast transcription factors

Specific transcription factors are characterised by at least two different functional domains, a DNA-binding and a regulator domain, respectively. One of many well characterised representative is yeast Gcn4p, and the homologous proteins of *A. nidulans* (Wanke *et al.*, 1997; Hoffmann *et al.*, 2001), and *N. crassa* (Bailey & Ebbole, 1998). In *S. cerevisiae GCN4* codes for a 281bp transcriptional activator protein. The Gcn4p activation domain (AD) consists of a 82 bp N-terminal activation domain (NTAD) and a 38 bp central acidic activation domain (CAAD), separated by a proline-, glutamine-, serine-, and threonine-rich stability area (PEST region). Gcn4p is contacting the RNA pol II complex with this activation domain, while the Pest region serves in the control of protein turnover (Rechsteiner & Rogers, 1996). The C-terminal end of Gcn4p contains a basic amino acid-rich DNA binding domain (BD) as well as a leucine rich region.

Under amino acid starvation conditions, Gcn4p is the main transcriptional activator of about 500 target genes of 12 amino acid biosynthetic pathways as well as numerous genes of the tRNA and the purine biosynthesis (Natarajan *et al.*, 2001; Hinnebusch & Natarajan, 2002). Most genes that are activated by Gcn4p dependent transcription carry a specific upstream activation site, the so-called Gcn4p responsive element (GCRE). This GCRE element is a 9bp palindrome consensus sequence 5'-ATGA(C/G)TCAT-3' (Oliphant *et al.*, 1989), which functions as a specific binding site for a Gcn4p activation (Arndt & Fink, 1986; Hope & Struhl, 1987; Struhl *et al.*, 1988). Recently, the GCRE half site was also identified as potential target of Gcn4p binding (Hollenbeck & Oakley, 2000; Meimoun *et al.*, 2000).

To manage a fine-tuned expression of all Gcn4p-dependent genes, not only expression of the target genes but also expression of the transcription factor has to be strictly controlled. It has been demonstrated that a 2-fold increased mRNA level occurs within a yeast cell under amino acid starvation. This results in both an elevated *GCN4* expression after 3h to 4h (Albrecht *et al.*, 1998) and 2- to 10-fold increased protein translation (Hinnebusch, 1992). This variability in the global Gcn4p amount is derived from influences on several regulation levels as described below.

Transcription factors can be further divided into several classes with respect to the DNA-binding motif and the number of monomers (Fig. 1.2). Yeast Gcn4p and Gal4p are two homodimeric representatives of different but typical classes of DNA binding proteins. The Gcn4p protein is member of the class of bZIP proteins (Landschulz *et al.*, 1988; McKnight *et al.*, 1988; Lee *et al.*, 2001; Hollenbeck *et al.*, 2002). These bZIP proteins are contacting the DNA backbone via the basic DNA binding domain (BD) that is built by a conserved DNA-responsive element present in each of the two monomer subunits. This part of the protein is separated from the leucine-rich dimerisation domain by a conserved spacing of the respective amino acids (Lee *et al.*, 2001). A characteristic repeat of at least seven leucine residues is involved in the dimerisation and forms the so-called zipper motif. For a better variation these zipper motifs cannot only be built by leucine residues but also by some unusual amino acid residues (Paluh & Yanofsky, 1991; Wanke *et al.*, 1997).

In contrast to Gcn4p, yeast Gal4p belongs to the wide class of homodimeric zinc-finger proteins that are contacting the DNA via globular domains that are formed around two zinc^{2+} ions (Rhodes & Klug, 1993; Urnov, 2002).
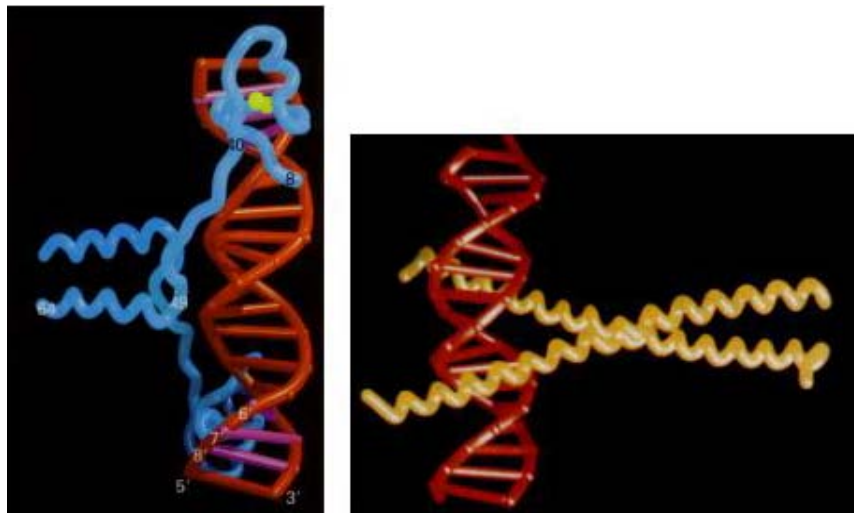
Fig. 1.2: Two ribbon model structures of the transcription factor Gal4p, and Gcn4p from the yeast *S. cerevisiae* are given. On the left site, the typical zinc-finger structure of the homodimeric Gal4p (blue) is given. The two monomers are forming a super-coil and bind to the DNA backbone (red) via two globular structures. These structures are build around two zinc^{2+}-ions that associated around two cysteine residues (yellow) of the DNA backbone. The picture was previously published from R. Marmorstein (Marmorstein *et al.*, 1992). On the right site, the ribbon structure of homodimeric yeast Gcn4p (yellow) is given, which is a representative of bZIP proteins. The two monomers of the bZIP proteins are thought to build a scissors-like structure that binds to the DNA backbone (red) via the DNA binding site that is referred to in the text. The picture was previously published from T.E. Ellenberger (Ellenberger *et al.*, 1992).

In contrast to the homodimeric Gcn4p or Gal4p proteins, the human GLI-type zinc-finger proteins (Pavletich & Pabo, 1993; Koebernick & Pieler, 2002) and the zinc-finger containing pacC of *Aspergillus nidulans* (Espeso *et al.*, 1997) are typical monomeric transcription factors.

Finally, heterodimeric transcription factors enlarge the number of possible regulators of the RNA pol II complex. Heterodimeric transcription factors can be exemplified by proteins that contain the winged-helix-motif (Zheng *et al.*, 1999). However, also many members of the *jun*-bZIP protein family are present (Kouzarides & Ziff, 1989; Chinenov & Kerppola, 2001).

In *S. cerevisiae* not only the transcriptional activation but also the transcriptional repression is an important mechanism for a regulated gene expression (Struhl *et al.*, 1998). Repression of transcription does not only occur under various nutritional (Trumbly, 1992; Carlson, 1999) but also under various environmental conditions (Brent, 1985; Reece & Platt, 1997; Bone & Roth, 2001). Typical repressor proteins also share two different functional domains, a DNA-binding domain and a dimerisation domain. Repression of transcription often occurs via a direct competition

of transcriptional activators at the upstream *cis*-elements (Fig. 1.1), but also indirect regulation mechanisms have been described. Repressors can disturb the binding of the transcriptional activators to the target sequence by interacting with their activation domain (Lodish *et al.*, 2001).

1.5.3 RNA polymerase II initiation complex

To date some general transcription factors have been characterised that are present in most transcription complexes and that are necessary for the positioning of RNA pol II at the transcriptional start site or for the assembly of the RNA pol II holoenzyme. These general transcription factors (TF) hereafter are designated as initiation factors (of transcription). The assembly of the RNA pol II has been studied extensively and the major steps of the assembly mechanisms are already known.

In vitro the formation of the RNA pol II initiation complex starts with the binding of the TATA binding protein (TBP) to the DNA TATA-box sequence element. The TBP is one of the various accessory subunits of all RNA polymerases and its existence is conserved among prokaryotes (σ-factors), archaea and eukaryotes (Cormack & Struhl, 1992; Cormack *et al.*, 1994; Rowlands *et al.*, 1994; Burgess *et al.*, 1998; Guo *et al.*, 2000; Anthony & Burgess, 2002). Different TB-proteins (TBPs) are able to distinguish between different promoter elements. Thus, TBPs are important for the transcriptional regulation of gene expression (Schultz *et al.*, 1992).

The initiation factors TFIIB and TFIIA join the DNA-bound TB-protein (Imbalzano *et al.*, 1994; Wang *et al.*, 2001). The presence of TFIIB is important for the regulation of the transcription within the yeast *S. cerevisiae* (Zhang *et al.*, 2000). Assembly of TFIIB allows the joining of a pre-formed complex of RNA pol II (+CTD) and TFIIF. This results in the formation of the transcriptional pre-initiation complex. Joining of TFIIE and TFIIH completes the *in vitro* assembly of the RNA pol II complex. In the presence of ATP, this RNA pol II initiation complex is able to unwind the DNA and to start the mRNA synthesis.

In vivo the complete formation of the RNA pol II holoenzyme seems to be more complex. It has been shown that at least two additional multi-enzymatic complexes are involved in a successful transcription initiation in *S. cerevisiae* and other eukaryotes.

First of all the TFIID-initiation complex of TBP and approximately a dozen TBP-associated factors (TAFs), which have not yet been entirely characterised, are required for transcription initiation (Struhl, 1997; Yatherajam *et al.*, 2003). *In vivo* it appears that TFIIA and the complex of TFIID/TAFs initially bind to the DNA and thereby form the start point and place for a one step joining of all other factors of the initiation complex pre-assembled within the nucleus (Stargell *et al.*, 2000). The functions of the TAF factors within the TFIID/TAF-complex are not completely known yet, since a TAF-dependent and a TAF-independent transcription initiation could be found within eukaryotes (Albright & Tjian, 2000; Kuras *et al.*, 2000; Kraemer *et al.*, 2001; Wu & Chiang, 2001).

On the other hand a big mediator complex is essential for a functional transcription initiation within yeast and higher eukaryotes (Myers & Kornberg, 2000; Bjorklund *et al.*, 2001; Gustafsson & Samuelsson, 2001; Rachez & Freedman, 2001; Boube *et al.*, 2002). Mediator proteins are directing the complex association of all *cis-* and *trans*-acting factors involved in the transcription initiation. Finally, the total association of all components of the RNA pol II holoenzyme results in a 3 MDa protein complex, almost as big as an entire ribosome. This complex is involved in a not yet assessed number of different interaction mechanisms that all have influence on transcription initiation.

In addition, other regulation mechanisms have been described, which are coupled to transcription initiation by the RNA polymerase II complex (Greenblatt, 1997). RNA II polymerases were found to mediate alterations in the chromatin structure (Edmondson & Roth, 1996; Kadonaga, 1998; Alen *et al.*, 2002) and play a key role in various regulatory mechanisms that influence the eukaryotic RNA chain elongation (Conaway *et al.*, 2000; Gnatt, 2002; Hartzog *et al.*, 2002).

Other important transcriptional regulation mechanisms are the phosphorylation of transcription factors by various signal cascade pathways (Whitmarsh & Davis, 2000), the phosphorylation of the CTD subunit within the RNA pol II complex (Kobor & Greenblatt, 2002; Proudfoot *et al.*, 2002) and the controlled expression of the general transcription factors.

1.6   Regulation of eukaryotic translation

After the pre-mRNAs of specific genes are transcribed and completely processed within the eukaryotic nucleus, these mRNAs have to be exported into the cytoplasm for translation at the ribosomes.

The stability and the degradation of mRNA have an important influence on the translational regulation of gene expression in the cytoplasm (Day & Tuite, 1998). In many eukaryotes the stability of mRNAs is controlled by specific intrinsic nucleotide sequences (AU-rich elements, ARE) that function as targets for regulatory proteins (Chen & Shyu, 1994). These regulatory proteins (ARE-binding protein, ARP) destabilise mRNAs by promoting poly(A) tail shortening. Other nucleotide sequences (iron-responsive elements, IRE) are able to prevent unstable mRNAs from degradation by endonucleolytic cleavage via binding of iron regulatory proteins (IRP)(Binder *et al.*, 1994). mRNA destabilising elements were also found in the mRNAs of *S. cerevisiae*, while stabilising elements are poorly documented (Herrick & Jacobson, 1992; Pierrat *et al.*, 1993; Muhlrad & Parker, 1994; Muhlrad & Parker, 1999). Finally, the regulation of mRNA surveillance through mRNA bound proteins (mRNP) have strong influence on the controlling of the eukaryotic translation machinery (Hilleren & Parker, 1999a; Hilleren & Parker, 1999b; Mitchell & Tollervey, 2000).

Among eukaryotes, targeting of mRNAs for nuclear export seems to start co-transcriptionally (Lei *et al.*, 2001; Hammell *et al.*, 2002). The major regulation of eukaryotic protein synthesis is achieved by a controlled translation initiation (Voorma *et al.*, 1994; Kozak, 1999; Kimball, 2001; Pestova *et al.*, 2001), whereas a controlled translation of *GCN4* mRNAs plays an important role in most fungi (Hinnebusch, 1994; Hinnebusch, 1997).

1.6.1 Regulation of the pre-initiation complex

Generally, regulation of translation initiation is directed by modifications of the eukaryotic initiation factors (eIF), several of which are phosphoproteins (Day & Tuite, 1998). A precise order of subsequent steps of pre-assemblies of many eukaryotic initiation factors at the small ribosomal subunit, are followed by the later joining of the 60S ribosomal subunit, finally building the 80S initiation complex that is competent for the translation.

Translation initiation starts with the association of eIF3 and the eIF1A to the 40S ribosomal subunit (Fig. 1.3). These steps are followed by the formation of the 43S pre-initiation complex. The initiation factor eIF2 has a critical role in the assembly of this complex. eIF2 recruits an initiator Met-tRNA$_i$ and a guanosine-tri-phosphate (GTP). Subsequently, a ternary complex of eIF2-GTP-Met-tRNA$_i$ is formed. This ternary complex (Fig. 1.3) joins the association of 40S, eIF3, and eIF1A.

At this level, the regulation of translation initiation mainly occurs via a regulated phosphorylation of the eIF2-GTP and the nucleotide exchange factor eIF2B (Pavitt *et al.*, 1998; Krishnamoorthy *et al.*, 2001). The heteropentameric eIF2B (Kimball *et al.*, 1998; Gomez & Pavitt, 2000; Nika *et al.*, 2000; Gomez *et al.*, 2002) catalyses the conversion of the inactive eIF2-GDP to the eIF2-GTP active form, which is required for the formation of the ternary complex. It has previously been demonstrated that phosphorylation of the α-subunit of eIF2 inhibits the exchange of GDP to GTP and thereby inhibits the translation initiation (Pavitt *et al.*, 1998). Recent studies on the structure and functions of the nucleotide exchange factor eIF2B demonstrated that a phosphorylation of the *SUI2* gene product is involved in the translational regulation mechanism. Sui2p is tightly bound to the two catalytic domains of the Gcd6p subunit of eIF2B. A phosphorylation of Sui2p blocks the eIF2B function probably by inhibition of the interactions with the β- and γ- subunits of eIF2-GDP (Krishnamoorthy *et al.*, 2001).

After the assembly the 43S pre-initiation complex binds to the mRNA, rewinds it and starts the scanning process. The pre-initiation complex identifies the first AUG codon of the mRNA that is located within the 'Kozak' consensus sequence 5'-ACCAUGG-3' (Kozak, 1981; Kozak, 1986; Kozak, 1999). Variations of this consensus sequence result in differences in the translation efficiency. The eIF3 proteins of the 43S pre-initiation complex are involved in the recognition of this specific consensus sequence, whereas the complete ATP consuming processes of mRNA binding and mRNA rewinding are under the control of the cap binding complex eIF4F.

80S-initiation complex

nucleotide exchange
[2]

ternary-complex

43S-pre-initiation complex

40S-pre-initiation complex

46S-pre-initiation complex
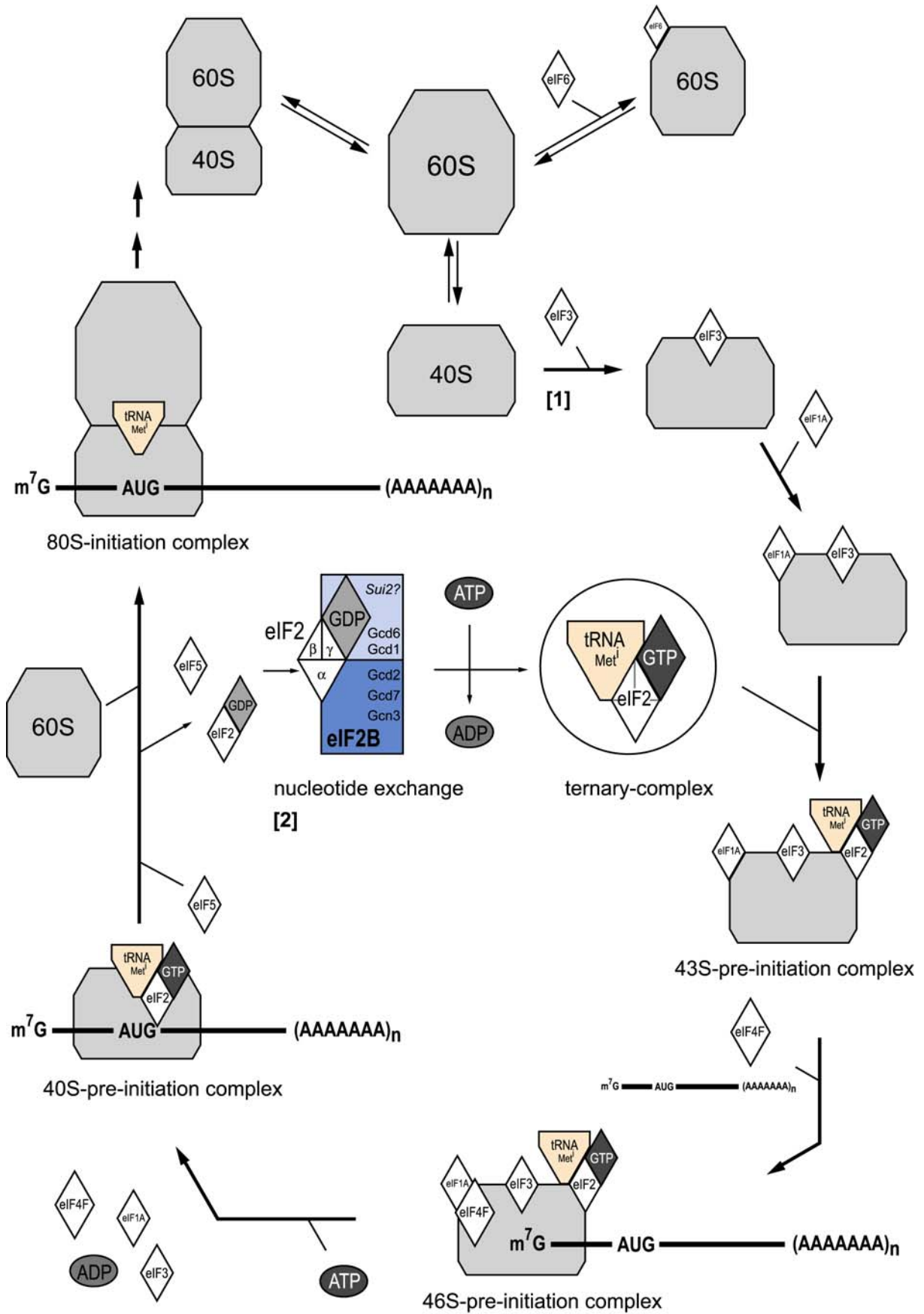
Fig. 1.3: Scheme of the eukaryotic translation initiation and the nucleotide exchange reaction of the eIF2 initiation factor at the eIF2B-complex. [1] Translation initiation starts with the binding of the multimeric initiation factor eIF3 to the 40S ribosomal subunit and is followed by subsequent binding of several other eIF-factors referred to in the text. A pre-association of the 60S subunit is prevented by the function of eIF6. One important step in controlling the translation initiation is the joining of the ternary complex to the 40S subunit to build the 43S pre-initiation complex. This step is under the control of the translational derepression that acts via the Gcn2p kinase activity. Under amino acid starvation conditions the Gcn2p kinase phosphorylated eIF2 prevents the GDP exchange, probably by phosphorylation of the α-subunit of eIF2-GDP (for details, see the text). After formation of the 80S initiation complex, the translation is continued by joining of a second amino-acyl-tRNA. **[2]** Regeneration of the ternary complex starts with the association of the transcription factor eIF2 at the eIF2B complex. While eIF2 (white rhombus) consists of three subunits (α-, β-, and γ-subunit), the eIF2B complex (light and dark blue) contains at least 5 subunits (Gcd1, Gcd2, Gcd6, Gcd7, Gcn3) and one tightly associated factor (*Sui2*?). The inital Met-tRNA$_i$ that is referred to in the text is given in light orange. The multimeric eIF4F complex referred to in the text is given by a big white rhombus. GDP (light grey rhombus) and GTP (dark grey rhombus), as well as ADP (light grey circle) and ATP (dark grey circle) are indicated, respectively.

1.6.2 Regulation of eukaryotic and fungal eIF4 translation complex

The multimeric eIF4F initiation complex is mainly composed of eIF4E and requires the assistant factors eIF4A and eIF4B for binding the 43S pre-initiation complex (Pestova *et al.*, 2001). The eIF4F complex (see figure 1.3) recognizes the 5'- m^{7}G-cap structure present in all eukaryotic mRNAs and subsequent helicase functions rewind the mRNAs secondary structures. In mammals the activity of the eIF4F protein complex is mainly regulated by a phosphorylation of the eIF4E subunit. A phosphorylated form of the mammalian eIF4E has a greater affinity to the m^{7}G-cap structure and the eIF4G factor when compared to non-phosphorylated eIF4E (Goss *et al.*, 1987; Minich *et al.*, 1994). In mammals differences in the phosphorylation state of eIF4E mediate changes in the overall rates of protein synthesis.

In contrast to the mammalian Ser209, the phosphorylation sites of yeast eIF4E are Ser2 and Ser15 (Joshi *et al.*, 1995; Zanchin & McCarthy, 1995). Only a small fraction of yeast eIF4E is phosphorylated *in vivo*. Therefore, the yeast translational regulation system appears to be less dependent on phosphorylation (Zanchin & McCarthy, 1995). In *S. cerevisiae* other mechanisms of regulating the eIF4E activity are more important, such as the translational repressor protein (p20), which competes with eIF4G for binding to eIF4E subunit. Phosphorylation of p20 increases the affinity of eIF4E to the m^{7}G-cap and results in a higher translation rate, whereas a dephosphorylated p20 is present under non-induced conditions (Altmann *et al.*, 1997). Thus, it seems to be clear that p20 is a repressor of the yeast cap-dependent translation initiation.

After the recognition of the m^7G-cap structure and the pre-scanning of the mRNA, the 43S pre-initiation complex is positioned at the AUG start codon. When the positioning of the 43S pre-initiation complex has been finished, the translation initiation factors eIF1A, eIF3 and eIF4 have left the pre-initiation complex. eIF1A enhances the ability of eIF1 to dissociate aberrantly assembled complexes from the mRNA (Pestova *et al.*, 2001). Dissociation of all these factors results in a smaller 40S intermediate complex that is temporarily associated with the eIF5 factor to induce the association of the 60S ribosomal subunit. The energy for the assembly of the competent 80S translation complex is delivered by the eIF2 bound GTP. The eIF2-GTP form is dephosphorylated to eIF2-GDP and dissociates from the complex together with eIF5. Subsequently, eIF2-GDP is recycled for a new assembly process (Fig. 1.3). Finally, the entire 80S initiation complex is ready for the translation elongation that starts with the entering of a second aminoacyl-tRNA.

1.6.3 Translational control of Gcn4p expression

Generally, the eukaryotic control of gene expression is regulated by translation initiation. In fungal organisms such as *S. cerevisiae* or *A. nidulans*, the gene expression is also regulated by control of the transcriptional regulator Gcn4p. This regulation process occurs on various levels and is termed 'translational derepression'.

The enhanced translation of *GCN4* mRNA is the result of the tricky arrangement of the four small upstream open reading frames (uORFs) within the 5'-non-coding region of the *GCN4* mRNA. The distance of these four uORFs is arranged in such way to regain the second competence of the 40S ribosomal subunit exactly at the translational start of the *GCN4* ORF under amino acid starvation conditions and with decreased levels of eIF2-GTP (Hinnebusch, 1997).

In contrast, the small 40S ribosomal subunit binds to *GCN4* mRNA and associates with eIF2-GTP and Met-tRNA$_i$ under non-starvation conditions to build a competent initiation complex (Fig. 1.3). This complex is scanning for the first ATG codon of the first uORF of *GCN4* mRNA. The translation is initiated and prolonged until dissociation at the first stop codon. During this process eIF2-GTP is hydrolysed to eIF2-GDP. For a further association of the small ribosomal subunit, eIF2-GDP has to be recycled (Fig. 1.3).

Under non-starvation conditions the recycling of eIF2-GTP takes place before the small subunit reaches the fourth uORF of *GCN4* mRNA. Subsequently, this fourth uORF is translated. Under non-starvation conditions this process results in a drastically reduced translation of the *GCN4* ORF, since ribosomal subunits cannot re-initiate at the downstream *GCN4* transcriptional start. According to this scheme, only the presence of the first and the fourth ORF of *GCN4* mRNA are essential for a function and controlled translation of *GCN4*, while the deletion of all four uORFs results in both a high *GCN4* gene expression and a higher Gcn4p protein level (Mueller & Hinnebusch, 1986; Mueller *et al.*, 1988).

The translation of *GCN4* mRNAs is drastically enhanced under amino acid starvation conditions due to the phosphorylation of eIF2. This translation derepression is effected by a not yet known signal that activates uncharged t-RNAs. Higher amounts of uncharged t-RNAs serve as signal for the Gcn2p sensor kinase of the Gcn4p-dependent transcriptional activation (Lanker *et al.*, 1992; Vazquez de Aldana *et al.*, 1994). In presence of high amounts of uncharged tRNAs, the activated Gcn2p kinase phosphorylates the α subunit of eIF2 (Fig. 1.3), which, in turn, results in a decreased eIF2-GTP concentration (Wek *et al.*, 1995; Zhu *et al.*, 1996; Zhu & Wek, 1998). Decreased amounts of eIF2-GTP result in an enhanced translation of *GCN4* mRNA via Gcn2p and Gcn20p (Marton *et al.*, 1993; Vazquez de Aldana *et al.*, 1995; Marton *et al.*, 1997). In contrast to this translational derepression, the translation of most other yeast mRNAs is reduced (Hinnebusch, 1994).

In addition to the translational derepression mechanism, the half live of Gcn4p is increased 6-fold under amino acid starvation conditions. This prolonged protein stability depends on the praline-, glutamic acid-, serine- and threonine-rich domain (PEST) of the protein (Kornitzer *et al.*, 1994). Furthermore, an inhibited phosphorylation of threonine 165 of Gcn4p seems to result in longer stability of Gcn4p, since threonine 165 functions as the target for ubiquitination and therefore for degradation of Gcn4p (Meimoun *et al.*, 2000; Pries *et al.*, 2002).

1.7   Eukaryotic and fungal ribosomes

Recently, many new insights into the structure of prokaryotic (Carter *et al.*, 2000; Gabashvili *et al.*, 2000; Wimberly *et al.*, 2000) as well as eukaryotic ribosomes have been described (Verschoor *et al.*, 1998; Gomez-Lorenzo *et al.*, 2000; Beckmann *et al.*, 2001; Spahn *et al.*, 2001).

Eukaryotic 80S ribosomes are RNA-based translation machines that are composed of a large 60S and a small 40S subunit (Fig. 1.4), including 50 to 80 ribosomal proteins (Green & Noller, 1997; Spahn *et al.*, 2001). In contrast, prokaryotic ribosomes exhibit a 70S sedimentation and are composed of a 50S and a 30S ribosomal subunit (Fig. 1.4) (Gabashvili *et al.*, 2000; Wimberly *et al.*, 2000). The eukaryotic 60S subunit is composed of the 28S, 5.8S, 5S rRNAs and approximately 50 proteins, whereas the small subunit (40S) comprises the 18S rRNA and 33 proteins (Spahn *et al.*, 2001).

Fungal ribosomes slightly differ from those of other eukaryotes. Yeast ribosomes only contain 46 proteins in the large and 32 proteins in the small subunit. In addition, yeast ribosomes contain a 25S rRNA (3400 nt) within the 60S subunit (Verschoor *et al.*, 1998). The yeast genome has been described to contain 137 genes encoding 78 different ribosomal proteins, with 59 duplicated proteins (Mager *et al.*, 1997). All yeast ribosomal proteins have mammalian counterparts beside the rat L28 protein, which cannot be found in yeast (Mager *et al.*, 1997).
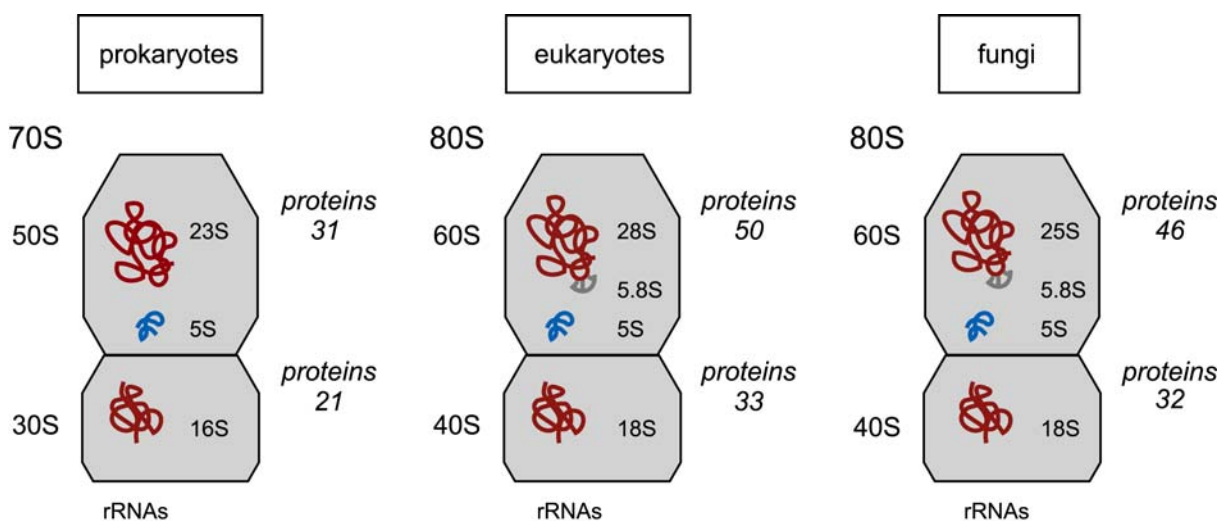
Fig. 1.4: General scheme of the ribosomes of prokaryotes, eukaryotes and fungi. Prokaryotic ribosomes sediment with 70S and are composed of a 50S and a 30S ribosomal subunit. Eukaryotic ribosomes are larger and contain subunits that sediment with 60S and 40S. Additionally, the large eukaryotic subunit contains a 5.8S rRNA not present within prokaryotic ribosomes. rRNAs are given in red, blue and dark grey.

Most information about eukaryotic ribosomes are derived from experiments performed in prokaryotes. It is assumed that ribosomal subunits are similar in function and fundamental mechanisms because of high homologies and evolutionary conservation (Spahn *et al.*, 2001). Due to a higher number of proteins eukaryotic ribosomes are larger than their prokaryotic counterparts (Wittmann-Liebold, 1986; Wool *et al.*, 1990; Planta & Mager, 1998). There also seem to be some functional differences in translation initiation (Sachs *et al.*, 1997), translation elongation (Triana-Alonso *et al.*, 1995; Chakraburtty & Triana-Alonso, 1998; Triana-Alonso *et al.*, 2000) and in the function of several antibiotics (Spahn & Prescott, 1996).

Apart from the crucial functions in translation, ribosomal proteins are also required for further cellular processes. The prokaryotic L2 protein has been described to be involved in association of the ribosomal subunits, tRNA binding and the peptidyl transfer (Diedrich *et al.*, 2000). Furthermore, *rpl16A* gene of *A. nidulans* has been demonstrated to be differentially expressed during sexual development (Jeong *et al.*, 2000). Ribosomes also play a function in translational regulation of *GCN4* mRNA. Deletion of one protein of the small yeast ribosomal subunit, Rps31p, decreases the eIF2B requirement (see above), and subsequently, the small subunit mediates this effect of low eIF2B activity by increased translation of *GCN4* mRNA (Mueller *et al.*, 1998).

1.8   The aromatic amino acid biosynthesis pathway of fungi

The synthesis of aromatic amino acid (Fig. 1.5) starts with a stereo-specific aldol-like condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) (Knaggs, 1999; Knaggs, 2000; Knaggs, 2001). This initial step of the shikimate pathway is catalysed by the DAHP synthase activity (E.C. 4.1.2.15).

In *S. cerevisiae* the two DAHP synthase isoenzyme activities are encoded by the *ARO3* and *ARO4* genes (Teshiba *et al.*, 1986; Künzler *et al.*, 1992). While the Aro3p is inhibited by phenylalanine, the *ARO4* gene product is inhibited by tyrosine (Paravicini *et al.*, 1988; Paravicini *et al.*, 1989; Schnappauf *et al.*, 1998a). In *A. nidulans aroF* and *aroG* encode for two similar DAHP synthases that are differentially regulated by tyrosine (aroFp) and phenylalanine (aroGp), respectively (Hartmann *et al.*, 2001). Although the prokaryotic and eukaryotic pathways of the aromatic amino acid biosynthesis are almost identical, most fungi only contain two instead of three enzymes for the initial step, whereas prokaryotes express one to three DAHPS enzymes for the same reaction (Ahmad & Jensen, 1988; Ahmad *et al.*, 1990). Among fungi, only *Neurospora crassa* has been described to contain a tryptophan-inhibited DAHP synthase activity for the initial step of the aromatic amino acid biosynthesis (Nimmo & Coggins, 1981a; Nimmo & Coggins, 1981b; Walker *et al.*, 1996).

In five subsequent reactions (Fig. 1.5) DAHP is converted into 5-enolpyruvyl-shikimate-3-phosphate (EPS3P) via four intermediates, namely 3-dehydroquinate (DHQ), 3-dehydroshikimate (DHS), shikimate, and shikimate-3-phosphate (S3P), respectively. In yeast these five reactions are catalysed by a pentafunctional enzyme complex that is encoded by the *ARO1* gene locus (Duncan *et al.*, 1988).

The chorismate synthase activity (E.C. 4.6.1.4) is the last common enzyme of the shikimate pathway. In the reaction that is catalysed by the chorismate synthase, 5-enolpyruvyl-shikimate-3-phosphate is converted into chorismic acid (CA) by a trans-1,2-elimination of phosphate (Knaggs, 1999). In *S. cerevisiae* this enzymatic activity is encoded by the *ARO2* gene product (Jones *et al.*, 1991). As described for the differences among the DAHP synthase (DAHPS) enzymes, also chorismate synthases are different between prokaryotes and eukaryotes. Most prokaryotes carry monofunctional enzymes, whereas the eukaryotic molds carry bifunctional enzymes

that also exhibit a flavin reductase activity with a not yet understood function (Macheroux *et al.*, 1999).

The end product of the shikimate pathway, chorismic acid (CA), is an important precursor of several biosynthetic pathways (Herrmann, 1995a; Herrmann, 1995b; Knaggs, 1999; Knaggs, 2001). Two main important of these are the subsequently strands of the aromatic amino acid biosynthesis, the tryptophan and the phenylalanine/tyrosine branch. Chorismic acid is also an important precursor of other secondary metabolic pathways that result in namely coumarins (*Trigonella spec.*), lignins and lignans (*Lithospermum erythrorhizon*), tannins (*Aspergillus niger*), betalains (*Amanita spec.*), ubichinone (yeast and *Escherichia coli*), flavanoids (*Arabidopis thaliana*), and vitamin K2 derivates (*Bacillus subtilis* and *E. coli*), respectively (Herrmann, 1995a; Herrmann, 1995b; Knaggs, 1999; Knaggs, 2001).

1.8.1 The tryptophan-branch of the aromatic amino acid biosynthesis

Downstream to shikimate (Fig. 1.5) the synthesis of tryptophan starts with the formation of anthranilic acid (AA). In the first reaction chorismate is transformed into 2-amino-2-deoxyisochorismate (ADIC). This reversible and ammonia consuming step is catalysed by a glutamine amido transferase activity that is followed by a an irreversible elimination of pyruvate. In yeast these reactions are catalysed by the heterodimeric *TRP2/3* encoded anthranilate synthase complex (E.C. 4.1.3.27). *TRP2* codes for the anthranilate synthase activity, while *TRP3* encodes the glutamine-amido-transferase activity and an associated indoleglycerolphosphate-synthase activity (IGP), which is not strictly necessary for this reaction step (Braus *et al.*, 1985; Prantl *et al.*, 1985). In five subsequent reactions the anthranilic acid is converted into tryptophan via phosphoribosylanthranilate (PRA), carboxy-phenylamino-1-deoxyribose-5-phosphate (CDRP), indoleglycerolphosphate (IGP) and indole. These five steps subsequently are catalysed by the *TRP4* encoded phosphoribosyl transferase activity (E.C. 2.4.2.18), the *TRP1* encoded PRA-isomerase activity, and the IGP-synthase activity of the *TRP3* encoded enzyme. The final two-step reactions from indoleglycerolphosphate via indole to the end product tryptophan are catalysed by the tryptophan synthase activity (E.C. 4.2.1.20).

In *S. cerevisiae* the tryptophan synthase activity is encoded by the bifunctional *TRP5* gene product (Bailey & Turner, 1983), whereas this enzymatic activity is encoded by two gene products in other organisms (Yanofsky, 1987; Hyde *et al.*, 1988).
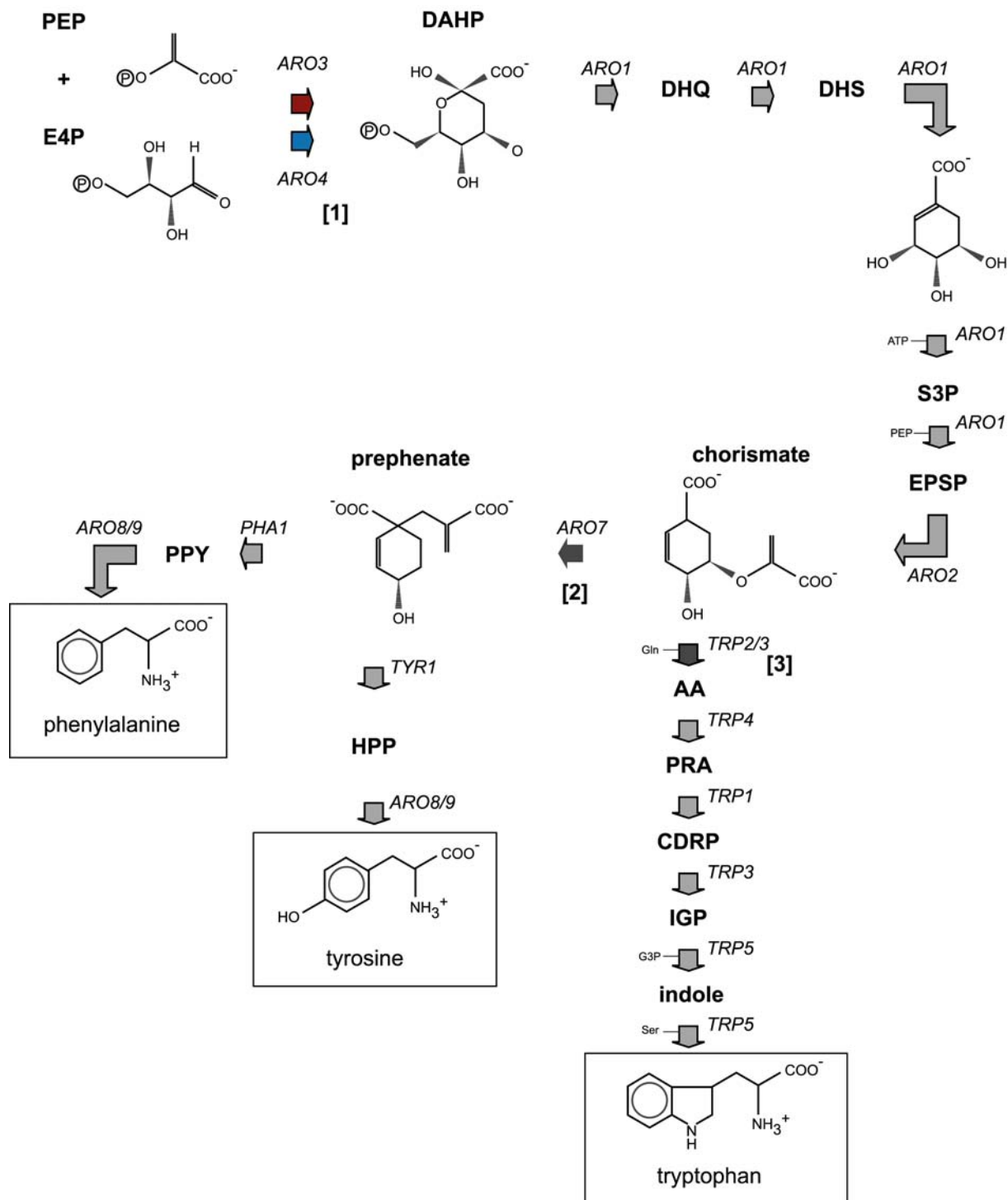
Fig. 1.5: Schematic drawing of the aromatic amino acid biosynthesis of *S. cerevisiae*. Enzyme activities referred to in the text are given in grey arrows, while some specific feedback inhibitable enzyme activities are given in black arrows. Genes encoding for enzyme activities are given in italic. **[1]** DAHP synthase activities (E.C. 4.1.2.15) are encoded by the two isogenes *ARO3* and *ARO4*. The *ARO3* encoded enzyme activity is feedback inhibitable by the end product phenylalanine, while the

ARO4 encoded activity is inhibited by tyrosine. **[2]** The chorismate mutase activity (E.C. 5.4.99.5) is encoded by the *ARO7* gene product, is feedback inhibited by the end product tyrosine, but activated by the end product tryptophan. **[3]** The heterodimeric anthranilate synthase activity (E.C. 4.1.3.27) is encoded by the *TRP2* and *TRP3* gene product and is feedback inhibited by the end product tryptophan. Abbreviations are: AA, anthranilic acid; CDRP carboxy-phenylamino-1-deoxyribulose-5-phosphate; DAHP, 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate; DHQ, 3-hydroquinate; DHS, 3-dehydroshikimic acid; E4P, erythrose-4-phosphate; EPSP, 5-enolpyruvyl-shikimate-3-phosphate; HPP, 4-hydroxyphenylpyruvic acid; IGP, indoleglycerole phosphate; PEP, phosphoenolpyruvic acid; PPY, phenylpyruvic acid; PRA, phosphoribosylanthranilic acid; S3P, shikimate-3-phosphate.

1.8.2 The phenylalanine- and tyrosine-branch

Synthesis of the other two end products of the aromatic amino acid biosynthesis, i.e. phenylalanine and tyrosine, starts with an intramolecular rearrangement of carboxyl groups of chorismic acid. This rearrangement of chorismic acid to prephenic acid (PA) is a Claisen reaction that is catalysed by a single enzyme, the chorismate mutase activity (E.C. 5.4.99.5). In *S. cerevisiae*, this enzyme activity is encoded by the *ARO7* gene (Ball *et al.*, 1986; Schnappauf *et al.*, 1998b; Helmstaedt *et al.*, 2002), whereas it is encoded by *aroC* in *Aspergillus nidulans* (Krappmann *et al.*, 1999; Krappmann & Braus, 2003), and HARO7 in *Hansenula polymorpha* (Krappmann *et al.*, 2000).

Among prokaryotic microorganisms, the chorismate mutase (CM) activity is often part of a bifunctional enzyme complex, of associated P- and T-proteins (Chen *et al.*, 2003). This bifunctional complex contains a prephenate dehydratase, a prephenate dehydrogenase, and a 3-DAHP-synthase activity.

In contrast, all yet characterised eukaryotic CM activities as well as the CM activities from *Bacillus subitilis* (Gray *et al.*, 1990), *Methanococcus jannaschii* (MacBeath *et al.*, 1998) or fungi (Krappmann *et al.*, 2000; Helmstaedt *et al.*, 2001) display a monofunctional character.

In *S. cerevisiae* prephenic acid then is converted into two similar chemical products, tyrosine and phenylalanine. A decarboxylation and a dehydration reaction are catalysed by the *PHA2* encoded prephenate dehydrogenase enzyme (E.C. 1.3.1.13) and yield the intermediate 4-hydroxyphenylpyruvate (4HP). The end product tyrosine is built by a transamination reaction of 4HP that is catalysed by an aminotransferase activity (E.C. 2.6.1.57). The end product of the other part of the second branchpoint, phenylalanine, is formed by the *TYR1* encoded prephenate dehydratase activity and a subsequent transamination of phenylpyruvate. This reaction is catalysed by the dehydratase activity (E.C. 4.2.1.51). The final

transamination reactions of these two strands of the second branch are catalysed by the *ARO8* and *ARO9* encoded gene products, respectively (Iraqui *et al.*, 1998; Urrestarazu *et al.*, 1998).

For other phylogenetic groups an alternative way of the synthesis of phenylalanine and tyrosine has been described. Higher plants prefer a formation of phenylalanine or tyrosine via the *L*-arogenate intermediate, and some microorganisms like *Xanthomonas spec.* or *Pseudomonas spec.* posses both synthesis pathways (Jensen & Fischer, 1987; Ahmad & Jensen, 1988).

1.8.3 Translational regulation of the aromatic amino acid biosynthesis

When compared to prokaryotes, most genes of the different amino acid biosynthetic pathways are under the control of a transcriptional regulation in *S. cerevisiae*, *A. nidulans*, or *N. crassa*. The basal expression of all genes of the amino acid biosynthesis is generally induced and therefore results in a relatively high basal level of intracellular pools of amino acid biosynthetic proteins. The fine-tuned control of these protein levels is regulated by the 'general control' or 'cross pathway control' of amino acid biosynthesis. This transcriptional control is acting through the transcription factor Gcn4p (Hinnebusch, 1988) and its related proteins among other fungi (Paluh *et al.*, 1988; Wanke *et al.*, 1997; Hoffmann *et al.*, 2001).

Comprehensive experiments demonstrated that most genes of the described above aromatic amino acid pathway, e.g. *ARO1* to *ARO4* and *TRP2* to *TRP5*, are derepressed under amino acid starvation (Miozzari *et al.*, 1978; Teshiba *et al.*, 1986; Duncan *et al.*, 1988; Jones *et al.*, 1991).

From the genes of the aromatic amino acid biosynthetic pathway, only *ARO7*, *TYR1* and *TRP1* are not regulated by the general control of transcription (Braus *et al.*, 1988; Mannhaupt *et al.*, 1989; Schmidheini *et al.*, 1990).

Generally, expression of all of the genes of the aromatic amino acid pathway is not only controlled by transcriptional and translational regulations. Activities of the encoded gene products also depend on allostery regulation mechanisms that were partially characterised in detail.

1.9   Allosteric control of enzyme activities

In general, allosteric regulations of the specific activity of a given enzyme are the result of 3-dimensional alterations within the enzyme structure. These conformational changes can be induced by substrates, ligands, and end products. All these 'effectors' can influence a regulatory site of the enzyme that is distinct from the active catalytic centre. In contrast, changes at the active site of the enzyme are defined as intrasteric regulations. In order to describe all possible conformational changes within an enzyme theoretically, several models of allosteric regulations have been established. The early MWC-model of Monod, Wyman, and Changeux (1965) describes the conformational changes of a symmetric enzyme by global shifts between two defined quaternary structures. The relaxed state of the enzyme (R-state) is defined as a condition that has high affinity towards the substrate (K-value) or increased catalytic activity (V-value). The tensed state of the enzyme (T-state) has low affinity towards the substrate or reduced catalytic activity. The allosteric equilibrium of a given enzyme is determined by the ratio between these two quaternary conformations and is described by a constant (L). In a later model of Koshland, Némethy and Filmer (KNF-model), the natural sequential changes of the entire enzyme structure was considered (Koshland *et al.*, 1966). It has been assumed that co-operative effects within one subunit of the enzyme subsequently change the conformation of the neighbouring subunits and thereby change the entire allosteric state. Finally, both models were combined in a general model for allostery regulation described by Eigen (1967).

1.9.1 DAHP synthase structure of fungi

Global conformational changes of given enzyme structures are the basis for allosteric regulation mechanism. DAHP synthase isoenzymes are known to catalyse the first step of the shikimate pathway of the aromatic amino acid synthesis (Ahmad *et al.*, 1986; Braus, 1991). Microorganisms derived from different phylogenetic groups have been described to contain between one and three DAHP synthase isoenzymes (Ahmad *et al.*, 1986). Not only the total number of enzymes for the initial step of the aromatic amino acid biosynthesis is variable among the different organisms, but also the enzymes themselves differ in their sensitivities towards feedback inhibition (Schnappauf *et al.*, 1998a; Hartmann *et al.*, 2001). The basis for

this conformational regulations are given by variations among the 3-dimensional structures of the DAHP synthase enzymes.

DAHPS enzymes belong to the wide class of TIM barrel enzymes (Wierenga, 2001; Nagano *et al.*, 2002). The name of this enzymatic group is derived from the t̲riose p̲hosphate i̲somerase (TIM) that catalyses the isomerisation of glycerinealdehyd-3-phospate and dihydroxyacetonephosphate within the glycolytic pathway (Alber *et al.*, 1981). The typical structure of TIM barrel folds consists of eight central, parallel or mixed β-sheets that are surrounded by eight α-helices. This $(\alpha/\beta)_8$ barrel structure has been found among many glycolytic enzymes as well as among functionally different enzymes such as binding proteins and transport metabolites (Hegyi & Gerstein, 1999).

The active site of TIM barrel enzymes (Fig. 1.6) is located at the C-terminal ends of the β-strands. Therefore, the geometry of the active site is shaped by residues of the eight α-loops that are following after the β-strands (Wierenga, 2001). These $\beta\alpha$-loops have a variable length and are important for the catalytic function of the TIM barrel enzymes (Larsen *et al.*, 1998; Maes *et al.*, 1999; Lang *et al.*, 2000).

In contrast to these, the $\alpha\beta$-loops (Fig. 1.6) at the backside of the $(\alpha/\beta)_8$ barrel structure are supposed to be important for the enzyme stability of all TIM barrel proteins (Urfer & Kirschner, 1992; Hocker *et al.*, 2001).

The DAHPS from *S. cerevisiae* and *Escherichia coli* are extended TIM barrel proteins, which carry some additional $\alpha\beta$-loops that are involved in the regulation of the feedback inhibition of DAHP synthase enzymes (Hartmann *et al.*, 2003). Namely, the $\beta0$, $\alpha0$, $\alpha00$, and the $\beta6a$ and $\beta6b$ strands are required for the formation of a 3-dimensional structure that is supposed to be the effector binding cavity of DAHP synthase enzymes. To date, the phenylalanine-inhibitable DAHPS from *E. coli* and the tyrosine-inhibitable DAHPS from *S. cerevisiae* were crystallised (Schneider *et al.*, 1999; Shumilin *et al.*, 1999; Shumilin *et al.*, 2002; Hartmann *et al.*, 2003). Comparison of various modelled DAHPS structures based on these data demonstrated that the additional $\alpha\beta$-loops of DAHPS are indeed located next to cavity assumed to be the effector binding site.
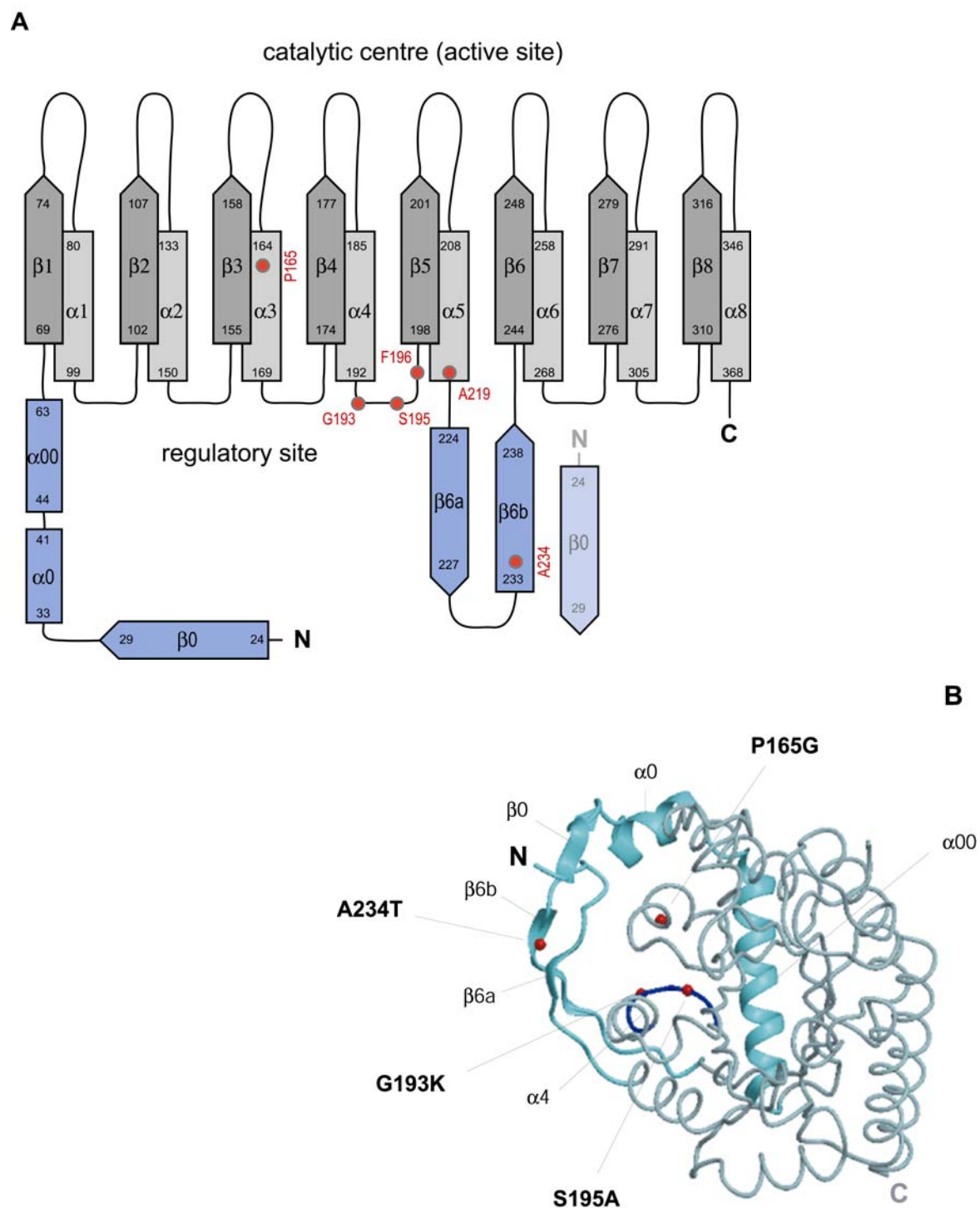
Fig. 1.6: A topology plot of the tyrosine-inhibitable DAHP synthase (Aro4p) from *S. cerevisiae* and a ribbon-model structure of one monomer of the Aro4p DAHP synthase are given. [A] The central $(\alpha/\beta)_8$ barrel structure common for all TIM barrel proteins is given in light and dark grey. Additional structure elements of the Aro4p DAHP synthase, located at the N-terminus are given in dark blue. An interacting $\beta0$-strand from second monomer is given in light blue. Amino acids residues are given by numbers. Some amino acid residues referred to in Chapter IV are marked by red dots. **[B]** A ribbon-model of one monomer of Aro4p is given. The structure was modelled on data collected from a crystallised yeast Aro4p enzyme and collected at a resolution of 2.3Å (Schneider *et. al.,* 1999) and 1.9Å (Hartmann *et. al.* , 2003), respectively. The projection of the four amino acid substitutions referred to in Chapter IV demonstrated that all four residues are distributed nearby a cavity assumed to be the effector-binding site of the DAHP synthase enzymes. The program MOLSCRIPT 2.1 (Kraulis, 1991) and RASTER3D (Merritt and Murphy, 1994) were used for this presentation.

1.9.2 Allostery regulation of the aromatic amino acid biosynthesis

In *S. cerevisiae* the enzyme activities that catalyse the initial step of the shikimate pathway, Aro3p and Aro4p, are strictly inhibited by the end products of the pathway, phenylalanine (Ki=75μM) and tyrosine (Ki=0.9μM), respectively. For Aro3p, phenylalanine acts as competitive inhibitor with respect to PEP and as a non-competitive inhibitor with respect to E4P. The inhibition behaviour is twisted for Aro4p, where tyrosine acts as competitive inhibitor to PEP and as a non-competitive inhibitor to E4P (Paravicini *et al.*, 1989; Schnappauf *et al.*, 1998a). In *A. nidulans* the allostery regulation of the homologous DAHPS enzymes is different. For the phenylalanine-inhibitable aroFp tyrosine acts as a competitive inhibitor in the reaction with PEP, while phenylalanine is the competitive inhibitor in the reaction with E4P for the tyrosine-inhibitable aroGp (Hartmann *et al.*, 2001). Although both enzymes are completely inhibited by the respective end products, the inhibition values are weaker when compared to those of the yeast enzymes (aroFp, Ki=8.4μM; aroGp, Ki=1.2μM).

A comparison of the deduced amino acid sequences of DAHPS derived from fungi with those derived from prokaryotes, revealed that all DAHPS among different phylogenetic groups share high sequence identities. The most obvious difference is the existence of a third tryptophan-inhibitable DAHPS among representatives of the *Enterobacteriacea* (Ahmad *et al.*, 1986). A gene for a tryptophan-inhibitable DAHPS is not present in the yeast genome (Mewes *et al.*, 1997). The allostery regulation of the tryptophan-inhibitable DAHPS activity is different when compared to the regulation of the two other enzymes. Neither phenylalanine, tyrosine, nor chorismic acid are competent inhibitors of this enzyme activity. Additionally, tryptophan only partially inhibits the tryptophan-inhibitable DAHPS (inhibition of 50% to 70%) (Ray *et al.*, 1988; Ray & Bauerle, 1991).

In contrast to this weak inhibition of the tryptophan-inhibitable DAHPS, tryptophan considerably affects other enzyme activities involved in the aromatic amino acid biosynthesis. With respect to the chorismate intermediate, tryptophan was demonstrated to be a competitive inhibitor of the anthranilate synthase activity (Braus *et al.*, 1985). In addition, tryptophan is also a strong activator of the chorismate mutase activity (Schnappauf *et al.*, 1998b; Schnappauf *et al.*, 1998c; Krappmann *et al.*, 2000). Furthermore, the end product tyrosine was demonstrated to inhibit the tyrosine-regulated DAHPS as well as the chorismate mutase activity

(Schnappauf *et al.*, 1998b), whereas phenylalanine only serves as an inhibitor of the phenylalanine-regulated DAHPS.

Allostery regulations of end products and intermediates of the aromatic amino acid pathway are the last step in a overall precise and fine-tuned regulation of enzyme activities among eukaryotes and prokaryotes, respectively (Helmstaedt *et al.*, 2001). Together with the two main regulations on the transcriptional and the translational level, these regulating systems result in a fine-tuned and balanced expression of the entire gene expression among every organism.

1.10   Aim of this work

In this work the three main levels of controlling a regulated gene expression, namely transcriptional regulation, translational regulation and allostery control of enzyme activity were partially investigated using some eukaryotic molds as microbial model systems. The filamentous fungi *Aspergillus nidulans* was the source for isolation of a new bZIP-like transcription factor. We were interested in the regulation of this specific transcriptional activator under various environmental conditions.

The yeast *Saccharomyces cerevisiae* was used for an analysis of a not yet described interaction between assumed components of the translation machinery on the one hand (e.g. the ribosomes) and a complex regulation of cell morphology on the other hand (e.g. haploid adhesive growth). We identified not yet described functions of ribosomal proteins, necessary for a regulation of growth under specific environmental conditions.

We were also interested in allostery regulation of enzyme activities. We used a heterologously expressed enzyme activities and the technique of protein engineering to determine some new aspects of the general regulation of DAHPS enzymes in *S. cerevisiae*.

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Induction of jlbA mRNA synthesis for a putative bZIP protein of *Aspergillus nidulans* by amino acid starvation

2.1   Abstract

The *jlbA* (jun-like-bZIP) gene of *Aspergillus nidulans* was isolated. The deduced amino acid motif of the C-terminal region of *jlbA* encodes a putative DNA-binding site composed of a basic amino acid domain and an adjacent leucine zipper motif. This region shares highest similarities to the C-terminal DNA binding domain and the basic zipper (bZIP)-motifs of transcription factors like CPCA from *A. niger*, Gcn4p from *Saccharomyces cerevisiae*, human JUNB and c-JUN. The putative *jlbA* protein contains a PEST-rich region described previously implicated in protein stability. The *jlbA* mRNA formation is elevated up to forty fold upon amino acid starvation induced by the addition of the false feedback inhibitor 3-amino-1,2,4-triazole (3AT). This induction is partially dependent and partially independent on the presence of the transcription factor CPCA. Therefore, *jlbA* is a novel gene of *A. nidulans*, which is transcriptional activated by amino acid starvation conditions.

2.2   Introduction

The `cross-pathway control´ (cpc) of the filamentous fungi *A. nidulans* (Sachs, 1996) and *Neurospora crassa* (Carsiotis & Jones, 1974; Carsiotis *et al.*, 1974; Paluh *et al.*, 1988), as well as the `general control of amino acid biosynthesis´ (gc) of the budding yeast *Saccharomyces cerevisiae* (Hinnebusch, 1988; Hinnebusch, 1997; Hinnebusch & Natarajan, 2002) are equivalent regulatory networks acting upon amino acid starvation conditions. Activated by starvation for at least one single amino acid, this control system finally leads to an increased synthesis of a transcriptional activator protein which binds to specific DNA sequence elements present in the upstream regions of more than 50 biosynthetic target genes (Hinnebusch, 1985; Arndt & Fink, 1986; Hinnebusch, 1997). The expression of the yeast transcription factor *GCN4* is mainly regulated on translational level (Natarajan *et al.*, 2001; Hinnebusch & Natarajan, 2002).

The primary signal for *GCN4* activation seems to be uncharged t-RNAs, which are recognized by the bi-functional Gcn2p protein located at ribosomes. Activation of this sensor kinase leads to increased translation of the final effector protein Gcn4p (Lopinski *et al.*, 2000). The complete signal transduction pathway from Gcn2p to the *GCN4* translational control requires more than 12 genes of the general control network. Genes of this network are either *GCN* – general control non-derepressible or *GCD* – general control derepressible. Finally, Gcn4p and its homolog CPCA from *A. nidulans* and *A. niger* act as transcriptional activator and increase the expression of more than 500 target genes (Natarajan *et al.*, 2001) with at least 12 biosynthetic pathways (Braus, 1991).

The *cpcA* encoded proteins of *A. nidulans* and *A. niger* and their yeast homolog Gcn4p as well as the eukaryotic transcription factors JUNB and c-JUN are members of the basic zipper (bZIP)-type proteins (Landschulz *et al.*, 1988; Vinson *et al.*, 1989; Hurst, 1994; Hollenbeck *et al.*, 2001; Lee *et al.*, 2001). These proteins are a subgroup of the AP-1 family of proteins. bZIP-type proteins are characterized by a DNA binding domain which interacts with the target sequences via basic amino acid residues (Ellenberger *et al.*, 1992) and an adjacent C-terminal leucine zipper motif at the end of the protein which functions in dimerisation of two bZIP-monomers. The leucine zipper motif was characterized by a helical heptads repeat of 3 to 5 leucine residues (Landschulz *et al.*, 1988), but to date also some unusual amino acids have been described in the zipper motif (Paluh & Yanofsky, 1991; Wanke *et al.*, 1997).

Beside the bZIP-domain, two distinct activation regions, a central acidic activation domain (CAAD), and a N-terminal activation domain (NTAD) are often present in the N-terminal part of these transcription factors. Additionally, in the yeast Gcn4p an instability region rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T), the PEST region, has been described (Kornitzer *et al.*, 1994). This region overlapping the two activation domains is proposed to function in regulation of protein stability and proteolytic degradation (Roth *et al.*, 1998; Roth & Davis, 2000). All jun-like bZIP transcription factors seem to be part of a general cellular protection system against damage and/or harmful environmental influences or stress conditions in general. For example, ultraviolet (UV) irradiation activates the transcription of the yeast *GCN4* as well as the mammalian *c-JUN* and resistance to UV irradiation is correlated to the level of Gcn4p in yeast (Zimmermann *et al.*, 1999).

Expression of *JunD*, as well as *JunB, c-Jun* and *Fos* are also involved in cell death processes (Woodgate *et al.*, 1999).

Here we present an additional member of the bZIP–type protein family from *Aspergillus nidulans* of unknown function, which is transcribed in response to amino acid starvation. The deduced amino acid sequence of *jlbA* codes for a protein containing a basic amino acid domain and an leucine zipper motif. Beside this DNA binding domain an adjacent PEST region is present. Comparison of the *jlbA* protein sequence shows highest homology to other eukaryotic bZIP proteins like JUNB from the fish *Cyprinus carpio*. The mRNA expression of *jlbA* is strongly induced by the addition of 3-amino-1,2,4-triazole (3AT) to the growth medium and induces an up to 40 fold increase of the *jlbA* mRNA level.

2.3   Experimental procedures

2.3.1 Strains

A. nidulans strain A234 (*yA2, pabaA1; veA1*) was provided from the Fungal Genetic Stock Center (FGSC, University of Kansas, USA). Strain GR5 (*wA3; pyrG89; pyroA4; veA1*) was obtained from G. May (Houston, TX, USA). Strains AGB10 (*pyrG89; pyroA4*) (Eckert *et al.*, 2000) and AGB13 (*wA3; pyrG89; pyroA4*) are descendants from a cross between GR5 (*wA3; pyrG89; pyroA4; veA1*) and FGSC A4 (Eckert *et al.*, 1999). Mutant strain AGB51 (*yA2, pabaA1; veA1; cpcA::phle^R*) is a derivative from A234 (Hoffmann *et al.*, 2001). A chromosome-specific recombinant DNA library from *A. nidulans* (Brody *et al.*, 1991) was obtained from the FGSC.

2.3.2 Media and growth conditions

All strains were grown in minimal liquid medium or plated on minimal medium (Bennett and Lasure, 1991) supplemented as described (Kafer, 1977b; Kafer, 1977a). Amino acid starvation in growth media was induced as follows. Strains were grown in minimal liquid medium for 24h at 30^{0}C. Mycelium was shifted to fresh medium and incubated for another 2h to certain logarithm growth. Mycelium was harvested again and distributed equally to fresh minimal medium without and with 10mM 3-amino-1,2,4-triazole (3AT). Cultures again were incubated for 2h, 4h and 8h, respectively. Finally, mycelium was harvested, frozen in liquid nitrogen and stored at -80^{0}C until further use.

2.3.3 Recombinant DNA techniques

Transformation of *A. nidulans* was performed according to the method of Punt and van den Hondel (Punt & van den Hondel, 1992). Transformants were selected on minimal medium containing 20µg/ml phleomycin and screened using three independent primers in parallel PCR experiments.

DNA sequencing of chromosomal and cDNA was performed by automated dye-labelled terminator DNA sequencing (Rosenblum *et al.*, 1997) using an ABI 310 sequencing machine (Applied Biosystems, Darmstadt, Germany) and custom oligo-nucleotides (Gibco BRL, Eggenstein-Leopoldshafen, Germany). Sequencing of the coding and the non-coding strand between positions -250 and +1000 (see Fig. 1B)

was performed using 16 independent primer and was repeated at least two times each for functional analysis.

Standard enzyme-restriction analysis and Southern blot analysis was performed as described earlier (Sambrook *et al.*, 1989).

For northern blot analysis, total RNA was isolated from harvested mycelium using the TRIzol reagent (Gibco BRL, Karlsruhe, Germany). 20 µg of total RNA was separated on an agarose / formaldehyd gel, electro blotted onto a membrane filter (Biodyne B, Pall, Portsmouth, UK) and hybridised with ^{32}P-radiolabelled DNA (Feinberg & Vogelstein, 1983; Feinberg & Vogelstein, 1984). Radiolabelled signals were quantified with a BAS 1000, Bio Imaging Analyser (Fuji Photo Film Co. Ltd., Tokyo, Japan). A 0.16-1.77kb RNA-ladder (Gibco BRL, Karlsruhe, Germany) was used as size standard.

The 5´-rapid amplification of cDNA ends (RACE) was performed using a 5´/3´-RACE Kit according to the distributers manual (Boehringer, Mannheim, Germany). *jlbA*-cDNA was synthesised from total RNA by reverse transcription using the gene specific primer junD (5´-CCCGACGAAGAAGCAGGATCC-3´). After mRNA degradation by RNase H activity, single stranded cDNA was tailed using terminal transferase in the presence of dATP and TdT. Finally the 5´-end of the *jlbA* transcript was amplified by PCR with the oligo dT-Anchor primer supplemented by the distributor and a gene specific primer SP3 (5´-GAGTTCGGGT-CGAGGTCAACGGG-3´). A distinct amplicon of about 400 bp was isolated and cloned into pBSKII$^{©}$ and was sequenced. This procedure was repeated two times, but lengths of amplicons did not change.

2.4   Results

2.4.1   Identification of a new *jun*-like bZIP encoding gene in *A. nidulans*

The *cpcA* gene of *A. niger* encodes the bZIP transcription factor which is required for the activation of amino acid biosynthetic genes (Wanke *et al.*, 1997). Similar genes had not yet been described in *A. nidulans*. To identify such proteins we screened partially digested DNA from *A. nidulans* in a heterologous Southern hybridisation experiment. Radioactive p[32] labelled *cpcA* from *A. niger* was used as probe. A 3.0 kb genomic *Bam*HI DNA fragment of *A. nidulans* was detected and subcloned into pBluescriptKSII[©] (pBSKII[©]). The DNA sequence was determined and revealed an open reading frame including a conserved region in the 5´-end of the cloned DNA. This conserved part of the gene encodes a putative DNA binding site compost of a basic amino acid rich domain and an adjacent leucine zipper motif. Upstream of the DNA-binding site a putative instability region (PEST-region) is localized. Since the cloned fragment did not include the 5´-region of the cloned gene, a second 2.1 kb DNA fragment containing the upstream DNA region was cloned using *Eco*RI / *Xho*I digested genomic *A. nidulans* DNA (Fig. 2.1.A). The putative DNA binding site and the adjacent leucine zipper show highest similarities with JUN-proteins suggesting that this gene corresponds to a *jun*-homologue. Therefore this gene was designated *jlbA* (*jun*-like-bZIP). The gene bank accession number assigned to this sequence is AF361222.
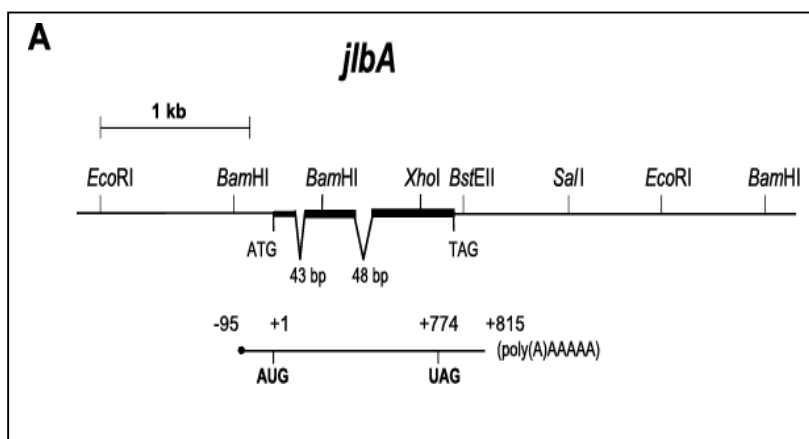
Fig. 2.1: The *jlbA* gene locus of *Aspergillus nidulans*. [A] Schematic view on the chromosomal gene locus of the *JUN*-like-bZIP gene, *jlbA*, of *A. nidulans*. Two introns of 43 bp and 48 bp are indicated. Below a schematic draw of the *jlbA* mRNA is shown. A transcriptional start site was identified at –95, relatively to the ATG start codon. Additionally, translational start and stop sites and the position of the poly (A)-tail are given.

[B] (next page) DNA sequence and deduced amino acid sequence of *jlbA* of *A. nidulans*. Putative CT boxes are given in bold, putative boxes homolog to the NIT-2 binding site are bold, italic and uppercase, putative GCRE binding sites are bold and underlined. Two putative stress response elements (STRE) are given in uppercase letters, one putative StuAp binding site is given in lowercase letters. The major transcriptional start site of *jlbA* is headed by a black square, the identified poly (A)-site is headed by a dot. Restriction sites used for cloning of the chromosomal DNA and referred to in the text are indicated. The amino acid sequence of a putative JLBA protein is indicated in bold letters and is interrupted by two marked introns.

B

<pre>
       EcoRI
-1373 GAATTCTCGGTTGAGAATACCCTAGAATTTGATCACCGGGACTT<i>TATCACA</i>GCGAGAAACTCCATGCTCTCGGGTTTG^{GT} -1294
-1293 ^{AGGGGC}AATCGGCTGCTGAGTTTTATTGCAAAATGGTTCTTCATCGGACGACTCTGGAACGGTATCTAATGCGGCTTTCG -1214
-1213 TCGTCTCTTCCACGGTCTGTAAACGCACTGCTAGGTCTCCAGCTTGGTTGAATTTTAAGGCTGATTGTGTCGGTGAATAT -1134
-1133 GTCTGTCGTTATAAGCGGAAGTATTGCTGGACGCTAAGTATTCCAATCGCGTAG^{AAAACAACATAATGGAACCCTTGGAA} -1054
-1053 GCTGTAAGCCAAGACAAAGATAACGCTGGTCGAATCTGGCAACCTGCGGACGCAAGTACAGCGCTTTCTGACTAAAGTCG -974
 -973 AGCAGTCT^{AAAGGGGC}TGAGCATACCCAGTGTCTCTTAGTTAAGCGGTGTAGGACTTGACAACTACGTCGCATCATGTGA -894
 -893 AACCCATCAAGCCAGGTGTGTTTGGAGGTCGAGACTCGGCACTCTTGGAGCCACCTTTGCGCCCCGCAGATTACAGGGCA -814
                                                                        BamHI
 -813 AACTTGATTGGAGGATCATAGATTATAGTAATA<u>A</u><u>ATGAGTCAT</u>TTTAGTACCGACAGATCGGGACTAGCCCTTGTTATTCT -734
 -733 GATACAATATCTTACGATACCGTTTGAAGATCATCATCCTCAGTCAGAATGAGTGATGCTGGGCAGCTGAGTATGGGCTA -654
 -653 CTTACAAAGGCTCTAGGTCTTAAGAGAGGGGAAGGCAAGAGGAATAAAGGAAC<i>TATCATC</i>TGA<i>TATCATG</i>TCAACTCATA -574
 -573 ATGTTGATATATAAGCGTTTCAACACTGTAGTTTTTAGTAGCAGAAAATATTGGTCTAAGCCTCTCTGCCGGCGGGAG<u>AT</u> -494
 -493 <u>GACTCCT</u>TGGATCCAAGGCATTCAATGATGCGGGAGCCAATATGATAATGGTATGTGATATTCATTACGTGTATTCTTTC -414
 -413 TGCTTGAGGATGAAGAAGCAGCCCGCCGCCGACCATCGCACTGAGGCGCTTAGAGATGAAGAATAGGAAGACGTCTCTAA -334
 -333 GATCACTTGGGCCGACATCATC<u>TGACTCAT</u>TATTCCCCAGCGATCTCAC<i>TATCGTT</i>TGACCCCCTCATAAAGAGATGGCT -254
 -253 AACTGGATCTATGTAAAGTATAGCGTGTAATATTACGGCTGTAAAGCTAATACAGACACTGCCCCACCCGTCTGCTAAA -174
                                                                               ■
 -173 CCCGATCAGCTCCCCGCAACCACGGCCTACTTAAAGATCAAGCCTCCTCTCCTGCTCCAGACTCTCTTTTTTCTCCCATA -94
 -93 CCATCCGCGAAGCGTCACCAGCGCCTCTCTCCCTCTCTTATAAACAGCCCGAGGCACCGGGTCGCCGCAAGACCCGGCTT -14
               +1
 -13 ATACTATCTCGCAATGGCCGCCGCCACTATATCCGCAGCGTCGGTCGCCCGCCAGATCACAGCAgtgtacctggtcggaa +67
               M A A A T I S A A S V A R Q I T A ----------------

 +68 gacaggtcactccagcagtctaatcagTACCTCGATATCTCTCCCCATTCCTTCAGCGACTCAGATTTTCTTTTCTCCTC +147
      ---INTRON1----------------Y L D I S P H S F S D S D F L F S S

+148 GAACTCCTCTTTATCCTCCTCTTCTACGCCGGCTCTTTTCCCCGATCTTTCAGCGGCCTTCCCTCCCGTTGACCTCGACC +227
      N S S L S S S S T P A L F P D L S A A F P P V D L D P
                                                                  BamHI
+228 CGAACGCACCACAGCTCTTCTATGACCCGCTTTTGGTCCCGTCTGTGTTCCCTGGGGATCCTGCTTCTTCGTCGGGTTCT +307
      N A P Q L F Y D P L L V P S V F P G D P A S S G S

+308 GATCAGTTCAACGACTTAACGACTGCGGCTGGATACCCGTCAATCACCACAACTACGGCGGACGACATCGACTGGACGGC +387
      D Q F N D L T T A A G Y P S I T T T T A D D I D W T

+388 CTTGGGCTGCTTGCCTCAAAGCGAGCATCCTGCCAATAGTCTTGGGAGTCAGAGCCAGGGACTTACTCCTAGCGTTGACT +467
      L G C L P Q S E H P A N S L G S Q S Q G L T P S V D S

+468 CTCCTGgtacgccatcgatttTccttcatcaatgacttcaactaactgtctcagATCCCCCGTTCAATTCCACCCAACCA +547
      P D-----------------INTRON2--------------------- P P F N S T Q P

+548 AGAATACTCACCACAGACCTATCCACGACCCATATCACAACTACAAAGCCGATACAACCTCAACCCATGCCCTCCGTGAC +627
      R I L T T D L S T T H I T T T K P I Q P Q P M P S V T

+628 ATCTCTTTCCACCTCCCGCGAATCATCTCCCAAAGAGAAGGAACACTTGTCACGCATCACTAAGCGCCAGCTTAACACCC +707
      S L S T S R E S S P K E K E H L S R I T K R Q L N T L
                                                              XhoI
+708 TGGCCGCAAGACGATACCGCCAGCGCAAGCTCGACAAGGTAGCTCAACTCGAGGAGGAACTGGCAGCCGTGAAGCGCGAA +787
      A A R R Y R Q R K L D K V A Q L E E E L A A V K R E

+788 CGGGACGAATTGAAGATGCGTGTTTCCAAGTTGGAGGGGGAGACTGAGGTGCTGAGGAGTATGGTCAAAGATAAGAATTA +867
      R D E L K M R V S K L E G E T E V L R S M V K D K N *
                                                   BstEII ●
+868 GGACTACGTAGTTAGGATTTGGAAATGGATTGTTCTCTGGTGACCTGAAAAACGTAGCATCAGTGTTTATGGTCCCGTAC +927
+938 TTGCGTTATCGCCTTCATTGACCCTCCAATAAAAGCAATGATGTTTCTAACCATGCCTGAAATGGAAATGGGATATGTGT +1007
+1008 ACATAACGCCTAGAAGGATACGTACACATTCAATGCTTTAACAGCAGTTATTTTTGACTGAGAAATTTCCAGCTCGATTA +1087
+1088 CGAGAAGGAACTCGGTGATCCGGGTATGCTACACACCTCTGATTCCGAAGAGGCTAGGTATGAACCTCGACACCACTCGACA +1167
</pre>

51

2.4.2 The *jlbA* gene encodes a transcript with two putative intervening sequences

In a northern hybridisation experiment *jlbA* mRNA was characterized using total RNA and poly (A)-enriched RNA, respectively. The length of the *jlbA* mRNA was determined as about 1.0 kb using a *Bam*HI / *Bst*EII DNA fragment of the conserved region as probe (Fig. 2.2A). To determine the precise 5´-end of the *jlbA* mRNA a 5´-rapid amplification of cDNA ends (RACE) was performed. A cDNA was synthesised, isolated, cloned into pBSK© and sequenced. This experiment identified a thymidine at position -95 as major start point of *jlbA* transcription (Fig. 2.1B).

The first ATG codon is located 95 bp downstream of this transcriptional start site. The DNA sequence suggests an open reading frame of 774 bp, which is interrupted by two introns (Fig. 2.1A / 2.1B). Starting with a commonly used GTG (Unkles, 1992) at +52, the first intron includes 43 bp, whereas the second intron at positon +474 includes 48 bp. The two GTCs at positions +58 and +73 are also possible intron start sites resulting in two introns with lengths of 34 bp and 22 bp, respectively. The usage of these splice sites seem to be unlikely, because introns smaller than 36 bp have not been described yet (Unkles, 1992). *In silico* analysis of the promoter region of the gene revealed five putative CT-boxes (5´-CAAT-3´) at nucleotide positions -1288, -1090, -729, -471, and -456, respectively (Fig. 2.1B). CT boxes have been demonstrated to function in transcriptional start site selection (Punt *et al.*, 1990) as well as transcription initiation (Adams & Timberlake, 1990; Unkles, 1992).

Three putative CPCA protein recognition elements (CPREs) are also present at nucleotide positions -780, -495, and -321, respectively (Fig. 2.1B). These sequence elements share high identity to the 5´-(A)TGA(G/C)TCA(T)-3´ consensus sequence found for the CPCA homolog protein Gcn4p of the yeast *S. cerevisiae* (Arndt & Fink, 1986; Hollenbeck & Oakley, 2000). Other transcription factor binding sites are also present within the *jlbA* promoter. Four sites homolog to the target of the positive-acting NIT-2 from *Neurospora crassa* and *A. nidulans* are present at positions -284, -590, -600 and -1329, respectively (Davis & Hynes, 1987; Fu & Marzluf, 1990; Hawker *et al.*, 1991; Tao & Marzluf, 1999). Two sequences similar to the binding sites of the stress-response elements STRE (Schuller *et al.*, 1994) are located at pos. -965 and -1293. Beside these, one StuAp specific binding site is present at pos. -1089 (Dutton *et al.*, 1997).
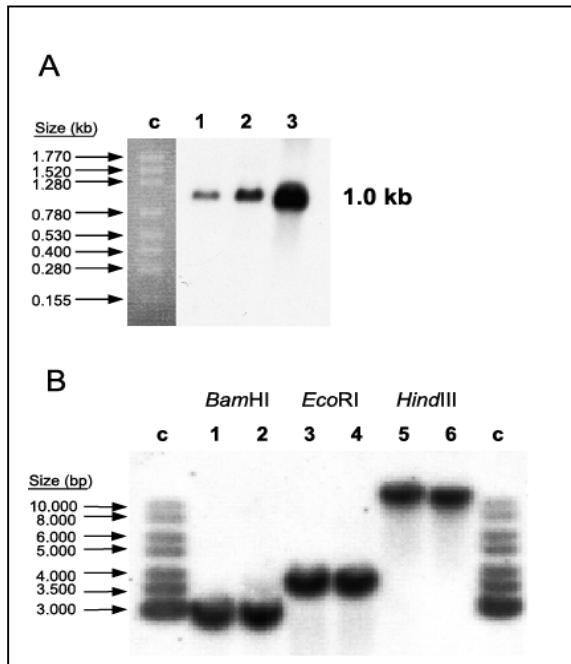
Fig. 2.2: Transcript length and Southern blot analysis of the *jlbA* gene. [A] In a Northern blot analysis different amounts of *jlbA* total RNA (lane 1 + 2) and poly (A) enriched RNA (lane 3) of *A. nidulans* was separated on an agarose / formaldehyde gel and hybridised with a *Bam*HI / *Bst*EII cut chromosomal DNA fragment containing the 3´- conserved region of the *jlbA* gene.
RNA size markers (in nucleotides) are indicated on the left. The mRNA transcript length was determined to about 1.0 kb. **[B]** Chromosomal copy number of *jlbA* was determined in a Southern blot analysis using DNA of the related *A. nidulans* strains A234 (lanes 1, 3, 5) and GR5 (lanes 2, 4, 6). DNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII, respectively and hybridised using a 600 bp *Bam*HI / *Bst*EII fragment of the gene. Size of the DNA marker (lanes c) is indicated on the left side.

To identify the exact 3'-end of the *jlbA* mRNA we screened for a *jlbA* cDNA. An inducible cDNA expression library of *A. nidulans* (Krappmann *et al.*, 1999) was screened by colony hybridisation. A 600 bp *Bam*HI / *Bst*EII radiolabelled fragment from the conserved region of the gene was used as a probe. We isolated and sequenced a shortened cDNA clone. Comparison of genomic and cDNA sequence revealed the second intron to be located as postulated. The exact 3´-end of the *jlbA* mRNA and a poly (A)-tail was identified at +911, 41 bp downstream of the translational stop TAG (Fig. 1B). Conserved poly (A)-signals as described for other genes from *A. nidulans* (Sienko & Paszewski, 1999) are not present in the identified 3´-sequences of *jlbA*.

2.4.3 The JLBA protein contains a DNA binding region that shares similarities with other proteins with a bZIP-DNA-binding motif

The deduced amino acid sequence of JLBA encodes for a bZIP-type protein of 258 amino acids with a calculated molecular weight of approximately 20 kDa (Fig. 2.3A). Comparison of the deduced amino acid sequence to other proteins revealed identities only in the C-terminal part. JLBA shares similarities with the bZIP-type family of transcription factors, like Gcn4 from *S. cerevisiae*, CPCA from *A. nidulans* and JUN protein in vertebrates. JunB of the fish *Cyprinus carpio* was identified as the most related protein (AdvancedBlast at NCBI). The conserved region of the *jlbA* gene encodes for a basic DNA-binding domain and an adjacent C-terminal leucine zipper motif with 4 leucine residues and one valine residue, respectively. The identities in this part of the protein are small when compared to the eukaryotic JUNB from the fish *Cyprinus carpio* (31.9%) or human (28.4%), but similarities are higher (51.3% and 52.6%, respectively).

When compared to other transcriptional activator proteins (Fig. 3B) the identities within the conserved part of JLBA range from 27.1% (c-JUN) to 37.3% (Gcn4p). The proposed N-terminus of JLBA shares no homology to any other proteins described so far. The basic amino acid rich region of JLBA which probably functions in DNA binding and recognition is conserved and shares a typical KR-NT-AAR-RK amino acid motif. This part of the protein contains only one atypical aspartat at the end of the domain (D197, Fig. 2.3A / 2.3B). The adjacent C-terminal zipper motif allows the dimerisation of two proteins. Zipper motifs are characterised by at least three repeats of seven amino acids (designated `a´ to `g´) forming an α-helical coiled-coil with the `d´ positions often occupied by leucine residues (Landschulz *et al.*, 1988). JLBA is conserved in all `d´ positions sharing hydrophobic amino acid residues (e.g. leucine) beside the second `d´, which is occupied by a valine. The `e´ and `g´ positions of zipper motifs are characterised by charged amino acid residues (Alber, 1992) as so in the deduced leucine zipper motif of JLBA. PEST regions have been described to function in rapid protein turnover and degradation (Kornitzer *et al.*, 1994; Salama *et al.*, 1994; Roth *et al.*, 1998; Roth & Davis, 2000) as well as in protein-protein interactions (Chu *et al.*, 1996).

A

```
 1                                    ▼                         40
M A A A T I S A A S V A R Q I T A Y L D I S P H S F S D S D F L F S S N S S L S
41                                                             80
 S S S T P A L F P D L S A A F P P V D L D P N A P Q L F Y D P L L V P S V F P G
81                                                            120
 D P A S S S G S D Q F N D L T T A A G Y P S I T T T T A D D I D W T A L G C L P
121              PEST region              ▼                   160
 Q S E H P A N S L G S Q S Q G L T P S V D S P D P P F N S T Q P R I L T T D L S
161                                                           200
 T T H I T T T K P I Q P Q P M P S V T S L S T S R E S S P K E K E H L S R I T K
201                      DNA binding site                     240
 R Q L N T L A A R R Y R Q R K L D K V A Q L E E E L A A V K R E R D E L K M R V
241                       258
 S K L E G E T E V L R S M V K D K N
```

B


```
                    basic amino acid domain              dimerisation = leucine zipper

(A.nid JLBA)   K R Q L N T L A A R R Y R Q R K L D K V A Q L E E E L A A V K R E R D E L K M R
(C.car JunB)   K R L R N R L A A T K C R K R K L E R I R S L E E K V K V L K N D N A G L S N T
(human JUNB)   K R L R N R L A A T K C R K R K L E R I A R L E D K V K T L K A E N A G L S S T
(hum. c-JUN)   K R M R N R I A A S K C R K R K L E R I A R L E E K V K T L K A Q N S E L A S T
(S.cer Gcn4)   K R A R N T E A A R R S R A R K L Q R M K Q L E D K V - - - - - E - - E L L S K
(A.nid CPCA)   K R A R N T E A A R K S R A R K L E R Q G D M E R R I A E L S K E L E E T R Q M
(A.nig cpcA)   K R A R N T E A A R K S R A R K L E R Q G E M E R R I E E L E R M L E E S K Q R

                                                     d e   g       d e   g       d e   g

                                                                    relative    relative
                                                                    identity    similarity
(A.nid JLBA)   V S K L E G E T E V L R S M V K D K N
(C.car JunB)   A S V L R D Q V A Q L K - - V L R H M N S G C Q L M L T S K M E A F    31.9%      51.3%
(human JUNB)   A G L L R E Q V A Q L K Q K V M T H V S N G C Q L L L G V K G H A F    28.4%      52.6%
(hum. c-JUN)   A N M L R E Q V A Q L K Q K V M N H V N S G C Q L M L T Q Q L Q T F    27.1%      48.7%
(S.cer Gcn4)   A N M L R E Q V A Q L K Q K V M N H V N S G C Q L M L T Q Q L Q T F    37.3%      59.3%
(A.nid CPCA)   V E F W K S Q A Q A R A R G A Z                                        28.8%      54.2%
(A.nig cpcA)   E E Y W R S M A K T G T N                                             35.0%      60.2%

               d e   g       d e   g
```

Fig. 2.3: Putative amino acid sequence of the JLBA protein. [A] Schematic view on the putative amino acid sequence of JLBA and its functional domains. Numbers above represent the numbers of the deduced JLBA protein. Triangles mark the positions of two introns referred to in the text. Amino acid sequence of an identified PEST region is given in bold letters. Conserved amino acids of the DNA binding site are also given in bold letters. **[B]** The second scheme shows a partial comparison of the JLBA DNA binding domain to other related DNA binding domains. Two sub domains of the identified DNA binding site are marked. The basic amino acid domain is given in light grey, the conserved positions of the dimerisation domain are given in dark grey. Below the sequence the conserved `d´, `e´ and `g´ positions of the leucine zipper motif are indicated in lowercase letters. Partial amino acid sequences are given from *A. nidulans* (*A. nid*), *Cyprinus carpio* (*C. car*), human (human, hum.), *S. cerevisiae* (*S. cer*) and *A. niger* (*A. nig*), respectively.

A putative PEST region of JLBA, rich in the amino acids proline, glutamic acid, serine, and threonine could be identified using PESTfind (EMBnet, Austria). The identified region has a PESTfind score of +6.12. Values bigger than +5.0 mark real PEST regions (Rogers *et al.*, 1986; Rechsteiner & Rogers, 1996). A conserved <u>c</u>entral <u>a</u>cidic <u>a</u>ctivation <u>d</u>omain (CAAD) or <u>N</u>-terminal <u>a</u>ctivation <u>d</u>omain (NTAD) as described for the proteins CPCA (Wanke *et al.*, 1997) and Gcn4p (Hope & Struhl, 1986; Hope *et al.*, 1988; Drysdale *et al.*, 1995) is not present in JLBA.

2.4.4 *jlbA* is a single copy gene and localized on chromosome VII

To determine the copy number of the *jlbA* gene, chromosomal DNAs from *A. nidulans* strain A234 and the related background of strain GR5 were digested with *Bam*HI, *Eco*RI and *Hin*dIII, respectively. In Southern hybridisation analysis the DNAs were hybridised using a *jlbA* gene fragment as radiolabelled probe. This resulted in a single band for each restriction and demonstrated that the *jlbA* gene is a single copy gene in both *A. nidulans* strains (Fig. 2.2B). To identify the chromosomal localisation of the *jlbA* gene, pools of chromosome I to VIII of a chromosome-specific recombinant DNA library from *A. nidulans* (Brody *et al.*, 1991) were used as templates for polymerase chain reactions (PCR) using 2 different pairs of *jlbA*-specific oligonucleotides. This experiment resulted in single amplicons of appropriate length only in reactions using chromosomal DNA of pool VII as template (data not shown). Therefore, the *jlbA* gene of *A. nidulans* is localised on chromosome VII.

2.4.4 The *jlbA* transcription level is strongly induced by addition of the amino acid analogue 3-amino-1,2,4 triazole (3AT)

The promoter region of *jlbA* contains five putative CT boxes and two sequence motifs similar to the yeast GCRE consensus sequence 5´-TGA(G/C)TCA-3´ upstream of the putative transcriptional start sites. GCREs function as Gcn4p recognition elements (Arndt & Fink, 1986; Hope & Struhl, 1987) and are located in promoter regions of genes that are transcriptional regulated by the general control of amino acid biosynthesis (Hinnebusch, 1992). In addition, these conserved sequence motifs are present not only in promoter regions of regulated genes but also in the *cpcA* promoter of *A. niger* (Wanke *et al.*, 1997) and in other regulators of the cross-pathway control. This prompted us to test whether *jlbA* expression is regulated by amino acid starvation. Starvation for one single amino acid is sufficient to induce the regulatory network of amino acid biosynthesis (Hinnebusch, 1992). Therefore we tested the expression of the *jlbA* gene in the presence of 10 mM of 3-amino-1,2,4-triazole (3AT), which induces starvation for the amino acid histidine.
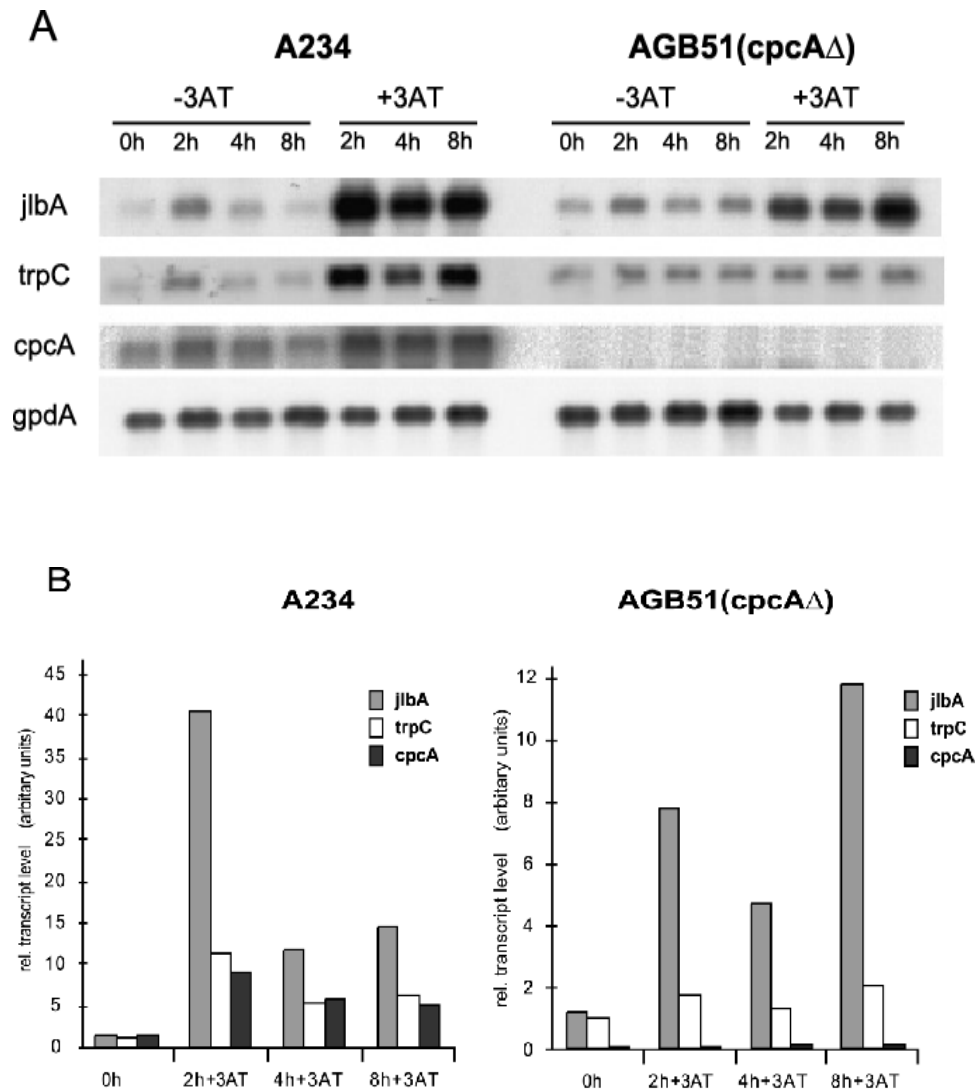
Figure 2.4 Induction of the *jlbA* mRNA expression under amino acid starvation conditions. Northern analysis of total RNA isolated from *A. nidulans* strains A234 and AGB51 (*cpcAΔ*) grown in minimal medium. At the indicated time points mycelium was harvested and distributed equally to fresh minimal medium with and without 10 mM 3-amino-1,2,4-triazole (3AT). RNA was isolated 2h, 4h, and 8h after induction. Steady-state levels of the *gpdA* gene transcript were used as internal standard. **[A]** Strong induction of the *jlbA* mRNA expression can be observed under amino acid starvation conditions after addition of 3AT. The *trpC* and *cpcA* mRNA expression were used as controls regulated by the general cross-pathway control network. **[B]** Quantification of the *jlbA, trpC* and *cpcA* mRNA levels normalised with respect to the *gpdA* levels using a FUJI-BAS 1500 phosphoimager. The induction of the mRNA level of strains A234 and AGB51 are indicated in two diagrams, representing the average of three independent measurements. Standard deviations did not exceed 15%.

When compared to total RNA isolated from identical liquid cultures without 3AT, the *jlbA* mRNA level of the *A. nidulans* wildtype strain A234 increased about 40-fold after 2h of exposure to 3AT (Fig. 3.4A). The induction effect was even approximately 80-fold in strain GR5 (data not shown).

After 4h of exposure to 3AT, the induction in strain A234 was around 10-fold and finally reached a 15-fold level after 8h (Fig. 2.4B). The *trpC* mRNA levels of this strain showed a similar induction pattern. After 2h of exposure to 3AT, transcription of *trpC* mRNA was induced 10-fold and showed a 6-fold induction after 8h of exposure to 3AT. These values are coincident to *trpC* values obtained in other studies (Busch *et al.*, 2001). In *Aspergillus* mutant strain AGB51 (*cpcA∆*) the induction of *jlbA* mRNA was 8-fold after 2h, 5-fold after 4h and finally reached a 10-fold induction level after 8h of exposure to 3AT. In contrast, *trpC* mRNA level of the mutant strain AGB51 was not effected under starvation conditions. Whereas *trpC* mRNA induction was fully dependent on the *cpcA* gene product, *jlbA* mRNA levels were partially increased even in the absence of CPCA and are therefore regulated additional.

2.5   Discussion

The cross-pathway control of amino acid biosynthesis is a conserved mechanism in cellular and filamentous fungi. Induction of mRNA expression of genes regulated by this system under amino acid starvation conditions have been shown in *S. cerevisiae* (Braus, 1991; Hinnebusch, 1997; Natarajan *et al.*, 2001), *N. crassa* (Sachs, 1996) (Kemp & Flint, 1982; Barthelmess, 1986; Flint & Wilkening, 1986), *A. niger* (Wanke *et al.*, 1997), and *A. nidulans* (Ventura *et al.*, 1997; Hoffmann *et al.*, 2001).

We have identified a new gene that is strongly induced by the addition of 3-amino-1,2,4-triazole (3AT) to growth medium, starving cells for histidine. Our results demonstrated an up to 40-fold induction of the *jlbA* mRNA transcript level after 2h of exposure to 3AT in strain A234. This transient induction peak in the mRNA expression of the *jlbA* gene is similar to the results obtained for the expression of the *GCN4* homolog *cpcA* in *A. niger*, although the induction effect for this gene was not so strong and did not exceed 3-fold (Wanke *et al.*, 1997).

Interestingly, the mutant strain AGB51 (*cpcA*Δ) also showed a significant induction of the *jlbA* mRNA expression. Despite the absence of CPCA, the mRNA level of *jlbA* still was partially induced after 2h of incubation in the presence of 3AT and finally reached a 10-fold level after 8h of exposure to 3AT. These values are similar to the measured values obtained from strain A234. In contrast, the *trpC* mRNA level varied only weak in strain AGB51. The *trpC* mRNA expression is regulated in response to the protein level of the transcription factor CPCA, probably because of the CPCA binding sites within the promoter region of the *trpC* gene (Hoffmann *et al.*, 2000; Busch *et al.*, 2001). Disruption of *cpcA* leads to a constant *trpC* mRNA level, indicating a strong dependence of the induction to protein levels of CPCA. Because of two deduced GCRE binding sites within the promoter region of the *jlbA* gene, results obtained for the *jlbA* mRNA levels in the *cpcA* mutant strain are surprising. Obviously, the *jlbA* expression is also regulated independently of CPCA.

This suggests that starvation using the histidine analogue 3-amino-triazole results in the induction of 'cross pathway' control genes and an additional induction process not yet described. Whether this effect is caused by a changed basal transcription or other transcriptional activators like NIT2p (Fu & Marzluf, 1990), StuAp (Dutton *et al.*, 1997) or factors binding to the STRE related elements (Schuller *et al.*, 1994) remains to be tested. The DNA binding domain of *jlbA* encodes for a typical basic amino acid domain and an adjacent leucine zipper motif.

This part of the deduced protein seems to be conserved when compared to other transcriptional activator proteins. In the middle of the putative JLBA protein, a PEST region was identified. This region might function in degradation processes of JLBA as described for other members of the bZIP-type protein family (Salama *et al.*, 1994; Roth *et al.*, 1998; Roth & Davis, 2000) or may be necessary for phosphorylation to alter protein-protein interaction (Chu *et al.*, 1996). The N-terminal part of JLBA shares no homology to other proteins described so far. Therefore, the JLBA protein might be a new member of the bZIP-type protein family. The function of JLBA in *A. nidulans* remains unknown. Neither the inactivation of *jlbA* mRNA function by antisense RNA, nor the overexpression of *jlbA* displayed any significant phenotype. The drastic induction of *jlbA* expression upon amino acid starvation suggests that JLBA might have a yet unknown role in the response to the starvation signal, maybe in combination to the CPCA transcription factor.

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Functional analysis of the ribosomal *RPS26* isogenes in the yeast *Saccharomyces cerevisiae*

3.1 Abstract

Commonly among eukaryotes, yeast ribosomes are built of a large 60S and a small 40S ribosomal subunit. While the large subunit is composed of 46 proteins, the small subunit only contains about 32 proteins. The *RPS26* isogenes of *S. cerevisiae* are located on chromosome V and VII, respectively, and encode for two proteins assumed to be located at the small 40S subunit. The two *RPS26* genes share high sequence identity and the deduced proteins differ by only two amino acid residues. In this work, we investigated the expression and the function of the two *RPS26* isogenes. Growth tests demonstrated that only *rps26AΔ* but not *rps26BΔ* yeast mutant strains display a reduced growth rate. By crossing experiments of *rps26AΔ* and *rps26BΔ* yeast mutant strains we could demonstrate that an *rps26AΔ rps26BΔ* double mutation is lethal for *S. cerevisiae*. Analysis of transcript levels demonstrated that the *RPS26A* gene product contributes about 70% of the cellular mRNA amounts, while the *RPS26B* gene product contributes only about 30%. Analysis of promoter strength of the *RPS26* isogenes demonstrated that although the *RPS26B* gene delivers only a minor amount of the cellular transcript levels, this gene is stronger inducible when compared to the *RPS26A* gene. Testing of *rps26A* or *rps26B* yeast mutant strains for influence on haploid adhesive and diploid pseudohyphal growth demonstrated that only the *RPS26A*, but not the *RPS26B* gene product is necessary for haploid adhesive and diploid pseudohyphal growth. Analysis of expression of the *FLO11* gene revealed that only *RPS26A* but not *RPS26B* is required for translation of the *FLO11* mRNA. Therefore, it was surprising that only the *rps26A* yeast mutant strains completely fail in translation of *FLO11* mRNA.

3.2   Introduction

Translation of messenger RNAs (mRNAs) is a basic function of all living cells, carried out by complex macromolecular machineries known as ribosomes (Green & Noller, 1997; Spahn *et al.*, 2001). Generally, ribosomes seem to be conserved between all prokaryotic and eukaryotic organisms (Verschoor *et al.*, 1998; Gabashvili *et al.*, 2000; Wimberly *et al.*, 2000; Spahn *et al.*, 2001). Most information about the ribosome has been obtained in prokaryotic systems. Ribosomes synthesise all of the proteins in the cell, using mRNAs as templates.

The 23S rRNA of the large subunit (50S) is responsible for the catalytic activity of the ribosome, which is the peptidyl-transferase activity (Nissen *et al.*, 2000). The 16S rRNA of the small subunits is used for selection and decoding of cognate tRNAs (Carter *et al.*, 2000). It has been assumed that protein synthesis follows three steps, an initiation, an elongation, and a termination reaction. During the elongation, each tRNA has to pass through three tRNA binding sites within the small ribosomal subunit, the acceptor- (A), peptidyl- (P), and exit- (E) site, respectively. Each of this site is occupied in succession by particular tRNAs during the protein synthesis. The tRNAs bridge the large and small subunits of the ribosomes, with the anticodon arm of the tRNA pointing towards the small 30S subunit for decoding. During elongation, the growing peptide chain is covalently attached to the tRNA bound in the P-site. The critical decoding event occurs, when charged tRNAs bind to the A-site. At this point, the cognate codon-anticodon interaction is sensed by the 30S subunit in a yet not understood way. The peptide formation is performed at the 50S catalytic site and the entire ribosome must shift down the mRNA for next round of peptide-bond formation (Carter *et al.*, 2000).

Yeast 80S ribosomes are composed of a large 60S and a small 40S subunit. The yeast 60S subunit is build by the 25S, 5.8S, 5S rRNAs and 46 proteins, while the small subunit is composed of the 18S rRNA and 32 proteins (Verschoor *et al.*, 1998). Among the yeast ribosomal proteins as well as in the complete yeast genome, many isogenes were identified with not yet clear functions (Mager *et al.*, 1997; Mewes *et al.*, 1997; Planta & Mager, 1998).

In *S. cerevisiae*, the *RPS26A* gene on chromosome VII codes for one protein of the small 40S subunit, while an isogene, *RPS26B* exists on chromosome V. The two *RPS26* genes share a high sequence identity and encode for two almost identical proteins of 119 amino acids. High x-ray resolution details of both, prokaryotic and eukaryotic ribosomes have not yet revealed the exact positioning of the two Rps26 proteins within the ribosomes (Verschoor *et al.*, 1998; Gabashvili *et al.*, 2000; Gomez-Lorenzo *et al.*, 2000; Spahn *et al.*, 2001; Yusupov *et al.*, 2001). Ribosomes not only function in protein translation, but also have been described to be required for a co-translational folding of some but not all nascent peptides (Hardesty & Kramer, 2001), and to function in a co-translational translocation of proteins at the endoplasmic reticulum (Menetret *et al.*, 2000; Beckmann *et al.*, 2001). The functions of the two Rps26 proteins within all these processes are not yet known.

The yeast *S. cerevisiae* is one representative of those fungi that are able to perform a dimorphic switch from unicellular to multicellular filamentous growth in response to various environmental stimuli (reviewed in Mösch, 2002). A complex regulatory network controls diploid pseudohyphal growth of *S. cerevisiae*. At least two environmental stimuli, a starvation for nitrogen and the presence of a fermentable carbon source are necessary to result in pseudohyphal development. Secondly, two main regulation pathways, the cAMP dependent protein kinase pathway and the STE mitogen-activated protein (MAP) kinase pathway, respectively, are involved in the complex regulation of pseudohyphal development (Gimeno *et al.*, 1992; Mösch & Fink, 1997; Rupp *et al.*, 1999; Mösch, 2000; Pan *et al.*, 2000).

Although the entire regulations are not completely identical, there are some evidences that diploid pseudohyphal and haploid adhesive growth share the same regulation mechanisms. For example, the cAMP pathway is activated in response to changes in the nutritional environment of yeast *S. cerevisiae*, e.g. when diploid yeast cells are starved for glucose or ammonia (Gancedo, 1998; Lorenz & Heitman, 1998; Versele *et al.*, 1999). Glucose depletion has also been demonstrated to induce haploid adhesive growth (Cullen & Sprague, 2000). Haploid yeast cells of strains those strains that are able to undergo pseudohyphal growth, highly adhere to the agar surface and form short filaments that can penetrate into the agar surface.

While haploid cells keep a round cell shape and can not grow outside of the colony border, diploid pseudohyphal cells display an elongated cell morphology and thereby can grow to the outside (Mösch & Fink, 1997). In a general view, the regulation mechanisms between haploid adhesive and diploid pseudohyphal growth seems to be highly conserved.

A family of cell wall proteins, the so called flocculins are necessary to secure cell-cell and cell-surface adhesion in *S. cerevisiae* (Caro *et al.*, 1997; Guo *et al.*, 2000). The expression of the *FLO11* gene is regulated by the Ras-cAMP and the MAPK-kinase cascade, respectively (Gagiano *et al.*, 1999; Palecek *et al.*, 2000). The ability to form pseudohyphae only occurs in those yeast strains that have retained a functional *FLO11* expression throughout the laboratory history, e.g. yeast ∑1278b strains and not the commonly used S288C laboratory strains (Mösch, 2002). Throughout laboratory selection S288C strains have picked up a mutation in the *FLO8* gene that leads to the formation of a non-functional protein (Liu *et al.*, 1996). Since Flo8p is a transcriptional activator of *FLO11* expression, natural *flo8* mutant strains (e.g. S288C) are unable to form pseudohyphae (Rupp *et al.*, 1999).

Here we present our finding that the *RPS26A* gene product as one part of the yeast translation machinery not only functions in the general translation but also is required for some differentiation processes. Rps26Ap functions in the regulation of haploid adhesive and diploid pseudohyphal growth of yeast ∑1278b strains. Additionally, under the tested conditions, the Rps26Ap seems to be strictly required for the translation of *Flo11* mRNA. In contrast, the *RPS26B* gene alone seems to deliver only a basal functionality within the translation machinery and seems be of minor importance for the regulation of differentiation processes.

3.3   Experimental procedures

3.3.1 Materials, media, and growth conditions

All chemicals used in this work were supplied by FLUKA/Sigma-Aldrich Chemie GmbH (Steinheim, Deisenhofen, Germany). Minimal vitamins (MV) and synthetic complete (SC) medium for the cultivation of yeast was described earlier (Miozzari *et al.*, 1978). Low ammonia medium for scoring of pseudohyphal growth (SLAD) was prepared as described (Gimeno *et al.*, 1992). Transformation of *S. cerevisiae* was performed by the LiOAc method as described (Ito *et al.*, 1983).

3.3.2 Strains and plasmids

All strains used for this study are derivatives of the yeast *S. cerevisiae* ∑1278b strain background and are listed in Table 1. All plasmids used for this work are listed in Table 2.

Table 1 - Yeast strains used in this work

Strain	Genotype	Reference
RH2584	*MATa, ura3-52, his3::hisG, trp1::hisG*	Mösch, 1998
RH2585	*MATα, ura3-52, his3::hisG, trp1::hisG*	Mösch, 1998
RH2586	*MATa ura3-52, his3::hisG, leu2::hisG*	Mösch, 1998
RH2587	*MATα, ura3-52, his3::hisG, leu2::hisG*	Mösch, 1998
RH2588	*MATa, rps26A::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2589	*MATa, rps26A::Kan^R, ura3-52,his3::hisG, trp1::hisG*	this work
RH2590	*MATa, rps26A::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2591	*MATα, rps26A::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2592	*MATa, rps26B::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2593	*MATα, rps26B::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2594	*MATa, rps26B::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2595	*MATα, rps26B::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2613	*MATa, (RPS26A, RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7900bp *Stu*I cut pME2144 integrated at the *ura3-52* locus; 750bp prom-*lacZ* of *RPS26A*	this work
RH2614	*MATa, (RPS26A, RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8500bp *Stu*I cut pME2146 integrated at the *ura3-52* locus; 1400bp prom-*lacZ* of *RPS26B*	this work
RH2615	*MATa, (RPS26A, RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8140bp *Stu*I cut pME2148 integrated at the *ura3-52* locus; 1040bp prom-*lacZ* of *RPS26B*	this work
RH2621	*MATa, rps26A::Kan^R, (RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7900bp *Stu*I cut pME2144 integrated at the *ura3-52* locus; 750bp prom-*lacZ* of *RPS26A*	this work

Table 1 - continued

Strain	Genotype	Reference
RH2622	*MATa, rps26A::Kan^R, (RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8500bp *Stu*I cut pME2146 integrated at the *ura3-52* locus; 1400bp prom-*lacZ* of *RPS26B*	this work
RH2623	*MATa, rps26A::Kan^R, (RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8140bp *Stu*I cut pME2148 integrated at the *ura3-52* locus; 1080bp prom-*lacZ* of *RPS26B*	this work
RH2629	*MATa, rps26B::Kan^R, (RPS26A), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7900bp *Stu*I cut pME2144 integrated at the *ura3-52* locus; 750bp prom-*lacZ* of *RPS26A*	this work
RH2630	*MATa, rps26B::Kan^R, (RPS26A), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8500bp *Stu*I cut pME2146 integrated at the *ura3-52* locus; 1400bp prom-*lacZ* of *RPS26B*	this work
RH2631	*MATa, rps26B::Kan^R, (RPS26A), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8140bp *Stu*I cut pME2148 integrated at the *ura3-52* locus; 1080bp prom-*lacZ* of *RPS26B*	this work
RH2662	*MATa, ura3-52, flo11::Kan^R, trp1::hisG*	Grundmann, 2000
RH2806	*MATa, µORF::Kan^R ura3-52, his3::hisG, trp1::hisG*	this work
RH2807	*MATα, µORF::Kan^R , ura3-52, his3::hisG, trp1::hisG*	this work
RH2808	*MATa, µORF::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2809	*MATα, µORF::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2810	*MATa, rps26A/µORF::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2811	*MATα, rps26A/µORF::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2812	*MATa, rps26A/µORF::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2813	*MATα, rps26A/µORF::Kan^R, ura3-52, his3::hisG, leu2::hisG*	this work
RH2814	*MATa, cdc55::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2815	*MATα, cdc55::Kan^R, ura3-52, his3::hisG, trp1::hisG*	this work
RH2816	*MATa, ura3-52, trp1::hisG, (his3::HIS3)*	this work
RH2817	*MATα, ura3-52, trp1::hisG, (his3::HIS3)*	this work
RH2818	*MATa, ura3-52, leu2::hisG, (his3::HIS3)*	this work
RH2819	*MATα, ura3-52, leu2::hisG, (his3::HIS3)*	this work
RH2854	*MATa, rps26A::HIS3, ura3-52, his3::hisG, trp1::hisG, His^+*	this work
RH2855	*MATα, rps26A::HIS3, ura3-52, his3::hisG, trp1::hisG, His^+*	this work
RH2856	*MATa, rps26A::HIS3, ura3-52, his3::hisG, leu2::hisG, His^+*	this work
RH2857	*MATα, rps26A::HIS3, ura3-52, his3::hisG, leu2::hisG, His^+*	this work
RH2858	*MATa, rps26B::HIS3, ura3-52, his3::hisG, trp1::hisG, His^+*	this work
RH2859	*MATα, rps26B::HIS3, ura3-52, his3::hisG, trp1::hisG, His^+*	this work
RH2860	*MATa, rps26B::HIS3, ura3-52, his3::hisG, leu2::hisG, His^+*	this work
RH2861	*MATα, rps26B::HIS3, ura3-52, his3::hisG, leu2::hisG, His^+*	this work
RH2862	*MATa, cdc55-100, ura3-52, leu2::LEU2, trp1::TRP1*	(Mösch & Fink, 1997)
RH2863	*MATα, cdc55-100, ura3-52, leu2::LEU2, trp1::TRP1*	(Mösch & Fink, 1997)
RH2866	*MATa/MATα (RPS26A/RPS26A), (RPS26B/RPS26B), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG*	this work
RH2867	*MATa/MATα rps26A::Kan^R/rps26A::Kan^R, (RPS26B/RPS26B), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG*	this work
RH2868	*MATa/MATα rps26B::Kan^R/rps26B::Kan^R, (RPS26A/RPS26A), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG*	this work

Table 1 - continued

Strain	Genotype	Reference
RH2869	*MATa/MATα rps26A::Kan^R/RPS26A, RPS26B/rps26B::Kan^R, ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG*	this work
RH2870	*MATa/MATα rps26A::Kan^R/RPS26B, (RPS26A/RPS26B), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG*	this work
RH2871	*MATa/MATα rps26B::Kan^R/RPS26A, (RPS26A/RPS26B), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG*	this work
RH2872	*MATa/MATα rps26A::Kan^R/RPS26A, RPS26B/rps26B::HIS3, ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG, His$^+$*	this work
RH2873	*MATa/MATα rps26A::HIS3/RPS26A, RPS26B/rps26B::Kan^R, ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG, His$^+$*	this work
RH2874	*MATa/MATα (RPS26A/RPS26A), (RPS26B/RPS26B), ura3-52/ura3-52, trp1::hisG/TRP1, LEU2/leu2::hisG, (his3::HIS3/his3::HIS3), His$^+$*	this work
RH2875	*MATa/MATα rps26A::HIS3/rps26A::HIS3, (RPS26B/RPS26B), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG, His$^+$*	this work
RH2876	*MATa/MATα rps26B::HIS3/rps26B::HIS3, (RPS26A/RPS26A), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG, His$^+$*	this work
RH2877	*MATa/MATα rps26A/µORF::HIS3/rps26A/µORF::HIS3, (RPS26B/RPS26B), ura3-52/ura3-52, his3::hisG/his3::hisG, trp1::hisG/TRP1, LEU2/leu2::hisG, His$^+$*	this work
RH2878	*MATa, (RPS26A, RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8150bp *StuI* cut pME2405 integrated at the *ura3-52* locus; 1050bp prom-*lacZ* of *RPS26A*	this work
RH2879	*MATa, rps26A::Kan^R, (RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8150bp *StuI* cut pME2405 integrated at the *ura3-52* locus; 1050bp prom-*lacZ* of *RPS26A*	this work
RH2880	*MATa, rps26B::Kan^R, (RPS26A), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 8150bp *StuI* cut pME2144 integrated at the *ura3-52* locus; 1050bp prom-*lacZ* of *RPS26A*	this work
RH2881	*MATa, (RPS26A, RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7650bp *StuI* cut pME2406 integrated at the *ura3-52* locus; 420bp prom-*lacZ* of *RPS26A*	this work
RH2882	*MATa, rps26A::Kan^R, (RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7650bp *StuI* cut pME2406 integrated at the *ura3-52* locus; 420bp prom-*lacZ* of *RPS26A*	this work
RH2883	*MATa, rps26B::Kan^R, (RPS26A), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7650bp *StuI* cut pME2406 integrated at the *ura3-52* locus; 420bp prom-*lacZ* of *RPS26A*	this work
RH2884	*MATa, (RPS26A, RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7640bp *StuI* cut pME2407 integrated at the *ura3-52* locus; 540bp prom-*lacZ* of *RPS26B*	this work
RH2885	*MATa, rps26A::Kan^R, (RPS26B), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7640bp *StuI* cut pME2407 integrated at the *ura3-52* locus; 540bp prom-*lacZ* of *RPS26B*	this work

Table 1 - continued

Strain	Genotype	Reference
RH2886	*MATa, rps26B::Kan^R, (RPS26A), ura3-52, his3::hisG, trp1::hisG, URA3,* carrying 7640bp *Stu*I cut pME2407 integrated at the *ura3-52* locus; 540bp prom-*lacZ* of *RPS26B*	this work
RH2887	*MATa, rps26A::HIS3, ura3-52, TRP1, LEU2, His⁺*	this work
RH2888	*MATα, rps26A::HIS3, ura3-52, TRP1, LEU2, His⁺*	this work
RH2889	*MATa, rps26B::HIS3, ura3-52, TRP1, LEU2, His⁺*	this work
RH2890	*MATα, rps26B::HIS3, ura3-52, TRP1, LEU2, His⁺*	this work

Table 2 - Plasmids used in this work

Plasmid	Description	Reference
pBluescript KSII®	2.96 kb vektor, *Amp^R*, (*bla*), *lacZ, ori*	Stratagene (La Jolla, USA)
pRS316	*URA3, CEN, Amp^R* (*bla*), *lacZ, ori*	(Sikorski & Hieter, 1989)
pRS426	*URA3, 2µm, Amp^R* (*bla*), *lacZ, ori*	(Sikorski & Hieter, 1989)
PRS426MET25	*URA3, 2µm, Amp^R* (*bla*), pRS426, carrying yeast *MET25* promoter and *CYC1* terminator as *Xho*I/*Kpn*I fragment	(Mumberg *et al.*, 1994)
YEp355	*URA3,* 8.2 kb *lacZ* shuttle vector	(Myers *et al.*, 1986)
YIp353	*URA3,* 7.1 kb *lacZ* shuttle vector	(Myers *et al.*, 1986)
YIp355	*URA3,* 7.1 kb *lacZ* shuttle vector	(Myers *et al.*, 1986)
BHUM74	593 bp C-terminal fragment of *FLO11* in *Xho*I of pBKS®	Mösch, pers. comm.
BHUM108	1.7 kb *Bam*HI *loxP::pTEF::Kan^R::tTEF::loxP* in *Eco*RV of pBKS®	Mösch, pers. comm.
B1679	2500bp *LEU2* gene in pBKS®	Hill, pers. comm.
B1683	1720bp *HIS3* gene in pBKS®	Hill, pers. comm.
B3782	3 kbp p*FLO11-lacZ* reporter in YEp355	(Rupp *et al.*, 1999)
pME637	1.1kb *URA3 Hind*III fragment in pUC19	(Künzler *et al.*, 1992)
pME2405	8150bp YIp355 carrying a 1050bp p*RPS26A-9bpORF-lacZ* fusion in the *Eco*RI site of the MCS	this work
pME2406	7650bp YIp355 carrying a 420bp p*RPS26A-9bpORF-lacZ* fusion in the *Eco*RI site of the MCS	this work
pME2407	7640bp YIp355 carrying a 540bp p*RPS26B-12bpORF-lacZ* fusion in the *Eco*RI site of the MCS	this work
pME2144	7900bp YIp355 carrying a 750bp p*RPS26A-9bpORF-lacZ* fusion in the *Eco*RI site of the MCS	this work
pME2146	8500bp YIp353 carrying a 1400bp p*RPS26B-12bpORF-lacZ* fusion in the *Bam*HI site of the MCS	this work
pME2148	8140bp YIp355 carrying a 1080bp p*RPS26B-12bpORF-lacZ* fusion in the *Eco*RI site of the MCS	this work
pME2449	pBKS® carrying a 1010bp (5´) and a 810bp (3´) PCR fragment of *RPS26A* ligated with *Bgl*II and cloned into *Eco*RV of the MCS	this work
pME2450	pBKS® carrying a 1010bp (5´) and a 560bp (3´) PCR fragment of *RPS26A/µORF* ligated with *Bgl*II and cloned into *Eco*RV of the MCS	this work

Table 2 - continued

Plasmid	Description	Reference
pME2451	pBKS® carrying a 770bp (5´) and a 560bp (3´) PCR fragment of the *μORF* ligated with *Bgl*II and cloned into *Eco*RV of the MCS	this work
pME2452	pBKS® carrying a 850bp (5´) and a 940bp (3´) PCR fragment of *CDC55* ligated with *Bgl*II and cloned into *Eco*RV of the MCS	this work
pME2453	pBKS® carrying a 570bp (5´) and a 660bp (3´) PCR fragment of *RPS26B* ligated with *Bgl*II and cloned into *Eco*RV of the MCS	this work
pME2454	pBKS® carrying a 1010bp (5´) and a 810bp (3´) PCR fragment of *RPS26A* and a 1600bp *Bam*HI cut *Kan^R*-cassette from BHUM108 cloned into the *Bgl*II site	this work
pME2455	pBKS® carrying a 1010bp (5´) and a 560bp (3´) PCR fragment of *RPS26A/μORF* and a 1600bp *Bam*HI cut *Kan^R*-cassette from BHUM108 cloned into the *Bgl*II site	this work
pME2456	pBKS® carrying a 770bp (5´) and a 560bp (3´) PCR fragment of the *μORF* and a 1600bp *Bam*HI cut *Kan^R*-cassette from BHUM108 cloned into the *Bgl*II site	this work
pME2457	pBKS® carrying a 850bp (5´) and a 940bp (3´) PCR fragment of *CDC55* and a 1600bp *Bam*HI cut *Kan^R*-cassette from BHUM108 cloned into the *Bgl*II site	this work
pME2458	pBKS® carrying a 570bp (5´) and a 660bp (3´) PCR fragment of *RPS26B* and a 1600bp *Bam*HI cut *Kan^R*-cassette from BHUM108 cloned into the *Bgl*II site	this work
pME2459	pBKS® carrying a 1010bp (5´) and a 810bp (3´) PCR fragment of *RPS26A* and a 1720bp *Bam*HI cut *HIS3*-cassette from B1683 cloned into the *Bgl*II site	this work
pME2460	pBKS® carrying a 1010bp (5´) and a 560bp (3´) PCR fragment of *RPS26A/μORF* and a 1720bp *Bam*HI cut *HIS3*-cassette from B1683 cloned into the *Bgl*II site	this work
pME2461	pBKS® carrying a 570bp (5´) and a 660bp (3´) PCR fragment of *RPS26B* and a 1720bp *Bam*HI cut *HIS3*-cassette from B1683 cloned into the *Bgl*II site	this work
pME2462	pRS316 carrying a 1700bp *Eco*RI-*Sac*I-*Eco*RI chimaric fusion of 750bp p*RPS26A* - 360bp CM *RPS26A-Sac*I-*RPS26B* - 590bp t*RPS26B*	this work
pME2463	pRS316 carrying a 2300bp *Eco*RI-*Sac*I-*Eco*RI chimaric fusion of 1080bp p*RPS26B* - 360bp CM *RPS26B-Sac*I-*RPS26A* - 850bp t*RPS26A*	this work
pME2464	pRS316 carrying a 1760bp *Eco*RI-*Sac*I-*Eco*RI chimaric fusion of 540bp p*RPS26B* - 360bp CM *RPS26B-Sac*I-*RPS26A* - 850bp t*RPS26A*	this work
pME2465	pRS316 carrying a 1950bp *Eco*RI fragment of 750bp p*RPS26A* - *RPS26A* - 850bp t*RPS26A*	this work
pME2466	pRS316 carrying a 2100bp *Eco*RI fragment of 1080bp p*RPS26B* - *RPS26B* - 590bp t*RPS26B*	this work
pME2467	pRS426 carrying a 2100bp *Eco*RI fragment of 1080bp p*RPS26B* - *RPS26B* - 590bp t*RPS26B*	this work
pME2468	pRS426MET25 carrying a 400bp *Bam*HI-*Xho*I PCR fragment of *RPS26A* fused to p*MET25*/t*CYC1*	this work
pME2469	pRS426MET25 carrying a 400bp *Bam*HI-*Xho*I PCR fragment of *RPS26B* fused to p*MET25*/t*CYC1*	this work

3.3.3 Crosses, sporulation, and tetrad dissection

Diploid homozygous and heterozygous yeast strains were obtained by mating of haploid yeast strains that were verified by Southern hybridizations experiments. Mating, sporulation and tetrad dissection was performed according to Sherman *et al.* (1986).

3.3.4 Wash test assay

Haploid adhesive growth was measured in a washing test. Haploid strains patched on SC media were incubated for 3 to 5 days at 30^0 C. Plates were photographed under white light. Then, plates were carefully washed under a weak stream of water, avoiding direct streaming of the patched cell surfaces. Washed plates were incubated for drying for 10 min at room temperature, and then plates were photographed again to document the remaining cells on the agar surface. Diploid pseudohyphal growth was tested the same way, but the diploid cells were photographed under the microscope.

3.3.5 Qualitative filamentous growth assay and determination of substrate invasion

The qualitative growth assay was performed as described previously (Gimeno *et al.*, 1992; Gimeno & Fink, 1994) using the modification described from Mösch and Fink (1997). Diploid strains to be tested were streaked to obtain single cells on fresh SLAD or SLAD + uracile (SLAD + Ura) media. The streaking technique was chosen to produce a gradient of colony density as described. Substrate invasion was followed under the microscope and representative colonies were photographed. To quantify invasiveness, the significant amount of a total of 50 cells that remained in the agar surface after washing, were counted. We defined five classes for invasiveness, with an average amount of all cells exhibiting invasiveness of < 5% in class I, 5% - 30% in class II, 30% - 50% in class III, 50% -70% in class IV, and of > 70% in class V, respectively.

3.3.6 Construction of gene-specific knockout cassettes

For the construction of the gene specific knockout cassettes referred to in the text, we used the following strategy. We amplified both, the 5'- and 3'- genomic regions of the respective gene using specific primers that were introducing a new 5'- and 3'- *Eco*RI restriction site as well as a *Bgl*II site not present in the amplified region. The PCR fragments were cloned into the multiple cloning site (MCS) of pBluescript KSII® (pBKS II®), using recommended restriction enzymes and joining the 5'- and 3'- fragments via the *Bgl*II site. Finally, a 1700bp *Kan^R* resistance marker (BHUM108) or a functional *HIS3* gene (B1863) were cloned into the *Bgl*II site, using *Bam*HI cut inserts. Thereby, the *Bgl*II or *Bam*HI sites of the joined fragments were eliminated. All constructed plasmids were tested for correct ligation by restriction analysis and by sequencing analysis, respectively (data not shown).

3.3.7 Isolation of yeast knockout-mutant strains and analysis of correct transformation

For isolation of haploid yeast knockout strains, linearised knockout cassettes carrying the 1.7kb *Kan^R*-resistance marker or the 1.6kb *HIS3* gene were transformed into the recommended yeast strains and were selected for the markers on selective SC media. Transformants were patched twice on fresh media and single integration was verified by Southern hybridisation analysis using ^{32}P-radio-labelled DNA probes (data not shown).

3.3.8 Construction of yeast *rps26A*, *rps26B*, and *cdc55* knockout mutant strains

In a mutation analysis, using a transposon mutagenised yeast genomic library (*Tn3::lacZ::LEU2*) (Burns *et al.*, 1994) and screening for yeast strains that exhibit a <u>d</u>efect in <u>f</u>ilamentous <u>g</u>rowth (dfg-phenotype), the *cdc55-100* yeast mutant strain RH2862 was isolated (Mösch & Fink, 1997). In this strain, the *Tn3* flanked *LEU2* gene was randomly integrated into the yeast genome in an intergenic region of two adjacent genes on chromosome VII, the *RPS26A,* and the *CDC55* gene, respectively (Fig. 3.1A).

While *RPS26A* (Wu & Chiang, 2001) encodes for a ribosomal protein of the small 40S subunit, the *CDC55* gene has been described to encode a regulatory B-type subunit of <u>p</u>rotein <u>p</u>hosphatase <u>2A</u> (PP2A) (Healy *et al.,* 1991).
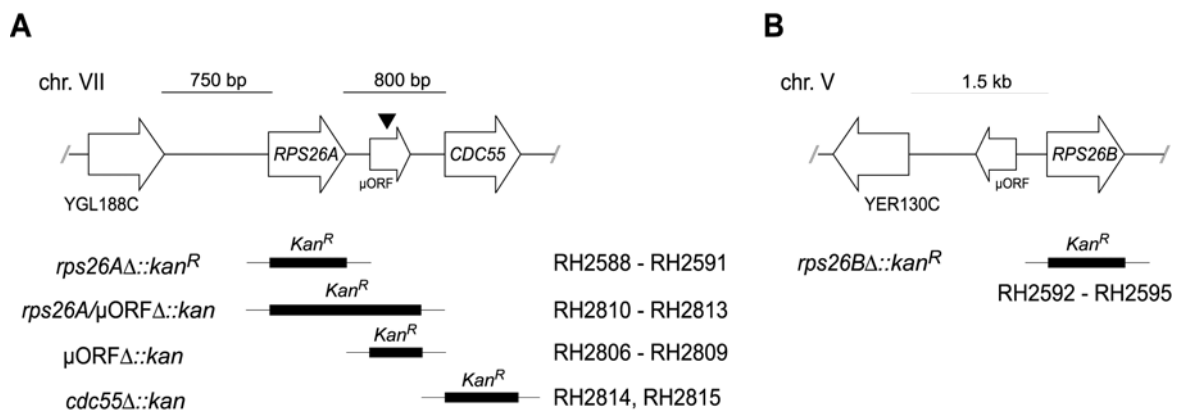

Fig. 3.1 : Schematic drawings of both, the *RPS26A* and the *RPS26B* gene locus of *S. cerevisiae*.
[A] The *RPS26A* gene locus on chromosome VII of *S. cerevisiae* is given, with the upstream YGL188C open reading frame (ORF) and the downstream *CDC55* gene. The intergenic region of the *RPS26A* and the *CDC55* gene carries a small ORF (µORF). This region was disrupted by a transposon insertion (black triangle) in the previously described *cdc55-100* yeast mutant strain RH2862 (Mösch & Fink, 1997). Several yeast mutant strains were constructed carrying specific gene knockouts as result of the integration of a selectable *Kan*[R]-resistance marker (Guldener *et al.*, 1996). Strains RH2588 to RH2591 carry a *rps26AΔ::Kan*[R] knockout (pME2454), RH2810 to RH2813 carry a *rps26A*/µORFΔ::*Kan*[R] knockout (pME2455), RH2806 to RH2809 carry a µORFΔ::*Kan*[R] knockout (pME2456), and strains RH2814 and RH2815 carry a *cdc55Δ::Kan*[R] knockout (pME2457), respectively (see Table 1 and Table 2 for details).**[B]** The *RPS26B* gene locus on chromosome V of *S. cerevisiae* is given, with two upstream ORFs, the YER130C ORF and the small ORF (µORF), respectively. Strains RH2592 to RH2595 carry a *rps26BΔ::Kan*[R]-resistance marker derived from plasmid pME2458.

Between both, the *RPS26A* and the *CDC55* gene, a small <u>o</u>pen <u>r</u>eading <u>f</u>rame (µORF) of 138bp is present in exactly that area, which was disrupted by the transposon-insertion (Fig. 3.1A). Upstream to the *RPS26A* gene, the 174 bp

YGL188C ORF is located, which has a yet unknown function in yeast (Fig. 3.1A). On chromosome V, an isogene to *RPS26A*, the *RPS26B* gene is located downstream to the 1332 bp YER130C ORF. This ORF is transcribed in reverse orientation when compared to *RPS26B* and encodes for a protein of a yet unknown function (Fig. 3.1B). In the intergenic region of *RPS26B* and YER130C, also a small open reading frame (µORF) of 212 bp is present (Fig. 3.1B).

Using specific primers and <u>p</u>olymerase <u>c</u>hain <u>r</u>eactions (PCR), we constructed five gene-specific knockout cassettes (Fig. 3.1A, 3.1B), which were cloned into pBluescriptKSII® (pBKSII®). These knockout cassettes are carrying the heterologous loxP-*Kan*MX-loxP marker gene (Guldener *et al.*, 1996) and were linearised by a *Bam*HI / *Kpn*I digestion. Next, all cassettes were chromosomally integrated into haploid yeast strain RH2584 (*MATa, ura3-52, his3::hisG, trp1::hisG*), respectively. Namely, we used the following cassettes: 3.5kb *RP26A*Δ (pME2454), 3.2kb *RPS26A/µORF*Δ (pME2455), 2.9kb µORFΔ (pME2456), 3.5kb *CDC55*Δ (pME2457) and 2.8kb *RPS26B*Δ (pME2458). Haploid transformants were selected on SC media supplemented with 150µg/ml geneticin G418 (Jimenez & Davies, 1980; Webster & Dickson, 1983). All transformants were verified by Southern hybridisation analysis (data not shown).

These transformation procedures resulted in a yeast *rps26A*Δ strain (RH2588, *MATa, rps26A::Kan^R, ura3-52, his3::hisG, trp1::hisG*), a µORFΔ strain (RH2806, *MATa, µORF::Kan^R, ura3-52, his3::hisG, trp1::hisG*), a strain carrying a double-knockout of both, the *RPS26A* gene and the downstream µORF (RH2810, *MATa, rps26A/µORF*Δ*::Kan^R, ura3-52, his3::hisG, trp1::hisG*), a *cdc55*Δ strain (RH2814, *MATa, cdc55::Kan^R, ura3-52, his3::hisG, trp1::hisG*), and a *rps26B*Δ strain (RH2592, *MATa, rps26A::Kan^R, ura3-52, his3::hisG, trp1::hisG*), respectively (Fig. 3.1A, 3.1B).

All knockout cassettes described above were also transformed into haploid yeast strains RH2585 to RH2587 carrying opposite mating types and differing in the *leu2-* and *trp1*-marker gene, respectively (see Table 1). Therefore, diploid homozygous yeast knockout strains could be constructed. Additionally, we constructed a second set of knockout cassettes using a functional *HIS3* gene instead of the *Kan^R* resistance marker (pME2459 to pME2461; see Table 2). The linearised *HIS3* knockout cassettes were also chromosomally integrated into yeast strain RH2584 (*MATa, ura3-52, his3::hisG, trp1::hisG*). Transformants were selected on SC media lacking histidine and uracile (SC -his -ura). Correct integration of the knockout

cassettes was followed by Southern hybridisation analysis (data not shown). This resulted in the histidine prototrophic (*His*⁺) yeast mutant strains RH2854 to RH2857 (*rps26AΔ::HIS3*) and RH2858 to RH2861 (*rps26BΔ::HIS3*) (see Table 1).

Therefore, two diploid yeast strains could be constructed, carrying two different combinations of marker genes at the original *RPS26* gene loci. Diploid strain RH2872 carries wildtype *RPS26A* and *rps26AΔ::Kan^R* as well as wildtype *RPS26B* and *rps26BΔ::HIS3*, respectively. Strain RH2873 carries wildtype *RPS26A* and *rps26AΔ::HIS3* gene as well as wildtype *RPS26B* and *rps26BΔ::Kan^R*, respectively (see Table 1).

3.3.9 Construction and integration of *RPS26A* and *RPS26B* promoter-*lacZ* fusions

With respect to the upstream-located μORF (Fig. 3.1), we fused three promoter fragments of the *RPS26A* gene locus inframe to the *lacZ* reporter gene on plasmids pME2405, pME2144, and pME2406, respectively. Accordingly, we also constructed three promoter-fragments of the *RPS26B* locus differing in length and fused inframe to the *lacZ* reporter gene on plasmids pME2146, pME2148, or pME2407.

To avoid varying ß-gal-activities as a sequence to varying copy numbers within transformed cells, all constructs were linearised by *Stu*I digestion and were chromosomally integrated in single copy into the yeast genome. For integration of *lacZ* reporter constructs, strains RH2584 (wildtype), RH2588 (*rps26AΔ::Kan^R*) and RH2592 (*rps26BΔ::Kan^R*) were used, respectively. All these strains are carry an *ura3-52* marker gene with a Ty-transposon element. Correct integration of the *lacZ*-reporter constructs restored functional expression of the *URA3* gene. Therefore, transformants were selected on SC-Ura media. Single integration was followed by Southern hybridisation analysis, using a 1.1kb *Hind*III *URA3* fragment (pME637) as radiolabelled probe (data not shown). For each transformation, two independent yeast mutant strains were isolated and were taken for further analysis. These haploid yeast strains were pre-incubated for 24h at 30 °C in SC-Ura media. Tester-cultures were inoculated to same OD values. After 6h of growth at 30 °C ß-gal-activities of all p*RPS26-lacZ*-reporter strains were determined.

3.3.10 Construction of yeast *RPS26A* and *RPS26B* complementation cassettes and chimera cassettes

Using specific primers, we amplified the entire *RPS26A* gene including both, a 750bp promoter, and an 850bp terminator fragment. Secondly, we amplified the *RPS26B* gene including a 1080bp promoter and a 590bp terminator fragment. While the *RPS26A* amplicon was cloned into the low-copy number plasmid pME2465, the *RPS26B* amplicon was cloned into both, the low-copy number plasmid pME2466, and the high-copy number plasmid pME2467 (Fig. 3.2). As control, we cloned a 400bp *Bam*HI/*Xho*I cut PCR fragment of the *RPS26B* ORF behind the inducible *MET25* promoter in pME2469 (Fig. 3.2).
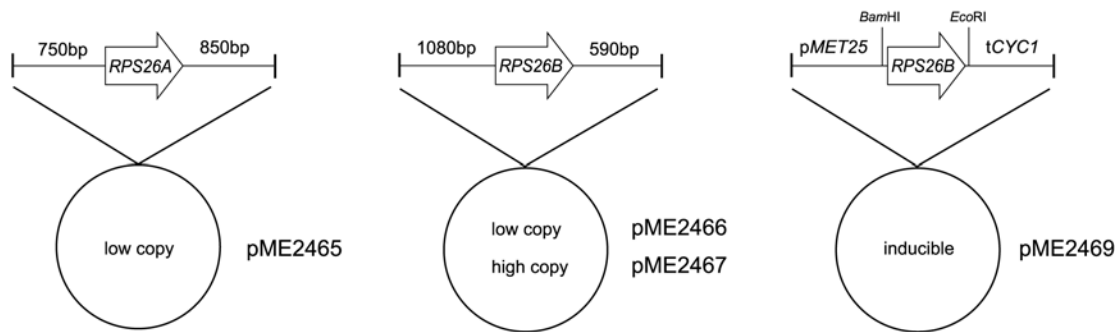

Fig. 3.2: Construction of four *RPS26A* or *RPS26B* extra chromosomal complementation cassettes. In this schematic drawing four plasmids are given that carry the *RPS26A* or the *RPS26B* gene. A 1.95kb PCR fragment of the *RPS26A* locus that carries a 750bp promoter fragment, the 360bp *RPS26A* ORF and a 850bp terminator fragment, was cloned into the low-copy number plasmid pME2465. Accordingly, plasmid pME2466 carries a 2.0kb PCR fragment of the *RPS26B* locus with a 1080bp promoter and a 590bp terminator fragment. Plasmids pME2465 and pME2466 are derivatives of the previously described pRS316 (ARS/CEN; (Sikorski & Hieter, 1989). Plasmid pME2467 carries the same 2.0kb PCR fragment of the *RPS26B* locus but cloned into high copy number plasmid pRS426 (2μm; Sikorsky & Hieter, 1989). As control, plasmid pME2469 carries a 400bp PCR fragment of *RPS26B* that was cloned behind the inducible *MET25* promoter (p*MET25*) and the *CYC1* terminator (t*CYC1*) on plasmid pRS426-*MET25* (Mumberg *et al.*, 1994).

Next, we constructed a set of three plasmids carrying chimera (CM) promoter (p*RPS26A,* p*RPS26B*) and open reading frame (ORF) fusions (Fig. 3.3). A 750bp p*RPS26A* and the original *RPS26A* ORF up to the internal *Sac*I site was fused in frame to the remaining part of the *RPS26B* ORF and a 590bp *RPS26B* terminator (t*RPS26B*) in plasmid pME2462. A 1080bp p*RPS26B* promoter and the original *RPS26B* ORF up to the internal *Sac*I site was fused in frame with the remaining part of the *RPS26A* ORF and the 850bp terminator (t*RPS26A*) fragment in plasmid

pME2463. Finally, we also fused a shortened 550bp p*RPS26B* promoter with the original *RPS26B* ORF up to the internal *Sac*I site in frame with the remaining part of the *RPS26A* ORF and an 850bp terminator (t*RPS26A*) fragment in plasmid pME2464.
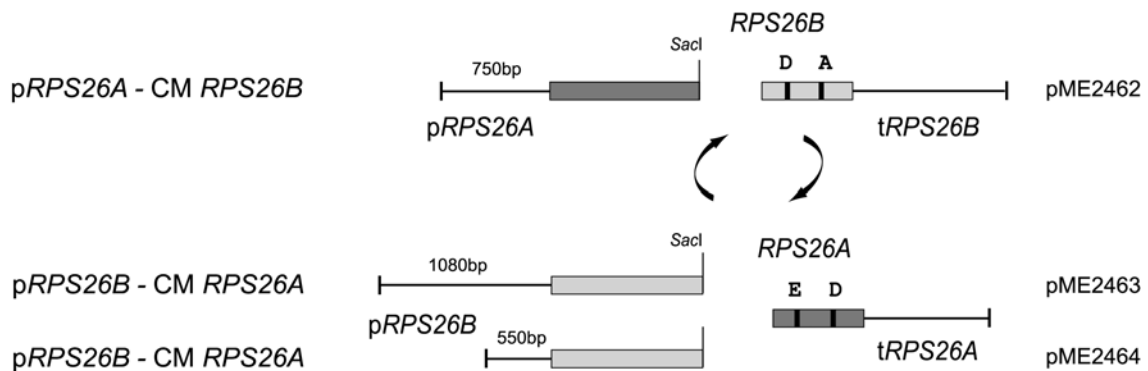
Fig. 3.3: Schematic drawings of three chimera promoter-open reading frame fusions of the *RPS26A* and *RPS26B* gene. Three plasmids were constructed, carrying chimera *RPS26A* and *RPS26B* gene fusions, respectively. A 750bp promoter fragment of *RPS26A* (p*RPS26A*) and the *RPS26A* gene up to an internal *Sac*I restriction site were fused to the C-terminal part of the *RPS26B* gene on the low-copy plasmid pME2462. The *RPS26B* 3´-fragment contains exactly that part of the *RPS26B* gene encoding for two variant amino acids in the highly conserved amino acid sequence. Also, a 1080bp and a 550bp promoter fragment of *RPS26B* (p*RPS26B*) and the part of the *RPS26B* ORF up to internal *Sac*I restriction site were fused to the C-terminal part of the *RPS26A* gene in low copy number plasmids pME2463 and pME2464, respectively. All three plasmids are derivatives of the previously described pRS316 (ARS/CEN; Sikorsky & Hieter, 1989).

3.4   Results

3.4.1 The *RPS26A* gene but not the *RPS26B* gene is required for normal growth in *S. cerevisiae*

The *RPS26A* and the *RPS26B* gene of *S. cerevisiae* encode for two, almost identical proteins of the small 40S ribosomal subunit (Wu & Tan, 1994; Coglievina *et al.*, 1997). The two 360 bp ORFs of the *RPS26A* and the *RPS26B* gene share 92% sequence identity, with a total of 28 different nucleotides. This different nucleotides result in only two different amino acids at position 106 and 113, respectively (Fig. 3.4). Therefore, both *RPS26* genes encode for a deduced protein with a calculated molecular weight of about 13.5 kDa. Although, the prokaryotic as well as the eukaryotic ribosomal structures have been resolved at high x-ray resolution, the exact positioning of the two Rps26p proteins is not yet known (Verschoor *et al.*, 1998; Gabashvili *et al.*, 2000; Gomez-Lorenzo *et al.*, 2000; Spahn *et al.*, 2001; Yusupov *et al.*, 2001; Yusupova *et al.*, 2001). We wanted to know, whetherthe *RPS26* gene products, the Rps26Ap and the Rps26Bp protein are equally required for growth of   *S. cerevisiae* under different nutritional conditions.
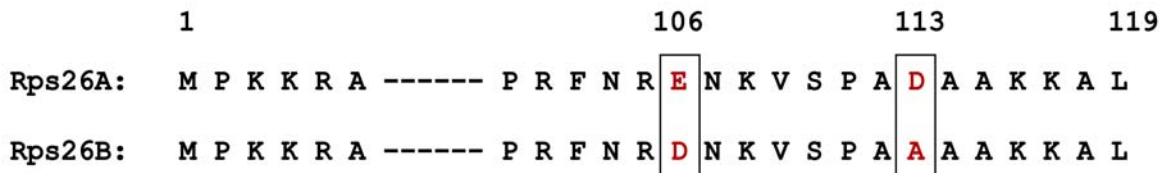
Fig. 3.4: A partial amino acid sequence alignment of the Rps26Ap and the Rps26Bp is given. The deduced Rps26Ap and Rps26Bp proteins, differ only in two amino acids at position 106 and 113 of 119, respectively. While the deduced Rps26Ap protein contains Glu[106] (E) and Asp[113] (D), the deduced Rps26Bp contains Asp[106] (D) and Ala[113] (A), respectively. Therefore, the two Rps26 proteins share a deduced amino acid sequence identity of 97%. Numbers above the amino acids give the positions relatively to the N-terminal ATG start codon.

We used the haploid yeast knockout strains RH2588 to RH2591 (*rps26AΔ::Kan^R*) and RH2592 to RH2595 (*rpS26BΔ::Kan^R*) to determine the growth rates of these strains under different nutritional conditions in <u>s</u>ynthetic <u>c</u>omplete (SC) and in <u>m</u>inimal <u>v</u>itamins (MV) medium, respectively. We also tested similar strains RH2854 to RH2857 (*rps26AΔ::HIS3*) and RH2858 to RH2861 (*rpS26BΔ::HIS3*), carrying the *HIS3* marker instead of the *Kan^R*-resistance marker. Surprisingly, only the *rps26AΔ* strains, but not the *rps26BΔ* strains exhibit a reduced growth rate under the tested conditions when compared to wildtype strains (RH2584 to RH2587).

The average of the growth rates of all *rps26AΔ* strains tested was μ=0.22 (h^{-1}), while it was μ=0.33 (h^{-1}) for the *rps26BΔ* and μ=0.34 (h^{-1}) for the wildtype strains, respectively (Fig. 3.5).

	SC	MV	strains
wildtype	0.35 h^{-1}	0.32 h^{-1}	RH2584 - RH2587
rps26AΔ::Kan^R	0.23 h^{-1}	0.19 h^{-1}	RH2588 - RH2591
rps26BΔ::Kan^R	0.33 h^{-1}	0.29 h^{-1}	RH2592 - RH2595
rps26AΔ::HIS3	0.24 h^{-1}	0.20 h^{-1}	RH2854, RH2855
rps26BΔ::HIS3	0.35 h^{-1}	0.31 h^{-1}	RH2858, RH2859

Fig. 3.5: Growth rates of different *rps26AΔ* and *rps26BΔ* yeast mutant strains. The growth rates depicted were determined in synthetic complete (SC) medium or SC medium lacking histidine (SC-His) to secure a functional expression of the *HIS3* marker gene. Names of strains referred to in the text are given in the right column.

Our result that only the *rps26AΔ*, but not the *rps26BΔ* yeast mutant strains exhibit a reduced growth rate gave rise to the question, whether we could identify further differences between both yeast mutant strains, respectively. Several antibiotics were previously identified to selectively influence gene expression (Berset *et al.*, 1998; Cardenas *et al.*, 1999). We wanted to know, whether the addition of paromomycin to growth media has an influence on the growth behaviour of the *rps26AΔ* or the *rps26BΔ* yeast mutant strains. Paromomycin has been described to increase the translational error rate by decreasing the dissociation of t-RNAs bound to the A-site of the ribosome (Carter *et al.*, 2000).

Therefore, the ribosomal *rps26AΔ::HIS3* and the *rpS26BΔ::HIS3* yeast mutant strains seem to be a good target for testing of paromomycin. As control, we transformed the *rps26AΔ* knockout strains RH2588 to RH2591 with the low-copy number plasmid pME2465 carrying a functional *RPS26A* gene (p*RPS26A*, Fig. 3.6). Accordingly, we transformed the *rps26BΔ* knockout strains RH2592 to RH2595 with the low-copy number plasmid pME2466 carrying a functional *RPS26B* gene (p*RPS26B*, Fig. 3.6). As wildtype control, the histidine prototrophic (*His$^+$*) strains RH2816 to RH2819 were used. In a serial dilution experiment starting off with 10^{-1} cells/ml, all transformed strains were spotted onto SC-His-Ura medium supplemented

with 0.1 mg/ml to 1.0 mg/ml of paromomycin. After 3 to 5 days of growth the spotted strains were compared to strains on media lacking the antibiotic drug.
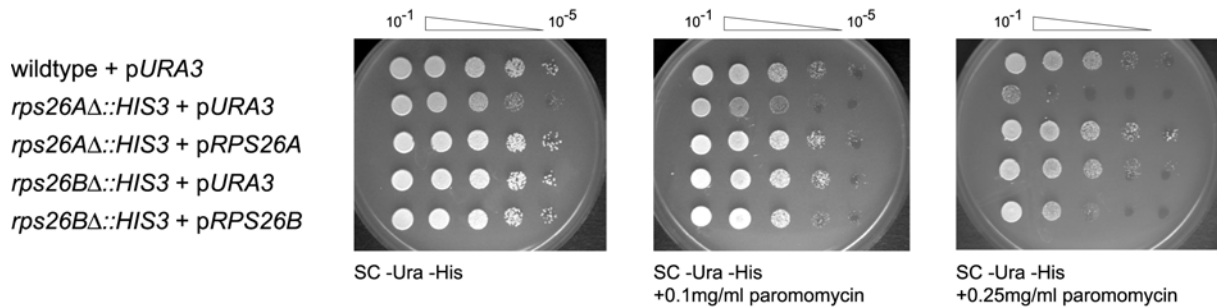

Fig. 3.6: A specific translation inhibition of *rps26A∆* or *rps26B∆* yeast mutant strains is shown. Plasmid harbouring yeasts, pre-cultured to same optical densities (OD$_{546}$=0.8) were spotted onto SC-his-ura medium or SC-his-ura medium supplemented with 0.1 mg/ml and 0.25 mg/ml paromomycin. Strains were spotted using a serial dilution and starting off with 10^{-1} cells/ml. Strains used were wildtype control RH2816, RH2846 (*rps26A∆::HIS3*), and RH2850 (*rps26B∆::HIS3*), respectively. Similar strains carrying the *rps26A∆::Kan^R* or *rps26B∆::Kan^R* marker could not be used, since a cross reaction between the antibiotic drug paromomycin and the resistance marker is possible. Tester strains were transformed with the control plasmid pRS316 (+ p*URA3*), with plasmid pME2465 carrying a 1.95kb PCR fragment of the *RPS26A* locus (+ p*RPS26A*), or with plasmid pME2466 carrying a 2.0kb PCR fragment of the *RPS26B* locus (+ p*RPS26B*). For details of plasmids see also figure 3.2.

We found, that all *rps26A∆::HIS3* yeast mutant strains (RH2588 to RH2591) are selectively inhibitable by the antibiotic paromomycin when compared to wildtype. Under the tested conditions, growth of the *rps26A∆* knockout strain RH2588 could be restored by expression of extrachromosomal *RPS26A* using a low-copy number plasmid (Fig. 3.6). Addition of 0.25 mg/ml of paromomycin to growth medium demonstrated that also the *rps26B∆* mutant strain RH2592 is inhibitable by the addition of paromomycin. At this concentration, it became obvious that strain RH2592 when expressing *RPS26B* from a low-copy number plasmid, is stronger inhibited by the antibiotic paromomycin than is the wildtype control. This is surprising, since strain RH2592 contains a functional *RPS26A* gene. The higher amount of Rps26Bp seems to compete with Rps26Ap within the ribosome under antibiotic induced conditions.

Our results from the growth tests prompted us to isolate a haploid yeast mutant strain completely lacking the *RPS26* gene function. Transformation of the *rps26AΔ::HIS3* cassette pME2459 into a *rps26BΔ* yeast mutant strain as well as of the *rps26BΔ::HIS3* cassette pME2461 into a *rps26AΔ* yeast mutant strain did not result in any transformants. Therefore, we tried to isolate haploid *rps26AΔ rps26BΔ* double mutant strains by sporulation of heterozygous diploid yeast mutant strains.

In a tetrad dissection assay, two diploid yeast strains, RH2872 (*rps26AΔ::Kan^R/RPS26A*, *RPS26B/rps26BΔ::HIS3*) and RH2873 (*rps26AΔ::HIS3/RPS26A*, *RPS26B/rps26BΔ::Kan^R*), were sporulated as described previously (Sherman *et al.*, 1986), and germinated strains were taken for an analysis of growth phenotypes. A total of 74 four-spore tetrads was isolated and was separated by micromanipulation. Viability of the germinated yeast strains was tested on solid SC media (data not shown).

Table 3:

phenotype of recovered spores	rps26AΔ::kan^R x rps26BΔ::HIS3	rps26AΔ::HIS3 x rps26BΔ::kan^R	genotype of recovered spores
	spores recovered from **35** tetrades (1)	spores recovered from **39** tetrades (2)	
His⁻, Kan^R ⁻	33	42	*RPS26A, RPS26B, his3Δ::hisG*
His⁻, Kan^R ⁺	37	36	*rps26AΔ::Kan^R, RPS26B, his3Δ::hisG* *RPS26A, rps26BΔ::Kan^R, his3Δ::hisG*
His⁺, Kan^R ⁻	37	36	*RP26A, rps26BΔ::HIS3, his3Δ::hisG* *rp26AΔ::HIS3, rps26BΔ::HIS3, his3Δ::hisG*
His⁺, Kan^R ⁺	0	0	*rps26AΔ::Kan^R, rps26BΔ::HIS3, his3Δ::hisG*

(1): RH2588: *Mat a, rps26A::kan^R, ura3-52, his3::hisG, trp1::hisG*
x RH2861: *Mat α, rps26B::HIS3, his3::hisG, leu2::hisG, ura3-52, His⁺*
19 TT : 7 NPD : 9 PD

(2): RH2857: *Mat a, rps26A::HIS3, his3::hisG, leu2::hisG, ura3-52, His⁺*
x RH2592: *Mat α, rps26B::kan^R, his3::hisG, trp1::hisG, ura3-52*
26 TT : 8 NPD : 5 PD

Table 3: Analysis of sporulation pattern of two diploid *rps26AΔ/rps26BΔ* yeast mutant strains. Diploid tester strains, RH2872 (Mat a/α, *rps26AΔ::Kan^R/RPS26A*, *rps26B::HIS3/RPS26A*, *ura3-52/ura3-52*, *his3::hisG/his3::hisG*, *trp1::hisG/TRP1*, *LEU2/leu2::hisG*, *His⁺*) and RH2873 (Mat a/α, *rps26AΔ:HIS3/RPS26A*, *rps26B::Kan^R/RPS26A*, *ura3-52/ura3-52*, *his3::hisG/his3::hisG*, *trp1::hisG/TRP1, LEU2/leu2::hisG, His⁺*) were sporulated as described. Germinated spores were taken for a growth test analysis on different SC medium (data not shown). From strain RH2872 35 four-spore tetrads were analysed, while 39 four-spore tetrads were analysed from strain RH2873. The germinated spores belong to three classes of segregants. Abbreviations are tetra type (TT), non-parental ditype (NPD) and parental ditype (PD).

Sporulation of 35 four-spore tetrads from diploid strain RH2872 (*rps26AΔ::Kan^R/RPS26A*, *RPS26B/rps26BΔ::HIS3*) yielded 19 x 3 viable spores of the tetra type (TT), 7 x 2 viable spores of the non-parental ditype (NPD) and 9 x 4 viable spores of the parental ditype (PD).

Sporulation of 39 four-spore tetrads from diploid strain RH2873 (*rps26A::HIS3/RPS26A*, *RPS26B/rps26B::Kan^R*) yielded 26 x 3 viable spores of the tetra type (TT), 8 x 2 viable spores of the non-parental ditype (NPD) and 9 x 4 viable spores of the parental ditype (PD).

We could only isolate haploid yeast mutant strains that were prototrophic for histidine (*His^+*) or were viable on media supplemented with geneticin G418 (*Kan^R+*). From 296 spores analysed no segregant was carrying a *rps26AΔ rps26BΔ* double mutation (see Table 3). This segregation pattern seems to be clear evidence that a deletion of both, the *RPS26A* and the *RPS26B* gene, is non-viable for the yeast *S. cerevisiae*.

In summary, the results obtained from all growth tests demonstrate that the yeast *S. cerevisiae* strictly requires at least one copy of the *RPS26* genes to fulfil its genetic functions. Although the *RPS26A* and the *RPS26B* gene products seem to be almost identical, differences in the protein function became obvious. It seems to be so, that the *RPS26A* gene product is of major importance in the growth of haploid yeast strains, while the *RPS26B* gene functions as a potential backup, when the *RPS26A* gene is damaged or malfunction. Therefore, the *RPS26B* gene seems to be the less important isogene partner. We also showed that a double deletion of both *RPS26* genes is non-viable for the yeast *S. cerevisiae*, thereby demonstrating an essential basal function of the two *RPS26* gene products.

3.4.2 Expression of *RPS26B* is slightly induced in *rps26A* yeast mutant strains, whereas expression of *RPS26A* does not change significantly in *rps26B* mutant strains

The two *RPS26* genes encode for almost identical Rps26p proteins that seem to share different cellular functions. We wanted to know whether these differences depend on different transcript levels of the two isogenes within haploid yeast wildtype strains and when compared to *rps26AΔ* and *rps26BΔ* yeast mutant strains, respectively.

We isolated total RNAs of two sets of slightly variant wildtype, *rps26AΔ*, and *rps26BΔ* mutant strains, respectively. Yeast strains RH2584 (wt), RH2588 (*rps26AΔ::Kan^R*) and RH2592 (*rps26BΔ::Kan^R*) as well RH2816 (wt), RH2846 (*rps26AΔ::HIS3*) and RH2850 (*rps26BΔ::HIS3*) were taken for a transcriptional analysis. Strains pre-incubated overnight, were incubated for 6h in SC media or SC-his media, and total RNAs were isolated. These RNAs were separated by gel electrophoresis and mRNAs were probed with *ACT1*, *RPS26A*, and *RPS26B* radiolabel probes, respectively. Since we already knew that yeast *rps26AΔ* mutant strains exhibit a growth delay, we loaded about 20 μg of total RNAs isolated of *rps26AΔ* strains, while we loaded 10 μg of total RNAs of yeast wildtype and yeast *rps26BΔ* strains, respectively. After exposure of membranes to imaging plates, the mRNA amounts were detected using a Fuji Bas-1500 imager (Fuji Photofilm & Co. Ltd., Japan). To avoid cross hybridisations as a result from almost identical mRNA targets we used specific *pre*-mRNA probes that exclusively hybridise 40 bp or 10 bp upstream to the translational start in the *RPS26A* or *RPS26B* mRNAs (Fig. 3.7A).

Using these specific probes, we found that the average amount of *RPS26B* mRNA is slightly induced to about 125% in the *rps26AΔ* yeast mutant strains RH2588 (*rps26AΔ::Kan^R*) and RH2846 (*rps26AΔ::HIS3*), when compared to wildtype strains RH2584 and RH2816, respectively. In contrast to the expression of *RPS26B*, the *RPS26A* mRNA levels did not change significantly within the *rps26BΔ* mutant strains RH2592 (*rps26BΔ::Kan^R*) and RH2850 (*rps26BΔ::HIS3*), in comparison to wildtype controls, respectively.
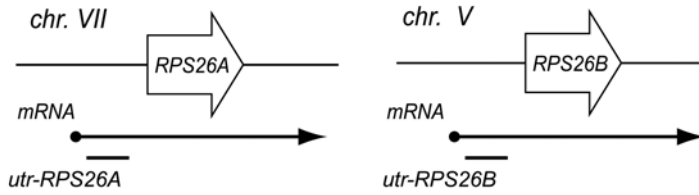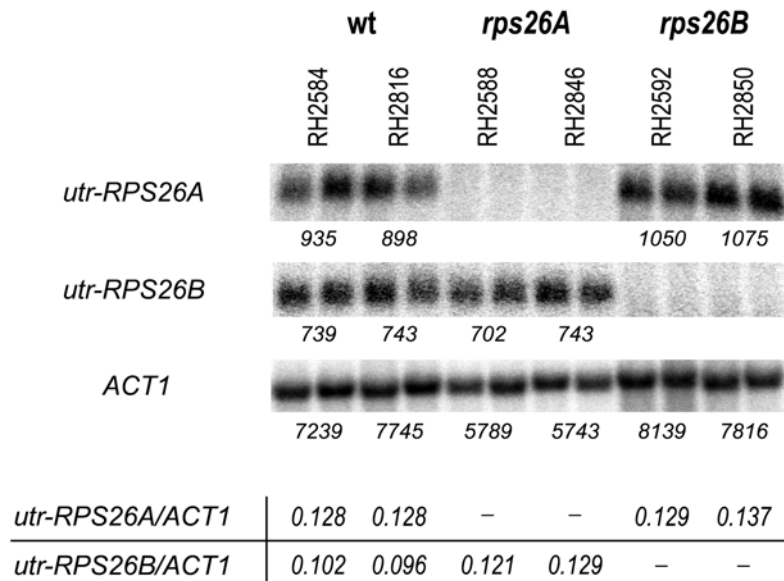
Fig. 3.7: A schematic drawing of a hybridisation pattern and a transcriptional regulation of the *RPS26A* or *RPS26B* gene within two sets of *rps26AΔ* or *rps26BΔ* yeast mutant strains is given, respectively. [A] *Utr*-mRNA probes comparable in length and GC content were amplified by PCR reactions, using specific primers that exclusively anneal within the upstream region of the *RPS26A* or the *RPS26B* gene. While the *RPS26A utr*-mRNA probe hybridises 40 bp upstream, the *RPS26B* probe hybridises 10 bp upstream to the translation start site within the deduced mRNAs. **[B]** Strains used for Northern hybridisation of mRNAs were grown in SC or SC-His media. RNA was isolated after 6h to 8h of growth and was hybridised with the specific *utr*-mRNA probes. Strains used were wildtype (wt) controls RH2584 and RH2816 (*His*[+]), *rps26AΔ* mutants RH2588 (*rps26AΔ::Kan*[R]) and RH2846 (*rps26BΔ::HIS3*, *His*[+]), and *rps26BΔ* mutants RH2592 (*rps26BΔ::Kan*[R]) and RH2846 (*rps26BΔ::HIS3*, *His*[+]), respectively. For better quantification, we loaded two lanes instead of one lane for each sample of RNA. The values depicted are the average of at least three independent measurements with a standard deviation not exiting 15%. Below, the calculated averages of *pre*-mRNA transcript levels from all measurements are given relatively to the *ACT1* transcript levels.

To verify these results, we also tested isolated total RNAs, using the 360bp *RPS26A* or *RPS26B* ORF as non-specific radiolabeled probe (Fig. 3.8A). With this testing we wanted to avoid differences within the labelling reaction of the mRNA probes by using identical radioactive probes for all RNA samples.

A

B

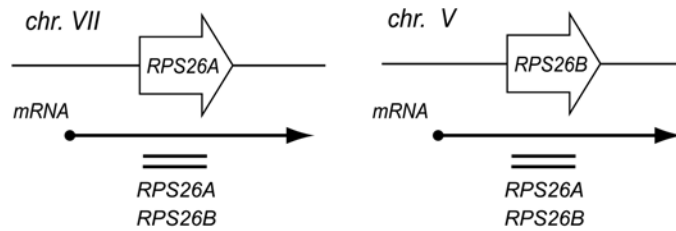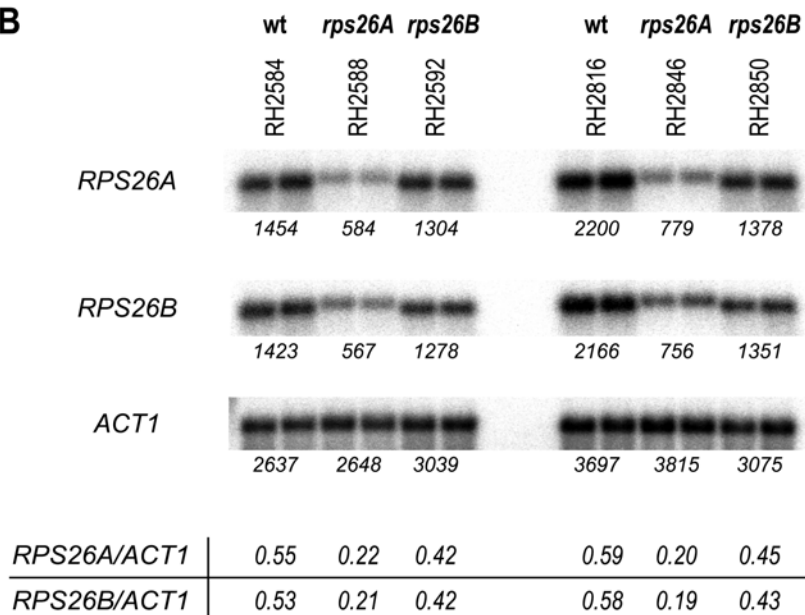|	wt	rps26A	rps26B		wt	rps26A	rps26B
	RH2584	RH2588	RH2592		RH2816	RH2846	RH2850
RPS26A	1454	584	1304		2200	779	1378
RPS26B	1423	567	1278		2166	756	1351
ACT1	2637	2648	3039		3697	3815	3075
RPS26A/ACT1	0.55	0.22	0.42		0.59	0.20	0.45
RPS26B/ACT1	0.53	0.21	0.42		0.58	0.19	0.43

Fig. 3.8: A schematic drawing of a hybridisation pattern and a transcriptional regulation of the *RPS26A* or *RPS26B* gene within two sets of *rps26A*Δ or *rps26B*Δ yeast mutant strains is given, respectively. [A] The *RPS26A* or the *RPS26B* gene was amplified by PCR reactions using DNA templates that derived from knockout mutant strains. Since both genes share a high sequence identity, the derived mRNA probes are suspected to hybridise in both, the *RPS26A* and the *RPS26B* mRNA, respectively. **[B]** Strains used for the Northern hybridisation experiment were grown in SC or SC-His media. RNA was isolated after 6h to 8h of growth and was hybridised with the non-specific labelled mRNA probes. Strains used were wildtype (wt) controls RH2584 and RH2816 (*His*[+]), *rps26A*Δ mutants RH2588 (*rps26A*Δ*::Kan*[R]) and RH2846 (*rps26B*Δ*::HIS3, His*[+]), and *rps26B*Δ mutants RH2592 (*rps26B*Δ*::Kan*[R]) and RH2846 (*rps26B*Δ*::HIS3, His*[+]), respectively. For better quantification, we loaded two lanes instead of one lane for each sample of RNA. The values depicted are the average of at least three independent measurements with a standard deviation not exiting 15%. Below, the calculated averages of three measurements are given relatively to the *ACT1* transcript levels.

We found that the *rps26BΔ* mutant strains RH2592 (*rps26BΔ::Kan^R*) and RH2850 (*rps26BΔ::HIS3*) express 70% to 80% of the complete mRNA level when compared to wildtype strains RH2584 and RH2816, respectively (Fig. 3.8B). Using the *RPS26A* gene for hybridisation, we detected between 30% and 40% of the mRNA signals of the wildtype strains among the *rps26AΔ* mutant strains RH2588 (*rps26AΔ::Kan^R*) or RH2846 (*rps26AΔ::HIS3*). These detected mRNAs are the result from the exclusive expression of the *RPS26B* gene, since both strains are *rps26AΔ* mutants. Therefore, the detected signals are the result from the cross-hybridisation. Since the expression of *RPS26B* seems to be induced to about 125% within the *rps26AΔ* mutant strains, these results were corroborating the results obtained with the specific mRNA probes.

Although the expression of the *RPS26B* gene is slightly induced in the *rps26AΔ* mutant strains, this enhanced transcription does not result in such protein levels to fulfil all genetic functions of the two *RPS26* gene products. A single *RPS26B* gene seems to deliver only a basal amount of mRNA. Since we do not now whether the *RPS26A* mRNA is more stable or is better translated than is the *RPS26B* mRNA, we cannot explain the reason for this effect. Additionally, it is unclear, whether the Rps26Ap is more stable than is the Rps26Bp. But the expression patterns seem to give hints towards the following model. A single *RPS26A* gene can deliver more than 70% of the total mRNA level of a yeast wildtype strain, while the *RPS26B* gene can deliver only up to 40%.

3.4.3 Expression of *RPS26B* is only weak induced in *rps26A* yeast mutant strains

Northern hybridisation analysis of mRNA transcripts gave first hints for differences in the expression of the *RPS26A* and *RPS26B* gene. This expression pattern could be the sequence of variable promoter strength of the two isogenic partners. The exact lengths of the *RPS26A* or the *RPS26B* promoters are not yet known. A typical promoter of *S. cerevisiae* is only about 300 bp to 500 bp in length (Mewes *et al.*, 1997). Nevertheless, some unusually long promoters are known in *S. cerevisiae*, e.g. the *FLO11* promoter, which is about 3 kb in length (Rupp *et al.*, 1999). Since we had no evidences for the exact promoter lengths, we used six promoter-*lacZ* fusion constructs to test for differences in the promoter activity of the two ribosomal isogenes *RPS26A* and *RPS26B*.

With respect to the upstream-located μORF, we fused three promoter fragments of the *RPS26A* gene locus inframe to the *lacZ* reporter gene on plasmids pME2405, pME2144, and pME2406, respectively (Fig. 3.9A). Accordingly, we also constructed three promoter-fragments of the *RPS26B* locus differing in length and fused inframe to the *lacZ* reporter gene on plasmids pME2146, pME2148, or pME2407 (Fig. 3.9B).

Determination ß-gal-activities of the integrated promoter-*lacZ* reporter constructs were performed as described (Bradford, 1976)(Rose & Botstein, 1983). For integration of *lacZ* reporter constructs, strains RH2584 (wildtype), RH2588 (*rps26A∆::Kan^R*) and RH2592 (*rps26B∆::Kan^R*) were used. We determined the transcriptional and the translational activities in twelve derivative strains (Fig. 3.9).

We found that a 420bp promoter fragment of the *RPS26A* gene when fused to the *lacZ* gene (p*RPS26A-lacZ*) results in very low ß-gal activities in strains RH2881 (wt), RH2882 (*rps26A::Kan^R*), and RH2883 (*rps26B::Kan^R*), respectively (Fig. 3.9A). Therefore a 420bp promoter fragment of *RPS26A* is too short for a functional expression of *RPS26A*. Promoter activities of both, the 750 bp and the 1050 bp p*RPS26A-lacZ* fusions resulted in almost identical ß-gal activity values in wildtype yeast strains RH2878 (750bp p*RPS26A-lacZ*), and RH2613 (1080bp p*RPS26A-lacZ*), as well as in *rps26B∆* mutant strains RH2880 (750bp p*RPS26A-lac*) and RH2629 (1080bp p*RPS26A-lacZ*), respectively (Fig. 3.9A).
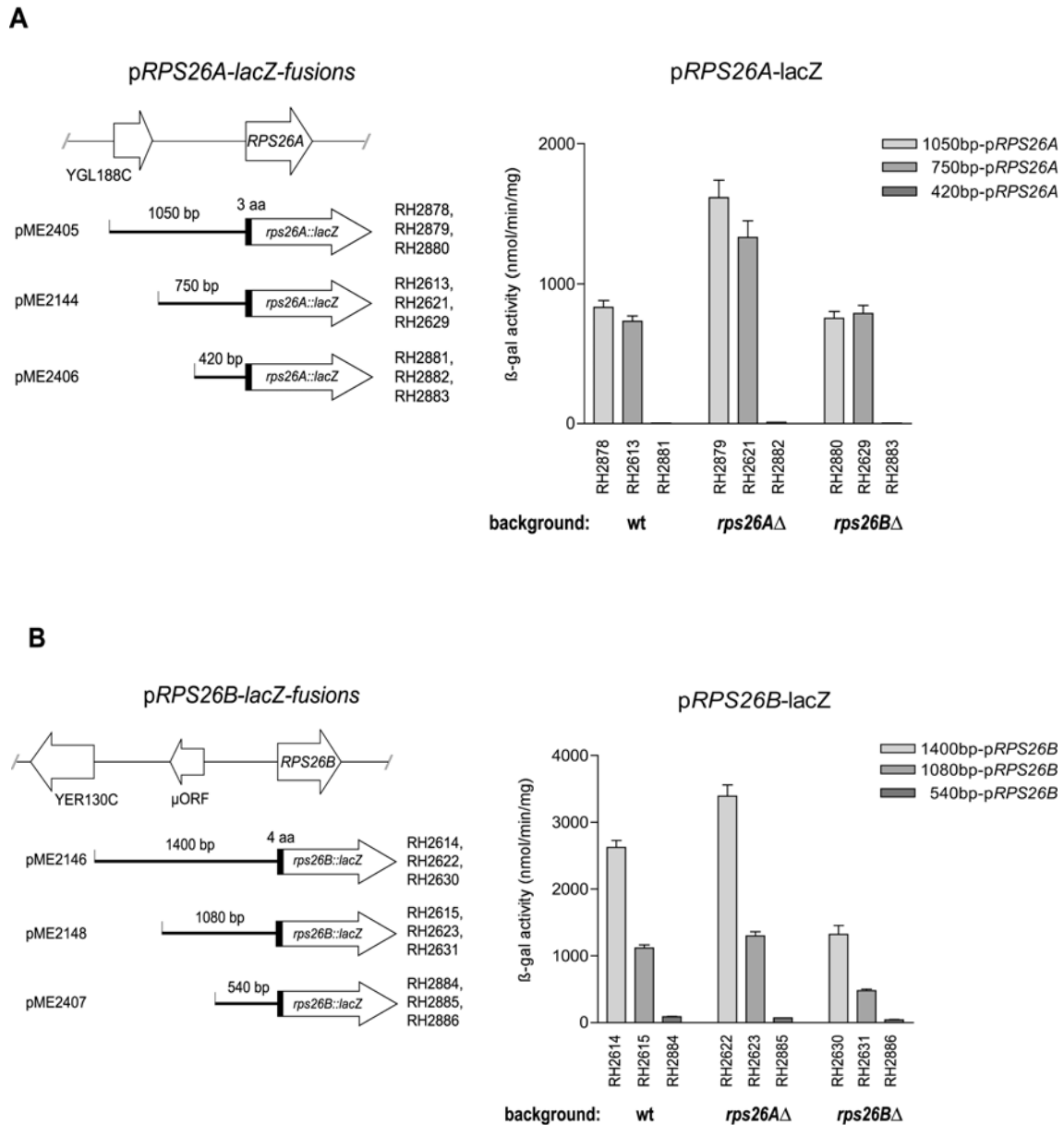
Fig. 3.9: Schematic drawings of six plasmids are shown, carrying *RPS26A* promoter-*lacZ* fusions or *RPS26B* promoter-*lacZ* fusions. On the right site two β-galactosidase activity assays of 18 derived yeast mutant strains are depicted, carrying the six variant promoter *lacZ*-fusions chromosomally integrated at the *URA3* gene locus. [A] Three plasmids were constructed carrying a 420 bp (pME2405), 750 bp (pME2144), or a 1050 bp (pME2405) promoter fragment of the *RPS26A* gene locused fused inframe to the *lacZ* reporter gene. These constructs were linearised by *Stu*I digestion and were chromosomally integrated into strains RH2584 (wildtype), RH2588 (*rps26AΔ::Kan^R*) and RH2592 (*rps26BΔ::Kan^R*), respectively. Resulting strains carrying the p*RPS26A*-*lacZ* reporter constructs at the *URA3* locus are given with RH-numbers. On the right side, the average values of three independent measurements of the β-galactosidase activities are depicted with indicated standard deviations. **[B]** Three plasmids were constructed carrying a 540 bp (pME2407), 1080 bp (pME2148), or a 1400 bp (pME2146) promoter fragment of the *RPS26B* gene locus fused inframe to the *lacZ* reporter gene. These constructs were linearised by *Stu*I digestion and were chromosomally integrated into strains RH2584 (wildtype), RH2588 (*rps26AΔ::Kan^R*) and RH2592 (*rps26BΔ::Kan^R*), respectively. Resulting strains carrying the p*RPS26A*-*lacZ* reporter constructs at the *URA3* locus are given with RH-numbers. On the right side, the average values of three independent measurements of the β-galactosidase activities are depicted with indicated standard deviations.

Therefore, at least one <u>u</u>pstream <u>a</u>ctivation <u>s</u>ite (UAS) of the *RPS26A* gene seems to be located between nucleotide position -420 and -750 relatively to the ATG start-codon of the *RPS26A* gene. Far upstream regions or the upstream µORF (YGL188C) seem to have no influence in correct transcription of the *RPS26A* gene.

Surprisingly, the ß-gal activity values of the p*RPS26A-lacZ* fusions were almost doubled in the *rps26A*Δ mutant background of strains RH2621 (750bp p*RPS26A-lacZ*) and RH2879 (1080bp p*RPS26A-lacZ*), when compared to wildtype strains RH2613 (750bp p*RPS26A-lacZ*) and RH2878 (1080bp p*RPS26A-lacZ*), respectively. This could be evidence for both, an autoregulation of the *RPS26A* gene expression via the Rps26Ap or for a general transcriptional activation of all genes within the *rps26A*Δ mutant strains.

A 540 bp p*RPS26B-lacZ* fusion including the complete DNA sequence of the intergenic region between the *RPS26B* and the upstream µORF exhibited higher ß-gal-activity values in strains RH2884 (wt), RH2885 (*rps26A*Δ::*Kan*^R), and RH2886 (*rps26B*Δ::*Kan*^R), respectively, when compared to the activities of the 420 bp p*RPS26A-lacZ* fusion in the corresponding *rps26A*Δ strains. Therefore, a 540 bp promoter fragment of *RPS26B* seems to induce a weak basal expression. A 1080 bp promoter-*lacZ* fusion that included the complete DNA sequence of the intergenic region and the sequence encoding for the deduced µORF resulted in high ß-gal-activities (1000 nmol/min/mg) in wildtype strain RH2615 and *rps26A*Δ mutant strain RH2623. There were hardly differences for the ß-gal-activity values obtained for the 1080 bp promoter-*lacZ* construct within strain RH2615 (wt) or RH2623 (*rps26A*Δ). Surprisingly, the same construct when integrated into the *rps26B*Δ mutant background of strain RH2631 also resulted in a 40% ß-gal-activity value, when compared to strains RH2615 (wt) or RH2623. This could to be also evidence for a autoregulation of the *RPS26B* expression via the Rps26Bp.

Finally, the ß-gal-activities obtained for the 1400 bp p*RPS26B-lacZ* fusion when integrated into the wildtype background of strain RH2614 raised 2.5 fold when compared to the integrated 1080 bp p*RPS26B-lacZ* fusion. Accordingly, the ß-gal-activities obtained for the 1400 bp p*RPS26B-lacZ* fusions raised also about 2.5-fold, when integrated into the *rps26A*Δ and the *rps26B*Δ mutant background of strains RH2622 and RH2630, respectively.

This seems to be evidence that at least one <u>u</u>pstream <u>a</u>ctivation <u>s</u>ite (UAS) of the *RPS26B* gene is located far upstream to *RPS26B* transcriptional start within the intergenic region of YER130C (-1400) and µORF (-550), respectively.

In summary, the results obtained from the promoter- and the transcription-analysis seem to demonstrate that the sum of both, transcription of the *RPS26B* gene and translation of *RPS26B* mRNA can reach a maximum of about 120% within *rps26AΔ* yeast mutant strains, when compared to wildtype strains. Mutations of the *RPS26A* gene seem to result in a general stress conditions within the yeast cell. As one consequence transcriptional and translational activities are raised within a *rps26AΔ* yeast mutant strain. Nevertheless, the induced activities at the *RPS26B* gene are not sufficient to complement all functions of Rps26Ap, since *rps26AΔ* yeast mutant strains exhibit a reduced growth rate. Although the transcription of *RPS26B* can be induced, this gene delivers weaker total amounts of mRNA than the *RPS26A* gene. This could be evidence for a normally weak expression of *RPS26B* in healthy yeast strains. In contrast to this, the knockout of the *RPS26B* gene seems to have only weak influence on regulation of the growth in the yeast *S. cerevisiae*.

3.4.4 The *RPS26A* but not the *RPS26B* gene product is required for haploid adhesive growth in the yeast *S. cerevisiae*

Our results from the Northern analysis demonstrated differences in the expression of the two isogenes *RPS26A* and *RPS26B* under normal conditions. Testing of transcriptional and translational activities demonstrated that expression of *RPS26B* could only be slightly induced in a *rps26AΔ* yeast mutant strain. We wanted to know whether the *RPS26A* and the *RPS26B* gene are involved in the regulation of haploid adhesive growth. This growth phenotype is part of a complex differentiation processes among Σ1278b yeast strains. To answer the question whether or not *RPS26* genes are involved in haploid adhesive growth, we used two different sets of haploid *rps26AΔ* or *rps26BΔ* mutant strains, respectively.

We used histidine prototrophic (*His⁺*) strains RH2816 to RH2819 (wt), RH2854 to RH2857 (*rps26AΔ::HIS3*) and RH2858 to RH2861 (*rps26BΔ::HIS3*) to perform a haploid adhesive growth test as described (Fig. 3.10A). Surprisingly, only the haploid *rps26AΔ* yeast mutant strains (RH2854 to RH2857) failed in adhesive growth, while the *rps26BΔ* yeast mutant strains (RH2858 to RH2861) displayed normal adhesive growth when compared to wildtype (data not shown).

We also tested whether strains RH2584 to RH2587 (wt), RH2588 to RH2591 (*rps26AΔ::Kan^R*) and RH2592 to RH2595 (*rps26BΔ::Kan^R*) display the same phenotypes, identified first for strains carrying the *HIS3*-resistance marker. We found, that also the *rps26AΔ* yeast mutant strain RH2584 (*rps26AΔ:: Kan^R*) did not exhibit haploid adhesive growth, while *rps26BΔ* yeast mutant strain RH2858 (*rps26BΔ:: Kan^R*) showed normal adhesive growth when compared to wildtype (data not shown).
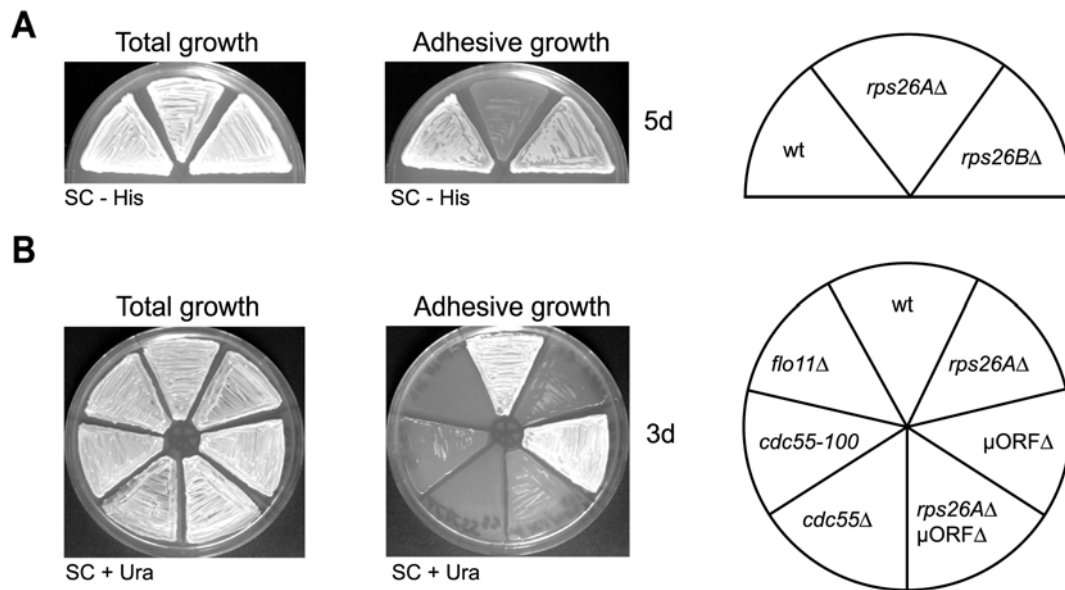
Fig. 3.10: A test for haploid adhesive growth of *rps26AΔ* or *rps26BΔ* yeast mutant strains is shown. [A] Strains to be tested were patched on SC-His medium, incubated, photographed after 5 days (5d) of growth (Total growth), washed under a steam of water, and were photographed again to document remaining cells on the agar surface (Adhesive growth). Strains used were wildtype (wt) control RH2816, *rps26AΔ* mutant RH2854 (*rps26AΔ::HIS3*) and *rps26BΔ* mutant RH2858 (*rps26BΔ::HIS3*), respectively. **[B]** A comparison of defects of haploid adhesive growth of various yeast mutant strains is shown. Strains to be tested were wildtype (wt) control RH2584, *rps26AΔ* mutant RH2588 (*rps26AΔ::Kan^R*), μORFΔ mutant RH2806 (*μORFΔ::Kan^R*), double knockout mutant RH2810 (*rps26A/μORFΔ::Kan^R*), *cdc55Δ* mutant RH2814 (*cdc55AΔ::Kan^R*), *cdc55-100* mutant RH2862 (*cdc55-100*), and *flo11Δ* mutant RH2662 (*flo11Δ::Kan^R*), respectively.

As further control we tested whether or not some other haploid yeast mutant strains fail in adhesive growth under the tested growth conditions. In former experiments the *CDC55* gene locus has been already demonstrated to be involved in haploid adhesive growth (Mösch & Fink, 1997). The *CDC55* gene is located downstream to *RPS26A* on chromosome VII. The isolated *cdc55-100* mutant strain carries a transposon insertion that is located between *CDC55* and *RPS26A*. (see also chapter 3.3.8). Therefore, we wanted to know, whether both genes are involved in haploid adhesive growth. We used strains RH2588, (*rps26AΔ::KanR*), RH2806 (*μORFΔ::KanR*), RH2810 (*rps26A/μORFΔ::KanR*), RH2814 (*cdc55Δ::KanR*), as well as RH2862 (*cdc55-100*) and RH2662 (*flo11Δ*) to test for adhesive growth phenotypes.

Deletion of the small μORF that is located upstream to the *CDC55* gene (RH2806) did not result in a significant change of adhesive growth (Fig. 3.10B). Therefore, the μORF is not involved in regulation of the adhesive growth phenotype. A yeast mutant strain carrying a *rps26A/μORFΔ::KanR* double deletion (RH2810) exhibits the same negative phenotype as a strain carrying only the *rps26Δ* gene deletion (Fig. 3.10B).

A yeast mutant strain carrying a *cdc55Δ* knockout (RH2814) completely failed in adhesive growth (Fig. 3.10B). Under a stream of water, cells of this strain were rapidly washed off and no cells were left on the agar surface after the washing procedure. With respect to adhesive growth, this phenotype is identical that displayed by the control strain RH2662 (*flo11Δ::KanR*). This *flo11Δ* mutant strain is lacking the main flocculin necessary for cell-cell and cell-surface adhesion (Guo *et al.*, 2000) and cells were also rapidly washed off the agar surface.

Furthermore, the *cdc55Δ* strain (RH2814) exhibits a strongly reduced growth rate (μ=0.10 h^{-1}) and displays a defect in cellular cytokinesis. Up to 30% of all cells of strain RH2814 form small cell chains with mother and daughter cells that do not separate (data not shown). This result is not surprising, since *CDC55* encodes for a B-type regulatory subunit of protein phosphatase 2A (Healy *et al.*, 1991), which has multiple functions in mitosis, the kinetochore and spindle checkpoint and in cellular morphogenesis, respectively (Blacketer *et al.*, 1993; Minshull *et al.*, 1996; Wang & Burke, 1997; Jiang & Broach, 1999).

This washing test demonstrated that the previously described *dfg*-phenotype of a haploid *cdc55-100* yeast mutant strain RH2862 seems to be identical to that phenotype displayed by the *rps26AΔ* yeast mutant strain RH2588. These two

mutants exhibit strongly reduced adhesive growth, but still left some cells on the agar surface after the washing (Fig. 3.10B). Therefore, a yeast mutant strain carrying the *cdc55-100* allele (RH2862) seems to cause an inhibition of the *RPS26A* gene expression that results in a phenotype identical to that displayed by the *rps26AΔ* mutant strain RH2588.

In summary, the haploid adhesive growth tests seem to demonstrate that there are differences in the regulatory functions of the two *RPS26* gene products in yeast wildtype strains. Basal functions like e.g. cell division and growth can be fulfilled by a single *RPS26B* gene expression. In contrast, the *RPS26B* gene cannot complete some special functions like the regulation of haploid adhesive growth. We have identified a not yet described function of the *RPS26A* in *S. cerevisiae* ∑1278b strains that point towards interesting aspects of translational regulation mechanisms.

3.4.5 Overexpression of *RPS26B* restores haploid adhesive growth in yeast *rps26A* mutant strains

Our finding, that only one isogene, e.g. the *RPS26A* gene, is strictly required for haploid adhesive growth, gave rise to the question whether overexpression of *RPS26B* in *rps26AΔ* yeast mutant strains can restore the ability of adhesiveness.

To answer this question, a functional *RPS26B* gene was cloned into low-copy number plasmid pME2466 or high-copy number plasmids pME2467 and was expressed in yeast *rps26AΔ* mutant strains, respectively. As control, a 400bp PCR fragment of the *RPS26B* ORF was cloned behind the inducible yeast *MET25* promoter (p*MET25*) on high-copy number plasmid pME2468. All plasmids (see Fig. 3.2) were transformed into the haploid *rps26AΔ* mutant strain RH2588 (*rps26AΔ::Kan^R*) or RH2854 (*rps26AΔ::HIS3*). To test for haploid adhesive growth, a wash test assay was performed as described.

We found that all *rps26AΔ* mutant strains restored haploid adhesive growth when *RPS26B* is expressed in high copy number from plasmid pME2467 (p*RPS26B*, high-copy). Expression of *RPS26B* in high copy number also restored adhesiveness of strain RH2588 (*rps26AΔ::Kan^R*) (Fig. 3.11A). Accordingly, the histidine prototrophic *rps26AΔ* strain RH2854 (*rps26AΔ::HIS3*) restored haploid adhesive growth when transformed with plasmid pME2467 (data not shown). Even expression of *RPS26B* in low-copy number from plasmid pME2466 (p*RPS26B*, low-copy) could restore haploid

adhesive growth of strain RH2588 (Fig. 3.11A) or strain RH2854 (*rps26AΔ::HIS3*) under the same conditions (data not shown).
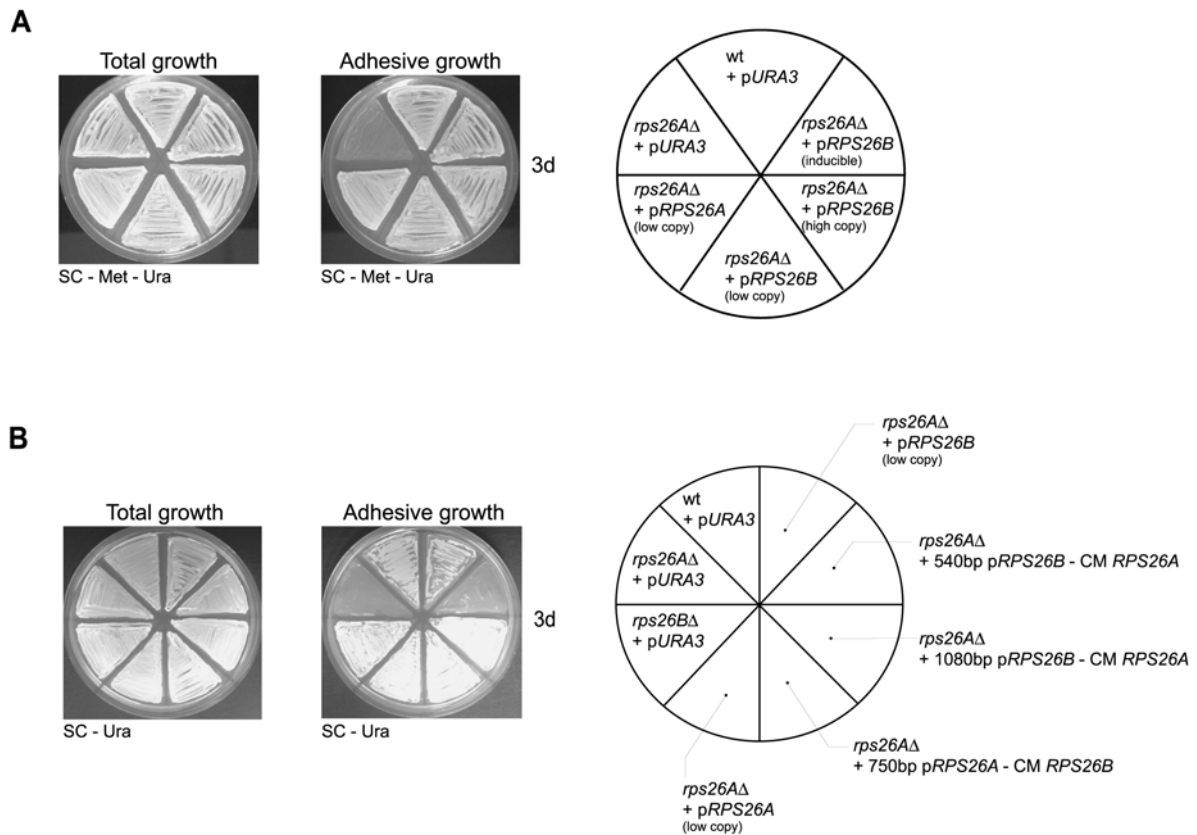

Fig. 3.11: A test for haploid adhesive growth of *rps26AΔ* or *rps26BΔ* yeast mutant strains is shown. [A] Strains to be tested were patched on SC-Met-Ura medium, incubated, photographed after 3 days (3d) of growth (Total growth), washed under a steam of water, and were photographed again to document remaining cells on the agar surface (Adhesive growth). Strains used were wildtype (wt) control RH2584, or *rps26AΔ* mutant strain RH2588 (*rps26AΔ::Kan^R^*). Yeast strains depicted were transformed with plasmid control pRS316 (+p*URA3*), *RPS26A* expressing plasmid pME2465 (+p*RPS26A*, low copy), *RPS26B* expressing plasmid pME2466 (+p*RPS26B*, low copy), *RPS26B* expressing plasmid pME2467 (+p*RPS26B*, high copy) and plasmid pME2469 expressing *RPS26B* in high copy number from the inducible *MET25* promoter (+p*RPS26B*, inducible). **[B]** Strains to be tested were patched on SC-Ura medium, incubated, photographed after 3 days (3d) of growth, washed under a steam of water, and were photographed again to document remaining cells on the agar surface. Strains used were wildtype (wt) control RH2584, *rps26AΔ* mutant strain RH2588 (*rps26AΔ::Kan^R^*), and *rps26BΔ* mutant strain RH2592 (*rps26BΔ::Kan^R^*). Depicted strains were transformed with plasmid control pRS316 (+p*URA3*), *RPS26A* expressing plasmid pME2465 (+p*RPS26A*, low copy), *RPS26B* expressing plasmid pME2467 (+p*RPS26B*, high copy). Additionally, *rps26AΔ* mutant strain RH2588 (*rps26AΔ::Kan^R^*) was transformed with plasmids carrying chimera promoter-ORF fusions. These plasmids were pME2462 expressing the Rps26B protein from behind a 750 bp *RPS26A* promoter fragment (+750bp p*RPS26A*-CM *RPS26B*), pME2463 expressing the Rps26A protein from behind a 1080 bp *RPS26B* promoter fragment (+1080bp p*RPS26B*-CM *RPS26A*), and pME2464 expressing the Rps26A protein from behind a 540 bp *RPS26B* promoter fragment (+540bp p*RPS26B*-CM *RPS26A*).

Next, we found that expression of *RPS26B* in high copy number from the inducible *MET25* promoter on plasmid pME2469 (p*RPS26B*, inducible) results in a hyper-adhesive phenotype of strain RH2588 (*rps26AΔ::Kan^R*) on SC-met-ura media (Fig. 3.11A). Under this tested conditions, the cells of strain RH2588 (*rps26AΔ::Kan^R*) strictly adhered to the agar surface even after a prolonged washing procedure (data not shown).

Our result that overexpression of Rps26Bp restores haploid adhesive growth in *rps26AΔ* mutant strains was contrary to the idea that the two variant amino acids of the Rps26Bp protein, e.g. Asp^{106} and Ala^{113}, are necessary for the differences in the regulation of adhesive growth. We tried to find further informations, by construction of chimera promoter-ORF fusions. In this chimera constructs, the *RPS26A* or the *RPS26B* promoter (p*RPS26A*, p*RPS26B*) and the main parts of the ORFs up to a *Sac*I restriction site are fused in frame to the downstream parts of the ORFs from the isogenic partner, respectively (see Fig. 3.3). With these constructs we should yield proteins that are under the control of the *RPS26B* promoter but encode for the Rps26Ap protein or are under the control of the *RPS26A* promoter but encode for Rps26Bp protein. Additionally, we cloned the shortened 540bp *RPS26B* promoter fragment to a chimera *RPS26A* ORF (Fig. 3.3).

When expressing the chimera constructs, we found that a chimera Rps26Bp when driven by a 750bp p*RPS26A*-promoter (pME2462) can suppress the *rps26AΔ* phenotype (750bp CM p*RPS26A-RPS26B*; Fig. 3.11B). We also found that low amounts of chimera Rps26Ap can suppress the *rps26AΔ* phenotype when expressed in low-copy number from behind the 1080bp p*RPS26B*-promoter on plasmid pME2462 (1080bp CM p*RPS26A-RPS26B*; Fig. 3.11B). Therefore the two variant amino acids of Rps26Bp seem to be not strictly required for the suppression of the non-adhesive phenotype, since additional amounts of Rps26Ap can bring back adhesiveness. In contrast, the Rps26Ap when expressed in low-copy number from a 540bp p*RPS26B*-promoter (540bp CM p*RPS26A-RPS26B*; Fig. 3.11B) cannot suppress the *rps26AΔ* phenotype. We concluded that this construct results in too low amounts of additional Rps26Ap to complement for the haploid adhesive growth phenotype.

In yeast wildtype strains a normal expression of *RPS26A* seems to result in such protein levels that the function of *RPS26B* is dispensable. Since most activities depend on *RPS26A*, the functions or importance of *RPS26B* can only be answered with the theory that *RPS26B* is an emergency system. Secondly, not the two different amino acids of the Rps26Ap or the Rps26Bp seem to be responsible for the different functions of both proteins.

3.4.6 Haploid *rps26A* yeast mutant strains do not exhibit an efficiently translation of *FLO11* mRNA

Regulation of *FLO11* expression is an important factor in the control of haploid adhesive and diploid pseudohyphal growth (Lambrechts *et al.*, 1996; Albrecht *et al.*, 1998). Since we found that haploid *rps26A*Δ yeast mutant strains exhibit a defect in the adhesive growth, we pointed the question whether we can find a functional *FLO11* expression within *rps26A*Δ yeast mutant strains.

To answer this question, we isolated *FLO11* mRNAs from haploid yeast wildtype strains RH2584 and RH2586 (*His*[+]), *rps26A*Δ mutant strains RH2588 (*rps26A*Δ*::Kan*[R]) and RH2590 (*rps26A*Δ*::HIS3*), and *rps26B*Δ mutant strains RH2592 (*rps26B*Δ*::Kan*[R]) and RH2594 (*rps26A*Δ*::HIS3*). Since all strains were carrying the *FLO11-lacZ* reporter construct (B3782, see Table 1), we split the identical cultures to determine the ß-galactosidase activities (Rose & Botstein, 1983). This analysis should result in both, the direct measurement of *FLO11* transcription and the indirect measurement of the translational of *FLO11* mRNAs.

While the *FLO11* mRNA is still present among *rps26A*Δ yeast mutant strains RH2588 (*rps26A*Δ*::Kan*[R]) and RH2590 (*rps26A*Δ*::HIS3*), the translational of the *FLO11-lacZ* reporter construct is completely abolished in these strains, when compared to wildtype and *rps26B*Δ yeast mutant strains RH2592 (*rps26B*Δ*::Kan*[R]) and RH2594 (*rps26A*Δ*::HIS3*), respectively (Fig. 3.12). Yeast wildtype strains RH2584 and RH2586 (Fig. 3.12, lanes 1+2) did not show a significant difference in the ß-gal-activity values of the *FLO11-lacZ* reporter constructs. Both strains yielded a specific activity of about 145 nmol/min/mg.

In contrast, *rps26B*Δ yeast mutant strains RH2592 (*rps26B*Δ*::Kan*[R]) and RH2594 (*rps26B*Δ*::Kan*[R]) (Fig. 3.12, lanes 5+6) exhibited 30% reduced ß-gal-values of about 90 nmol/min/mg.
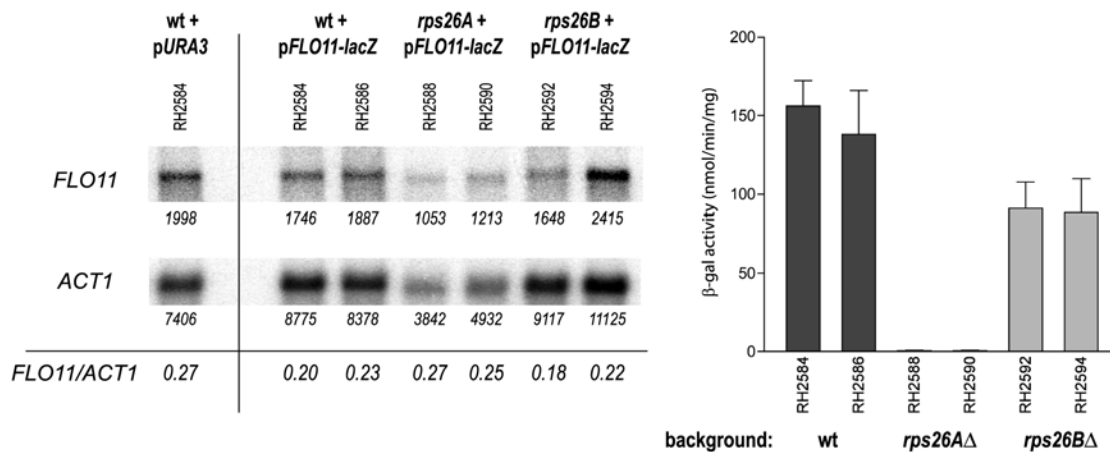
Fig. 3.12: Regulation of *FLO11* transcription and translation in *rps26AΔ* and *rps26BΔ* yeast mutant strains. Strains taken for Northern hybridisation experiments were grown for 6h in SC-ura medium, total RNAs were isolated and were separated by gel electrophoresis. Total RNA was prepared from haploid yeast strains RH2584 and RH2586 (wt), RH2588 (*rps26AΔ::Kan^R^*) and RH2590 (*rps26AΔ::Kan^R^*), and RH2592 (*rps26BΔ::Kan^R^*) and RH2594 (*rps26BAΔ::Kan^R^*), respectively, all carrying the *FLO11* reporter construct B3782 (+p*FLO11-lacZ*) or the plasmid control pRS316 (+p*URA3*). For measurements of *FLO11* and *ACT1* transcript levels, 10μg of total RNA was subjected to Northern hybridisation. For labelling of *FLO11* mRNA, a 593bp C-terminal fragment of *FLO11* (BHUM74) was used as probe. Signals were quantified using a Phosphor-Imager. Numbers depicted give average counts of three independent measurements, with standard deviations not exciting 15%. Below, the average quotients of the *FLO11/ACT1* levels of three measurements are given. On the right site the average of at least three independent measurements of the β-galactosidase activities of the six strains are depicted, with indicated standard deviations.

Therefore translational of the *FLO11* mRNAs is reduced in *rps26BΔ* yeast mutant strains when compared to wildtype strains.

We could not detect significant ß-gal-activities in the *rps26AΔ* yeast mutant strains RH2588 (*rps26AΔ::Kan^R^*) and RH2590 (*rps26AΔ::Kan^R^*) (Fig. 3.12, lanes 3+4). The average values measured were about 3 nmol/min/mg. These values were not significantly higher than the values measured for a yeast wildtype control that was transformed with an *URA3* vector control (data not shown).

In summary, *rps26AΔ* mutant strains seem to fail in an efficient translation of *FLO11* mRNAs, due to the missing protein Rps26Ap of the small ribosomal subunit. When compared to wildtype this analysis of *FLO11* translation demonstrated that also the Rps26Bp seems to be important for an efficient translation activity, since *rps26BΔ* mutant yeast strains exhibit a 30% reduced translation of *FLO11* mRNA.

3.4.7 The *RPS26A* gene but not the *RPS26B* gene is required for pseudohyphal growth in the yeast *S. cerevisiae*

Diploid yeast ∑1278b strains induce filamentous growth under nitrogen starvation conditions and form long elongated cells, the so-called pseudohyphae (reviewed in Mösch, 2000 and 2002). Pseudohyphal development is both, partially dependent and independent to *FLO11* transcription (Lo & Dranginis, 1998; Palecek *et al.*, 2000) and is also dependent on alterations of the cellular polarity (Taheri *et al.*, 2000).

Since we could not detect translation of *FLO11* mRNAs in haploid *rps26A∆* yeast mutant strains, we wanted to know whether diploid and homozygous *rps26A∆/rps26A∆* yeast mutant strains exhibit filamentous growth when starved for nitrogen on medium contain low concentrations of ammonia (SLAD-medium). We constructed two diploid homozygous *rps26A∆* mutant strains, RH2867 (*rps26A∆::Kan^R^ / rps26A∆::Kan^R^*) and RH2875 (*rps26A∆::HIS3 / rps26A∆::HIS3*), two homozygous *rps26B∆* mutant strains RH2868 (*rps26B∆::Kan^R^ / rps26B∆::Kan^R^*) and RH2876 (*rps26B∆::HIS3 / rps26B∆::HIS3*), as well as four diploid mutant strains that are heterozygous for the two *RPS26* genes, respectively (RH2869 to RH2873, see Table 1).

These diploid strains were grown on SLAD-medium and were incubated for 3 - 5 days at 30°C. In a quantitative filamentous growth assay micro-colonies that had invaded the agar were scored under the microscope (see material & methods).

We found that the diploid homozygous *rps26B∆* mutant strains RH2868 (*rps26B∆::Kan^R^/rps26B∆::Kan^R^*) and RH2876 (*rps26B∆::HIS3/rps26B∆::HIS3*) exhibited almost the same amounts of pseudohyphae as yeast wildtype strains RH2866 and RH2874 (*His^+^*) carrying two intact copies of the *RPS26* genes (Fig. 3.13A). The average number of cells that displayed pseudohyphal development when starved for nitrogen on SLAD+Ura media was only between 30% and 70% under the tested conditions.

The diploid homozygous *rps26A∆* mutant strains RH2867 (*rps26A∆::Kan^R^/rps26A∆::Kan^R^*) and RH2875 (*rps26A∆::HIS3/rps26A∆::HIS3*) displayed strongly reduced amounts of pseudohyphae when starved for nitrogen on SLAD+Ura media. Only a few cells (<5%) of both strains displayed short pseudohyphae and invaded into the agar. Most cells did not show any substrate invasion (Fig. 3.13A).
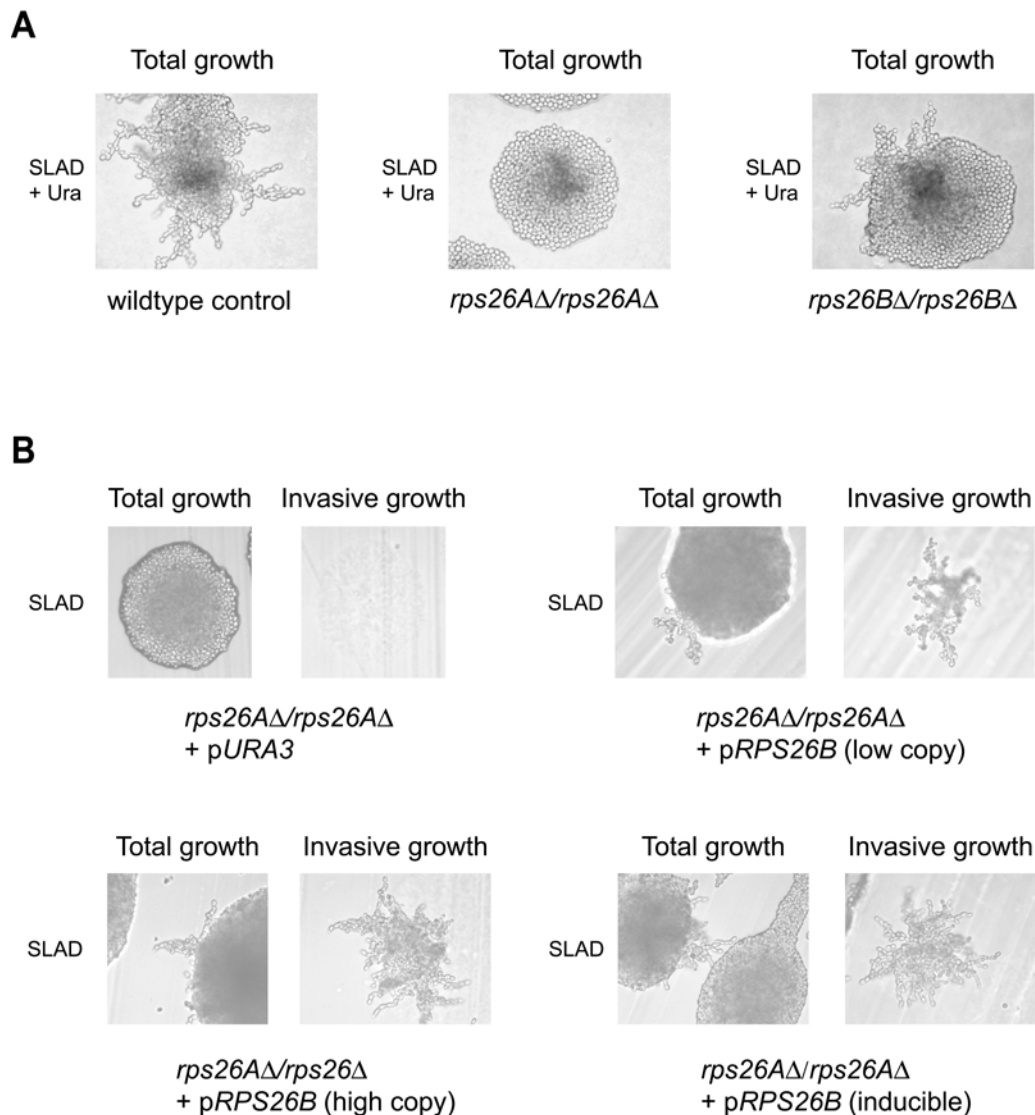
A

Total growth	Total growth	Total growth
SLAD + Ura	SLAD + Ura	SLAD + Ura
wildtype control	*rps26AΔ/rps26AΔ*	*rps26BΔ/rps26BΔ*

B

Total growth	Invasive growth		Total growth	Invasive growth
SLAD			SLAD	
rps26AΔ/rps26AΔ + p*URA3*			*rps26AΔ/rps26AΔ* + p*RPS26B* (low copy)	

Total growth	Invasive growth		Total growth	Invasive growth
SLAD			SLAD	
rps26AΔ/rps26Δ + p*RPS26B* (high copy)			*rps26AΔ/rps26AΔ* + p*RPS26B* (inducible)	

Fig. 3.13: Pseudohyphal development of diploid homozygous yeast mutant strains and a wash test assay is shown. [A] Diploid homozygous strains were streaked out on SLAD+Ura medium, were incubated for 5 days at 30 ^{0}C, and were photographed under the microscope. Diploid homozygous strains used were RH2866 (wildtype control), *rps26AΔ* mutant RH2867 (*rps26AΔ::Kan^R/rps26AΔ::Kan^R*) and *rps26BΔ* mutant RH2868 (*rps26BΔ::Kan^R/rps26BΔ::Kan^R*), respectively. **[B]** Strains used for a wash test assay were grown on SLAD medium, were incubated for 5 days at 30 ^{0}C, and were photographed under the microscope (Total growth). Colonies were washed off the agar surface and were photographed again (Invasive growth). The strain depicted was the diploid homozygous *rps26AΔ* yeast mutant strain RH2875 (*rps26AΔ::HIS3/rps26AΔ::HIS3*) that was transformed with various plasmids referred to in the text. Plasmids used were vector control pME316 (+p*URA3*), *RPS26B* expressing plasmid pME2466 (+p*RPS26B*, low copy), *RPS26B* expressing plasmid pME2467 (+p*RPS26B*, high copy) or plasmid pME2469 expressing *RPS26B* in high copy number from the inducible *MET25* promoter (+p*RPS26B*, inducible).

We also tested whether the heterozygous yeast mutant strains RH2872 (*rps26AΔ::Kan^R/rps26BΔ::HIS3*) or RH2873 (*rps26AΔ:HIS3/rps26BΔ::Kan^R*), display a pseudohyphal development. Both strains are carrying only one intact copy of the two *RPS26* genes, and exhibited a significant reduced amount of pseudohyphae. Only 10% to 30% of all cells invaded into the agar (data not shown).

The diploid, heterozygous yeast strains RH2870 (*rps26AΔ::Kan^R/RPS26A*) and RH2871 (*rps26BΔ::Kan^R/RPS26B*) carried only one copy of the *RPS26A* or the *RPS26B* gene, but two intact copies of the respective isogene. With respect to the pseudohyphal development, these strains could not be distinguished from diploid yeast wildtype strains homozygous for both, *RPS26A* and *RPS26B*, respectively (data not shown).

We tested next, whether we could restore pseudohyphal development of diploid *rps26AΔ* mutant strains by expression of different amounts of Rps26p. The diploid homozygous *rps26AΔ* yeast mutant strain RH2875 (*rps26AΔ::HIS3/rps26AΔ::HIS3*) was transformed with the *RPS26B* carrying plasmid pME2466 (p*RPS26B*, low copy), pME2467 (p*RPS26B*, high copy), or pME2469 (p*RPS26B*, inducible). These plasmids express the *RPS26B* in various copy numbers or in high copy number from the inducible *MET25* promoter. Therefore, the expression *RPS26B* rate is variable over a wide range.

Expression of these plasmids in diploid homozygous *rps26AΔ/rps26AΔ* mutant strains could restore diploid filamentous growth under nitrogen starvation conditions (Fig. 3.12B). After 5 days of growth on SLAD media the amount of cells that invaded the agar raised from below 5% in strain RH2875 (*rps26AΔ::HIS3/rps26AΔ::HIS3*) transformed with the vector control, to over 30% in strain RH2875 when transformed with plasmid pME2467 (p*RPS26B*, high copy). Accordingly, up to 50% of all cells displayed pseudohyphae in strain RH2875 when expressing the *RPS26B* gene in high copy number from the inducible *MET25* promoter on plasmid pME2469 (p*RPS26B*, inducible).

In summary, the *RPS26A* gene product on chromosome VII seems to be strictly required for haploid adhesive and diploid filamentous growth in the yeast *S. cerevisiae*, while the *RPS26B* gene product on chromosome V seems to play a other role under the tested nutritional conditions. Using the pseudohyphal development as a representative genetic function, we could demonstrated that the expression level of the two Rps26 proteins seems to be important in the regulation of

different cellular functions of *rps26A* or *rps26B* mutant strains. Testing for pseudohyphal development also demonstrated that there seems to be no clear differences in the protein functionality of the Rps26 proteins of the small ribosomal subunit, since overexpression of Rps26Bp restored the deduced Rps26Ap functions.

3.5   Discussion

Although the yeast genome seems to be strictly condensed when compared to other eukaryotic genera, many isogenes can be found among the entire genome. The yeast *S. cerevisiae* kept the presence of two copies of apparently redundant genetic information over a long evolutionary time. This 'backup' system seems to have general advantages for yeast strains to survive all genetic and environmental influences on the complex genome. Gene products encoded by isogenes function in the regulation of many different processes, namely the aromatic amino acid biosynthesis pathway by e.g. *ARO3* and *ARO4* (see chapter IV), the regulation of the cAMP/MAP kinase pathway by e.g. *RAS1* and *RAS2* (Mösch *et al.*, 1999), and the formation of ribosomes (Mager *et al.*, 1997; Planta & Mager, 1998; Verschoor *et al.*, 1998).

First it was surprising that the deletion of one of the isogenes, *RPS26A*, results in a drastically reduced growth rate, whereas deletion of *RPS26B* seems to have no influence on growth. When compared to the transcriptional regulations of other yeast isogenes, it seemed to be logical that some answers for differences in growth should be given by differences in the expression rate and the promoter strength of the two isogenes partners. In part, this theory was corroborated by the analyses of mRNA transcript levels in Northern hybridisation experiments. The *RPS26A* gene alone delivers about 70% of the entire transcript level, while expression of *RPS26B* alone can only supply a maximum of 40%. With this 40% *RPS26B* encoded transcript level, the cell is able to secure one main important function of the *RPS26* genes, the functionality of the entire ribosome. On the other hand, the 40% *RPS26B* transcript level and the resulting Rps26Bp level seems to be unable to completely overtake all *RPS26A* gene functions. In sequence to this, a reduced growth rate of *rps26AΔ* mutant strains is not very surprising, since one important ribosomal protein is missing within the translation machinery.

Secondly, we have to expect additional effects for an *rps26AΔ* yeast mutant strain. Indeed, we could demonstrate that the gene products of *RPS26A* and *RPS26B* are essential for *S. cerevisiae*, since an *rps26A rps26B* double mutation is synthetically lethal for yeast. Therefore, the function of at least Rps26Ap within the yeast ribosomes cannot be insignificant.

To yield more information about the strength of the *RPS26A* and the *RPS26B* promoter we started an analysis of the two promoters using various promoter-*lacZ* fusion constructs. The results of these β-galactosidase activity tests demonstrated that the sum of transcription and translational of *RPS26A* does not significantly rise (1.2-fold) within the *rps26BΔ* mutant strains, whereas the translational activity of *RPS26B* seems to be slightly induced within *rps26AΔ* mutant strains when compared to wildtype. This could be evidence that yeast ribosomes without Rps26Ap are not exactly functioning. Whether this results in a general translational defect has to be proven for the other gene transcripts in further experiments. Using the promoter-*lacZ* fusion constructs we could also demonstrate that expression of the *RPS26A* gene seems to be drastically induced in *rps26AΔ* mutant strains when compared to wildtype. The cell somehow is sensing the missing ribosomal subunit and tries to upscale both the transcription and translation of *RPS26A*. Whether this is an index for an auto regulation mechanism has to be proven. Such auto-regulation mechanism has been described for expression of ribosomal Rps14Bp of *S. cerevisiae*. Rps14Ap represses the translation of Rps14B by binding to *RPS14B* pre-mRNA (Fewell & Woolford, 1999).

Beside some few exceptions like e.g. *FLO11* (Rupp *et al.*, 1999), yeast promoters seem to be very short (Mewes *et al.*, 1997). It was a little astonishing that the *RPS26A* gene has the main upstream activation site located between position – 500 and –750 relatively to the ATG transcriptional start. Within the *RPS26B* promoter we could also identify at least two far upstream activation sites. One site is located between positions –500 and –1000, while the second site seems to be located between –1000 and –1400 relatively to the ATG transcriptional start. At this point we don't have evidences why the expression of the two *RPS26* genes need such long promoters. One possible explanation seems to be given by the fact that the expression of ribosomal proteins exhibits a biphasic response to nutritional changes (Griffioen *et al.*, 1994; Griffioen *et al.*, 1996). Therefore, longer promoter areas seem to be capable for binding of many different transcription factors.

Searching for further differences among the cellular functions, we could demonstrate that expression levels of *RPS26A* are important not only for growth but also for differentiation processes. *RPS26A* seems to be strictly required for both, haploid adhesive and diploid pseudohyphal development. Therefore, deletion of *RPS26A* results in the lack of haploid adhesiveness and the lack of diploid

pseudohyphal growth, while the basal translational machinery still works. At this point it seemed to turn out that there are both, identical functions and different functions between the two *RPS26* gene products.

Haploid adhesive and diploid pseudohyphal growth partially depends on the expression of *FLO11* mRNA (Lo & Dranginis, 1998; Rupp *et al.*, 1999; Palecek *et al.*, 2000). The main important result of this thesis is given by the finding that one of the specific functions of Rps26Ap is the coordinated translation of *FLO11* mRNA. *rps26AΔ* mutant strains completely fail in translation of *FLO11*-lacZ reporter constructs, whereas *rps26BΔ* mutant strains exhibit a reduced translation, when compared to wildtype strains. Expression of the *RPS26B* gene within the *rps26AΔ* yeast mutant strains seems to result in such a Rps26Bp protein level that is unable to keep a functional translation of the *FLO11* mRNA. We do not know yet, why the presence of Rps26Bp protein alone does result in a general functionally translation machinery but not in a functional translation of *FLO11* mRNAs. It is speculative, whether the yeast ribosomes without Rps26Ap cannot correctly interact with the *FLO11* mRNA, or whether the Rps26Ap is required as an activator or interacting partner of other gene products like e.g. Rps26Bp. Our results seem to implicate a function of both, the Rps26Ap and the Rps26Bp on translational regulation of *FLO11* mRNA, since *rps26BΔ* yeast mutant strains also exhibit a reduced *FLO11* mRNA translation. This seems to be further evidence for the finding that there are specific differences between the Rps26Ap and the Rps26B protein, respectively. It also seems to demonstrate that Rps26Bp could be a regulatory protein that enhances translation efficiency but cannot complement all of the Rps26Ap function under specific conditions. Since a measurement of *FLO11-lacZ* activity is only an indirect demonstration of Rps26Ap function, transcription of *FLO11-lacZ* or measurements of Flo11p protein levels have to be tested in further experiments.

Furthermore, it seems to be possible that the cellular Rps26p protein level, has to reach a not yet identified level to secure all genetic functions with the yeast cells   (Fig. 3.14). Using the *FLO11* expression as one specific translational target, we could demonstrate that *rps26AΔ* yeast mutant strains fail in some of these functions.
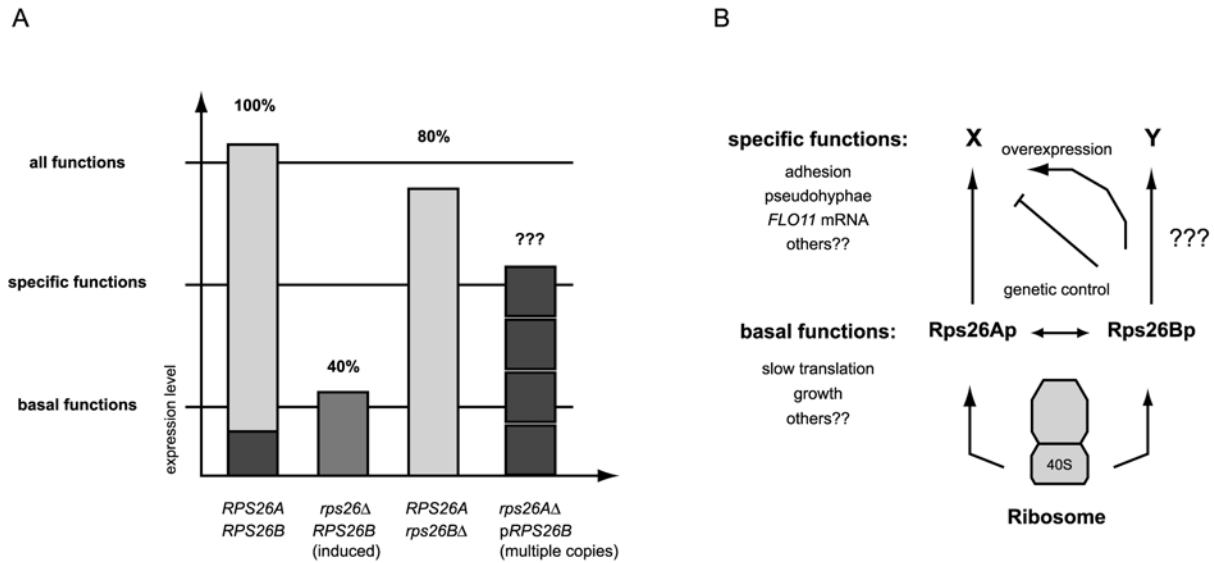
Fig. 3.14: Two models of expression levels and translational regulation mechanisms of *rps26AΔ* and *rps26BΔ* yeast mutant strains are given. [A] A model of the *RPS26* expression level is given. Squares in light grey represent *RPS26A* other squares represent *RPS26B* expression levels. **[B]** A putative model of the different Rps26 protein functions is given.

All these findings became complicated by the results that Rps26Bp when overexpressed in *rps26AΔ* yeast mutant strains, could complement most of the specific *RPS26A* functions within the differential processes. With overexpressed *RPS26B*, *rps26AΔ* mutant strains restored adhesive growth and seem to restore the pseudohyphal development. But the own yeast derived transcriptional and translational regulation mechanisms are not able to complement the *rps26AΔ* gene defect. Therefore, the *RPS26B* is clearly not a simple backup of the *RPS26A* gene but could be a regulator of translation with not yet identified other functions. When the *RPS26A* gene is malfunctioning, single yeast cells can complement some of the functions of this necessary gene (e.g. the basal translation) by enhancing the expression of *RPS26B*. But the cells fail in the specific functions of Rps26Ap within the differentiation processes like e.g. the translation of *FLO11* mRNA. In contrast to this finding, we could not yet identify specific functions that are exclusively directed by the Rps26Bp.

Exposure of *rps26AΔ* and *rps26BΔ* yeast mutant strains to media containing paromomycin revealed that both mutants are selectively inhibited by the enhanced translation error rate at the ribosome caused by the antibiotic drug (Carter *et al.*, 2000). In case of the *rps26AΔ* mutant strains it seems to be not so clear whether an exposure to paromomycin further enhanced the already malfunctioning translation

machinery or whether this is a specific effect. More interesting was the fact, that paromomycin targets the expression of Rps26Bp in an *rps26B∆* mutant strain that expresses extra chromosomal copies of a functional *RPS26B* gene fragment. If it is assumed that the *RPS26B* promoter is very long, the use of a 1080 bp fragment within this specific *RPS26B* complementation construct may be to short for binding of other regulatory elements. On the other hand addition of paromomycin to growth medium seems to be a good possibility to screen for suppressors of *rps26A* mutant phenotypes.

At this state of the work, some main important information about *RPS26A* and *RPS26B* is still missing, the exact measurements of Rps26Ap and Rps26Bp protein levels within all the processes described above. Tagging of both proteins was started within this work (data not shown) but measurements of protein levels could not be finished yet. With the results described above we could demonstrate that there are interesting differences between the two *RPS26* isogenes. In summary the *RPS26A* function of *S. cerevisiae* seems to be important for all cellular processes, whereas the *RPS26B* function is not important. Therefore it will be intriguing to find out why the yeast *S. cerevisiae* kept this two isogene over a long evolutionary time.

3.6 Acknowledgements

3.7 References

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Evolution of DAHP synthase encoding genes in the yeast *Saccharomyces cerevisiae*

4.1   Abstract

Differently regulated isoenzymes characterise various specific biochemical control points in living cells. The initial step of the shikimate pathway resulting in three aromatic amino acids is catalysed in different organisms by two and three isoenzymes, respectively. Aro3p and Aro4p are the only yeast enzymes and are feedback-inhibitable by phenylalanine (Aro3p) and tyrosine (Aro4p). A yeast strain deficient in the general control system of amino acid biosynthesis is unable to live in the presence of high amounts of phenylalanine and tyrosine. Here, we show that this yeast strain can be rescued by the expression of the tryptophan-regulated a*roH* from *Escherichia coli* (Ec AroHp) as third isoenzyme. Yeast, carrying Ec AroHp as the only enzyme for the initial step of the shikimate pathway can grow in the absence of tryptophan. If phenylalanine and tyrosine are missing, this yeast strain survives only when the yeast *ARO3* instead of the *ARO4* promoter drives *E. coli aroH*. The analyses of various Ec AroHp mutant enzymes in yeast demonstrated that a single Ala177Lys amino acid substitution resulted in an Ec AroHp enzyme, which is still regulated by tryptophan but in addition is effectively inhibitable by tyrosine. The careful analysis of yeast strains carrying either *ARO3* or *ARO4* genes revealed a similar dual control for both enzymes. This knowledge was used for engineering of Aro4p Pro165Gly as an enzyme, which is under the dual and efficient control of tyrosine and tryptophan. Our data suggest that yeast possess only two instead of three isogenes because both isoenzymes can be fine-tuned by Tryptophan as additional effector. Transcriptional regulation by the general control system is induced as backup when aromatic aminos in the cultivation medium are imbalanced.

4.2   Introduction

The first step of the shikimate pathway, the stereo specific condensation of erythrose-4-phosphate and phosphoenolpyruvate (Herrmann & Poling, 1975), is catalysed by the 3-<u>d</u>eoxy-D-<u>a</u>rabino-<u>h</u>eptulosonate-7-<u>p</u>hosphate synthase (DAHP synthase, DAHPS, EC 4.1.2.15). By subsequent enzyme reactions, DAHP is converted to chorismate (Herrmann, 1995; Knaggs, 1999; Knaggs, 2000; Knaggs, 2001), which is a precursor for the aromatic amino acids phenylalanine (phe), tyrosine (tyr), and tryptophan (trp). Various fungi including the yeast *S. cerevisiae* and the filamentous fungus *Aspergillus nidulans* express two differently regulated DAHP synthases. In *E. coli*, the three isogenes *aroF* (Shultz *et al.*, 1984), *aroG* (Davies & Davidson, 1982) and *aroH* (Zurawski *et al.*, 1981) code for three DAHPS isoenzymes, each feedback-regulated by one of the aromatic amino acids phenylalanine (AroF), tyrosine (AroG), and tryptophan (AroH), respectively (Byng & Jensen, 1983). The filamentous fungus *Neurospora crassa* has been described to possess three DAHPS isoforms, each negatively regulated by one of the three aromatic amino acids (Halsall & Catcheside, 1971; Hoffmann *et al.*, 1972; Nimmo & Coggins, 1981a; Nimmo & Coggins, 1981b; Walker *et al.*, 1996). However, actual genomic data reveals only two conserved isogenes present among the to date completely sequenced *Neurospora crassa* genome (Whitehead Institute, Center for Genome Research; www-genome.wi.mit.edu / annotation / fungi / neurospora/).

The two *S. cerevisiae* DAHP synthases are encoded by *ARO3* (Teshiba *et al.*, 1986) and *ARO4* (Künzler *et al.*, 1992). While the *ARO3* encoded DAHP synthase is inhibitable by phenylalanine, the *ARO4* gene product is inhibitable by tyrosine (Paravicini *et al.*, 1989a; Paravicini *et al.*, 1989b; Schnappauf *et al.*, 1998). In *A. nidulans aroF* and *aroG* code for two DAHP synthases that are differentially regulated by tyrosine (aroFp) and phenylalanine (aroGp) (Hartmann *et al.*, 2001).

Total cellular DAHPS activities are unequally distributed to the three isoenzymes. In *E. coli*, the phe-inhibitable AroG (79%) and the tyr-inhibitable AroF (20%) deliver nearly all of the DAHP synthase activity for the cell. The trp-inhibitable AroH contributes only 1% of the cellular enzyme activity (Tribe *et al.*, 1976). Inhibition of AroG (phe-inhibitable) and AroF (tyr-inhibitable) is highly effective and abolishes more than 95% of their specific enzyme activity (Schoner & Herrmann, 1976;

McCandliss *et al.*, 1978), whereas the trp-inhibition of AroH is only about 60%, thereby resulting in a 40% constitutive activity (Pittard *et al.*, 1969).

There is no counterpart for the trp-inhibitable AroH of *E. coli* in *Saccharomyces* or *Aspergillus* (Mewes *et al.*, 1997). In addition to the feedback control of Aro3p and Aro4p, the transcription of the corresponding genes is part of a complex regulatory network, which couples transcriptional derepression of more than 500 genes to the availability of amino acids (Natarajan *et al.*, 2001). This regulatory network is conserved between several fungi and known as '*cross-pathway control*' in molds and as '*general control*' of amino acid biosynthesis in yeast (Hinnebusch, 1997; Hoffmann *et al.*, 2001; Hinnebusch & Natarajan, 2002). A key factor is the transcriptional activator Gcn4p of yeast and its counterparts including e.g. CPCA of *A. nidulans* (Hoffmann *et al.*, 2001). Gcn4p binds to the promoter region of target genes to a well-characterised upstream activation site. These upstream sites are present in both *ARO3* and *ARO4* of yeast as well as in the corresponding genes of *A. nidulans*. During amino acid deprivation, *ARO3* and *ARO4* transcription is increased by a factor of two and four, respectively (Paravicini *et al.*, 1989a; Künzler *et al.*, 1992).

Alignment studies (Centre National de la Recherche Scientifique, IBCP, Lyon, France; www.ibcp.fr) of amino acid sequences of DAHP synthases from *E. coli* and *S. cerevisiae* show almost the same sequence identity between tryptophan-regulated AroH and phenylalanine-regulated Aro3p (63%) as between AroH and tyrosine-regulated Aro4p (64%). To date, the structures of the *aroG* gene product (phenylalanine-inhibitable, phe-inhibitable) from *E. coli* (Shumilin *et al.*, 1996; Shumilin *et al.*, 1999) and the *ARO4* gene product (tyrosine-inhibitable, tyr-inhibitable) from *S. cerevisiae* (Schneider *et al.*, 1999; Hartmann *et al.*, 2003) are resolved. These data suggest that the overall structures of AroH and Aro4p are highly conserved.

Here, we found that both yeast DAHP isoenzymes can be fine-tuned by tryptophan as a second effector. A single Pro164Gly substitution resulted in an Aro4p mutant enzyme, which is equally well controlled by tyrosine as well as by tryptophan.

4.3 Experimental procedures

4.3.1 Media, Materials and Supplements

All chemicals were supplied by Fluka / Sigma-Aldrich Chemie GmbH (Seelze, Germany). Minimal vitamins (MV) and synthetic complete (SC) medium for the cultivation of yeast has been described earlier (Miozzari *et al.*, 1978). Transformation of *S. cerevisiae* was performed by the LiOAc method as described (Ito *et al.*, 1983).

4.3.2 Strains and Plasmids

For overexpression, purification and as recipient for the allelic Ec *aroH* mutations, yeast strain RH2424 (*MATa, can1-100, GAL⁺, aro3Δ::HIS3, aro4Δ::LEU2, ura3-1, his3⁻, leu2⁻*) was used. Strain RH2424 (*aro3Δ::HIS3, aro4Δ::LEU2*) was transformed with a 3.1 kb *Bam*HI / *Xho*I *GCN4Δ* knockout cassette originating from plasmid pME2389. Transformants were selected on SC media supplemented with 150 µg / ml geneticin. Correct transformation was verified by Southern hybridisation analysis (data not shown) and resulted in strain RH2804 (*gcn4Δ::Kan^R, aro3Δ::HIS3, aro4Δ::LEU2*). For general control regulation experiments, strains RH1408 (*MATa, ura3-52, gcn4-103*) and RH2804 were used. All strains used for the genetic analyses in this study are listed in Table 1. Plasmids used in this work are shown in Table 2.

Table 1 – Yeast strains used in this work

Strain	Description	Reference
RH730	*Mat α* wild-type yeast strain	(Kohlhaw *et al.*, 1980)
RH1313	*MAT α aro4-1*	Paravicini, 1989
RH1316	*MATa aro3-2*	Paravicini, 1989 ATCC 204131
RH1408	*MATa ura3-52, gcn4-103*	(Hinnebusch, 1985)
RH1416	*MATa ura3-52*	(Berben *et al.*, 1988)

Table 1 - continued

Strain	Description	Reference
RH2424	*MAT a, can1-100, GAL$^+$, aro3Δ::HIS3, aro4Δ::LEU2, ura3-1, his3$^-$, leu*	M. Hartmann pers. comm.
RH2487	*MAT a, can1-100, GAL$^+$, aro3Δ::aroH, aro4Δ::LEU2, his3, ura3-1, leu2$^-$*	this work
RH2803	*MAT a, can1-100, GAL$^+$, aro3Δ::HIS3, aro4Δ::aroH, leu2, ura3-1 his3$^-$*	this work
RH2804	*MAT a, can1-100, GAL$^+$, gcn4Δ::Kan^R, aro3Δ::HIS3, aro4Δ::LEU2, ura3-1, his3$^-$, leu2$^-$*	this work
RH2805	*MAT a, can1-100, GAL$^+$, gcn4Δ::Kan^R, aro3Δ::HIS3, aro4Δ:aroH, ura3-1, his3$^-$, leu2$^-$*	this work

Table 2 – Plasmids used in this work

Plasmid	Description	Reference
pBluescript®KS	2.96 kb vektor, *Amp^R*, (*bla*), *lacZ*, *ori*	Stratagene (La Jolla, USA)
pRS416	*URA3*, *CEN*, *Amp^R* (*bla*), *lacZ*, *ori*	(Sikorski & Hieter, 1989)
pRS426	*URA3*, *2µm*, *Amp^R* (*bla*), *lacZ*, *ori*	(Sikorski & Hieter, 1989)
pRS416MET25	pRS416, *URA3*, *CEN*, *Amp^R* carrying yeast *MET25* promoter and *CYC1* terminator *Xho*I / *Kpn*I fragment	(Mumberg *et al.*, 1994)
pRS426MET25	pRS426, *URA3*, *2µm*, *Amp^R* carrying yeast *MET25* promoter and *CYC1* terminator *Xho*I /*Kpn*I fragment	(Mumberg *et al.*, 1994)
pCHA3	*E. coli aroH*-ORF under the control of the *tac*-promotor in the phagemid-vector pttS9	(Akowski & Bauerle, 1997)
pME1083	2.8 kb genomic *S. cerevisiae GCN4 Sal*I / *Eco*RI-fragment in pYCP50	(Hinnebusch, 1985)
pME1200	pJDB207, carrying an 2.0 kb *Acc*I / *Eco*RV *ARO4* fragment as *Xba*I / *Bam*HI fragment	(Künzler *et al.*, 1992)
pME1513	pRS426MET25, *URA3*, *2µm*, *Amp^R* with altered MCS (*Sac*I-p*MET25*-*Xba*I *Spe*I *Bam*HI *Sal*I *Sfi*I *Not*I *Xho*I-t*CYC1*-*Kpn*I)	(Krappmann *et al.*, 1999)
pME1870	1.1 kb *aroH* from pCHA3 cloned *Bam*HI / *Eco*RI to 300bp yeast *ARO3* promoter and 350bp yeast *ARO3* terminator in pBluescript®KS	this work
pME1873	1.7 kb *Hin*dIII / *Not*I *aroH*-Fragment from pME1870 cloned to pRS416 (*ARO3* prom. / term.)	this work
pME1874	1.7 kb *Hin*dIII / *Not*I *aroH*-Fragment from pME1870 cloned to pRS426 (*ARO3* prom. / term.)	this work
pME1877	1.1 kb *Bam*HI / *Eco*RI *aroH*-Fragment under the control of the yeast *MET25* promoter in pRS416*MET25*	this work

Table 2 - continued

Plasmid	Description	Reference
pME1878	1.1 kb *Bam*HI / *Eco*RI *aroH*-Fragment under the control of the yeast *MET25* promoter in pRS426MET25	this work
pME1879	like pME1878, carrying a single amino acid substitution in the *aroH* gene; exchange in codon 149: GGC_{Gly} -CCC_{Pro}	this work
pME1880	like pME1878, carrying a single amino acid substitution in the *aroH* gene; exchange in codon 179: TCC_{Ser} -GCC_{Ala}	this work
pME1881	like pME1878, carrying a single amino acid substitution in the *aroH* gene; exchange in codon 218: ACC_{Thr} - GCC_{Ala}	this work
pME1882	like pME1878, but carrying two amino acid substitution in the *aroH* gene; exchanges in codons 179: TCC_{Ser} -GCC_{Ala} and 218: ACC_{Thr} -GCC_{Ala}	this work
pME1931	pME1513 carrying an 1.1 kb *Bam*HI / *Xho*I *ARO4* allele under the control of the *MET25* promoter, single amino acid substitution in codon 193: GGT_{Gly} -AAG_{Lys}	this work
pME2384	pME1513 carrying a 1.1 kb *Bam*HI / *Xho*I *ARO3* wild-type ORF under the control of the *MET25* promoter	this work
pME2385	pME1513 carrying a 1.1 kb *Bam*HI / *Xho*I *ARO4* wild-type ORF under the control of the *MET25* promoter	this work
pME2389	pBluescript®KS carrying a 3.1 kb *Bam*HI / *Xho*I p*ARO4* (1.2kb) - *aroH* (1.1kb*)* - t*ARO4* (0.8kb) fusion	this work
pME2390	3.1 kb *Bam*HI / *Xho*I p*ARO4* (1.2kb) - *aroH* (1.1kb*)* - t*ARO4* (0.8kb) fusion in pRS416	this work
pME2391	3.1 kb *Bam*HI / *Xho*I p*ARO4* (1.2kb) - *aroH* (1.1kb*)* - t*ARO4* (0.8kb) fusion in pRS426	this work
pME2395	pME1513 carrying an 1.1 kb *Bam*HI / *Xho*I *ARO4* allele under the control of the *MET25* promoter. Single amino acid substitution in codon 165: CCT_{Pro} -GGT_{Gly}	this work
pME2397	pME1513 carrying an 1.1 kb *Bam*HI / *Xho*I *ARO4* allele under the control of the *MET25* promoter. Single amino acid substitution in codon 234: GCT_{Ala} -ACT_{Thr}	this work
pME2400	pRS426*MET25* carrying an 1.1 kb *Bam*HI / *Eco*RI *aroH* allele under the control of the *MET25* promoter. Single amino acid substitution in codon 177: GCA_{Ala} - AAA_{Lys}	this work
pME2402	pRS426*MET25* carrying an 1.1 kb *Bam*HI / *Eco*RI *aroH* allele under the control of the *MET25* promoter. Single amino acid substitution in codon 180: TGT_{Cys} - TTT_{Phe}	this work
pME2404	pRS426*MET25* carrying an 1.1 kb *Bam*HI / *Eco*RI *aroH* allele under the control of the *MET25* promoter. Single amino acid substitution in codon 203: CGC_{Arg} - GCC_{Ala}	this work

4.3.3 Mutations of AroHp

Site-directed mutations of *AroH* and *ARO4* were introduced using polymerase chain reactions (PCR) with specific oligo-nucleotides carrying single nucleotide exchanges (Leung *et. al.,* 1989) and supplied from Invitrogen (Karlsruhe, Germany). All mutated alleles were cloned into pRS416*MET25* and pRS426*MET25*, respectively (Mumberg *et al.*, 1994). The DNA sequence of all plasmids described in this work was determined by sequencing using an ABI310 capillary sequencer (Applied Biosystems Europe, Darmstadt, Germany) and the method described by B.B. Rosenblum (Rosenblum *et al.*, 1997). After transformation of the allelic plasmids into yeast strains RH2424 and RH2804, transformants were selected on SC medium lacking aromatic amino acids to screen for functionally intact DAHP synthases.

4.3.4 Enzyme assays

The DAHP synthase activity was determined by the stop assay described by Takahashi and Chan (Takahashi & Chan, 1971), with the modifications described by Schnappauf, 1998 (Schnappauf *et al.*, 1998). The pH value of the enzymatic reaction was adjusted to pH 6.8 instead of pH 6.5 and sodium periodate was dissolved in 0.25 M instead of 0.125 M H_2SO_4. Both PEP and E4P concentrations were kept at 0.5 mM, respectively. After pre-testing of different substrate concentrations, assays were performed with appropriate substrate concentrations, where the absorbance of the product was proportional to the amount of enzyme added. Measurements of enzyme activities were performed at 550 nm using an Ultrospec 4000 photometer (Pharmacia Biotech, Cambridge, GB). Enzymatic activities were determined at least three times with two parallel measurements and the resulting average values were displayed. Specific enzyme activities are quantified in Units per mg (U/mg) equaling 1 nmole product formed in 1 min turnover by 1 mg of total protein. Protein contents of crude extracts were measured twice by the method of Herbert (1971) using the Bradford assay (Bradford, 1976).

4.4   Results

4.4.1   A tryptophan-inhibitable DAHP synthase regenerates growth of a *gcn4-103* mutant yeast strain in the presence of the two aromatic amino acids, phenylalanine and tyrosine

Numerous fungi including *Saccharomyces cerevisiae, Aspergillus nidulans,* and *Neurospora crassa* possess a transcriptional control system ('*general control*' of amino acid biosynthesis, '*cross-pathway control*'), which regulates amino acid biosynthetic genes including those for the initial step of aromatic amino acid biosynthesis by the transcriptional activators Gcn4p, CPCA, and CPC-1, respectively (Paluh & Yanofsky, 1991; Hinnebusch, 1997; Wanke *et al.*, 1997). The yeast mutant strain RH1408 (*gcn4-104*) is unable to activate the general control of amino acid biosynthesis due to the lack of the transcriptional activator Gcn4p. We wanted to know, how a *gcn4* yeast mutant strain carrying the wild type *ARO3* and *ARO4* genes responds to imbalanced amino acid supply. This yeast is unable to grow on medium that is supplemented with 5 mM phenylalanine and 5 mM tyrosine (Fig. 4.1). This amino acid concentration inhibits the activity of both DAHP synthases and thereby blocks the metabolic flux into the shikimate pathway. Addition of tryptophan regenerates growth of strain RH1408 suggesting that under these conditions this strain is starving for tryptophan. In the presence of only phenylalanine and tyrosine, strain RH1408 can be rescued by the expression of a plasmid carrying a copy of the *GCN4* gene (pME1083). In response to the tryptophan starvation, Gcn4p increases the expression of *ARO3* and *ARO4* as well as that of the other genes of the general control network and therefore regenerates growth (Fig. 4.1).

We wondered whether yeast strain RH1408 could grow in the presence of high amounts of phenylalanine and tyrosine when a trp-regulated DAHP synthase as a third enzyme for the initial step of shikimate biosynthesis is present, simulating the natural *E. coli* situation.
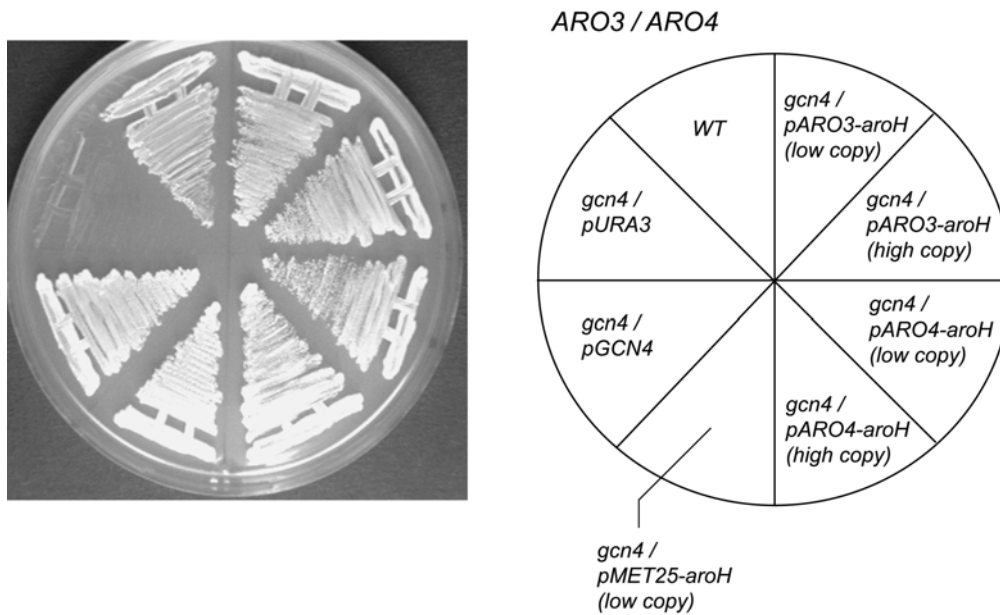
Fig. 4.1: Suppression of the growth defect of a *gcn4-103* mutation of *S. cerevisiae* on phenylalanine- and tyrosine-supplemented medium. Strain RH1408 (*gcn4-103*) transformed with various plasmids was streaked out for growth on minimal vitamin (MV) medium lacking tryptophan and supplemented with arginine, histidine, leucine, isoleucine and valine, respectively, to exclude other growth phenotypes. Additionally, 5 mM phenylalanine and 5 mM tyrosine were added for inhibition of both yeast DAHP synthase activities. Strain RH1408 (*gcn4-103*) was transformed with the *GCN4* carrying plasmid (pME1083), with the *URA3* carrying control-plasmid (pRS416), by high- and low-copy number plasmids carrying *E. coli aroH* fused to the *ARO*3 promoter (p*ARO3-aroH* (high-copy, pME1874); p*ARO3-aroH* (low-copy, pME1873) and by plasmids carrying *E. coli aroH* fused to the *ARO4* promoter (p*ARO4-aroH* (low-copy, pME2390) and p*ARO4-aroH* (high-copy, pME2391). One plasmid was transformed into strain RH1408 that carries *aroH* regulated by the *MET25* promoter (pME1877). As additional control the yeast wildtype (wt) strain RH730 was streaked out carrying the intact *ARO3*, *ARO4,* and *GCN4* genomic loci.

Therefore, the *E. coli aroH* gene encoding the trp-regulated DAHPS was heterologously expressed in yeast (protein here after termed as Ec AroHp). The *aroH* coding sequence was driven either by the *ARO3*, the *ARO4* or by the *MET25* promoter and expressed on low-copy or high-copy number plasmids in yeast strain RH1408. Expression of all *aroH* constructs rescued growth of strain RH1408 on media supplemented with 5 mM phenylalanine and 5 mM tyrosine. Therefore, Ec AroHp suppresses this specific yeast *gcn4Δ* phenotype by regenerating the metabolic flux into the shikimate pathway (Fig. 4.1).

It is unclear, whether *S. cerevisiae* or *A. nidulans* did loose the gene for a third trp-regulated DAHP synthase, or whether they never acquired this third isogene during the course of evolution. Here we show that in the presence of a specific unbalanced amino acid diet and in the absence of a functional general control system as backup, the lack of a third DAHPS enzyme is of disadvantage for yeast. We

focused on subtle differences between the two genes *ARO3* and *ARO4*, their gene products and wondered whether we could find traces of a tryptophan specific regulation of the initial step of the aromatic amino acid biosynthesis in the yeast *S. cerevisiae*.

4.4.2 Expression of a heterologous DAHP synthase is significantly higher when driven by the yeast *ARO3* promoter when compared to the yeast *ARO4* promoter

The result that a trp-inhibitable third DAHPS isoenzyme improves the physiological potential of yeast *gcn4Δ* mutant strain in the presence of an unbalanced aromatic amino acid diet, prompted us to construct a yeast strain with a trp-regulated enzyme as the only enzyme for the initial step of the shikimate biosynthesis. Strain RH2804 (*gcn4Δ::Kan^R, aro3Δ::HIS3, aro4Δ::LEU2*) no DAHPS activity and no possibility to adapt to imbalances in the amino acid nutrition. Strains RH2804 and as control RH2424 were transformed with high-copy number plasmids expressing the *E. coli aroH* gene driven by the *ARO3* promoter (p*ARO3*) and the *ARO4* promoter (p*ARO4*), respectively. As control, the *E. coli aroH* gene was fused to the *MET25* promoter (p*MET25*) that is constitutively expressed on media lacking methionine. A growth test on supplemented MV media demonstrated that a yeast strain expressing the trp-inhibitable DAHP synthase is able to survive on media without aromatic amino acids (Fig. 4.2A). Even without the transcriptional regulator Gcn4p, these artificial yeasts channelled enough flux into the shikimate pathway and were able to grow on supplemented MV-media (Fig. 4.2B).
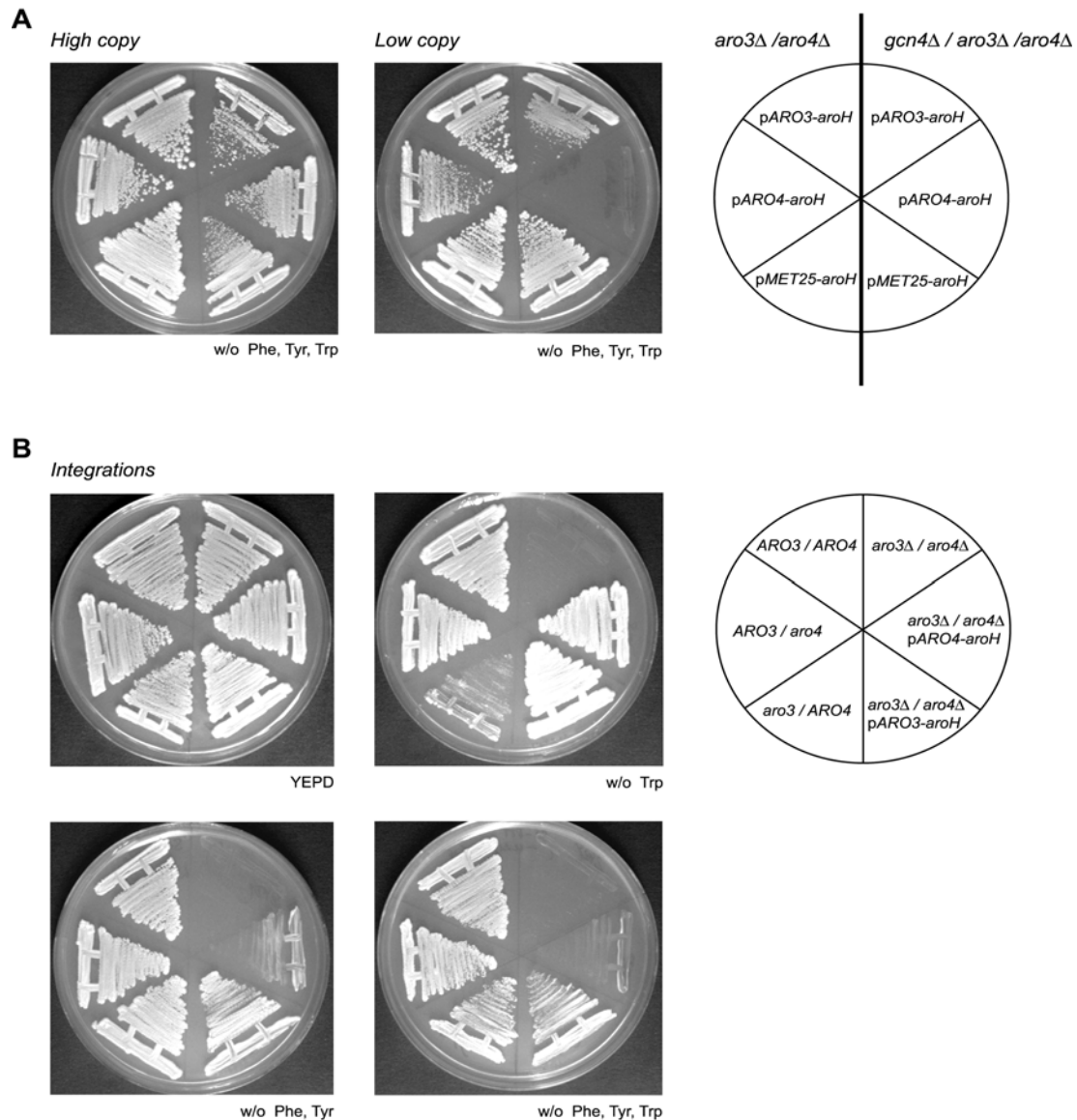
Fig. 4.2: Growth of different *S. cerevisiae* strains carrying various plasmids expressing the *E. coli aroH* gene or carrying *E. coli aroH* integrated into the genome. [A] High copy and low copy: Strains RH2424 (*aro3Δ*, *aro4Δ*) and RH2804 (*gcn4Δ*, *aro3Δ*, *aro4Δ*) lacking any DAHP synthase activity were transformed with high- and low-copy number plasmids carrying
E. coli aroH fused to the *ARO3* promoter (p*ARO3-aroH* (pME1874, pME1873)), *ARO4* promoter (p*ARO4-aroH* (pME2391, pME2390)) and *MET25* promoter (p*MET25-aroH* (pME1878, pME1877)), respectively. Plasmid carrying yeast strains were streaked out on MV media supplemented with arginine, histidine, leucine, iso-leucine, valine, and lacking all three aromatic amino acids (w/o Phe, Tyr, Trp). **[B]** Integrations: Strain RH2424 lacking any DAHP synthase activity was transformed with the *aroH* gene that was integrated in the genome and driven by the original *ARO3* promoter (p*ARO3-aroH;* RH2487) and the original *ARO4* promoter (p*ARO4-aroH;* RH2803), respectively. As controls, a yeast wild-type strain with intact *ARO3*, *ARO4* (RH1416), an *ARO3*, *aro4-1* mutant strain (RH1313), an *aro3-2*, *ARO4* mutant strain (RH1316), and an *aro3Δ*, *aro4Δ* mutant strain (RH2424) was plated out. All strains were plated on synthetic complete (SC) medium lacking tryptophan (w/o Trp), on SC lacking phenylalanine and tyrosine (w/o Phe, Tyr) and on SC lacking phenylalanine, tyrosine, and tryptophan (w/o Phe, Tyr, Trp), respectively.

We then asked whether the same promoter-*aroH* fusions are sufficient to maintain enough flux into the shikimate-pathway when expressed from low-copy number plasmids. Growth tests demonstrated that *E. coli aroH*, when expressed from p*ARO3* and p*ARO4* is able to maintain the flux into the shikimate pathway, when the yeast still possesses the master regulator of amino acid biosynthesis, Gcn4p. In a yeast *gcn4Δ* mutant strain, only the p*ARO3-aroH* fusion but not the p*ARO4-aroH* fusion resulted in normal growth (Fig. 4.2A). These data suggested that the basal promoter of *ARO3* is stronger than the basal promoter of *ARO4*. This seems to be contradictory to the previously described requirement of Gcn4p for basal *ARO3* expression (Paravicini *et al.*, 1989a).

We constructed yeasts where single copies of p*ARO3-aroH* or p*ARO4-aroH* fusions were integrated into the yeast genome to verify these results. Successful strain construction was proven by Southern hybridisation analysis (data not shown). The growth behaviour of these strains carrying an intact *GCN4* gene was examined in the presence of all amino acids (YEPD), on SC media lacking tryptophan (w/o Trp), lacking phenylalanine and tyrosine (w/o Phe, Tyr), lacking all three aromatic amino acids (w/o Phe, Tyr, Trp) and on supplemented MV media, respectively (Fig. 4.2B).

The *E. coli aroH* gene integrated at the *ARO4* locus and driven by the *ARO4* promoter (RH2803) resulted in a specific DAHPS activity that was hardly sufficient to rescue the *aro3Δ / aro4Δ* double deletion on SC media lacking all three aromatic amino acids (Fig. 4.2B). Under these conditions, strain RH2803 was nearly unable to grow showing a bradytrophic phenotype ($\mu \approx 0.10$ h^{-1} in comparison to $\mu = 0.32$ h^{-1} for wildtype). Growth defects induced by the lack of tryptophan could be complemented by the integrated p*ARO4-aroH*, whereas growth induced by the lack of phenylalanine and tyrosine could only be restored slow growth. Integration of p*ARO3::aroH* (RH2487), however, resulted in normal growth under all conditions ($\mu = 0.31$ h^{-1}, Fig. 4.2B). The same p*ARO4-aroH* fusion was also integrated into a yeast strain lacking the Gcn4p protein (RH2805, *gcn4Δ::Kan^R, aro3Δ::HIS3, aro4Δ:aroH*). This strain is unable to grow in the absence of aromatic amino acids and is auxotrophic on SC media with phenylalanine and tyrosine (data not shown).

In summary, the results obtained for the integrated *E. coli aroH* gene corroborated the results obtained for the extra-chromosomal growth tests. In the presence, as well as in the absence of Gcn4p, the *ARO4* promoter seems to be weaker when compared to the *ARO3* promoter, suggesting that the yeast cell possesses more Aro3p than Aro4p molecules.

These results prompted us to analyse yeast strains with intact Gcn4p and carrying only one of the two isogenes driven by their own promoters. A yeast strain with intact *ARO4* and deficient in *ARO3* (RH1316, *aro3-2*) is retarded in growth on SC media containing phenylalanine and tyrosine. However, a strain carrying an intact *ARO3,* but a defective *ARO4* (RH1313, *aro4-1*) shows normal growth under same conditions (Fig. 4.2B). All these data suggest that the yeast *ARO3* promoter is more efficiently transcribed when compared to the *ARO4* promoter.

4.4.3 A single Ala177Lys substitution changes the tryptophan-inhibitable DAHP synthase towards a tryptophan- and tyrosine-inhibitable enzyme

A trp-inhibitable DAHP synthase (Ec AroHp) as the only enzyme for the initial step of the shikimate biosynthesis in yeast is critical in environments with unbalanced amounts of aromatic amino acids, especially when high amounts of tryptophan are present. We used this artificial yeast strain carrying an *E. coli* derived trp-inhibitable Ec AroHp enzyme (DAHP^{Trp} synthase; DAHPS^{Trp}) for *in vitro* evolution.

Our goal was to construct a DAHPS^{Trp} enzyme, which is not only regulated by tryptophan but also by a second aromatic amino acid as additional effector. The strategy that was pursued included an amino acid sequence alignment of native *E. coli* AroH (trp-inhibitable) and yeast Aro4p (tyr-inhibitable), a comparison of the 3-dimensional structures modelled in *E. coli aroG* (Shumilin *et al.*, 1999; Shumilin *et al.*, 2002), and yeast Aro4p (Schneider *et al.*, 1999; Hartmann *et al.*, 2003), as well as results from various genetic screens (data not shown). We concentrated our efforts on codons encoding for amino acid residues, which are located nearby an area of the modelled DAHPS enzymes supposed to be the substrate-binding site of both, *E. coli* AroH and yeast Aro4p DAHP synthase, respectively.

All residues shown in Fig. 4.3 were mutated by site-directed mutagenesis of the corresponding sites within the gene. The codons for glycine 149, alanine 177, serine 179, cysteine 180, arginine 203 and threonine 218 from the *aroH* gene were exchanged by single amino acid substitutions. Enzyme activity assays from crude extracts with 0.5 mM of phenylalanine, tyrosine, or tryptophan were performed as described. Exchange of Ser179Ala (TCC→GCC), Cys180Phe (TGT→TTT), and Arg203Ala (CGC→GCC) did not significantly affect the trp-regulation of the DAHPS^{Trp} when compared to the heterologously expressed *aroH* wild-type enzyme (Fig. 4.4).
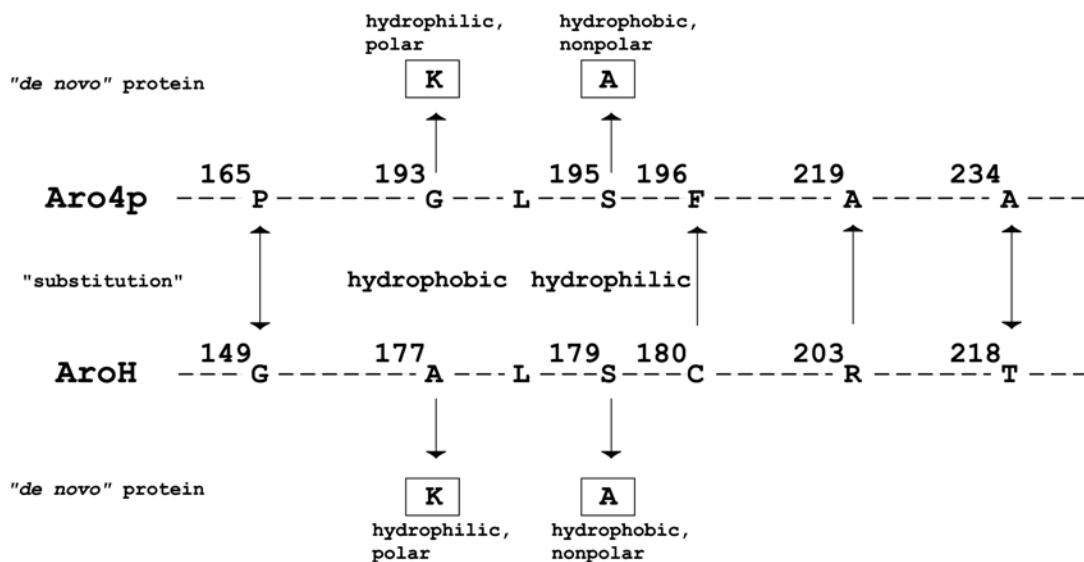

Fig. 4.3: A schematic partial amino acid alignment of AroH (*E. coli*) and yeast Aro4p is shown. The specific amino acid residues from yeast Aro4p and *E. coli* AroH used for mutation experiments are depicted in a partial amino acid sequence alignment. Numbers above give positions relatively to the first methionine of the amino acid sequence. Arrows indicate amino acid substitutions using amino acids from the homologues protein. Amino acids in squares were used to construct "*de novo*" proteins disturbing the original amino acid arrangements by polar or non-polar interactions.
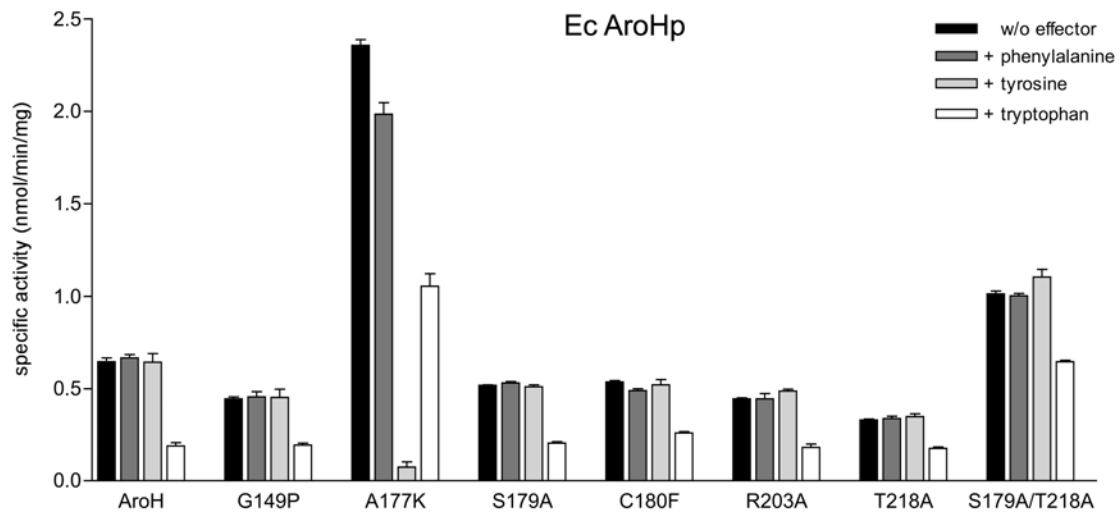
Fig. 4.4: Specific enzyme activities of seven allelic and heterologously expressed Ec AroHp mutant enzymes are shown referred to in the text. Yeast strain RH2424 (*aro3Δ, aro4Δ*) lacking any DAHPS activity was transformed with plasmids expressing *E. coli aroH* derived DAHPS activity from the inducible *MET25* promoter. All plasmids except the wild type Ec AroH (AroH, pME1878) and the double mutant Ser179Ala/Thr218Ala (S179A/T218A, pME1882) carried single amino acid substitutions. Substitutions of Ec AroHp were Gly149Pro (G149P, pME1879), Ala177Lys (A177K, pME2400), Ser179Ala (S179A, pME1880), Cys180Phe (C180F, pME2402), Arg203Ala (R203A, pME2404), and Thr218Ala (T218A, pME1881), respectively. Activities from yeast crude extracts are given, measured without effector (w/o effector), with 0.5mM of phenylalanine, 0.5mM of tyrosine and 0.5mM of tryptophan, respectively. Standard deviations of at least three independent measurements are indicated.

In part, this results were surprising since e.g. an exchange of the homologous Ser195Ala in the tyr-inhibitable yeast DAHP synthase (DAHPS^{Tyr}, Aro4p) did result in the loss of the tyrosine regulation (Hartmann *et al.*, 2003). The exchange of a Gly149Pro had only slight effect in decreasing the trp-sensitivity of the mutant DAHPS^{Trp} (Fig. 4.4). The same substitutions in *E. coli* AroH, a Gly149Asp and a Gly149Cys exchange, respectively, resulted in DAHP^{Trp} synthases that are completely resistant to trp-inhibition (Ray *et al.*, 1988). Exchange of Thr218Ala (ACC→GCC) resulted in an enzyme that is less sensitive (55%) to its main effector tryptophan when compared to wild type. Finally, we constructed a double substitution of the trp-regulated DAHPS^{Trp}. While the single point mutations had only weak effects on the enzyme regulation, a DAHPS^{Trp} Ser179Ala / Thr218Ala variant resulted in a trp-inhibitable enzyme that is sensitive towards tryptophan and showed a slightly higher activity at 0.5 mM tryptophan (Fig. 4.4).

We also exchanged the alanine 177 by a basic and polar lysine residue in DAHPS^{Trp} to disturb this non-polar enzyme area to test for effects on enzyme regulation. This amino acid residue is located next to a cavity assumed to be the substrate-binding site of both, *E. coli* DAHPS^{Phe} (Shumilin *et al.*, 2002) and *S. cerevisae* DAHPS^{Tyr} (Hartmann *et al.*, 2003). Furthermore, this residue is a glycine residue in all other DAHP synthases. By this polar substitution, we constructed a mutant enzyme that is more sensitive towards tyrosine than to tryptophan. With an exchanged alanine 177, a mutant DAHPS^{Trp} becomes yeast DAHPS^{Tyr} like, but is still a tryptophan-responsive enzyme (Fig. 4.4). The projection of all exchanged residues on a modelled structures of the trp-inhibitable DAHP synthase from *E. coli*, using the crystal structure data described for DAHPS^{Phe} (Shumilin *et al.*, 1999), showed that all altered residues are indeed located next to the assumed regulatory site of this DAHPS enzyme (Fig. 4.5).

By exchanging a non-polar against a hydrophobic amino acid residue at position 177 of heterologously expressed Ec AroHp, we identified an important amino acid residue. When mutated, this residue allows a second feedback control of Ec AroHp regulated by tryptophan as well as by tyrosine.
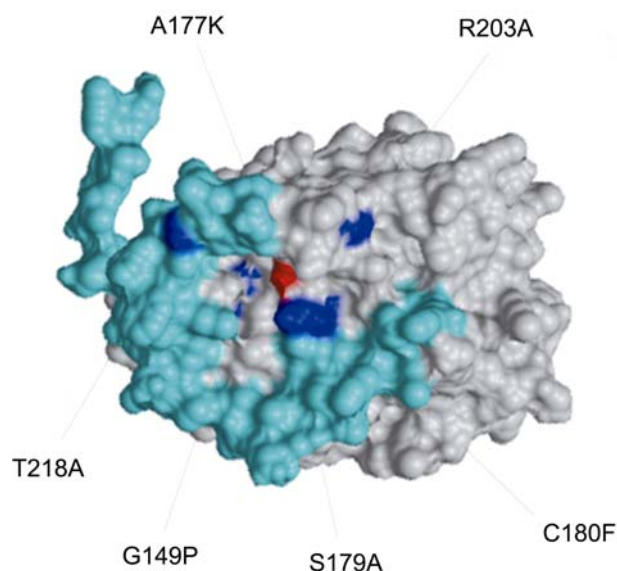

Fig. 4.5: A modelled surface blot of one *E. coli* AroH monomer. A view on the modelled structure of one monomer of the tryptophan-inhibitable DAHP synthase (AroH) with marked positions (blue, red) of amino acid residues exchanged by site-directed mutations is given. The structure depicted was modelled on data accessible for the phenylalanine inhibitable DAHPS enzyme (Schumilin *et. al.,* 1999). In the arranged position a view into the cavity of the tryptophan-inhibitable DAHP synthase is given, assumed to be the effector binding site and the regulation hot spot of the enzyme.

4.4.4 The yeast Aro4p DAHP synthase is similarly regulated by tryptophan and tyrosine as an Ala177Lys Ec AroHp mutant enzyme

The result from the Ala177Lys exchange of Ec AroHp with a strong tyrosine and less efficient tryptophan inhibition prompted us to ask whether the yeast phe- and tyr-regulated DAHP synthases (Aro3p, Aro4p) are similarly regulated by tryptophan under these growth conditions. We prepared crude extracts from yeast liquid cultures harvested at nearly the same optical density (OD_{546}=1.9), using the identical host strain RH2424 (*aro3Δ::HIS3, aro4Δ::LEU2*) and the same expression vector pME1513 (2 µm). A modified enzyme stop assay was performed as described (Schnappauf *et. al.,* 1998) using 0.1 mM, 0.25 mM, 0.5 mM, and 1.0 mM of phenylalanine, tyrosine, and tryptophan as effector concentration, respectively. Enzyme activities without effectors (e.g. with water) were taken as 100% reference values.

The tryptophan-inhibitable DAHPS (Ec AroHp) did not show an altered inhibition at the different tested effector concentrations. When compared to probes without effectors, specific enzyme activities of crude extracts were always about 100% for all tested concentrations of phenylalanine or tyrosine, while it was between 32% and 25% at the different tryptophan concentrations (data not shown). Both Aro3p and Aro4p DAHP synthases showed an inhibition of catalytic turnover at higher tryptophan concentrations. While activities of yeast DAHP synthases drastically drop in presence of their specific effectors phenylalanine and tyrosine (Takahashi & Chan, 1971; Schnappauf *et al.*, 1998), respectively, they appeared to be also inhibitable in dependence to the tryptophan concentration. The effects measured first from protein crude extracts could be demonstrated for purified Aro3p and Aro4p DAHP synthase enzymes (Fig. 4.6).

The phe-inhibitable DAHP synthase (Aro3p) exhibited 95% of specific activity in presence of 0.1 mM tryptophan, but only 22% of specific activity at 1.0 mM tryptophan. The tryptophan inhibition was even more obvious for the tyr-inhibitable DAHP synthase (Aro4p). In presence of 0.1 mM tryptophan, the DAPH^{Tyr} synthase exhibited only 50% of specific activity, while it displayed 20% of specific activity at 1.0 mM tryptophan (Fig. 4.6). At 0.1 mM of tryptophan the Aro4p enzyme is reduced to half activity, while the isoenzyme Aro3p displays full activity. It was previously described that the *E. coli* DAPH^{Trp} synthase activity is reduced to half at a

concentration of 2 µM (Ray & Bauerle, 1991), while the activity of the yeast *TRP2/3* encoded anthranilate synthase activity is even inhibited at 1 µM (Prantl *et al.*, 1985).
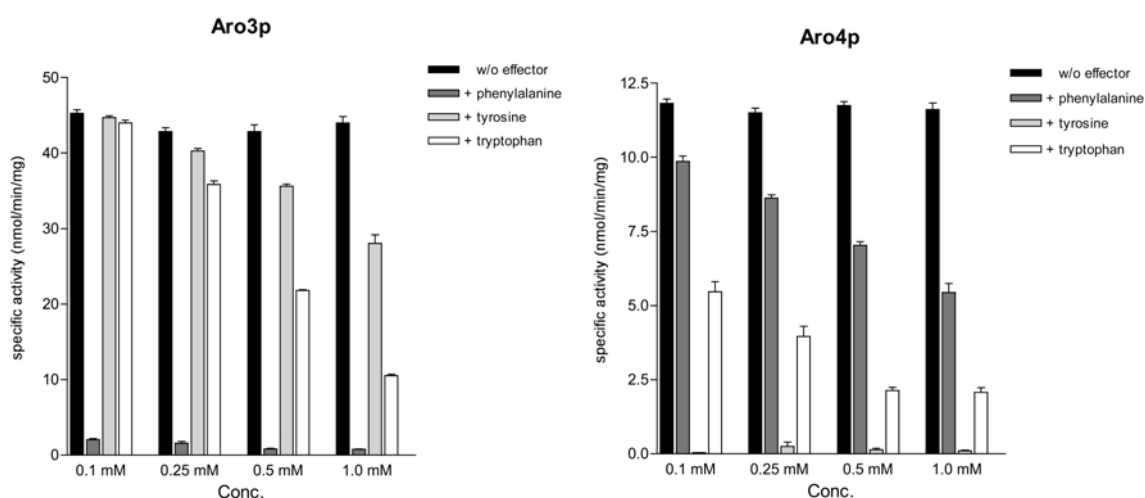

Fig. 4.6: An enzyme activity assay with purified Aro3p and Aro4p DAHP synthases is shown. The specific phenylalanine- (Aro3p) and tyrosine- (Aro4p) inhibitable DAHP synthase activities of purified enzymes are given, measured without effector (w/o effector), and with 0.1 mM, 0.25 mM, 0.5 mM, and 1.0 mM of phenylalanine, tyrosine and tryptophan, respectively. Standard deviations of at least three independent measurements are given.

In summary, these data suggest that the two isoenzymes Aro3p and Aro4p are moderately feedback-inhibitable by tryptophan as third effector. In addition to their known control by phenylalanine (Aro3p) and tyrosine (Aro4p), respectively, both enzymes are also regulated when high amounts of tryptophan are present in the diet. In contrast to the yeast enzymes, the third Ec AroHp cannot be regulated by a second effector as seen before at 0.1 mM to 1.0 mM of effector concentration. Additionally, the regulation of Aro3p and Aro4p but not of Ec AroHp is also influenced by tyrosine and phenylalanine at this tested concentrations. This dual regulation of the two yeast isoenzymes might counteract the lack of the third enzyme as long as the Gcn4p dependent general control is functional as further backup system.

4.4.5 A single Pro165Gly substitution results in an Aro4p yeast DAHP synthase, which is efficiently inhibitable by tyrosine and tryptophan

In amino acid sequences alignments, the yeast tyr-inhibitable DAHP synthase (Aro4p) is more similar to the trp-inhibitable DAHP synthase (*E. coli* AroH) than is the yeast phe-inhibitable DAHP synthase (Aro3p). Since we could demonstrate that it is possible to shift regulation of a trp-inhibitable DAHPS towards tyrosine- and tryptophan-regulation, we wanted to change regulation of a yeast tyr-inhibitable DAHPS towards tryptophan-inhibition. By site-directed mutagenesis, we exchanged the codons of proline 165, glycine 193, serine 195, and alanine 234 in the yeast *ARO4* encoded DAHP^{Tyr} synthase, respectively. Specific enzyme activities from crude extract measurements with effector concentrations of 0.5 mM revealed that the exchange of an Ala234Thr (GCT→ACT) did not result in significant alteration of Aro4p enzyme activity. Therefore, this amino acid residue seems to be not involved in the regulation of the yeast DAHP^{Tyr} (Fig. 4.7).
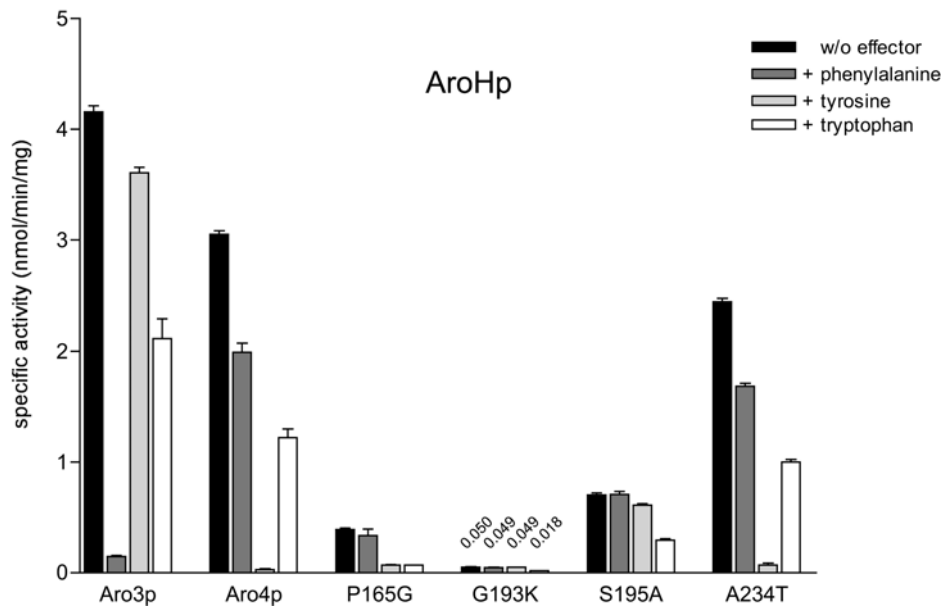

Fig. 4.7: The specific crude extract activities of four allelic Aro4p mutant enzymes constructed by site directed mutagenesis are shown. Yeast strain RH2424 (*aro3Δ*, *aro4Δ*) lacking any DAHPS activity was transformed with various plasmids expressing DAHP synthase activity from the inducible *MET25* promoter. All plasmids beside wild-type Aro3p (Aro3p, pME2384) and wild-type Aro4p (Aro4p, pME2385) carried single amino acid substitutions in the resulting Aro4p mutant proteins. Site-directed mutations of Aro4p were Pro165Gly (P165G, pME2395), Gly193Lys (G193K, pME2395), Ser195Ala (S195A, pME2396), and Ala234Thr (A234T, pME2397), respectively. The specific enzyme activities from yeast crude extracts are given without effector, and meassured with 0.5 mM of phenylalanine, tyrosine, and tryptophan, respectively. Standard deviations of at least three independent measurements are given.

Exchange of a Gly193Lys (GGT→AAG) in yeast Aro4p abolished most of DAHPS activity. The enzyme no longer regulated by any of its original effectors, still exhibits weak sensitivity to tryptophan at 0.5 mM of effector concentration. An exchange of a Ser195Ala (TCT→GCT) in the yeast tyr-inhibitable DAHPS (Aro4p) resulted in an enzyme that no longer is inhabitable by its main effector tyrosine, is no longer sensitive for phenylalanine, but is still inhibitable by tryptophan. Thereby this yeast DAHPS^{Tyr} mutant becomes DAHPS^{Trp} (Ec AroHp) like. In contrast, the exchange of a Pro165Gly (CCT→GGT) in the yeast tyr-inhibitable DAHP synthase did result in an enzyme that is strongly inhibitable by tyrosine and additionally became tryptophan sensitive. Total enzyme activity was drastically reduced, indicating strong influence of this amino acid substitution on the global DAHPS structure. A projection of all four amino acid substitutions on a modelled structure of the tyr-inhibitable DAHPS from *S. cerevisiae* basing on the yeast Aro4p crystal structure (Schneider *et al.*, 1999; Hartmann *et al.*, 2003) demonstrated that all four residues are located at the putative effector-binding cavity of the yeast *ARO4* encoded DAHPS^{Tyr} (data not shown). The structure of this effector-binding cavity is also very similar to the structure published for the phe-inhibitable *aroG* from *E. coli* (Shumilin *et al.*, 1999).

By evolutionary processes, some microorganisms like *S. cerevisiae* have evolved only two DAHP synthase enzymes, while others like *E. coli* and *N. crassa* carry three differently inhibitable DAHPS enzymes. Our data suggest that the fungus *S. cerevisiae* does renounce a third tryptophan-inhibitable enzyme due to the fine-regulation of its two isoenzymes Aro3p and Aro4p and the presence of the general control of amino acid biosnthesis (Gcn4p), respectively.

4.5   Discussion

Evolution of DAHPS enzymes in prokaryotes has been described in detail (Jensen, 1985; Ahmad *et al.*, 1986; Ahmad *et al.*, 1987; Ahmad & Jensen, 1988; Jensen & Ahmad, 1988; Ahmad *et al.*, 1990). The evolution of prokaryotic DAHP synthases presumably started at the level of an allosterically insensitive DAHPS^0 and a tyr-sensitive DAHPS^{Tyr}. To date, this situation can still be found in species of *Acinetobacter*, or in *Oceanospirillum minutulum* (Byng *et al.*, 1985). It was proposed that evolutionary processess changed the DAHPS^0 enzyme towards a trp-inhibitable DAHPS^{Trp} enzyme. Some micro-organisms still represent this state of evolution harbouring both, a DAHPS^{Tyr} and a DAHPS^{Trp} enzyme as e.g., some representatives of the *group I* pseudomonads (Byng *et al.*, 1983; Byng *et al.*, 1985). In secondary evolutionary processes, some prokaryotes seem to have lost the DAHPS^{Tyr} carrying only one, highly efficient DAHPS^{Trp} enzyme. This seems to be the case for some *group V* pseudomonades (Whitaker *et al.*, 1981; Whitaker *et al.*, 1984).

All DAHPS enzymes described above share one catalytic feature. The enzyme activity is regulated not only by tyrosine or tryptophan but also by chorismate as additional effector. Among those microbial representatives with two enzymes, a wide variability of chorismate sensitivity of DAHPS^{Trp} has been found (Ahmad *et al.*, 1986; Ahmad *et al.*, 1990). In the evolutionary processes, a loss of the chorismate sensitivity of the DAHPS^{Trp} synthases seems to occur at that the same time when micro-organisms have acquired a third phe-regulated enzyme activity (DAHPS^{Phe}). Only those prokaryotes carry three DAHP synthases, which have a DAHPS^{Trp} enzyme that is inhibitable only by tryptophan but not by chorismate, while the allosterical chorismate dependent regulations of DAHPS^{Phe} or DAHPS^{Trp} are variable (Ahmad *et al.*, 1990). All representatives with three catalytic enzymes all are members of an evolutionary young lineage of the prokaryotic super family B, e.g. *E. coli* and some closely related organisms like *Klebsiella pneumoniae, Erwinia carotovora, Salmonella typhimurium* and *Yersinia enterocolitica*. Among this group, the DAHP^{Tyr} activity seems to be more specialised. Higher sequence similarities implicate that the procaryotic DAHP^{Phe} synthases have evolved by a gene duplication of DAHP^{Trp} synthase encoding genes and not of genes encoding for DAHP^{Tyr} synthase enzymes (Jensen & Byng, 1981; Jensen & Ahmad, 1988).

The amino acid sequences of yeast Aro3p and Aro4p share 76% identical amino acid residues. Both enzymes also share sequence identities of up to 65% to the three *E. coli* DAHPS isoenzymes. The overall identities of all other enzymes of the shikimate pathway are significantly lower when compared between different groups of micro organisms (Braus, 1991). A high degree of identity between different isoenzymes is not unusual. More remarkable is the high identity between isoenzymes of two unrelated micro organisms from different phylogenetic groups like prokaryotes (*E. coli*) and eukaryotes (*S. cerevisiae*).

It is unknown, whether *S. cerevisiae* has lost a third DAHPS^{Trp} activity or never acquired one, but the known yeast genome definitely contains only two DAHPS loci (Mewes *et al.*, 1997). Amino acid sequence alignments implicate that yeast DAHPS^{Tyr} (Aro4p) and *E. coli* DAHPS^{Trp} (AroH) are more similar than are DAHPS^{Phe} (Aro3p) and DAHPS^{Trp} (AroH). We have recently shown that a single amino acid substitution (Gly219Ser) is sufficient to switch the *ARO3* encoded DAHPS^{Phe} into an Aro4p-like DAHPS^{Tyr} and *vice versa* the exchange of Ser226Ala of the *ARO4* encoded DAHPS^{Tyr} results in an Aro3p-like DAHPS^{Phe} (Hartmann *et al.*, 2003). We now extended this study and show here that a single amino acid substitution (Ala177Lys) in *E. coli* DAHPS^{Trp} (Ec AroHp) results in an Aro4p-like DAHPS^{Tyr} enzyme. Amino acid sequence alignments suggest that Ala177 of *E. coli* (trp-inhibitable) corresponds to Gly193 in yeast Aro4p (tyr-inhibitable). A Gly193Lys exchange abolished enzyme activity of yeast Aro4p suggesting that evolution of yeast DAHPS might be different to that described in prokaryotes.

Since we find weak tryptophan regulation for both fungal isoenzymes, we suggest that fungi evolution might have started with a trp-inhibitable DAHPS precursor enzyme. Gene duplication and the transcriptional regulation of the general control network of amino acid biosynthesis finally resulted in two Gcn4p regulated genes encoding for highly effective and closely related DAHPS activities, a DAHPS^{Tyr} (Aro4p) and a DAHPS^{Phe} (Aro3p), respectively. Since both yeast enzymes still share a slight tryptophan-regulation, this scenario seems to be possible.

The presence of two initial DAHPS enzymes and the Gcn4p regulated general control system implicate some direct consequences for the subsequent regulations within the aromatic amino acid biosynthesis. In *S. cerevisiae*, the last common enzyme activity of the shikimate pathway, the *ARO7* encoded chorismate mutase (E.C. 5.4.99.5) is not under the control of the general amino acid biosynthesis

(Schmidheini *et al.*, 1990). The chorismate mutase activity, negatively regulated by tyrosine and activated by tryptophan, directs the biosynthetic flux towards tryptophan or tyrosine and phenylalanine, respectively. In the presence of high tyrosine and phenylalanine concentrations, these end products inhibit the activity of the two isoenzymes Aro3p and Aro4p, respectively. The function of Gcn4p protects the cell from shutting down the shikimate pathway and starving from tryptophan. In *S. cerevisiae*, a third trp-regulated DAHPS seems to be not necessary, due to the regulatory mechanisms on the three genetic levels. Transcriptional and translational activities supervise the general gene expression via the general control. A major allostery regulation of the trp-inhibitable chorismate mutase and a minor allostery trp-inhibition of Aro3p and Aro4p, respectively, result in an overall balanced enzyme activity. Here we demonstrated that the expression of a trp-inhibitable DAHP synthase from *E. coli* (Ec AroHp) is beneficial for a mutant *S. cerevisiae* strain when the general control is malfunctioning.

The '*status quo*' in the regulation of the *S. cerevisiae* DAHPS enzymes is the result of an evolutionary fine-tuning of two instead of three DAHPS enzymes. Further investigations among other fungi should demonstrate whether the here described regulatory scenario could also be found in other species. A next step will concern a comparison of DAHPS activities from other eukaryotes, including the enzymes from plants. Results from these investigations will have further implications for the protein engineering of the variously regulated DAHP synthase enzymes.

4.6   Acknowledgements

4.7 References

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