Chromatin Structure and Regulation of Gene Expression at the Histidine/Adenine Branch Point in Yeast and Aspergillus

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Oliver Valerius
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Table of Contents

- **Summary** 1
- **Zusammenfassung** 2
- Chapter 1:
  Introduction 3

1. The biosynthesis of histidine is metabolically linked to the *de novo* biosynthesis of purines 4

1.1 Reactions and enzymes in histidine biosynthesis 4

1.2 The AICAR cycle metabolically links the histidine and purine biosynthetic pathways 6

2. Genes encoding IGP synthase, the enzyme at the juncture 9

2.1 In prokaryotes two structural genes of the *his*-operon encode the bifunctional IGP-synthase 9

2.2 In eukaryotes both activities of the IGP synthase are encoded from one single gene 12

3. Regulation of IGP-synthase encoding genes in prokaryotes and eukaryotes 12

3.1 The majority of the *his* genes of prokaryotes are clustered in operons and are simultaneously regulated upon histidine availability 12

3.2 In eukaryotes the metabolic link between the histidine and the purine biosynthesis is reflected by the regulation of the gene at the juncture 14

3.2.1 The transcriptional activation of the *HIS7* gene upon amino acid starvation – the 'general control system' of *S. cerevisiae* 15

3.2.2 'Cross-pathway control' and amino acid biosynthesis of the filamentous fungus *Aspergillus nidulans* 18

3.2.3 Purine limitation activates the *HIS7* expression in yeast 18

4. Modulation of the chromatin structure is an essential step for the regulation of gene expression in eukaryotes 20

4.1 The transcriptional regulation of gene expression strongly depends on the local chromatin structure 20

4.2 Chromatin modifying activities diversify the chromatin structure and enable regulated gene expression 22
4.2.1 Swi/Snf mediated ATP-dependent nucleosome remodeling 23
4.2.2 The SAGA complex (Spt/Ada/Gcn5 Acetyltransferase) 26

5. Transcriptional interference of tandemly transcribed genes 28
5.1 Adjacent genes in close vicinity are susceptible to transcriptional interference 28
5.2 Natural barriers to transcriptional interference 29

6. Metabolism and differentiation in fungi 31

Aim of this work 35

References 36

- Chapter 2:
Regulation of *hisHF* Transcription of *Aspergillus nidulans* by Adenine and Amino Acid Limitation 46

Abstract 46
Introduction 47
Experimental Procedures 49
Results 52
Discussion 63
References 66

- Chapter 3:
Different Transcriptional Activators Regulate *HIS7* Expression by Different Nucleosome-mediated Activation Mechanisms 70

Abstract 70
Introduction 71
Experimental Procedures 75
Results 79
Discussion 90
References 95

➢ Chapter 4:
A Positioned Nucleosome Prevents Transcriptional Interference Between the Adjacent *ARO4* and *HIS7* Genes of Yeast **99**

Abstract 99

Introduction 100

Experimental Procedures 102

Results 106

Discussion 117

References 120

➢ **Curriculum Vitae** **122**

Summary

Imidazole glycerole phosphate (IGP) synthase (E.C.2.4.2.14 & E.C.4.3.2.4) catalyzes the fifth and the sixth reaction step of the histidine biosynthesis. The byproduct of the reactions, AICAR, is also an intermediate compound of the *de novo* biosynthesis of purines. Metabolic flux in this biosynthesis generates the so-called purine salvage pathway. In the yeast *Saccharomyces cerevisiae* the transcription of the IGP synthase encoding gene, *HIS7*, is regulated upon amino acid and purine availability. Therefore the metabolic link of both biosynthetic pathways is also reflected on the level of regulation of the gene at the crossways.

In this thesis, the regulation of the homologous gene of a filamentous fungus was shown to respond to amino acid and purine availability as in yeast. Overexpression of this *Aspergillus nidulans hisHF* gene results in a block of formation of sexual fruiting bodies at an early step of development. The appropriate regulation of *hisHF* expression is therefore linked to the complex developmental program of cleistothecia formation.

All eukaryotic genes are part of chromatin that *per se* acts repressive on gene expression. Therefore chromatin-modifying activities are required for accurate gene expression by overcoming this repression. The transcriptional activation of the yeast *HIS7* gene upon amino acid starvation requires the chromatin remodeling complex Swi/Snf. Together with the transcription factors Gcn4p and Abf1p this complex changes the nucleosomal promoter structure. In comparison, the activation of *HIS7* transcription upon purine limitation by the transcription factor Bas1p/Bas2p requires a chromatin modifying activity that acetylates nucleosomes in a *GCN5*-dependent manner.

A nucleosome located immediately upstream of the *HIS7* promoter seems to represent the border to the proceeding *ARO4* gene. Possibly this nucleosome prevents transcriptional interference between the tandemly orientated genes and permits such a short intergenic region as it is typical for this eukaryotic microorganism.

Zusammenfassung

Das Enzym Imidazol-glyzerol-phosphat (IGP) Synthase (E.C.2.4.2.14 & E.C.4.3.2.4) katalysiert den fünften und sechsten Reaktionsschritt der Histidinbiosynthese. Das bei diesen Reaktionen entstehende Nebenprodukt AICAR ist gleichzeitig ein Intermediat der *de novo* Biosynthese von Purinen und fließt in diese mit ein. Diese metabolische Verknüpfung beider Biosynthesen spiegelt sich in der Hefe *Saccharomyces cerevisiae* auch auf der Ebene der Regulation des für die IGP-Synthase codierenden *HIS7*-Gens durch Aminosäure- und Purinverfügbarkeit wider.

In der vorliegenden Arbeit wurde zunächst das homologe Gen eines filamentösen Pilzes isoliert und seine Regulation durch Aminosäure- und Purinverfügbarkeit nachgewiesen. Eine starke Überexpression dieses *hisHF*-Gens aus *Aspergillus nidulans* führt zu einer Blockierung der sexuellen Fruchtkörperbildung in einem frühen Stadium der Entwicklung. Eine genaue Regulation der *hisHF*-Expression ist daher Voraussetzung für das Durchlaufen des komplexen Entwicklungsprogrammes der Cleistothecien-Bildung.

Eukaryotische Gene liegen im Chromatinverbund, vor welches *per se* repressiv auf die Genexpression wirkt. Die Zelle benötigt chromatinmodifizierende Aktivitäten, die diese Repression überwinden können. Die transkriptionelle Aktivierung des *HIS7*-Gens durch Aminosäuremangel benötigt dafür die Anwesenheit des Swi/Snf-Komplexes und der Transkriptionsaktivatoren Gcn4p und Abf1p, die gemeinsam eine Veränderung der Nukleosomenverteilung an dem *HIS7*-Promotor bewirken. Im Vergleich dazu benötigt die *HIS7*-Aktivierung bei Purinmangel durch den Transkriptionsaktivator Bas1p/Bas2p eine chromatinmodifizierende Aktivität, die Nukleosomen *GCN5*-abhängig azetyliert.

Darüberhinaus stellt ein Nukleosom, das sich direkt stromaufwärts des *HIS7*-Promotors befindet, eine Grenze zum vorhergehenden *ARO4*-Gen dar. Möglicherweise dient es dem Schutz vor transkriptioneller Interferenz zwischen beiden gleichgerichteten Genen, die nur durch eine kurze intergenische Region getrennt sind.

Chapter 1

Introduction

Recent genome sequencing projects have shown that a living cell, whichever organism is taken into account, can synthesize different proteins in a range of 35.000 (man) to 12.000 (nematodes as *Caenorhabiditis elegans*) to 6.000 (yeasts as *Saccharomyces cerevisiae*) to 4.000 (bacteria as *Escherichia coli*). Some of these proteins have to be frequently synthesized whereas others rather scarcely. In principle each protein consists of 20 different amino acids, and sometimes of additional modified amino acids formed by extra mechanisms (e.g. selenocysteine; Böck, 2000).

Therefore the cell has to cope with the logistical problem to provide all different protein biosyntheses with sufficient amounts of the required amino acids. Since amino acids like e.g. tryptophan are incorporated only infrequently into proteins, for others there is a great demand like e.g. glycine or the translational starter amino acid methionine. The logistical problem gets even more complicated by the fact that for economical reasons the cell must discriminate two different nutritional states. In one state the cell can fill its internal amino acid(s) pool by the uptake of amino acid(s) present in the growth medium. In the other state the required amino acid(s) is/are not present in the environment driving the cell into its own amino acid biosyntheses. However, not each organism is capable of synthesizing all required amino acids by itself but depend on the uptake of one or several amino acids from the environment to maintain the internal pool(s). Such amino acids therefore are termed ‘essential amino acids’ for the concerned organism, as for e.g. the amino acids histidine and methionin and eight further amino acids are essential for man.

In consideration of the costs for amino acid biosyntheses measured as turnover of the energy unit ATP, it is obvious that the expenses for biosyntheses of different amino acids heavily vary. The biosyntheses of several amino acids are organized in families with the first reaction steps in common. Such families are e.g. the oxalacetate family, the α-ketoglutarate family, or the very expensive family of aromatic amino acids. The amino

acid histidine is also rather expensive but its biosynthesis is not organized in a family. Histidine biosynthesis is connected to the *de novo* biosynthesis of purines.

The 'logistical masterstroke of in-time biosynthesis/uptake' of amino acids requires a complex network of genetically and enzymatic organized transport and regulation mechanisms. Histidine biosynthesis serves as example for studying the link between the involved biosynthetic genes and enzymes of the unicellular fungus *Saccharomyces cerevisiae* and that of the filamentous fungus *Aspergillus nidulans*.

1. The biosynthesis of histidine is metabolically linked to the *de novo* biosynthesis of purines

1.1 Reactions and enzymes in histidine biosynthesis

The biosynthesis of the amino acid histidine in bacteria, fungi and plants is an unbranched pathway formed by ten complex biochemical reactions catalyzed by eight distinct enzymes (Fig. 1) (Alifano *et al*., 1996). In contrast to the biosyntheses of many other amino acids, evolution has probably developed the biosynthetic pathway leading to histidine only once. The first reaction is the condensation of ATP and 5-phosphoribosyl-1-pyrophosphate (PRPP) to form *N´*-5´-phosphoribosyl-ATP (PRATP). This reaction is catalyzed by PRATP transferase that itself is feedback controlled by the pathway endproduct histidine together with the product of the reaction PRATP, and moreover by AMP (Klungsoyr *et al*., 1968; Ohta *et al*., 2000). Thermodynamic calculations revealed that the synthesis of one molecule histidine requires 41 ATP molecules (Brenner and Ames, 1971).

The following two reaction steps are the irreversible hydrolysis of PRATP to PRAMP and the opening of the purine ring leading to the production of the imidazole intermediate *N´*-((5´-phosphoribosyl)-formimino)-5-aminoimidazole-4 carboxyamide-ribonulceotide (5´-ProFAR, or BBM II). The first of these reactions is catalyzed by the carboxy-terminal domain whereas the second one by the amino-terminal domain of one bifunctional enzyme (Smith and Ames, 1965). The fourth step of the pathway is an internal redox

reaction involving the isomerization of the aminoaldose 5´-ProFAR to the aminoketose *N´*-((5´-phosphoribulosyl)-formimino)-5-aminoimidazole-4 carboxamide-ribonucleotide (5´-PRFAR, or BBM III) (Margolies and Goldberger, 1966).

The fifth reaction of the pathway is the transformation of 5´-PRFAR to imidazole-glycerole-phosphate (IGP) catalyzed by the bifunctional IGP synthase. This enzyme fulfills a glutamine amidotransferase activity providing the emerging IGP with the amide nitrogen, and a cyclase activity for the formation of the imidazole ring (Rieder *et al.*, 1994). Beside IGP the compound 5´-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) is formed as a byproduct. AICAR (also ZMP) itself is also an intermediate compound of the *de novo* biosynthesis of purines and thus flows into this pathway leading to the recycling of the initially invested ATP (Klem and Davisson, 1993) (see chapter 1.2).

The enzymatically dehydrated IGP results in an enol that is further ketonized nonenzymatically to imidazole-acetol-phosphate (IAP) (Brenner and Ames, 1971). The seventh step consists of a reversible transamination involving IAP and a nitrogen atom from glutamate, leading to α-ketoglutarate and L-histidinol-phosphate (HOL-P), catalyzed by a pyridoxal-P-dependent aminotransferase. HOL-P is then converted to L-histidinol (HOL) by the phosphatase activity of a bifunctional enzyme (Brenner and Ames, 1971). During the last two steps of histidine biosynthesis, HOL is oxidized to the corresponding amino acid L-histidine (His).

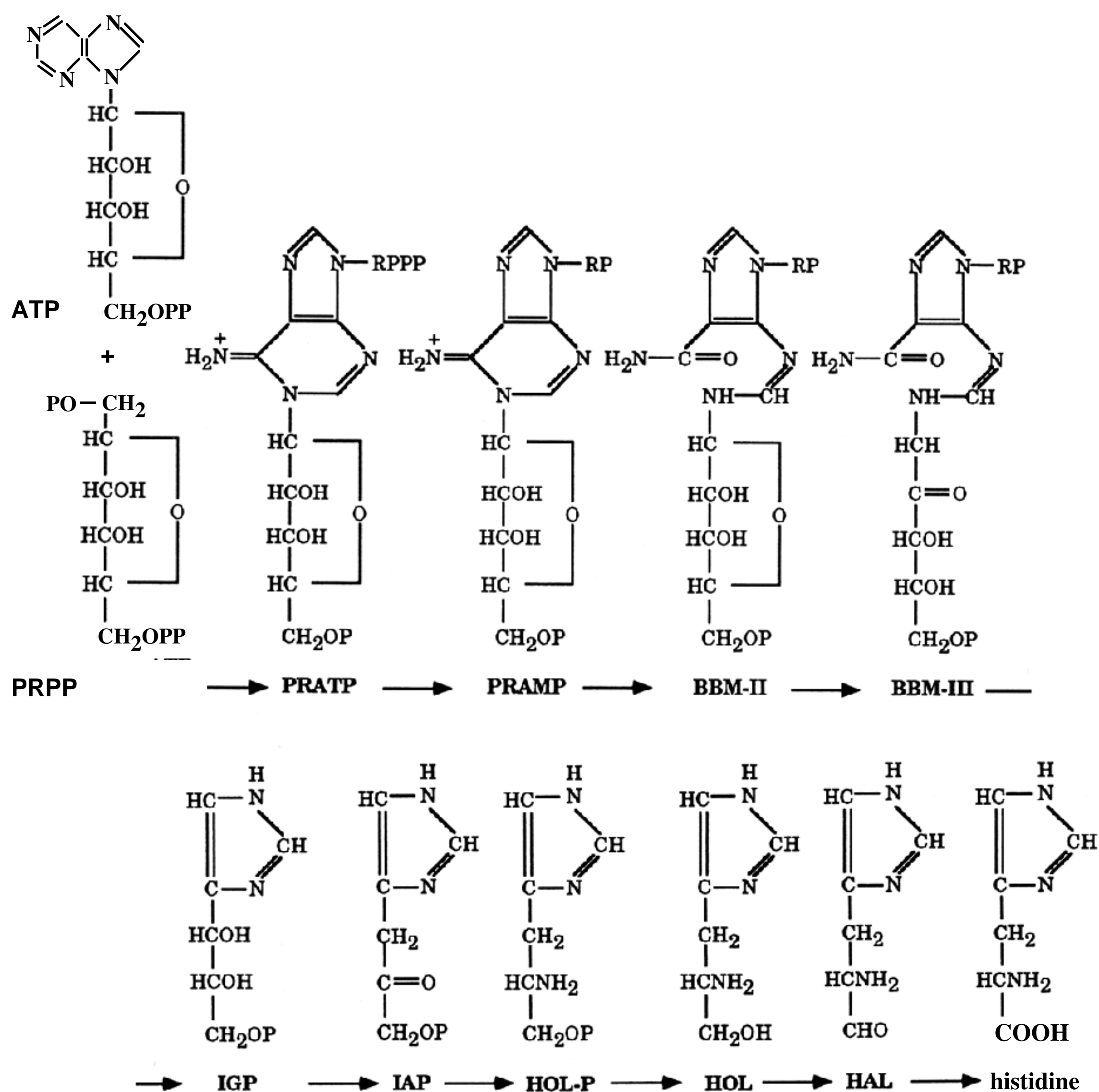

Figure 1. Metabolic pathway of histidine biosynthesis. Order of the reactions and the intermediates in the pathway. PRPP: ribosyl triphosphate; PR: ribosyl phosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; PRATP: *N*´-5´-phosphoribosyl-ATP; PRAMP, *N*´-5´-phosphoribosyl-AMP; BBM-II (5´-ProFAR): *N*´-((5´-phosphoribosyl)-formimino)-5-aminoimidazole-4-carboxamide-ribonucleotide; BBM-III (5´-PRFAR): *N*´-((5´-phosphoribulosyl)-formimino)-5-aminoimidazole-4-carboxamide-ribonucleotide; IGP: imidazole glycerol-phosphate; AICAR (ZMP): 5´-phosphoribosyl-4-carboxamide-5-aminoimidazole; IAP: imidazole acetol-phosphate; HOL-P: L-histidinol-phosphate; HOL, L-histidinol; HAL: L-histidinal (not found as free intermediate).

1.2 The AICAR cycle metabolically links the histidine and purine biosynthetic pathways

AICAR, the by-product of the reaction catalyzed by the IGP synthase, is also an intermediate of the *de novo* purine biosynthesis. As such an intermediate it directly

boosts the purine biosynthesis to synthesize more imidazole monophosphate (IMP) that is further metabolized to AMP or GMP (Fig. 2 A).

Mutant strains of different organisms bearing nonfunctional enzymatic activities in the early steps of histidine biosynthesis have been shown to be auxotroph not only for histidine but also for purines (Johnston and Roth, 1979). Since the biosynthesis of histidine requires a carbon and a nitrogen equivalent from the purine ring of ATP, the metabolic link represented by the production of AICAR is an important aspect of the following purine salvage pathway, originated during a central step of the histidine pathway (Fig. 2 A)(Guetsova *et al.*, 1997). The conversion of AICAR to IMP solely involves a folic acid-mediated one-carbon (C1) transfer (Neuhard and Nygaard, 1987).

However, under folate starvation conditions also the formation of the unusual nucleotide 5-aminoimidazole-4-carboxamide-riboside-5´-triphosphate (ZTP) has been reported (Fig. 2 B)(Bochner and Ames, 1982). It was proposed that ZTP acts as an alarmone signaling C-1-folate deficiency and mediating a physiologically beneficial response to folate stress. This hypothesis is supported by several findings concerning ZTP synthesis. In contrast to other triphosphate ribotides, whose synthesis involves a two-step process controlled by specific monophosphate kinases and a nonspecific diphosphate kinase, ZTP is made by pyrophosphate transfer onto ZMP in a single enzymatic reaction catalyzed by PRPP synthase (Fig. 2) (Sabina *et al.*, 1984).

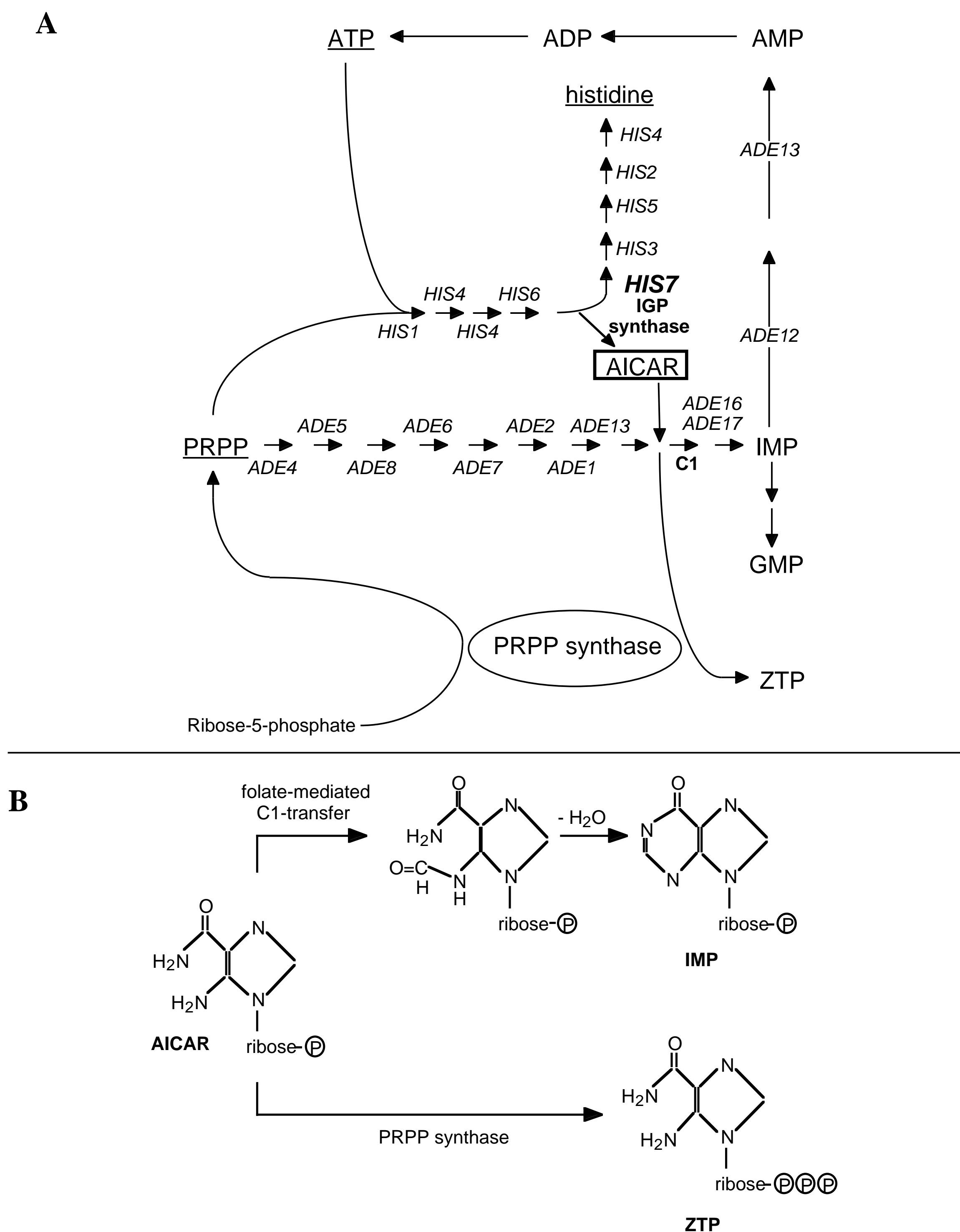

Figure 2. A. Scheme of the purine salvage pathway. Indicated and written in italic are the *HIS* and the *ADE* genes encoding the histidine and adenine biosynthetic enzymes of *S. cerevisiae*, respectively. AICAR (ZMP): 5´-phosphoribosyl-4-carboxamide-5-aminoimidazole; ZTP: 5´-phosphoribosyl-4-carboxamide-5-aminoimidazole-triphosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; IMP: imidazole monophosphate; AM/D/TP: adenosine mono/di/tri-phosphate; GMP: guanosine monophosphate. **B**. Reactions from AICAR to either IMP or ZTP.

Interesting effects on gene expression have been observed in folate-deficient *S. typhimurium* cells. The availability of 10-formyl-tetrahydrofolate influences the mode of derepression – sequential or simultaneous – of the genes clustered in the *his* operon, possibly by affecting the mechanism of translation coupling at the intercistronic barriers (Petersen *et al.*, 1976a; Petersen *et al.*, 1976b). Addition of inhibitors of folate metabolism induces polarity and affects the rate of decay or processing of several polycistronic mRNAs (Alifano *et al.*, 1994). Other bacteria as *Bacillus subtilis* respond to folate shortage by initiating sporulation (Freese *et al.*, 1979). The involvement of AICAR as a metabolic regulator in different aspects of bacterial life is also suggested by the fact that 5-aminoimidazole-4-carboxamide-riboside or, more likely, its phosphorylated form, AICAR, is required throughout development during the process of nodule formation by many *Rhizobium* species (Newman *et al.*, 1994). However, the involvement of ZTP in these processes is speculative, and the evidence for a folate stress regulon controlled by ZTP remains elusive.

2. Genes encoding IGP synthase, the enzyme at the juncture

2.1 In prokaryotes two structural genes of the *his*-operon encode the bifunctional IGP-synthase

The IGP synthase is a bi-enzyme complex and comprises two enzymatic activities namely a glutamine amidotransferase and a cyclase activity (Beismann-Driemeyer and Sterner, 2001). Both subunits reside stoichiometrically in the holoenzyme (Lang *et al.*, 2000). In bacteria the genes encoding both enzymes are typically structural genes of the *his* operons (Alifano *et al.*, 1996). *hisH* is the structural gene encoding the glutamine amidotransferase activity which is identified for the gram-negative bacteria *Escherichia coli*, *Salmonella typhimurium, Pseudomonas aeruginosa, Haemophilus influenzae*, *Azospirillium brasilense*, and the cyanobacterium *Synechocystis* PCC6803 as well as for the gram-positive bacteria *Lactococcus lactis* and *Streptomyces coelicolor* (Tab. 1). Recently the *hisH* gene from the low GC content *Thermoanaerobacter ethanolicus* and

the hyperthermophilic *Thermotoga maritima* were isolated and characterized (Beismann-Driemeyer and Sterner, 2001; Erbeznik *et al*., 2000). The lengths of the respective coding regions of the *hisH* genes from these organisms range from 557 base pairs (bp) to 666 bp and the predicted amino acid sequences are highly conserved among them (Fani *et al*., 1998).

The gene encoding the cyclase activity is named *hisF*. It was identified for the bacteria as listed in Table 1. The coding regions consist of 732 bp to 786 bp in the different bacteria and the deduced amino acid sequences again share high similarities (Fani *et al*., 1998). The only archaeon for which the *hisH* and *hisF* genes have so far been identified is *Methanococcus jannaschii* (Bult *et al*., 1996).

In the bacteria *E. coli*, *S. typhimurium*, *K. pneumoniae*, *H. influenzae* the *hisH* and *hisF* genes are clustered in single compact *his* operons. On the other hand in *P. aeruginosa* both genes are separated from the other *his* sequences. Also for *Streptomyces coelicolor* the *his* genes are partially scattered on the chromosome (Limauro *et al*., 1992). Interestingly the nine histidine biosynthetic genes of the archaebacterium *Methanococcus jannaschii* are scattered throughout the chromosome and are transcribed from both strands (Bult *et al*., 1996). Moreover, their relative order is not at all reminiscent of the operon organization among bacteria.

Organism (b: bacteria a: archaea e: eukarya)	ORF encoding glutamine amidotransferase activity (length/bp)	Gene encoding cyclase activity (length/bp)	Reference
E. coli (b)	*hisH* (588)	*hisF* (774)	Carlomagno *et al*., 1988
S. typhimurium (b)	*hisH* (582)	*hisF* (774)	Carlomagno *et al*., 1988
P. aeruginosa (b)	*hisH* (558)	*hisF* (755)	Burrows *et al*., 1996
C. glutanicum (b)	*hisH* (nd)	*hisF* (771)	Jung *et al*., 1998
A. brasilense (b)	*hisH* (576)	*hisF* (786)	Fani *et al*., 1993
L. lactis (b)	*hisH* (606)	*hisF* (732)	Delorme *et al*., 1992
S. coelicolo (b)	*hisH* (666)	-	Limauro *et al*., 1990
T. ethanolicus (b)	*hisH* (nd)	*hisF* (762)	Erbeznik *et al*., 2000
T. maritima (b)	*hisH* (nd)	*hisF* (nd)	Thoma *et al*., 1998
K. pneumoniae (b)	*hisH* (nd)	*hisF* (776)	Rieder *et al*., 1994
M. jannaschii (a)	*hisH* (nd)	*hisF* (nd)	Bult *et al*., 1996
S. cerevisiae (e)	*HIS7* (1656)		Kuenzler *et al*., 1993
A. thaliana (e)	*hisHF* (1774)		Fujimori and Ohta, 1998

Table 1. Identified and characterized genes of various organisms encoding glutamine amidotransferase and cyclase subunits of IGP synthase. All *his* genes of bacteria identified so far are structural genes of either a histidine or another operon. Both activities of the IGP synthase are encoded by individual structural genes and are transcribed from a single promoter that regulates the respective operon. In contrast in the eukaryotes *S. cerevisiae* and *A. thaliana* single *his* genes with their own promoters encode both activities of the IGP synthase. nd written in brackets behind the gene name means that they have not yet been described in further detail in literature.

2.2 In eukaryotes both activities of the IGP synthase are encoded by one single gene

The budding yeast *Saccharomyces cerevisiae* is the only eukaryotic organism whose entire histidine biosynthetic genes have been isolated and characterized. In contrast to most of the bacterial *his* genes, the yeast *HIS* genes are scattered throughout the genome distributed over six chromosomes. The two enzymatic activities of the IGP synthase are encoded in *S. cerevisiae* from the single *HIS7* gene on chromosome II (Kuenzler *et al*., 1993). The coding sequence of the gene consists of 1656 bp and is not interrupted by any introns. This is little more than the additive lengths of the bacterial *hisH* and *hisF* genes. Alignments of its deduced amino acid sequence with the bacterial gene products of *hisH* and *hisF* revealed a linear composition of both enzymes in His7p and its encoding gene in yeast, separated only by a linker region of 21 amino acids in length. This linker region shares homology with neither bacterial hisHp/hisFp, nor with any other yeast proteins (Kuenzler *et al*., 1993).

Arabidopsis thaliana is the only further eukaryotic organism for which a homologous gene, encoding a protein with the same function as the yeast HIS7p, has been so far identified (Fujimori and Ohta, 1998). In dependence on the notation of the bacterial *hisH* and *hisF* genes the *A. thaliana* gene has been named *hisHF*. The deduced amino acid sequence of *hisHF* is highly similar to that of yeast *HIS7*. The *A. thaliana hisHF* cDNA complements a histidine auxotrophic *his7Δ* mutant strain (Fujimori and Ohta, 1998).

3. Regulation of IGP-synthase encoding genes in prokaryotes and eukaryotes

3.1 The majority of the *his* genes of prokaryotes are clustered and organized in operons simultaneously regulated upon histidine availability

The considerable metabolic cost of the histidine biosynthesis accounts for the evolution of multiple and complex strategies to finely tune the rate of synthesis of this amino acid in different organisms to the changeable environment. In *S. typhimurium* and *E. coli*, the biosynthetic pathway is under the control of distinct regulatory mechanisms that operate at different levels.

As mentioned in chapter 1.1. the metabolic flux through the histidine biosynthesis is mainly regulated by the first enzyme of the biosynthetic pathway, the *N*´-5´-phosphoribosyl-ATP transferase. The abundant presence of the end product of the catalyzed reaction PRATP together with the end product of the pathway histidine strongly feedback inhibits the enzyme. Moreover, high levels of AMP indicate a disadvantageous economical starting point for the histidine biosynthesis and therefore inhibit the transferase activity.

Besides the strong regulation of the first enzyme of the pathway, the variegated transcriptional regulation of the *his* operon and the subsequent mRNA turnover are of significant importance for the controlled biosynthesis of histidine. The *his* operons of *S. typhimurium* and *E. coli* are transcribed into a polycistronic mRNA extending from the primary $E\sigma^{70}$ promoter, *hisp1*, to the bi-directional terminator located at the end of the gene cluster (Carlomagno *et al.*, 1988; Frunzio *et al.*, 1981). The transcription of the operon is strongly regulated by the availability of histidine. This transcriptional control works by an attenuation mechanism at the level of the leader region preceding the first structural gene and depends on the availability of charged histidyl-tRNAs (Artz and Broach, 1975; Barnes, 1978; Kolter and Yanofsky, 1982). A his-specific translational control of transcription termination is the essence of attenuation control. It is mainly achieved by a short coding region in the mRNA leader that includes numerous tandem codons specifying histidine (7 histidine codons in a row of 16) and overlapping regions of dyad symmetry that may fold the mRNA into alternative secondary structures, one of which includes a rho-independent terminator. In the termination configuration a stable stem-loop structure constitutes a strong intrinsic terminator whereas in the antitermination configuration this constitution is prevented. The equilibrium between these alternative configurations is determined by the ribosome occupancy of the leader region, which in turn depends on the availability of charged histidyl-tRNA. High levels of charged histidyl-tRNA cause the termination configuration leading to the loss of

ribosomes that in consequence results in premature transcription termination (Yanofsky, 2000).

A positive transcriptional control of this operon in *S. typhimurium* upon amino acid starvation in the presence of sufficient histidine has been reported (Venetianer, 1969). They found that guanosine 5´-diphosphate 3´-diphosphate (ppGpp), which is the effector of the stringent response (Cashel and Rudd, 1987), positively regulates the *his* operon expression by stimulating its transcription initiation under conditions of moderate amino acid starvation and in cells growing in minimal medium.

A further regulation of the *his* operon takes place at the level of mRNA processing and decay. The unstable primary 7.300-nucleotide-long transcript has a half-life of about 3 minutes. The 5´-to-3´directed decay process generates three major processed species, 6.300, 5.000, and 3.900 nucleotides in length that encompass the last seven, six, and five cistrons, respectively, and have increasing half-lives (5, 6, and 15 min. respectively). As the shortest species spans the distal cistrons that are not only involved in the histidine but also in the purine biosynthesis this uncommon stability suggests a functional link of both biosyntheses to the mRNA turnover (Alifano *et al*., 1994).

3.2 In eukaryotes the metabolic link between the histidine and the purine biosynthesis is reflected by the regulation of the gene at the juncture

S. cerevisiae is the only eukaryotic organism whose histidine biosynthesis and the regulation of its genes has been investigated in detail. The organisation and regulation of these genes in yeast seem to be generally different to that of bacteria. As described in chapter 2.2 the histidine biosynthetic genes in *S. cerevisiae* are scattered throughout the genome with each gene possessing its own promoter. Moreover, in contrast to bacteria the processes of transcription and translation are temporally and spatially separated in eukaryotes due to the compartmentation of the cell with a nucleus distinguished from the cytoplasm.

In *S. cerevisiae* starvation for only one of most of the amino acids leads to the coordinate derepression of more than 50 biosynthetic genes in 11 different biosynthetic pathways, mainly resulting in increased biosynthesis of amino acids. Such a regulatory network has also been described for other several fungi, e.g. for the filamentous fungus *Aspergillus nidulans* that is by reason of its cell differentiation and development a model organism of great interest (Adams *et al*., 1998; Yager, 1992). The expression of the gene encoding the IGP synthase in yeast, *HIS7*, is moreover activated upon starvation for purines. This various transcriptional regulation of the *HIS7* gene reflects the position of its enzyme at the juncture of two biosynthetic pathways.

3.2.1 The transcriptional activation of the *HIS7* gene upon amino acid starvation – the 'general control system' of *S. cerevisiae*

The 'general control system' of *S. cerevisiae* up-regulates the expression of amino acid biosynthetic genes during amino acid starvation conditions in order to counteract the shortage by increasing the own amino acid production. The key player of this regulatory network is the transcriptional activator Gcn4p whose expression is elevated during starvation conditions, and that activates the transcription of biosynthetic target genes through binding to their promoters.

Uncharged tRNAs in the cytoplasm as consequence of shortcoming for any or several amino acid(s) are detected by a sensor protein, Gcn2p, that in turn phosphorylates the translation elongation initiation factor eIF-2 (Dong *et al*., 2000; Qiu *et al*., 2001). eIF-2 is a trimeric protein complex formed by three different subunits, α, β, and γ. Together with GTP that binds to the γ-subunit, and an initiator tRNA charged with methionine it forms a ternary complex (Fig. 3 A). This complex associates with a small ribosomal subunit to form a 43 S preinitiation complex, which binds to mRNAs near its capped 5´-end and migrates downstream to the AUG start codon. Following AUG recognition, the GTP bound to eIF-2 is hydrolysed to GDP and eIF-2 is released as an eIF-2-GDP binary complex. To re-form the ternary complex for further translational initiation events, the

GDP bound to eIF-2 must be replaced by GTP, and this nucleotide exchange reaction is catalysed by a factor known as eIF-2B (Fig. 3 A) (Merrick, 1992).

Phosphorylation of the α-subunit of eIF-2 by the sensor kinase Gcn2p inhibits the guanine nucleotide exchange on eIF-2 that in turn diminishes the formation of new ternary complexes (Fig. 3 B). This in turn lowers the translation of most mRNAs at the ribosomes, and in accordance with the harsh nutritional conditions during amino acid starvation the result is reduction of protein syntheses. Because of its specific 5´ untranslated sequence (UTR) upstream of the actual coding sequence for the protein, the mRNA of the transcription factor Gcn4p behaves quite converse to most of the other mRNAs. Under those circumstances the *GCN4* mRNA becomes much more efficiently translated and therefore appears in greater abundance in the cell (Hinnebusch, 1994; Qiu *et al*., 2000).

Following the migration into the nucleus of the cell, Gcn4p binds to the promoters of more than 50 target genes from different biosynthetic pathways and thereby activates their transcription. The promoter of the *HIS7* gene possesses two Gcn4p recognition elements (GCREs) and gene transcription is activated through the transcription factor via both *cis*-elements during amino acid starvation (Springer *et al*., 1996). Besides the *HIS7* gene also the transcription of *HIS1*, *3*, *4*, and *5* increases upon amino acid starvation conditions (see also Fig. 2) (Arndt *et al*., 1987; Hill *et al*., 1986; Hinnebusch and Fink, 1983; Nishiwaki *et al*., 1987). Altogether the derepression of the histidine biosynthetic genes increases the flux through the pathway finally leading to increased amounts of histidine in the cell.

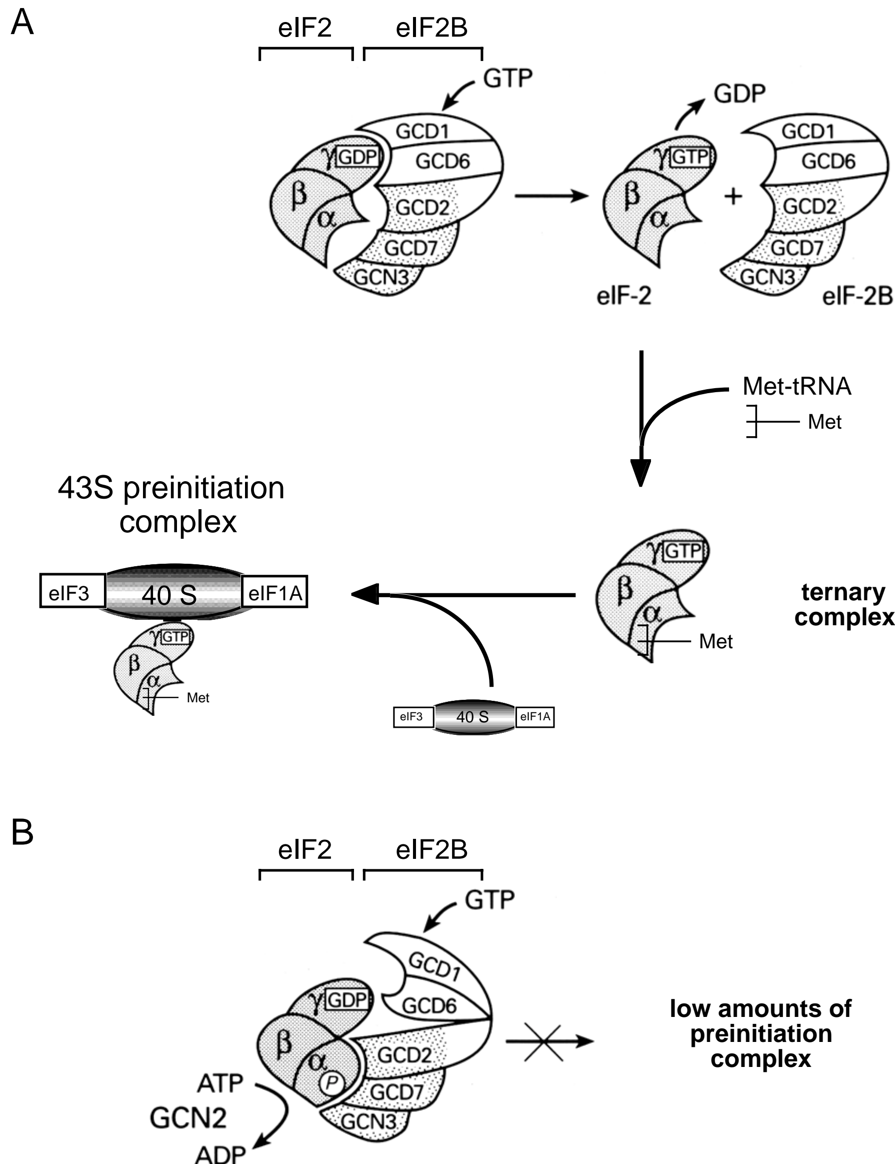

Figure 3. Model for inhibition of the guanine nucleotide exchange activity of eIF-2B by phosphorylated eIF-2. The heterotrimeric eIF-2 complex is shown shaded with a binding site for GDP or GTP on the γ–subunit. The five subunits of the eIF-2B complex are labeled by their gene designations in yeast. **A** The exchange of GDP for GTP on eIF-2 catalysed by eIF-2B and the subsequent formation of the ternary complex, finally leading to the 43S preinitiation complex. **B** The α-subunit has been phosphorylated on Ser52 by Gcn2p. This leads to a stronger interaction between eIF-2α and the Gcn3p, Gcd7p and Gcd2p subunits of eIF-2B; it also leads to a structural change in the Gcd6p and Gcd1p subunits that prevents GDP-GTP exchange on eIF-2. Therefore eIF-2B cannot catalyse nucleotide exchange on phosphorylated eIF-2. The greater affinity of eIF-2B for phosphorylated versus unphosphorylated eIF-2 prevents the nucleotide exchange. As result only small amounts of ternary complex and sequentially 43S preinitiation complex turn out. Scheme adapted from (Hinnebusch, 1994).

3.2.2 'Cross-pathway control' and amino acid biosynthesis of the filamentous fungus *Aspergillus nidulans*

Only some amino acid biosynthetic genes have been identified in other fungi besides yeast so far. The first *his* gene encoding IGP synthase of filamentous fungi has been described during the course of this work (Valerius *et al*., 2001). Work with e.g. *Neurospora crassa*, *Aspergillus niger*, *Cryphonectria parasitica* and *Aspergillus nidulans* has demonstrated that a similar regulatory response upon amino acid starvation conditions exists as described for *S. cerevisiae* in the preceding chapter (Eckert *et al*., 2000; Nishiwaki *et al*., 1987; Wang *et al*., 1998; Wanke *et al*., 1997). In filamentous fungi this regulatory network is called 'cross-pathway control'. In *A. nidulans* the transcription of the histidine and tryptophan biosynthetic genes *hisB* and *trpB*, respectively, has recently been demonstrated to be activated upon starvation conditions (Busch *et al*., 2001; Eckert *et al*., 2000).

Whereas the expression of the yeast *GCN4* gene is mainly translationally regulated and only weakly on the transcriptional level (Albrecht *et al*., 1998), the corresponding gene encoding the similar and exchangeable transcription factor in *A. nidulans*, *cpcA*, is significantly regulated on both levels (Hoffmann *et al*., 2001).

3.2.3 Purine limitation activates the *HIS7* expression in the yeast *S. cerevisiae*

The *HIS7* gene encodes the enzyme at the juncture of the histidine and the *de novo* biosynthesis of purines. The metabolic linkage between both pathways described before is genetically represented by the transcriptional regulation of this gene upon the availability of exogenous purines. The access to plenty amounts of purines represses the most portion of the basal *HIS7* transcription that exists in the absence of amino acid starvation (Springer *et al*., 1996). This transcriptional regulation is mediated through the joint action of the two proteins Bas1p and Bas2p (= Pho2p = Grf10p). Bas1p contains a region that is related to the DNA-binding motif of the myb oncoproteins and has been shown to have a similar DNA-binding specificity as Gcn4p. The homeodomain protein

Bas2p has been implicated in phosphate regulation of diverse genes such as *TRP4* (Braus *et al*., 1989), and *PHO5* (Vogel *et al*., 1989), and is apparently involved in the regulation of yeast mating type switching (Brazas and Stillman, 1993). The basal *HIS7* transcription during purine limitation conditions is driven by the heterodimeric complex formed by Bas1p and Bas2p that also recognizes the *HIS7* promoter at one of the two Gcn4p recognition elements. Recent work has strengthen the assumption of former studies that in the presence of adenine the complex formation of the heterodimeric Bas1p/Bas2p is prevented and therefore *HIS7* activation impaired (Pinson *et al*., 2000; Zhang *et al*., 1997).

Primarily Bas1p and Bas2p regulate the transcription of all genes required for the ten enzymatic reaction steps of the *de novo* biosynthesis of purines (see also Fig. 2). Dependent on Bas1p and Bas2p, adenine limitation increases the expression of these *ADE* genes finally leading to the increased flux through the purine biosynthetic pathway (Denis *et al*., 1998). Not only the *HIS7* gene at the branch-point to the purine pathway itself but also two of three genes encoding the preceding enzymes of the histidine pathway, *HIS1* and *HIS4*, are co-regulated with the purine biosynthetic genes (see also Fig. 2). The substantial availability of these preceding activities thereby provides the IGP synthase with sufficient substrates finally leading to increased amounts of AICAR which then feed the purine biosynthetic pathway. In accordance with this assumption the *HIS* genes encoding the activities succeeding that of the IGP synthase are not derepressed by adenine limitation (Denis *et al*., 1998). In contrast to the general control system that regulates *HIS7* expression during amino acid starvation, the control system for the basal expression of purine or histidine biosynthetic genes during purine limitation has neither been described for fungi nor for other eukaryotic organisms yet.

4. Modulation of the chromatin structure is an essential step for the regulation of gene expression in eukaryotes

4.1 The transcriptional regulation of gene expression strongly depends on the local chromatin structure

The transcriptional regulation of gene expression is not confined to activators (as described above for CPCA; Gcn4p; Bas1p/Bas2p) but also depends on repressors and antirepressors of transcription. The ubiquitous repressors in eukaryotic organisms are nucleosomes. DNA of eukaryotes is tightly associated with and thoroughly packaged by protein complexes together forming the so-called chromatin. This chromatin is organized in a hierarchy of structures, from the basic repeat unit called nucleosome to the complex appearance of metaphase chromosomes (Fig. 4 A) (Wolffe, 1995). The nucleosomes consist of an octameric protein complex surrounded by about 120 to 200 base pairs of DNA that wraps around the protein core in 1.65 left-handed turns of a superhelix (Fig. 4 B left) (Luger *et al.*, 1997). The octameric complex consists of two of each histone proteins H2A, H2B, H3, and H4, and is also called histone octamer (Fig. 4 B right). The nucleosomes are connected with each other by the linker DNA and thereby form structures like beads on a chain resulting in the 100 Å (10nm) chromatin fiber. On a higher order level these nucleosome chains form densely packed 300 Å (30 nm) chromatin fibers that themselves are highly ordered to the tightly condensed chromatin of the nucleus (Fig. 4).

Each level of chromatin organization contributes to the dense packaging of DNA, effectively repressing gene expression. However, all nuclear processes, including transcription, require that enzymes gain access to the DNA template despite the fact that it is associated with histone and also non-histone proteins. The presence of nucleosomes on DNA generally restricts the access of proteins to the DNA, thereby suppressing gene expression. Nucleosomes inhibit both the binding of RNA polymerase II to initiation sites and transcriptional elongation (Kornberg and Lorch, 1999).

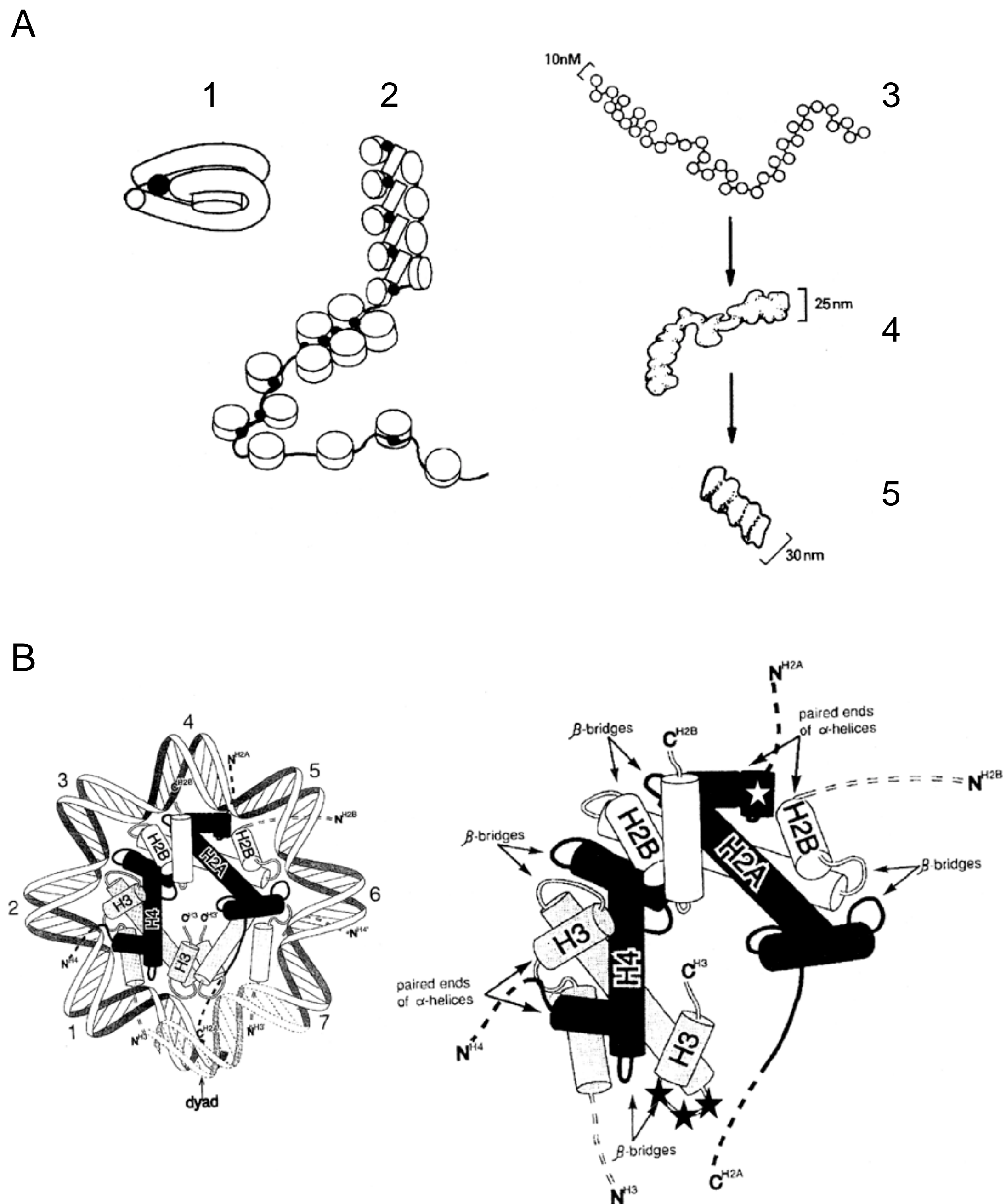

Figure 4. Chromatin and nucleosome structure. A Different levels of chromatin packing. A model (top left) of nucleosome with associated proteins (black ball). In the middle a model of the 'beads-on-a-string' form of chromatin. At right folding intermediates from 'beads-on-a-string' to the 30-nm fiber. **B** Left: Structural model for the interaction of the core histones with DNA in the nucleosome. The view is one turn of DNA. For clarity only one molecule of H2A, H2B and H4 is shown. Right: Scheme of the interactions between heterodimers of H2A, H2B and H3-H4. The sites of primary interaction of the histone fold domains with DNA are indicated (the paired ends of helices and bridge motifs). Adopted from (Perez-Martin, 1999).

Mapping of chromatin structure using a variety of nucleases has shown that the 5´ regulatory regions of active genes often appear to be nucleosome-free. Such regions are referred to as ‘hypersensitive sites’ (Elgin, 1988). In some cases hypersensitive promoters of inducible genes maintain in this configuration even during the repressed state. Such genes are referred to as ‘pre-set’, as no change in the nucleosome array at the promoter is required for activation. It has been suggested that housekeeping genes might fall into this group, although relatively few studies have been performed on such genes. In other cases, 5´ regulatory regions, including the promoter, are packaged into a nucleosome array when the gene is in an inactive state. In this case remodeling of the chromatin structure is an integral part of the process of specific gene activation (Wallrath *et al*., 1994).

Besides the repressive role of nucleosomes in the primary chromatin fiber, higher-order packaging is of critical importance. While higher-order structures are not well defined, the genome is subdivided by boundaries that limit the regulatory effects of positive and negative elements such as enhancers or repressors (Bell and Felsenfeld, 1999). Further, one can identify large domains that are either permissive or restrictive for gene expression. This level of gene regulation apparently depends on DNA packaging in scaffold/matrix-attached regions, so called S/MAR elements (Bode *et al*., 2000; Gasser *et al*., 1998).

4.2 Chromatin modifying activities diversify the chromatin structure and enable regulated gene expression

Recent advances highlight two important chromatin remodeling systems involved in the transcriptional process. One system includes several members of the evolutionary conserved *SW2=SNF2* family found in distinct multiprotein complexes with ATP-dependent nucleosome destabilizing activity. The other is the enzymatic system that governs histone acetylation and deacetylation. Identification of the catalytic subunits of

these opposing histone-modifying activities reveal conserved proteins defined genetically as transcriptional regulators.

Further regulation systems of gene expression that work by modifying histones are phosphorylation and methylation. Phosphorylation and dephosphorylation of the Ser10 residue of histone H3 might regulate chromosome condensation and the associated changes of gene expression during entry into mitosis. The enzymes regulating these modifications, the kinases as well as the phosphatases, have been identified in yeast and have been shown to have homologs in higher cells. Methylation of the Lys9 residue of histone H3, which was shown to interfere with phosphorylation of H3 Ser10, is another histone modification with consequences on gene expression, however poorly understood at present time.

4.2.1 Swi/Snf mediated ATP-dependent nucleosome remodeling

Swi/Snf is a protein complex that activates expression of several genes in yeast. The description of the Swi/Snf complex was the result of a convergence of genetic and biochemical studies. The *SWI* genes (*SWI* = *swi*tch in mating type) were identified as being important for transcription of the *HO* gene that encodes an endonuclease required for mating type switching (Stern *et al.*, 1984). On the other hand, *SNF* (*SNF* = sucrose non-fermentable) genes were identified to be required for transcription of the *SUC2* gene that encodes invertase, the enzyme required by *S. cerevisiae* to catabolize sucrose (Neigeborn and Carlson, 1984).

The first indication of the function of *SWI* and *SNF* genes outside the transcription of *HO* and *SUC2* was that mutant strains defective in some of these genes exhibit slow growth and other phenotypes like inositol auxotrophy or inability to use galactose as carbon source (Peterson and Herskowitz, 1992). It was found that *SWI2* was identical to *SNF2*, and hence, the gene is referred to as *SWI2*/*SNF2*. Further experiments revealed a functional interdependence among some of the Swi and Snf proteins which indicated that they may act together as a complex (Laurent *et al.*, 1991). Biochemical studies of

Swi/Snf proteins led to the purification of a 2-MDa protein complex, which is known as the Swi/Snf complex (Cairns *et al.*, 1994). This complex is composed of the Swi Proteins. Swi2p/Snf2p, Swi3p, Snf5p and Snf6p and five additional polypeptides: Swp82p, Swp73p, Swp61p, Swp59p, and Swp29p (Swp = protein of the Swi/Snf complex).

The connection between the function of the Swi/Snf complex and chromatin was first established through genetic studies. The chromatin structure surrounding the *SUC2* promoter changes in response to the induction of transcription of this gene. Mutations in either *SWI2/SNF2* or *SNF5* result in a decrease in transcription and in a chromatin structure more resistant to digestion by micrococcal nuclease, even in induced conditions (Matallana *et al.*, 1992). This result was an indication of a failure of the mutant cells to antagonize nucleosomal organization at the promoter region.

The biochemical characterization of the purified yeast Swi/Snf complex provides direct evidence that the complex might function by disrupting nucleosome structure. Binding of Gal4p derivatives to a reconstituted mono-nucleosome carrying a single Gal4p binding site is substantially facilitated by purified Swi/Snf complex in a reaction that requires ATP hydrolysis and is independent of the presence or absence of activation domains in various Gal4p derivatives (Cote *et al.*, 1994). Purified Swi/Snf complex is also able to disrupt an array of pre-assembled nucleosomes reconstituted with purified histones in an ATP-dependent manner (Owen-Hughes *et al.*, 1996).

The mechanisms by which the Swi/Snf complex facilitates the accessibility to nucleosomal DNA is not known. It is clear that the activity of the complex requires continuous ATP hydrolysis. The *SWI2* gene encodes a protein that contains motifs similar to those found in DNA-stimulated helicases. In fact, the Swi2 protein has a DNA-dependent ATPase activity (Laurent *et al.*, 1993). A model suggests that these factors may function as ATP-driven motors that translocate along DNA and destabilize DNA-protein interactions. A DNA translocation protein might use the energy derived from ATP hydrolysis to transverse a nucleosome in a wave-like manner that results in only

partial disruption of the nucleosome at any particular point. The transcription factors use this transitory disruption to reach its DNA target. This model is similar to the 'spooling mechanism' that has been suggested for the procession of polymerases through nucleosomes (Studitsky *et al.*, 1995). However, this model of Swi/Snf action does not explain the observation that the action of this complex on nucleosomes results in a stable remodeled form of nucleosomes (Schnitzler *et al.*, 1998). An alternative model proposes that the Swi/Snf complex interacts with nucleosomal DNA and uses the energy of ATP hydrolysis to alter DNA-histone interaction. In its original conception it was supposed that the action of the complex promotes the loss of one or both H2A-H2B dimers from the nucleosome core (Peterson and Tamkun, 1995), but recent data about *in vitro* Swi/Snf-altered nucleosomes indicate that there is no loss of histones (Burns and Peterson, 1997).

The question of how the Swi/Snf complex is targeted to the correct chromosomal position is also poorly understood. Since the complex with approximately 100 copies per cell is not abundant, the possibility that it is a general chromatin component is ruled out. One possibility is that the Swi/Snf complex associates with activator proteins subsequently targeting them to specific genes. However, recent results suggest that it is rather the chromosomal context of the binding site of the activator that determines the Swi/Snf dependence of transcription (Burns and Peterson, 1997). Another possibility would be that the complex is recruited to promoters along with the transcriptional machinery (Wilson *et al.*, 1996). However, a strong or stable association with the RNA polymerase holoenzyme has been questioned (Cairns *et al.*, 1996). Still another alternative would be to assume that one of the Swi/Snf subunits has sequence-specific DNA binding affinity that provides promoter specifity (Quinn *et al.*, 1996). An interesting clue is the interaction of the Swi/Snf complex with the SAGA complex, which is recruited by specific activators.

Several candidates for functional homologs of *SWI2/SNF2* have been identified as part of large multiprotein complexes in higher eukaryotes. Therefore complexes that belong to

the Swi/Snf family have been suggested for *Drosophila melanogaster* (named brahma, *brm*) or human cells (*hbrm*) (Dingwall *et al*., 1995; Tamkun *et al*., 1992).

4.2.2 The SAGA complex (Spt/Ada/Gcn5 Acetyltransferase)

Histone acetylation plays an important role in chromatin assembly and transcription. Acetylation occurs *in vivo* only at specific lysines in the amino-terminal tail of histones. Although these tails are not required to maintain the structural integrity of the nucleosome, they are implicated in arranging nucleosomes into higher order chromatin structures. Acetylation of the histone tails may introduce allosteric changes in nucleosome conformation and inhibit the higher order folding of nucleosome arrays that are repressive to transcription (Bauer *et al*., 1994).

Two major histone acetyltransferase (HAT) activities have been described in eukaryotic cells. The first is a cytoplasmic enzyme complex, called HAT-B, involved in the deposition-related acetylation of H4 onto replicated DNA.

The second type of acetyltransferase activity, HAT-A, has been associated with the nucleus and is responsible for transcription-associated acetylation. The HAT-A activity is encoded by the *GCN5* gene and was initially identified in a genetic screen designed to isolate mutants unable to grow under conditions of amino acid limitation (Brownell *et al*., 1996; Georgakopoulos and Thireos, 1992). *gcn*-mutations result in strains unable to induce the general control system (general control non-derepressable). A second screen selecting for mutants that reversed the toxicity (squelching) caused by overproduction of the strong activator Gal4-VP16 also gave this gene and in addition the two genes *ADA2* and *ADA3* (Pina *et al*., 1993). Their three gene products are required for the function of several activators. Genetic and biochemical studies revealed that Gcn5p, Ada2p and Ada3p form a complex, called the ADA complex (ADA for adaptor) (Marcus *et al*., 1994). Purified Gcn5 protein shows histone acetyltransferase activity and is able to acetylate free histones at specific lysine positions (K14 in H3; K8, K16 in H4) (Kuo *et al*., 1996). However, purified Gcn5 protein is unable to acetylate *in vitro* histones already

assembled in nucleosomes, suggesting the possibility that other proteins are required to direct Gcn5p in the acetylation of nucleosomes. A biochemical search for native complexes able to acetylate *in vitro* nucleosomes yielded the isolation of two high molecular mass complexes, 0.8 and 1.8 MDa (Grant *et al.*, 1997). Both complexes contain Gcn5p, Ada2p and Ada3p. The larger of these two complexes turned out to contain Spt proteins (Spt20p, Spt3p, Spt8p and Spt7p) and is called SAGA (Spt/Ada/Gcn5 acetyltransferase). Spt proteins have originally been isolated as suppressors of transcriptional initiation defects caused by promoter insertions of the transposon Ty and are supposed to play a role in TATA-binding protein (TBP) function (Winston and Sudarsanam, 1998). The relationship between ADA and SAGA is not yet clear. One possibility is that ADA is a subcomplex of the larger SAGA complex. An alternative possibility is that each complex might represent quite distinct nucleosomal HAT activities with unique functions in the cell.

Overexpression of *GCN5* leads to increased acetylation of core histones. Moreover, Gcn5p increases histone acetylation at promoter regions in a manner that is correlated with Gcn5p-dependent transcriptional activation and histone acetylase activity *in vitro*. The way in which the histone acetylase complex selectively affects gene expression is poorly understood. Gcn5p might be selectively recruited to promoters as it has been shown to interact directly or indirectly through Ada2p with a number of transactivators, for example VP16 (= virus protein 16), Gcn4p, and Adr1p (Chiang *et al.*, 1996; Drysdale *et al.*, 1998; Silverman *et al.*, 1994). In addition to the ability of acidic activators to physically interact with purified native SAGA complex, it has been shown that a Gal4-VP16 fusion targets acetylation and transcriptional enhancement by SAGA (Utley *et al.*, 1998).

An additional link between histone acetylation, activators and the basal transcription machinery is the recent characterization of several TBP-related proteins as components of the SAGA complex. The first group of these components comprises all members of the TBP-related set of Spt proteins (Spt3p, Spt7p, Spt8p and Spt20p), with the exception

of TBP itself (Grant *et al.*, 1998b). The second group is composed of several TAF_{II} (TATA binding protein-associated factors) (Grant *et al.*, 1998a). This association of multiple transcriptional regulatory proteins may confer upon SAGA the ability to respond to a range of stimuli and to interact with numerous activators, with the potential to regulate a broad range of promoters.

5. Transcriptional interference of tandemly transcribed eukaryotic genes

5.1 Adjacent yeast genes in close vicinity are susceptible to transcriptional interference

In compact genomes as e.g. of *S. cerevisiae*, genes often are located in close proximity adjacent to each other (Kruglyak and Tang, 2000). Accordingly, small intergenic regions separate the open reading frames. The average size in-between tandemly transcribed open reading frames of yeast genes which are transcribed into the same direction is 517 base pairs (Dujon, 1996). These intergenic regions contain most of the regulatory elements required for efficient mRNA 3´ end processing and transcription termination (terminator, calculated average length: 163 bp), but also for the controlled initiation of transcription (promoter, calculated average length: 309). As these DNA elements are parts of the respective genes one sometimes can hardly define any intergenic regions (Springer *et al.*, 1997).

For the downstream promoter activity of tandemly repeated genes it is essential that efficient termination of transcription of the adjacent upstream gene has taken place. Incomplete termination of transcription, characterized by RNA polymerase II-transcription complexes still bound to the DNA template and scanning downstream, interferes with the process of assembly of the transcriptional initiation complex at the downstream initiator element. This phenomenon is called ‘transcriptional interference’ and transcription complexes that read far beyond the 3´ end of the initial gene have indirectly demonstrated by Northern hybridisations detecting long read-through transcripts. Transcription ‘run-on’ experiments have directly stated the exact position

where the RNA pol II leaves its template. The prevention of initiation of transcription at the promoter as consequence of transcriptional interference has also been termed ‘promoter occlusion’.

5.2 Natural barriers to transcriptional interference in eukaryotes

During environmental circumstances, i.e. when the upstream one of two adjacent genes is strongly transcriptionally activated it could become a severe disadvantage for the cell if it failed to prevent transcriptional interference. Thus, promoter occlusion might abolish the expression of a possibly essential function of a downstream located gene. Natural barriers and mechanisms that signalize the transcribing RNA polymerase II complex the completion of the original gene in order to terminate and finally release the DNA template are poorly understood.

DNA binding proteins that act as transcription termination factors and therefore as transcriptional barriers were found for RNA polymerase I genes encoding rRNAs. In yeast, mouse and mammals the efficient transcription of downstream rDNA units by RNA polymerase I has been shown to depend on binding of the homologous termination factors Reb1p (yeast) or TTF-I (mouse, man) to the preceding terminator element that belongs to the upstream rDNA transcription unit (Längst *et al.*, 1997; Mason *et al.*, 1997). These termination factors primarily block the transcribing polymerase complex and function thus by pausing the transcription complex on its template. As binding of TTF-I to its terminator element is strictly required to maintain a positioned nucleosome at this locus, a link between transcription termination and chromatin structure for RNA polymerase I genes was suggested (Längst *et al.*, 1997). It remains elusive whether this positioned nucleosome itself or rather TTF-I is the actual barrier for the transcribing complex. The release of the nascent transcript from the ternary complex and the final release of the polymerase from the DNA requires a further protein, the transcription release factor PTRF (Jansa and Grummt, 1999). This protein has been found and isolated from mouse and is also able to liberate the pre-rRNA and the Reb1p-paused transcription

complex from ternary transcription complexes isolated from yeast (Jansa and Grummt, 1999).

For termination of RNA polymerase III transcription *cis*-elements have been described (Hamada *et al.*, 2000). The enzyme terminates transcription at a run of four or more thymidine (T) residues. As some pol III genes contain runs of T residues that are not recognized as termination signals, the adjacent sequence context also seems to be of importance (Gunnery *et al.*, 1999). The pols III of different organisms including *S. cerevisiae*, *S. pombe*, or human exhibit quite distinctive properties. Termination occurs in *S. pombe* in a manner that is functionally more similar to human than it is in *S. cerevisiae*. DNA binding transcription termination factors for pol III genes or the requirement of certain chromatin structures at the terminator elements have not yet been described.

The sites where transcription termination of RNA polymerase II takes place and the enzyme leaves the DNA template are e.g. pause sites for RNA pol II. Functional poly(A) signals in the 3´ end of the respective gene were suggested to be required for the termination process, thus coupling it with mRNA processing that is transcript cleavage and subsequent polyadenylation (Hamada *et al.*, 2000). Recent work has demonstrated that the poly(A) signals themselves rather than the mRNA processing are required for termination activity. mRNA processing itself consists of pre-mRNA cleavage at the poly(A) site and subsequent polyadenylation of the mRNA. Both steps were previously demonstrated to be separable processes (Egli and Braus, 1994). The observation that the phenomena of termination and cotranscriptional RNA cleavage can be uncoupled supports the assumption, that there are template-specific elongation and/or RNA processing factors associated with the transcribing pol II, which are altered upon passage through a poly(A) signal, resulting in termination somewhere downstream, more or less close to the poly(A) site (Osheim *et al.*, 1999). Also DNA regions that pause the transcribing pol II complex can also be involved in termination of transcription by facilitating the release of the complex (Birse *et al.*, 1997). Although there is quite a lot of

knowledge about the relation between initiation/elongation of transcription and the impact on chromatin structure at pol II genes, there are no reports that link the process of termination of pol II transcription to the chromatin structure at the respective terminator.

6. Metabolism and differentiation in fungi

The regulation of biosyntheses within cells and the availability of nutrients from the growth medium have a great impact on the life cycles of fungi. On rich medium, in the presence of sufficiently well utilizable carbon and nitrogen sources, as e.g. glucose and ammonium sulfate, diploid cells of *S. cerevisiae* propagate by budding (Fig. 5). Shifting the cells to minimal medium of poor carbon source, e.g. very low amounts of glucose, leads to meiosis and the formation of asci termed tetrades (tetrade = 4) that carry four meiotic spores inside, two of each mating type. On rich medium each of these spores can proceed in haploid budding cycles or, if bringing cells of opposite mating type together, conjugate to form a diploid zygote that again can proceed propagating by budding. Low amounts of fermentable nitrogen compounds but rich carbon sources drive the diploid cells into another growth form that is called pseudohyphal growth. Under these conditions cells are long shaped and bud unidirectional to form long filaments comparable to hyphe of filamentous fungi (Taheri *et al.*, 2000). Upon such growth conditions the general control system of amino acid biosynthesis is inactivated and the expression of the transcription factor Gcn4p strongly diminished (Grundmann *et al.*, 2001). This points to a close relationship between regulation of amino acid biosyntheses and developmental programs of the yeast life cycle. When diploid cells are starved for nitrogen and carbon they enter the stationary phase.

The life cycle of *A. nidulans* can be subdivided into three stages, an asexual, a parasexual, and a sexual cycle. In respect of cell differentiation mainly the asexual and sexual life cycles are of major interest. The conidiophores, outcome of the asexual reproduction cycle, are multi-cellular structures that finally produce the conidiospores or conidia (Fig. 6). During asexual development the fungus growths radially with long

filaments that form a networked entity called mycelium. Out of these filaments vesicles differentiate that afterwards themselves produce a further individual cell type on their tips, the so-called metulae. Afterwards the phialides differentiate out of the tip of the metulae. These phialides finally produce long rows of conidiospores on their tops.

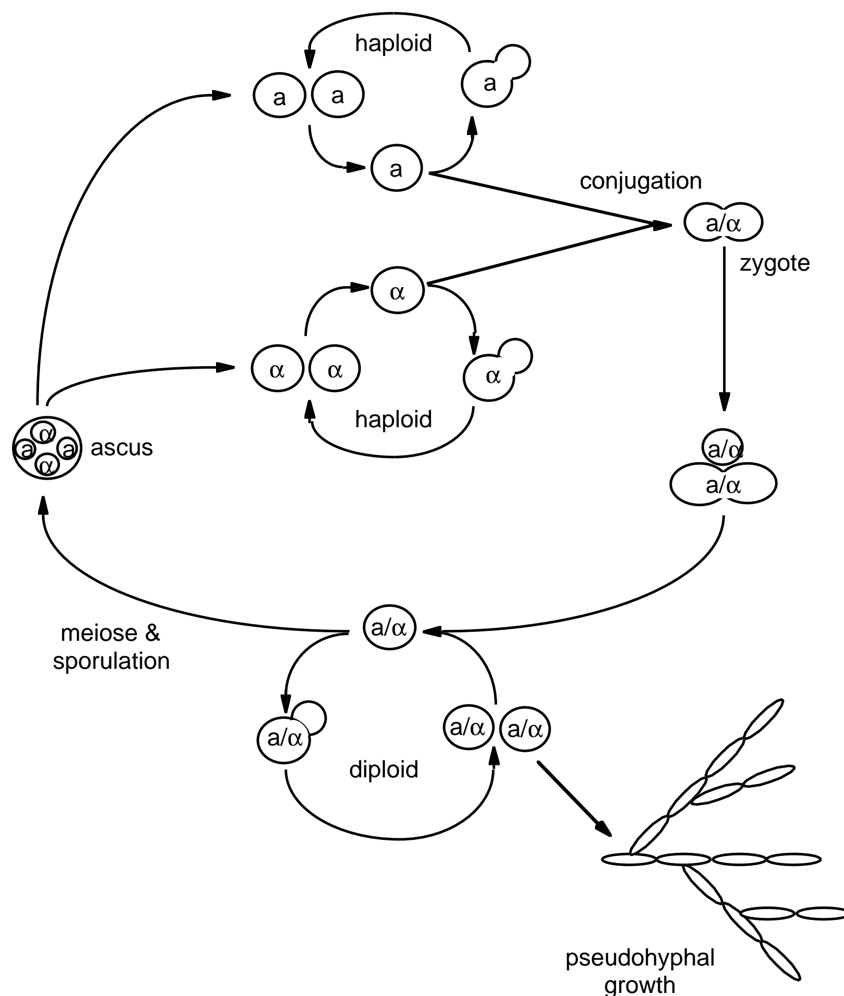

Figure 5. Scheme of the life cycle of *S. cerevisiae*. Presented are the different states through which yeast cells can pass during the life cycle. Cells of the two mating types are indicated with a and α. A poor nitrogen source and a rich carbon source drives the diploid cells into pseudohyphal growth, whereas a poor carbon source drives it into meiosis and sporulation. Haploid cells of opposite mating type deriving from the ascus can either propagate by budding or conjugate to form a diploid zygote.

Results of the sexual life cycle are the ascospore containing cleistothecia (Fig. 6). This cycle is initiated by the cytoplasmic fusion of two individual hyphal cells of either identical strain (selfing) or of two different strains with unequal genotypes (crossing).

This specialized dicaryotic fusion cell with the nuclei of both starting cells in close proximity, divide profusely (up to 10.000 divisions), and form numerous dikaryotic cells in which afterwards fusion of nuclei occur (Hoffmann *et al.*, 2001). After meiosis and following mitosis eight nuclei lay site by site inside of a bi-layered membrane, later on individually packaged as meiospores. These eight meiospores together form one so-called ascus, with about 80.000 of them filling one sexual fruiting body (cleistothecium). The first visible structure that the fungus develops after the fusion of hyphal cells is the so-called nest or primordium, a densely packaged aerial mycelium together with specific globular Hülle cells that are thought to be required for nutrient supply during the development of the fruiting body. The mycelium around the dikaryotic hyphal cells condenses further and finally forms a tight wall around the maturing asci.

Connections between amino acid syntheses and cell differentiation during sexual development of *A. nidulans* have already been shown. Amino acid limitation as well as heterologous overexpression of *GCN4* in *A. nidulans* has been demonstrated to generally impair the development of fertile cleistothecia. Furthermore the deletion of the *cpcB* gene that encodes a repressor of the cross-pathway control also impairs fruiting body formation (Hoffmann *et al.*, 2000). Moreover tryptophan auxotrophic mutant strains require very high exogenous amounts of tryptophan to partially restore the formation of fertile fruiting bodies (Eckert *et al.*, 2000). The genetic and enzymatic requirements for those regulated developmental processes are poorly understood so far and the correlation between the regulatory network for amino acid biosynthesis and cell differentiation is of special interest.

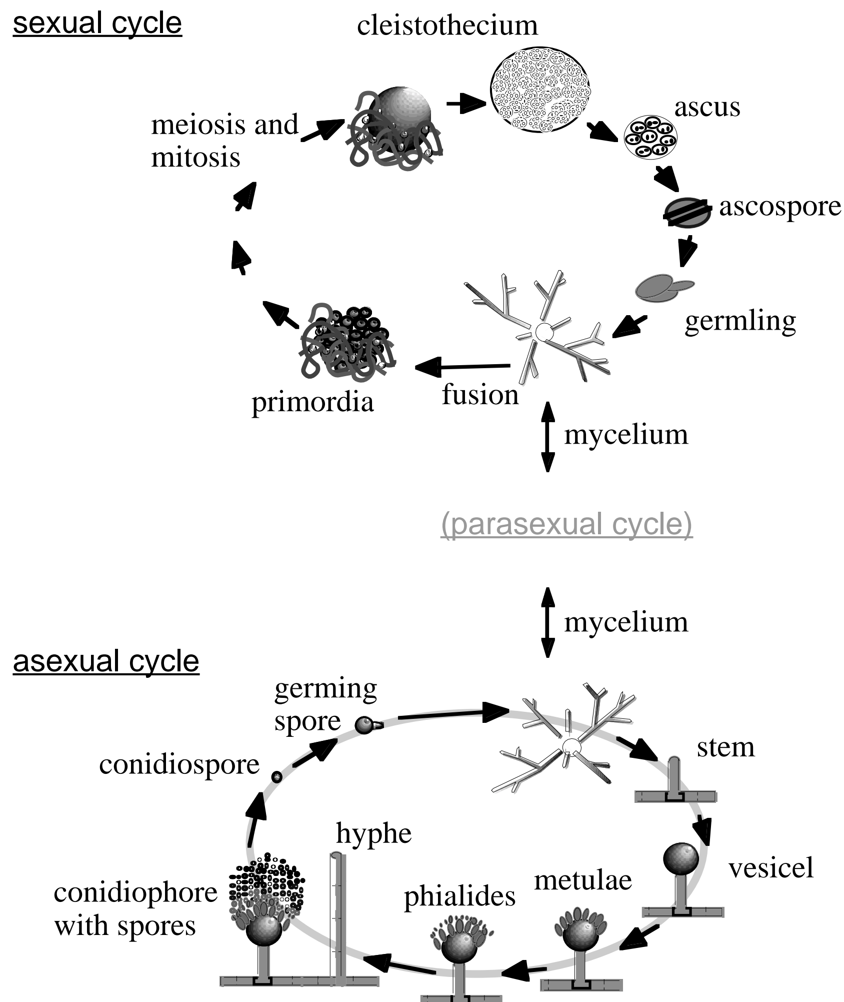

Figure 6. Scheme of the life cycle of *A. nidulans*. On the top the sexual life cycle of the fungus with the final formation of the sexual fruiting body (cleistothecium) is drawn. The arrows indicate the time course. From germination of a spore to the completion of a fertile cleistothecium it takes approximately 200 hours. The bottom sketches the asexual life cycle from a germing spore to the complete conidiophore that carries long rows of conidiospores. The development from the germination of an ascospore to a mature conidiophore takes about 20 hours. The parasexual cycle is only suggested in light grey (Adams *et al*., 1998).

Aim of the work

In this work, the regulation of expression of the gene encoding IGP synthase, an enzyme involved in the biosyntheses of the amino acid histidine and of purines, was investigated. The studies were performed with the genes of two model organisms, *HIS7* of the unicellular fungus *S. cerevisiae* and its homologue *hisHF* of the filamentous fungus *A. nidulans*. The *hisHF* gene from *A. nidulans* should be isolated and its regulation should be investigated with respect to those environmental conditions that are known to regulate the expression of the homologous yeast gene *HIS7*. Since the progression of *A. nidulans* through its developmental life cycle depends on the state of a genetic regulatory network that controls the biosyntheses of amino acids, the impact of *HIS7* expression on the development of the fungus should be analyzed. With the yeast *HIS7* gene as an example for a housekeeping gene, the chromatin structure of its promoter for different environmental conditions that influence its expression should be studied. We were interested in mechanisms that regulate *HIS7* transcription on the level of chromatin. Furthermore, we addressed the question, how an eukaryotic cell avoids transcriptional interference of two genes that are located in close proximity and transcribed into the same direction. Therefore we analyzed to which extend the *HIS7* expression is influenced by the transcription of its upstream gene *ARO4*, and what elements within the *ARO4*-*HIS7* region prevent interference.

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Chapter 2

Regulation of *hisHF* Transcription of *Aspergillus nidulans* by Adenine and Amino Acid Limitation

Abstract

The *hisHF* gene of *Aspergillus nidulans* encodes imidazole-glycerole-phosphate (IGP) synthase consisting of a glutamine amidotransferase and a cyclase domain. The enzyme catalyzes the fifth and sixth step of histidine biosynthesis which results in an intermediate of the amino acid and an additional intermediate of purine biosynthesis. An *A. nidulans* *hisHF* cDNA complemented a *Saccharomyces cerevisiae his7*$^{-}$ strain as well as *Escherichia coli hisH* and *hisF* mutant strains. The genomic DNA encoding the *hisHF* gene was cloned and its sequence revealed two introns within the 1659 bp long open reading frame. The transcription of the *hisHF* gene of *A. nidulans* is activated upon amino acid starvation suggesting that *hisHF* is a target gene of *cross pathway control*. Adenine but not histidine, both end products of the biosynthetic pathways connected by the IGP-synthase, represses *hisHF* transcription. In contrast to other organisms HISHF overproduction did not result in any developmental phenotype of the fungus in hyphal growth or the asexual life cycle. *hisHF* overexpression caused a significantly reduced osmotic tolerance and the inability to undergo the sexual life cycle leading to acleistothecial colonies.

Introduction

The filamentous fungus *Aspergillus nidulans* has become an eukaryotic model organism for metabolic diversity and the genetic regulation of cell differentiation during asexual and sexual development (Adler *et al*., 1996). *A. nidulans* is able to synthesize histidine as well as all other amino acids *de novo*. Studies with several prokaryotic microorganisms gave insight to the biosynthesis of histidine in general (Alifano, et al., 1996). Within the eleven reactions, starting with phosphoribosylpyrophosphate (PRPP) and ATP, leading to histidine, a connection to the *de novo* biosynthesis of purines exists, but none to other amino acids. The histidine and purine biosynthetic pathways are connected by the compound 5-aminoimidazole-4-carboxamide ribotide (AICAR), a byproduct of histidine synthesis and an intermediate of purine biosynthesis. During histidine biosynthesis the intermediate compound imidazole-glycerole-phosphate (IGP) and its byproduct AICAR are formed by two reactions catalyzed by the heterodimeric IGP-synthase.

This IGP synthase comprises a glutamine amidotransferase activity (EC 2.4.2.14) and a cyclase activity (EC 4.3.2.4). In the eubacteria *Escherichia coli* and *Salmonella typhimurium* they are encoded by the structural genes *hisH* and *hisF*, respectively, both part of histidine operons (Beckwith, 1987; Brener, 1971). Regulation studies with these organisms have shown that histidine in the growth medium completely shuts down the expression of the *his* operon. Starvation for histidine results in a strong transcriptional and translational induction, leading to an increased internal histidine pool (Alifano *et al*., 1996; Verde *et al*., 1981). However, enhanced overexpression of the *his* operon in *S. typhimurium* causes growth inhibition at 42°C, a changed cell morphology leading to long filaments, and growth inhibition on increased salt concentrations (Gibert and Casadesus, 1990; Murray and Hartman, 1972; Roth *et al*., 1966). In *E. coli* a similar response was observed (Frandsen and D'Ari, 1993). This pleiotropic response is due to the surplus of hisHp and hisFp that possibly causes a defect in septum formation leading to cell division inhibition and to cell filamentation (Cano *et al*., 1998).

The identified *HIS* genes of eukaryotes are scattered throughout the respective genomes. For *Saccharomyces cerevisiae* or *Arabidopsis thaliana* the heterodimeric IGP-synthases are encoded from the single bifunctional genes *HIS7* and *hisHF*, respectively (Fujimori and Ohta, 1998; Kuenzler *et al.*, 1993). Even with plenty of external histidine the yeast *HIS7* gene is transcribed at a significant basal level. Starvation for any amino acid activates in yeast the *general control system of amino acid biosynthesis* resulting in the transcriptional activation of many target genes from different biosynthetic pathways (Hinnebusch, 1986). The transcription factor that triggers this control system is Gcn4p, whose mRNA is more efficiently translated under those conditions (Mueller and Hinnebusch, 1986). The yeast *HIS7* promoter contains two Gcn4p binding sites, which were shown to be targets for this transcription factor. One of these *cis*-elements is also the binding site for the heterodimeric transcription factor Bas1p/Bas2p that activates the *HIS7* gene and several genes of purine biosynthesis upon adenine or guanine limitation and thereby reflects biosynthetic connection of both pathways (Springer *et al.*, 1996).

For filamentous fungi a regulatory network similar to the general control of yeast has been described as *cross pathway control* (Piotrowska, 1980). The *GCN4* homologous genes of *Neurospora crassa*, *Aspergillus niger* and *Cryphoectria parasitica*, *cpcA*, *cpc1* and *CpCPC1*, respectively, were cloned and characterized (Paluh *et al.*, 1988; Wang *et al.*, 1998; Wanke *et al.*, 1997). The corresponding transcription factor of *A. nidulans* has not yet been described. Homologous proteins to the yeast Bas1 and Bas2 proteins are also not known for this fungus.

In this work we describe the isolation and characterization of the bifunctional *hisHF* gene of *A. nidulans*. We investigated its transcriptional regulation upon amino acid starvation and purine limitation. Furthermore we studied the consequences of *hisHF* overexpression by fusing the gene to the inducible *alcA* promoter. Overproduction of HISHF led to a reduced salt tolerance of the fungus. Furthermore the *hisHF* overexpression impaired the formation of sexual fruiting bodies at an early stage of sexual development.

Experimental Procedures

Strains, Media and General Techniques.

The *S. cerevisiae* strain RH1616 (*MATα aro3-2, ura3-52, his7Δ::his7*^{P}*-lacZ, gcn4-101*) (Kuenzler *et al*., 1993) was used as recipient for a *his7*$^{-}$ complementing cDNA of a galactose inducible *A. nidulans* expression library. The *E. coli* strains HfrG6 (λ^{-}, *hisA323*) (Matney *et al*., 1964), SB3931 (λ^{-}, *hisF860*) and UTH1767 (λ^{-}, *hisH1767*) (Goldschmidt *et al*., 1970) with stable mutations in *hisA*, *hisF* and *hisH*, respectively, were obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT) and served as recipients for the cDNA found by the yeast complementation experiment. The *A. nidulans* strain GR5 (*wA3*, *pyrG89*, *pyroA4*, *veA1*) from G. May (Houston, USA) was recipient for the plasmids pME1565 and pME1608. *A. nidulans* strain AGB121 corresponds to GR5 with an ectopic integrated empty overexpression vector pME1565. Transformants with the *hisHF* overexpression plasmid pME1608 were called AGB122 and AGB123, carrying one and two further ectopic *hisHF* alleles, respectively.
E. coli strain DH5α (Hanahan, 1983) was used for the propagation of plasmid DNA. Enzymatic manipulations and cloning of DNA were performed as described in (Sambrook, 1989). Yeast strains were cultivated in YEPD complete medium (Rose, 1989) or MV minimal medium (Miozzari *et al*., 1978), and *A. nidulans* strains in minimal medium according to (Bennett, 1991). Transformation of *A. nidulans* was carried out according to Punt and van den Hondel (Punt and van den Hondel, 1992). Yeast transformation was performed as described in (Elble, 1992). DNA isolation from yeast (Braus *et al*., 1985), *A. nidulans* (Kolar *et al*., 1988), and Southern analysis (Southern, 1975) were previously described.

Race tube growth tests for *A. nidulans*.

In order to compare growth rates of different *A. nidulans* strains, 25 ml pipettes were sealed at one end, filled with a layer of the respective medium, and inoculated at the open

end with about 2 µl from a conidia suspension of about $1x10^9$ conidia/ml. In a time course of 40 days the distance from the inoculation spot to the growth front was followed.

cDNA Library and *hisHF* Overexpression Plasmid.

The cDNA library was constructed after mRNA isolation from *A. nidulans* strain FGSC A234 (*yA2, pabaA1, veA1*) using the Superscript^{TM} cDNA Synthesis Kit from Life Technologies, Inc. (Gaithersburg, MD) as described (Hoffmann *et al.*, 2000). cDNAs were ligated as *Sal*I/*Not*I fragments in the shuttle vector pRS316-*GAL1* (Liu *et al.*, 1992) and propagated in *Escherichia coli*.

The *hisHF Sal*I/*Not*I cDNA fragment was inserted into the *Sma*I linearized overexpression vector pME1565 (*Amp*^{R}, *pyr4*, *alcA*^{P} pBluescript-MCS *his2B*^{T}) between the inducible *alcA* promoter and the *his2B* terminator by blunt end ligation leading to plasmid pME1608. The orientation of the integrated cDNA fragment was tested by restriction analysis.

Sequencing, Sequence Alignment and Homology Modeling Studies.

DNA sequencing was performed using an ABI PRISM^{TM} 310 Genetic Analyzer from Perkin Elmer (Foster City, CA) via primer walking. DNA sequences were analyzed using the LASERGENE Biocomputing software from DNAstar (Madison, WC). Alignments were created as cluster as described (Higgins and Sharp, 1989).

RNA Preparation and Analysis.

Total RNAs were prepared from vegetatively growing *A. nidulans* cultures using TRIzol^{TM} reagent from Life Technologies, Inc. following the supplier`s instructions. For Northern hybridization analysis 20 µg of total RNA were separated on a formaldehyd agarose gel, transferred to a positively charged nylon membrane (Biodyne B, PALL) by electroblotting and hybridized with the respective ^{32}P-labeled DNA fragments. The DNA fragments were randomly radiolabeled using the HexaLabel DNA Labelling Kit from

MBI Fermentas. Transcript levels were visualized by autoradiography and quantified by using a Bio-Imaging Analyzer from Fuji Photo Film C. Ltd. (Tokyo, Japan). Transcript length was determined using the 0.16-1.77 kb[1] RNA ladder from Life Technologies, Inc.

Microscopic Technics.

A. nidulans strains were grown on solid medium at 30°C. The growth of colonies was followed by using a Zeiss Stemi 2000-C binocular with an eightfold enlargement and cleistohecia were investigated with a Zeiss Axiolab microscope (Jena, Germany) with a 40-fold enlargement. Cryoscanning electron microscopy was performed as previously described (Adler *et al*., 1996).

Nucleotide Sequence Accession Number.

The DNA sequence of the 4.3 kb *Eco*RV genomic fragment containing the complete *hisHF* gene and its cDNA sequence has been submitted to the GenBank sequence database (Accession Number: AF159463).

Results

Isolation and Characterization of the *hisHF* Gene of *A. nidulans* Encoding a Bifunctional Histidine Biosynthetic Enzyme.

The *hisHF* gene encoding a bifunctional glutamine amidotransferase and cyclase (EC 2.4.2.14 and EC 4.3.2.4) of *A. nidulans* was isolated by functional complementation of the histidine auxotrophic *S. cerevisiae* $his7^-$ strain RH1616 (Kuenzler *et al*., 1993). This strain RH1616 ($his7\Delta::his7^{p}$-*lacZ*, *ura3-52*) was transformed with an *A. nidulans* cDNA library expressed from the *GAL1* promoter (Hoffmann *et al*., 2000) and transformants were selected by growth on minimal medium lacking histidine and containing 2% galactose as sole carbon source. The plasmid DNA pME1611 from one of the transformants was retransformed into RH1616 to verify its ability to complement the histidine auxotrophy. Plasmid pME1611 was further analyzed by transforming it into the histidine auxotrophic *E. coli* strains HfrG6 (Matney *et al.*, 1964), UTH1767 and SB3931 (Goldschmidt *et al*., 1970) containing mutations in the *hisA*, *hisH* and *hisF* genes, respectively. Although no functional *E. coli* promoter was present on the plasmid, the transformed DNA was able to complement the mutations in *hisH* and *hisF*, encoding a glutamine amidotransferase and a cyclase, respectively, but not in *hisA*, encoding imidazole-carboxamide isomerase.

The length of the cDNA fragment of plasmid pME1611 was determined as 1849 bp without the poly(A) tail. The 5` untranslated region consisted of 64 bp, the 3` untranslated region of 126 bp. The open reading frame was 1659 bp in length corresponding to 553 codons. Cluster alignments (Higgins and Sharp, 1989) of the deduced amino acid sequence showed strong similarities to bifunctional IGP-synthases from *S. cerevisiae* (59% identity (i) and additional 24% similarity (s)) and *A. thaliana* (i: 42% and s: 27%) as well as to the respective prokaryotic monofunctional enzymes from *E. coli* (i: 30% and s: 29%), *L. lactis* (i: 32% and s: 28%) or *S. typhimurium* (i: 29% and

s: 27%). In analogy to the nomenclature of the homologous genes in these organisms we named the gene of *A. nidulans hisHF*.

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-438   CGGAGGTGAA TTCAGTGCGA ATGGTCAGTT GTAATCACGT GACTGTACGA GCTCTACCTA AGCTCGAAAA GGAAATCCCT
-358   CCATTAGCCG GTGGTATACT TAATTCTGCT GTAATACATT TGCGGCCAAG ATTGTATTTG TGAAAGACAT TGTGTTTGTC
-278   CGGAGCTTTC TGCTAGTATG ACAAAGCACC GTTGTCTAGT GGTTAATGAT ATCTTTTCCG TTGAGCACAT GATAGCTCTC
-198   GACTGTAGGA TCGATACACT CAAAAAAAAA AAAAACTTGT ATGAGTCAGC CCCTCCGTTC CTCTGGTGGG GTGGCGTGGA
-118   AAGATACGAT GGTATTGAGT AGTCAAAACT TGGGTCTACG AAGCCATTGA GCTTTTCTAT CAAACCTAAT TTCTGTTACT
 -38   ACGCATCTCA AACGTAGTAG GAGCCAGCTA TTATCACTAT GCCCACTGTT CATCTGCTTG ATTATGTCGC TGGAAATGTG
   1                                            M  P  T  V  H  L  L  D  Y  V  A  G  N  V
  43   CGTTCCTTGG TGAATGCCAT CAACAAGGTT GGATATGAAG TTGAGTGGGT GAGATCGCCC AGCGATTTGA AAGATGTCGA
  15   R  S  L  V  N  A  I  N  K  V  G  Y  E  V  E  W  V  R  S  P  S  D  L  K  D  V  E
 123   GgtaagGAAA TTATCGTCGT CACCATTGTG CCTTGTGCCT CATTATGTAT CTGTTTCCGG AAATTCAGGC TAACGTGGGG
        --------------------------------INTRON-------------------------------------------
 203   GCTAtagAAG CTCATACTTC CAGGAGTCGG CCACTTCGGC CATTGCCTTT CTCAGCTTTC AAGCGGTGGC TATTTGCAGC
  42   -------K  L  I  L  P  G  V  G  H  F  G  H  C  L  S  Q  L  S  S  G  G  Y  L  Q  P
 283   CGATAAGAGA GCATATTGCT TCCGGGAAAC CGTTTATGGG AATATGTGTT GGTTTACAAT CCCTTTTCGA GAGTTCCGAG
  67     I  R  E  H  I  A  S  G  K  P  F  M  G  I  C  V  G  L  Q  S  L  F  E  S  S  E
 363   GAGGATCCCA ACATTCCTGG TTTGGGTACA ATCCCAGCGC GGTTGCGCAA ATTTGACGCA AAAACGAAGA GCGTACCTCA
  93   E  D  P  N  I  P  G  L  G  T  I  P  A  R  L  R  K  F  D  A  K  T  K  S  V  P  H
 443   TATCGGTTGG AACTCGGCAA CAGATACCCG CATTGACTCG ACTGGAGGCC AGACCTTCTA TGGATTGAGC CCAAGCAGCA
 120     I  G  W  N  S  A  T  D  T  R  I  D  S  T  G  G  Q  T  F  Y  G  L  S  P  S  S  K
 523   AGTACTACTA CGTACACTCA TACGCCGCGC CATATGAGCC GGGAATTCTT GAGAAAGATG GTTGGTTGGT CGCACGGCGA
 147     Y  Y  Y  V  H  S  Y  A  A  P  Y  E  P  G  I  L  E  K  D  G  W  L  V  A  R  R
 603   GTTTATGGGG AGGAGAAATT CATCGGCGCG ATAGCACGAG ATAACATCTT TGCGACACAA TTCCATCCCG AGAAGAGCGG
 173   V  Y  G  E  E  K  F  I  G  A  I  A  R  D  N  I  F  A  T  Q  F  H  P  E  K  S  G
 683   CCAAGGCAGG CCTACGCACC CTTCGCGCTT TCTTGGACGG AGCTCAGCTC CATTCTGTCA CATTAGAGAC TCGATTTTGA
 200     Q  G  R  P  T  H  P  S  R  F  L  G  R  S  S  A  P  F  C  H  I  R  D  S  I  L  T
 763   CAGGAGAGAA AAACGGTCTT ACCCGTAGGA TCATCGCCTG TCTTGATGTT CGTACGAATG ATGTCGGCGA TCTCGTTGTG
 227     G  E  K  N  G  L  T  R  R  I  I  A  C  L  D  V  R  T  N  D  V  G  D  L  V  V
 843   ACTAAGGGCG ATCAATATGA TGTTCGCGAG AAGGATGGTG CGGATGCTGG AGGGCAAGTG AGGAACCTGG GAAAGCCGGT
 253   T  K  G  D  Q  Y  D  V  R  E  K  D  G  A  D  A  G  G  Q  V  R  N  L  G  K  P  V
 923   TGATATGGCT AAGAAATATT ACGAACAGGG GGCAGATGAG GTGACGTTTT TAAACATCAC CTCTTTCAGA AACTGTCCGT
 280     D  M  A  K  K  Y  Y  E  Q  G  A  D  E  V  T  F  L  N  I  T  S  F  R  N  C  P  L
1003   TAGCCGACCT CCCTATGCTC GAGATTCTCC GAAGAACGTC GGAGACCGTC TTCGTACCTT TGACTATTGG TGGCGGCATT
 307     A  D  L  P  M  L  E  I  L  R  R  T  S  E  T  V  F  V  P  L  T  I  G  G  G  I
1083   AGGGACACTG TGGATACAGA CGGTACTCAC ATCCCAGCTC TAGACGTGGC ATCGATGTAC TTCAAATCTG GGGCTGACAA
 333   R  D  T  V  D  T  D  G  T  H  I  P  A  L  D  V  A  S  M  Y  F  K  S  G  A  D  K
1163   AGTCAGCATT GGTTCGGATG CCGTTGTTGC TGCGGAAGAT TATTACGCAG CTGGCAAAGT TCTGTCTGGC AAAACTGCCA
 360     V  S  I  G  S  D  A  V  V  A  A  E  D  Y  Y  A  A  G  K  V  L  S  G  K  T  A  I
1243   TTGAAACTAT TTCTAAGGCG TATGGAAACC AGGCTGTCGT TGTAAGCGTT GACCCGAAGC GCGTTTATGT CAGCCAACCA
 387     E  T  I  S  K  A  Y  G  N  Q  A  V  V  V  S  V  D  P  K  R  V  Y  V  S  Q  P
1323   GAAGACACGA AACACCGTAC GATAGAAACG AAATTTCCTA ACGCCGCCGG GCAAAATTTC TGTTGGTACC AGTGTACTAT
 413   E  D  T  K  H  R  T  I  E  T  K  F  P  N  A  A  G  Q  N  F  C  W  Y  Q  C  T  I
1403   AAAGGGTGGC AGAGAGACCA GAGACTTAGA TGTCTGCCAG CTGGTGCAAG CCGTCGAGGC AATGGGTGCT GGGGAGATTC
 440     K  G  G  R  E  T  R  D  L  D  V  C  Q  L  V  Q  A  V  E  A  M  G  A  G  E  I  L
1483   TGCTGAATTG CATTGATAAA GACGGGAGCA ACAGTGGTTC GATCTTGACT GATCAACCAC CGTCAAAGCG TGCAGTAAAA
 467     L  N  C  I  D  K  D  G  S  N  S  G  S  I  L  T  D  Q  P  P  S  K  R  A  V  K
1563   ATACCCGTGA TTGCCTCTAG CGGAGCTGGT ATGCCGAAGC ATTTTGAGGA AGTTTTTGAT CAAACGACGA CAGATGCTGC
 493   I  P  V  I  A  S  S  G  A  G  M  P  K  H  F  E  E  V  F  D  Q  T  T  T  D  A  A
1743   TCTGGGTGCT GGGATGgtat gtATATATGT GTACCTTTTC CTGTTTCTGT CTACTACAGC AATACTAATT GAGGTAcagT
 520     L  G  A  G  M  -----------------------------INTRON-------------------------------F
1823   TCCATCGTGG TGAGTATACT GTTGGTGAAG TCAAGCAATA CCTTGAGGAT AGAGGTTTCC TTGTTCGACG ATTCGAGCCT
 526     H  R  G  E  Y  T  V  G  E  V  K  Q  Y  L  E  D  R  G  F  L  V  R  R  F  E  P
1903   GATGTCTGAG TGGGTTATGC GTCAAATTCC GCTGCTGGTC CCCAGGTTTA GTTTGTCAGA TTCAGATATA GATTTTAGAG
 552   D  V
1983   GCAGAATTAT ATCGTTTTGT TGAGCTCGTT GCCAACGGTG CCTTCACTGA GACAATCCTC TAACCCTATC ATATTCCCGA
2062   GGTAAAACTC TTGCCTGAAA GCTGTGAAAC CACAAAGAAA ATGCAATTCT CGCTTAGCTC TCCCTCCTAT GTGAGGTATT
```

Figure 7. DNA and deduced amino acid sequences of the *hisHF* gene of *A. nidulans*. Sequence analysis of the genomic *Eco*RV and the cDNA fragment revealed a promoter with two elements similar to the consensus *cis*-sequence for Gcn4p (underlined), the transcriptional activator of the general control system in *S. cerevisiae*, a putative transcriptional initiation site (first solid triangle), two introns (dashed lines), a poly(A) site (second solid triangle) and the deduced amino acid sequence. The region between the glutamine amidotransferase and the cyclase (underlined) did not show any similarity to sequences of other proteins, neither of *A. nidulans* nor of other organisms and was called linker. 5’ and 3’ splice motifs are shown in small underlined letters.

Southern hybridisation experiments with the cDNA fragment of plasmid pM1611 as probe and genomic *A. nidulans* DNA, digested with different restriction enzymes, suggested, that the *hisHF* gene exists as a single copy in the *A. nidulans* genome (Fig. 9

A, lane 1 for *Eco*RV). A genomic clone including *hisHF* was isolated by colony hybridisation as a 4.3 kb *Eco*RV fragment from an *A. nidulans* genomic sublibrary with *Eco*RV fragments of 3.5-4.5 kb in size. The isolated plasmid pME1633 was subjected to DNA sequence analysis. Two introns interrupted the coding region, intron I from postions 124 to 209 and intron II from 1759 to 1822 relative to the translational start codon (Fig. 7). Both introns showed the conserved internal 5`splicing and 3`splicing motifs described for *A. nidulans* (May *et al.*, 1987). The open reading frame of the genomic fragment was flanked by a 519-bp 5`-region and a 1.9-kb 3`-region. In the putative promoter region upstream of the cDNA 5`end, two elements similar to the motif 5`-TGACTC-3` were present at positions –157 and –399 relative to the AUG translational start codon (underlined in Fig. 7). This regulatory *cis*-element has been described in yeast as Gcn4p recognition element (GCRE), the binding site for the transcriptional activator of the amino acid biosynthetic network, Gcn4p. In filamentous fungi this network was called *cross pathway control* and the *GCN4* homologous genes of *A. niger* and *N. crassa*, *cpc1* and *cpcA* respectively, were identified (Paluh *et al.*, 1988; Wanke *et al.*, 1997). Therefore the binding site was named CPRE for cross pathway recognition element (Fig. 8 B).

Activation of *hisHF* Transcription by Amino Acid Starvation.

Northern hybridisation analysis of the *hisHF* transcripts revealed a length of approximately 1.8 kb (not shown). In yeast the homologous gene *HIS7* is regulated by the *general control of amino acid biosynthesis* and its transcription is activated by the transcription factor Gcn4p upon amino acid starvation (Kuenzler *et al.*, 1993). As the *hisHF* promoter contains two putative binding sites resembling the yeast Gcn4p binding site, we investigated *hisHF* transcription during conditions of amino acid starvation. The *A. nidulans* strain strain GR5 (*wA3*, *pyrG89*, *pyroA4*, *veA1*) was cultivated in liquid medium for 20 h and mycelia were transferred to fresh medium containing 20 mM 3-amino-1,2,4-triazole (3AT). 3AT acts as a feedback-inhibitor signal of histidine

biosynthesis and therefore depletes the histidine pool of the cells leading to the amino acid starvation (Hilton *et al.*, 1965). Mycelia were harvested at three time points and total RNAs were isolated. *hisHF* transcript levels were determined by hybridizing with the radioactively labeled cDNA probe. A specific probe for *gpdA* transcripts was used as an internal control. Transcription of *gpdA* encoding glyceraldehyde-3-phosphate dehyrdogenase (EC 1.2.1.12) (Punt *et al.*, 1988) was not affected upon addition of 3AT. Quantification of the northern signals by Phospho Imager analysis revealed up to a 3.0 fold increase in the level of *hisHF* in cells incubated with 3AT (Fig. 8 A). We confirmed this result by inducing amino acid starvation with 5-methyltryptophan, a tryptophan analogue acting as false feedback inhibitor of this amino acid (not shown). Therefore our results strongly suggests that the transcription of the *hisHF* gene is regulated by the cross-pathway-control system in *A. nidulans.*

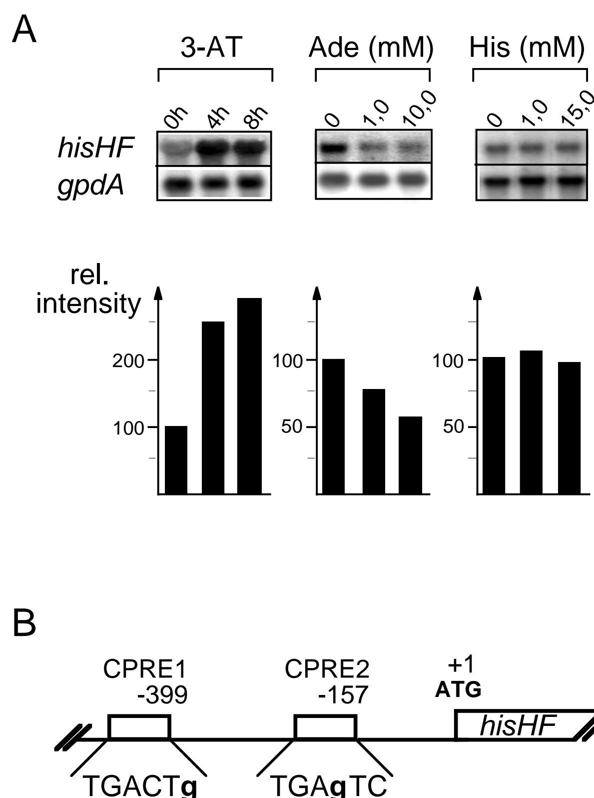

Figure 8. Expression of the *hisHF* gene of *A. nidulans* under amino acid starvation, adenine and histidine supplementation conditions. A Each autoradiography is an example of at least four independent Northern hybridization experiments with total RNA of mycelia of strain GR5 and the *hisHF* cDNA as probe. The addition of 3AT led to an increased level of *hisHF*

transcripts. Supplementation with adenine reduced *hisHF* mRNA levels and external histidine had no effect. The *gpdA* mRNA was used as internal standard. Quantification by Phospho Imager revealed a 3.0 fold increased *hisHF* transcript level after 4 hours of growth with 3AT. It did not significantly increase during the next 4 hours of cultivation. Growth in medium supplemented with 1.0 mM adenine reduces the *hisHF* transcript level to approximately 75 % and with 10.0 mM adenine to 50 %. Supplementation with histidine displays no effect on *hisHF* transcript levels. The standard deviation did not exceed 15 %. **B** shows a scheme of the putative CPREs and their sequences within the promoter region of the *hisHF* gene. Small letters printed in bold are mismatches of the postulated consensus sequence for the respective Gcn4p binding sites in yeast.

Repression of *hisHF* Transcription by Adenine but not Histidine.

The byproduct AICAR of the reaction catalyzed by the IGP-synthase is a common intermediate of histidine and purine biosyntheses and thereby connects both pathways. We investigated whether end products of either pathways, histidine or adenine/guanine, influenced the expression of the *hisHF* gene. The *hisHF* mRNA levels in *A. nidulans* strain GR5 grown in the presence of different amounts of histidine and adenine were determined. Mycelia from liquid cultures containing up to 15.0 mM histidine were harvested and total RNAs were isolated. The same *hisHF* mRNA levels were found for these cultures as for a culture without histidine supplementation. The autoradiography shown in Fig. 8 A illustrates the situation for 1.0 or 15.0 mM histidine supplementation. In contrast, supplementation with adenine to final concentrations of 1.0 mM or 10.0 mM reduced the *hisHF* mRNA levels to 75% and nearly 50%, respectively (Fig. 8 A), in comparison to mycelia from cultures without adenine. Higher concentrations did not increase this effect. Thus *hisHF* transcription of *A. nidulans* is repressed in the presence of external adenine but not by histidine.

Reduction of Salt Tolerance and Inhibition of Sexual Development by high *hisHF* Transcription

Strong overproduction of the IGP-synthase in the eubacteria *S. typhimurium* or *E. coli* resulted in growth deficiencies and morphological changes (Casadesus and Roth, 1989; Flores *et al*., 1993; Gibert and Casadesus, 1990; Murray and Hartman, 1972). Here we

investigated the consequences of *hisHF* overexpression for growth and development of *A. nidulans*. We cloned the *hisHF* cDNA between the inducible *alcA* promoter and the *his2B* terminator of vector pME1565 leading to pME1608. The *alcA* gene of *A. nidulans* is highly expressed and codes for alcohol dehydrogenase I (EC 1.1.1.1). Transcription of *alcA* is extensively repressed during growth in medium containing glucose but strongly induced in medium containing ethanol as sole carbon source (Waring *et al*., 1989). The overexpression plasmid pME1608 and the vector pME1565 without the *hisHF* gene, both carrying the *pyrG* marker gene, were transformed into *A. nidulans* strain GR5. Transformants were selected by growth on uridine deficient medium. Ectopic integration events and the number of *hisHF* copies in the genome were analyzed by Southern hybridization analysis (Fig. 9 A). Transformation with the empty vector resulted in strain AGB121 with only the wild type *hisHF* allele. Strains AGB122 and AGB123 were transformants with pME1608 and carry one or two *alcA-hisHF* alleles, respectively.

hisHF transcription levels of the strains AGB122 and AGB123 were compared with that of strain AGB121. Northern hybridization experiments with total RNA from mycelia grown in glucose or ethanol containing medium were performed. Glucose repressed and ethanol activated the *hisHF* transcription from the *alcA* promoter of the transformants as shown in Fig. 9 B. In medium containing ethanol as sole carbon source *hisHF* transcription of AGB122 increased five-fold and of strain AGB123 eleven-fold (Fig. 10 B).

On glucose the growth rate of strain AGB123 (2x *alcA-hisHF* + wt-*hisHF*) and that of AGB121 (wt-*hisHF*) was similar as measured with race tubes in a time course of 40 days. Induction of *hisHF* overexpression by cultivating strain AGB123 on ethanol led to a growth rate of 70 % relative to that of AGB121 on ethanol. On glucose medium increasing concentrations of NaCl impaired colony growth of AGB123 to the same extent as of the wild-type strain AGB121 (Fig. 10, rows 1,2). High salt concentrations combined with *hisHF* overexpression, induced by growth on ethanol, drastically diminished colony growth of strain AGB123 (Fig. 10, row 4). As measured in race tubes

with ethanol medium containing 0.9 M NaCl, the growth rate dropped to 30 % of the wild type at this conditions, and even to 10 % on 1.5 M NaCl. On 1.8 M NaCl hardly any growth was observed after several days of incubation. As shown for bacteria earlier, overexpression of *hisHF* also impaired salt tolerance of *A. nidulans*.

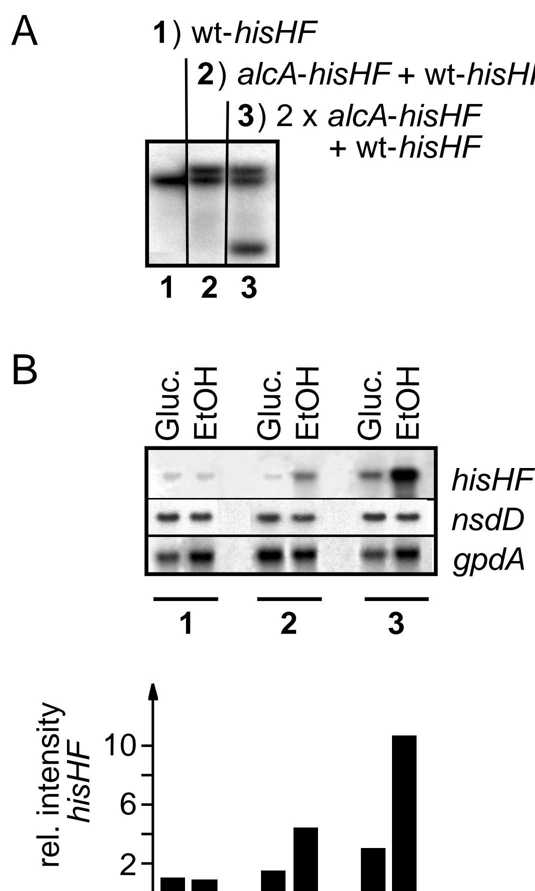

Figure 9. Overexpression of the *hisHF* gene in *A. nidulans*. The autoradiography **A** shows a Southern hybridisation experiment with *Eco*RV digested genomic DNA probed with the radioactive labeled *hisHF* cDNA. The strain AGB121 had the wild type *hisHF* allele (lane 1), transformant AGB122 had one and transformant AGB123 two ectopic *alcA-hisHF* (lanes 2,3). The number of copies was confirmed by Southern analysis with *Nhe*I digested DNA (not shown). **B** The respective *hisHF* mRNA levels of these strains were determined by Northern hybridisation experiments and are illustrated in the autoradiography and quantified by Phospho Imager analysis. All quantifications are the average of four different RNA isolations and the standard deviation did not exceed 15%. AGB122 exhibited a five-fold and AGB123 an eleven-fold increased *hisHF* mRNA level if cultivated on medium with ethanol as carbon source. *nsdD* mRNA levels did not change upon *hisHF* overexpression.

HISHF overproduction in *A. nidulans*, in contrast to that in *S. typhimurium*, did not reduce growth at elevated temperatures. Moreover the colony morphology of *A. nidulans* did not change upon *hisHF* overexpression as it was observed in bacteria (Fig. 10). Electron microscopical studies revealed that the cell morphology of the distinct cell types of the asexual formed conidiophores did not change upon the *hisHF* overexpression (Fig. 11 A+B top). Cultivation of both *hisHF* wild type or an overexpressing strain on solid glucose medium under oxygen limitation conditions led to mature sexual fruiting bodies after 10 days of growth. On ethanol medium this sexual development took 13-15 days for the wild type (Fig. 11 A bottom) leading to more than 100 mature cleistothecia on a square centimeter. The *hisHF* overexpression strain AGB123 did not form any sexual fruiting bodies on ethanol and the number of nests was reduced and showed accumulations of globular hulle cells (Fig. 11 B bottom). Even after 30 days of growth these nests were found. Eleven-fold overexpression of the *hisHF* gene therefore prevented the sexual life cycle in *A. nidulans*. Shifting these *alcA-hisHF* cultures to repressing glucose medium resulted in the completion of the sexual life cycle and the formation of cleistothecia after another four to five days (not shown). This suggested that the ectopic integration of the construct did not result in a secondary mutation. In contrast to the eleven-fold *hisHF* overexpression of AGB123, the five-fold overexpression of AGB122 did not impair the formation of cleistothecia.

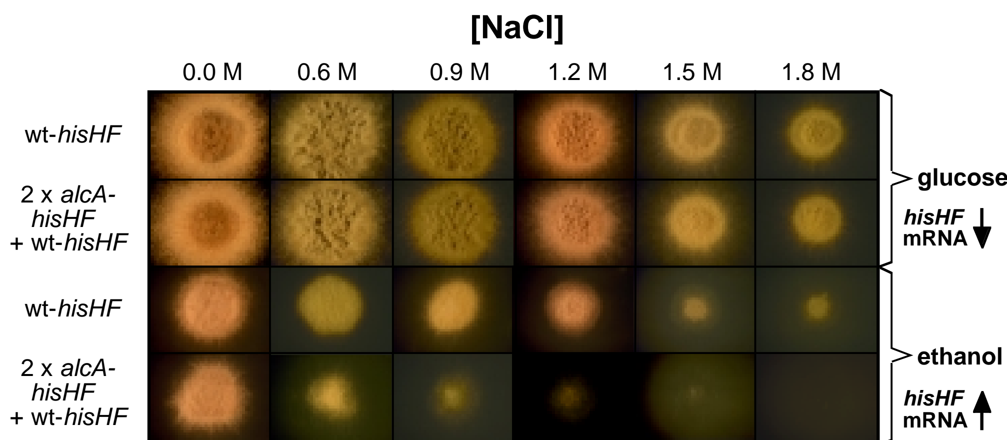

Figure 10. Salt sensitivity of the *hisHF* overexpressing *A. nidulans* strain AGB123. Colonies of the *A. nidulans* strain AGB121 with the *hisHF* wild type allele and strain AGB123 with its two *alcA-hisHF* copies were grown on raising NaCl concentrations. Growth at salt concentrations up

to 1.8 M is shown for repressing glucose medium and inducing ethanol medium. Whereas both strains grew similar on glucose (rows 1,2), on ethanol the *hisHF* overexpression strain AGB123 displayed an increase in sensitivity for raising salt concentrations resulting in much smaller colonies (row 4) compared to AGB121 (row 3). These results were confirmed on media with similar concentrations of sorbitol (not shown).

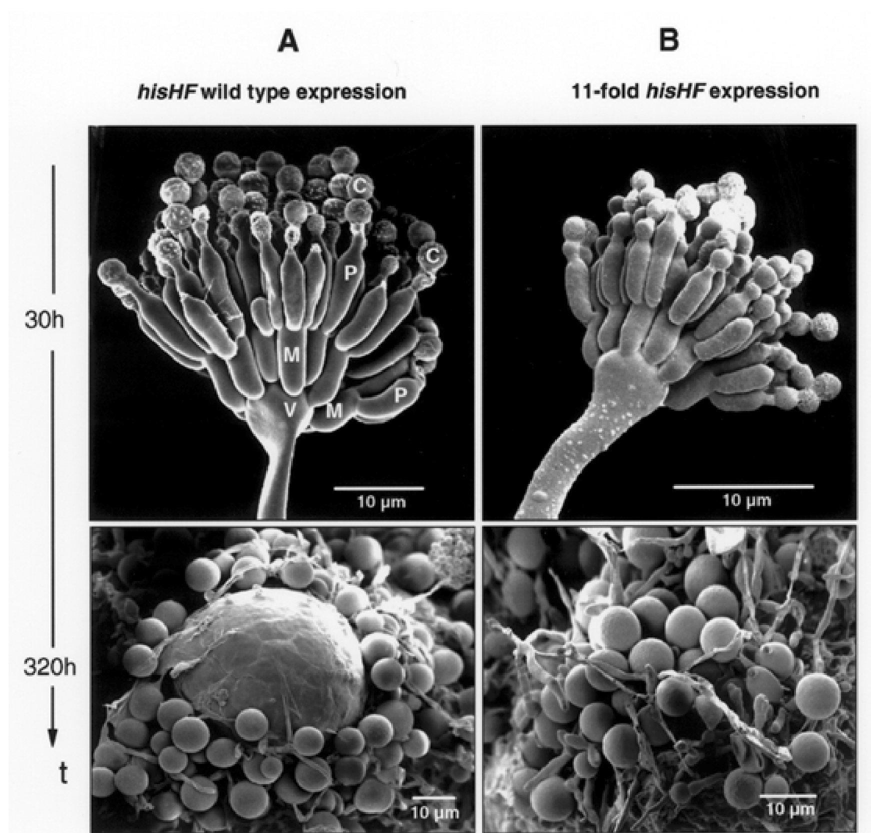

Figure 11. Sexual development is prevented by *hisHF* overexpression. Scanning electron microscopy of AGB121 (wt-*hisHF*) and AGB123 (2x *alcA-hisHF* + wt-*hisHF*). **A** Conidiophores of both strains for growth on ethanol medium. The individual cell types are vesicle (V), metulae (M), phialide (P) and conidium (C). *hisHF* overexpression did not change the morphology of the individual cell types. **B** Cultivation of wt-*hisHF* strain under oxygen limitation led to the formation of sexual fruiting bodies. The photograph on the left side shows a cleistothecium as they were visible after about 300 hours of growth on ethanol medium. 11-fold overexpression of the *hisHF* gene only led to nests with accumulations of hulle cells, as shown on the right photograph. Even further 200 hours of growth did not led to cleistothecia. Only the shift to repressing glucose medium raised the block of the developmental process and led to mature cleistothecia (not shown).

hisHF Overexpression did not Activate the Cross Pathway Control.

One reason for a block in the formation of cleistothecia could be the activation of the *cross pathway control system* (Eckert *et al.*, 1999; Hoffmann *et al.*, 2000). We

investigated whether the strong overexpression of the *hisHF* gene in AGB123 led to an activation of this regulatory network, i.e. by causing a severe imbalance of the internal pool of histidine in relation to the other amino acids. In Northern hybridisation experiments we determined the transcript levels of the biosynthetic genes *hisB* and *argB* of AGB123, both known target genes of cross pathway control (Busch *et al.*, 2000; Goc and Weglenski, 1988) (Fig. 12). Although the transcription of *hisHF* in strain AGB123 is strongly activated if cultivated in medium with ethanol as carbon source (EtOH-lanes in Fig. 12), the *argB* and *hisB* mRNA levels did not increase. We conclude that the acleistothecial phenotype caused by the strong *hisHF* overexpression was not linked to an activated *cross pathway control.*

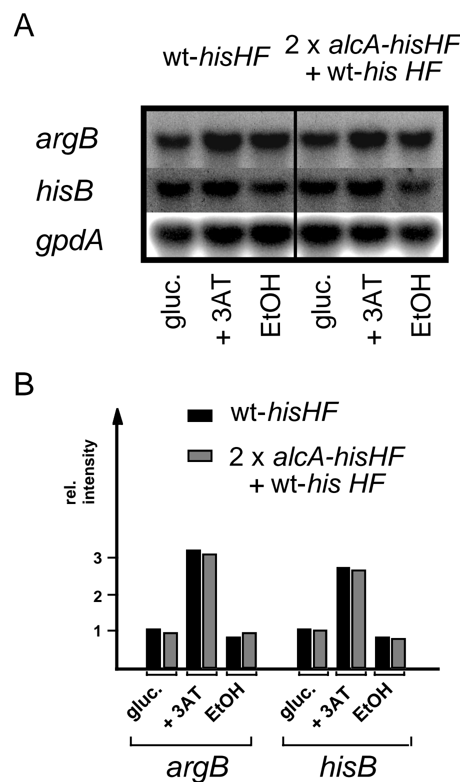

Figure 12. *hisHF* overexpression did not induce the *cross pathway control*. The autoradiography in **A** shows an example of three independent Northern hybridisation experiments with total RNA of mycelia of strains AGB121 (wt-*hisHF*) and AGB123 (2x *alcA-hisHF* + wt-*hisHF*). Both strains were cultivated in glucose, 3AT supplemented or ethanol medium. The blots were probed against *argB* and *hisB*, and RNA amounts equalized in relation to *gpdA* mRNA levels. The quantification **B** confirmed the transcriptional activation of the known target genes of the *cross pathway control argB* (Goc and Weglenski, 1988) and *hisB* (Busch *et al.*, 2000) upon 3AT addition. However, the overexpression of *hisHF* in AGB123 on ethanol medium did not

give rise to higher mRNA levels for *argB* and *hisB* and therefore did not activated this regulatory network.

As the expression of the *nsdD* gene is essential for the formation of cleistothecia but not for asexual development (Chae, 1995) we analyzed its transcript levels during basal and ethanol-induced *hisHF* expression. The amount of *nsdD* transcripts in AGB123 did not change upon *hisHF* overexpression and therefore it was not involved in the acleistothecial phenotype (Fig. 9 B).

Discussion

Metabolic enzymes are among the best-studied examples of protein function and structure. In this work we report the structure and regulation of the *hisHF* gene of the filamentous fungus *A. nidulans* as the first gene identified encoding an IGP-synthase in filamentous fungi. These proteins were proposed to be the result of a twofold gene dublication and gene fusion. The proteins are ß/α barrels and their ancestor might have been a half barrel enzyme (Lang, et al., 2000). The transcription of the fungal gene is regulated upon amino acid as well as adenine limitation. Eleven-fold overexpression of the *hisHF* gene resulted in increased osmosensitivity. In addition, cleistothecia formation was impaired in this strain, although we could not find any amino acid stress, which is normally reflected by the induction of the cross pathway network.

The deduced amino acid sequence of the *A. nidulans* HISHF displayed high similarities to homologous bifunctional gene products of other eukaryotes including *S. cerevisiae* or *A. thaliana*, and slightly reduced similarities to homologous proteins coded from separate genes in prokaryotes including *E. coli*, *S. typhimurium* or *L. lactis*. These data further support the idea that all genes encoding enzymes for this reaction of histidine biosynthesis are the descendants of a common ancestor gene. Accordingly, the fungal cDNA was able to complement a *HIS7* deletion of *S. cerevisiae* as well as *hisH* or *hisF* mutations in *E. coli*. The linker region between both catalytic subunits did not display any similarity to either hisHp or hisFp and was 21 amino acids in length. In comparison the *A. thaliana* linker comprised 17 amino acids (Fujimori and Ohta, 1998) and the *S. cerevisiae* linker 23 (Kuenzler *et al*., 1993). Therefore it seems that the function of the linker is primarily to guarantee appropriate spacing between the different domains.

Amino acid starvation in *A. nidulans* led to a threefold activation of the *hisHF* transcription. Although the final transcription factor of a regulatory network for the biosynthesis of amino acids has not been described for *A. nidulans* yet, a *cross pathway control* system similar to the *general control* system of yeast was described (Piotrowska,

1980). Also for *Aspergillus niger* a similar *cross pathway* regulation with its transcription factor cpcAp was described (Wanke *et al*., 1997). We assume that *A. nidulans* possesses a homologous transcription factor to *A. niger* cpcAp and yeast general control factor Gcn4p, both recognizing the DNA consensus sequence 5'-TGACTC-3'. The *hisHF* promoter has two putative binding sites for this kind of regulation. One of these cross pathway recognition elements (CPRE1) was at position –399 relative to the translational AUG start codon and CPRE2 at position –157. Both CPREs are separated by 242 bp. Within the yeast *HIS7* promoter the two functional Gcn4p recognition elements (GCREs) at the positions –231 and –145 were only 86 bp apart from each other. Moreover GCRE2 mediated the activation of *HIS7* transcription by binding of the heterodimeric transcription factor Bas1p/Bas2p. Bas1p/Bas2p act as transcription activators upon purine limitation (Springer *et al*., 1996). For *A. nidulans* Bas1p/Bas2p homologous factors are not yet identified. Since transcription of *hisHF* raised upon purine limitation, it remains to be shown whether CPRE1 or CPRE2 as putative promoter sites are also involved.

Overexpression of the *hisHF* gene driven from the inducible *alcA* promoter led to an increased sensitivity to high osmolarity of the fungus. Raising concentration of NaCl or sorbitol resulted in reduced growth rates and smaller colonies in comparison to wild type *hisHF* expression. A similar osmotic phenotype was previously observed for *S. typhimurium* and *E. coli* as part of a pleiotropic response caused by hisHp and hisFp overproduction (Flores and Casadesus, 1995). Further overexpression phenotypes in the eubacteria were a reduction in growth at elevated temperatures and a changed cell morphology that manifested in long filaments instead of small rods. In contrast *hisHF* overexpression in *A. nidulans* did not affect growth at elevated temperatures or the morphology of hyphae or conidiophores. However, cleistothecia were no longer formed during strong *hisHF* overexpression. The molecular reasons for the osmotic and developmental phenotypes of *A. nidulans* remain to be shown. It will be interesting to

compare them on the molecular level with those of the pleiotropic response observed in *S. thyphimurium* and *E. coli* upon overproduction of hisHp and hisFp.

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Chapter 3

Different Transcriptional Activators Regulate *HIS7* Expression by Different Nucleosome-mediated Activation Mechanisms

Abstract

ARO4 and *HIS7* are two tandemly orientated genes of *Saccharomyces cerevisiae* which are transcribed into the same direction. The *ARO4* terminator and the *HIS7* promoter regions are hypersensitive to Micrococcus nuclease (Mnase) and separated by a positioned nucleosome. The *HIS7* promoter is target for the transcription factors Gcn4p and Bas1p/Bas2p that activate its transcription upon amino acid starvation and purine limitation, respectively. Activation of the *HIS7* gene by Gcn4p but not Bas1p/Bas2p releases the defined nucleosomal distribution to yield increased Mnase sensitivity throughout the entire intergenic region. This change in chromatin structure requires the intact *HIS7* promoter with both Gcn4p recognition elements and the binding site for an additional protein, Abf1p. The remodeling is *SNF2*-dependent but *GCN5*-independent. Accordingly *SNF2* is necessary for the Gcn4p-mediated transcriptional activation of the *HIS7* gene. *GCN5* is required for activation upon adenine limitation by Bas1p/Bas2p and to maintain the resulting basal Gcn4p-independent *HIS7* expression. Our data suggest that activation of *HIS7* transcription by Gcn4p and Bas1p/Bas2p is supported by two distinct mechanisms acting on the nucleosomes. Whereas Gcn4p activation causes Swi/Snf-mediated remodeling of the nucleosomal architecture at the *HIS7* promoter, theBas1/Bas2p complex activates via the Gcn5p acetyltransferase without changing the chromatin structure.

Introduction

Regulation of transcription involves specific transcription factors and multiple complexes that act directly or indirectly by changing the nucleosomal order of the chromatin (Armstrong and Emerson, 1998). In average, 147 base pairs (bps) of DNA coil around a histone octamer and jointly form the nucleosome. Inclusion of promoters and/or upstream activation sequences (UAS) into fixed nucleosomes represses gene transcription and result in silent or basal transcribed genes (Cavalli and Thoma, 1993; Moreira and Holmberg, 1998b; Svaren and Horz, 1995). The process of destabilizing this chromatin structure in order to facilitate access of the general transcription machinery requires not only sequence specific transcription factors, but also cooperation with histones and with cofactors that help to remodel or displace nucleosomes. Genetic studies and subsequent biochemical analyses have identified a number of factors required for transcriptional regulation in relation to chromatin. The multitude of these proteins function as part of large complexes, such as the Swi/Snf complex and the RSC (from *r*emodeling the *s*tructure of *c*hromatin), Ada and SAGA complexes and the Srb/mediator/holoenzyme complex (reviewed in Perez-Martin, 1999).

Swi/Snf was purified as a 2-MDa protein complex that is composed of the Swi1, Swi2/Snf2, Swi3, Snf5 and Snf6 proteins plus five additional polypeptides (Cairns *et al.*, 1994). Swi/Snf can bind to nucleosomes and DNA either and thereby creates loops in nucleosomal arrays or naked DNA, respectively, bringing distant sites into close proximity (Bazett-Jones *et al.*, 1999). In an ATP-dependent fashion it can reposition nucleosomes in *cis* on the same DNA molecule (Whitehouse *et al.*, 1999) and the *SWI2/SNF2* gene itself encodes the DNA-dependent ATPase activity (Richmond and Peterson, 1996). Recent transcriptome analyses with a *swi2/snf2* mutant strain have estimated that Swi/Snf controls transcription of only 6% of all *S. cerevisiae* genes and that the control is exerted at the level of individual promoters rather than over chromosomal domains (Sudarsanam *et al.*, 2000). Swi/Snf thereby both activates and

represses transcription of different target genes. Recruitment of Swi/Snf to specific promoters by DNA-binding regulatory proteins as well as targeting of the complex by the general transcription machinery has been suggested (Yudkovsky *et al*., 1999).

Two high molecular mass Ada-Gcn5 complexes (0.8 and 1.8 MDa) have been biochemically isolated from *S. cerevisiae* and shown to be able to acetylate nucleosomes *in vitro* as well as *in vivo* at specific lysine residues of histones H3 and H4 (Grant *et al*., 1997). Both complexes share Gcn5p, Ada2p and Ada3p, whereas the larger one additionally contains Spt proteins (Spt20p, Spt3, Spt8 and Spt7) and is called SAGA (Spt/Ada/Gcn5 acetyltransferase). Gcn5p comprises the histone acetyltransferase (HAT) activity to acetylate histones in promoter regions in a manner that is correlated with Gcn5p-dependent transcriptional activation and HAT activity *in vitro* (Kuo *et al*., 1998). SAGA interacts with both TATA-binding protein (TBP) and acidic transcriptional activators such as the herpes virus VP16 activation domain and yeast Gcn4p, suggesting that the complex also might have a transcriptional adaptor function for some promoters (Grant *et al*., 1998).

HIS7 is a typical house keeping gene of yeast and its gene product is necessary for the biosynthesis of the amino acid histidine as well as purines (Künzler *et al*., 1993). Its expression has previously shown to be activated by two major stimuli, that are lack of amino acid availability and limitation of external purines, respectively (Springer *et al*., 1996). Starvation for amino acids triggers increased expression of Gcn4 protein that in turn activates transcription of amino acid biosynthetic genes (*general control of amino acid biosynthesis*, reviewed in Braus, 1991; Hinnebusch, 1997). Upon starvation, two Gcn4p-recognition elements (GCREs) within the *HIS7* promoter are targeted by the transcription factor thereby mediating an eight-fold increase of *HIS7* expression (Fig. 13). Purine limitation causes a two-fold increase in *HIS7* expression and is mediated by the heterodimeric transcription factor Bas1/Bas2p that shares a common binding site with Gcn4p, the TATA-proximal GCRE. Nevertheless, both activation pathways act independently of each other and, moreover, are additive upon simultaneous amino acid

and purine limitation (Springer *et al.*, 1996). In-between the two GCREs another *cis*-element is occupied by the ubiquitous transcription factor Abf1p that contributes to the basal *HIS7* expression in the absence of starvation conditions (Springer *et al.*, 1997).

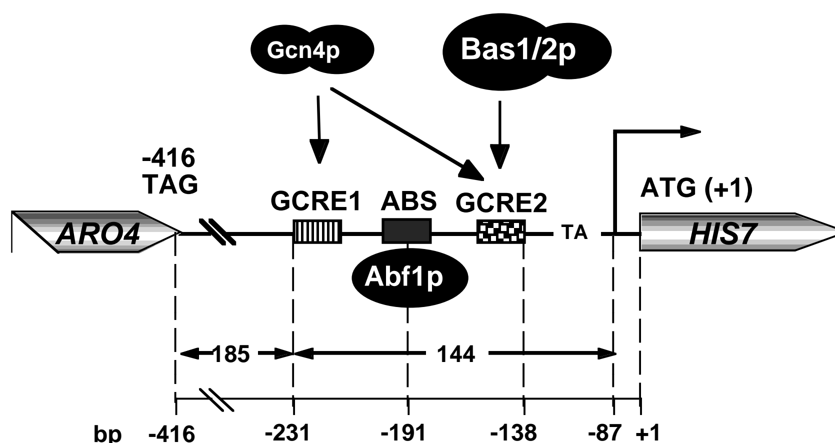

FIGURE 13. Scheme of the yeast *HIS7* promoter. Two binding sites for Gcn4p (GCRE1+2) with a binding site for Abf1p (ABS) in-between are functional parts of the promoter. GCRE2 additionally functions as recognition element for the heterodimeric transcription factor Bas1p/Bas2p. TA at position (-120) reflects the putative TATA element. The arrow at (–87) indicates the initiator element of the major transcriptional start site. Positions are relative to the translational start codon ATG which is shown as +1.

Up to now there is only little knowledge about how different chromatin modifying complexes like Swi/Snf or HATs act together in regulating one gene that is regulated by different activation systems. Whether different activators demand for different possibly independently acting chromatin modifying activities, or whether they claim for their simultaneous presence is poorly understood.

In this work we show that two different transcription factors though sharing a common binding site use different chromatin modifying activities to achieve their goal of transcriptional gene activation. Transcriptional *HIS7* activation by Gcn4p requires the Swi/Snf-dependent rearrangement of otherwise positioned nucleosomes whereas Bas1/Bas2p mediated *HIS7* transcription demands for the presence of the HAT activity

of Gcn5p. Both activation processes and moreover their accompanying effects on chromatin seem to be independent of each other.

Experimental Procedures

Yeast strains and growth conditions

Yeast strains and their genotypes used in this work are listed in table 2. For high *GCN4* expression, strains were transformed with plasmid p238, harboring a constitutively high expressed *GCN4* allele (Müller and Hinnebusch, 1986). To enable starving strains FY1553 and 1360 for histidine with its analog 3-AT, histidine auxotrophy was initially regained by transforming them with plasmid pRS303 (*HIS3*) (Sikorski and Hieter, 1989) yielding strains RH2561 and RH2563. Strains RH2569 and RH2570 with deleted *GCN5* and translational ^{P}*his7-lacZ*-fusions were obtained by transforming RH1615 and RH1616 with the deletion cassette of plasmid pME1236. The inserted *kan*^{nx} marker gene was afterwards removed with the Cre-LoxP recombination system as described by (Güldener *et al.*, 1996).

Strains were cultivated in minimal vitamins (MV) medium (Miozzari *et al.*, 1978). Adenine repression was achieved by supplementation with 0.3 mM adenine (Springer *et al.*, 1996).

Plasmids

Plasmids used in this work are listed in table 2. Plasmid pME1236 carrying the *gcn5::kan*^{nx} deletion cassette was created by replacement of the *GCN5* coding sequence for the *kan*^{nx} kanamycin resistance cassette using a PCR-based three step cloning strategy, with plasmids pME1234 and pME1235 as intermediates. Other plasmids used here have been described previously.

Genomic chromatin preparation and nuclease digestions

These methods have been described previously (Thoma, 1996). Biodyne B nylon membranes (Pall, Dreieich, Germany) were used for Southern transfer. Probes were labeled by the random primer method (Feinberg and Vogelstein, 1983).

Indirect end-labeling

Chromosomal DNA from the nuclease digestion was digested with *Xba*I and *Mlu*I and fractionated on 1.2% agarose gels. The fractionated DNA was blotted on a Hybond-N nylon membrane by the alkaline blotting method and hybridized with a radioactively labeled 250 bp PCR-amplicon, generated with the oligonucleotides HIS7-CHR1 (5´gagattaaagaaattgtcaga3´) and HIS7-CHR2 (5´caagtattgaggagaaatggta3´), annealing just downstream of the *Xba*I site. A DNA ladder consisting of multiples of 256 bp was used for calibration (Thoma *et al*., 1984).

Primer extension

Oligonucleotides CHR-PE1 (5´gccaattcggtattatttaattgtgtttagcgc3´ for top strand) and CHR-PE2 (5´cctaaactggatactgctacttcaatagctgcc3´ for bottom strand) were purchased by Gibco BRL and radioactively 5´ end labeled by Hartmann Analytik GmbH (Braunschweig, Germany). The Mnase-digested DNA was cut with *Xba*I/*Mlu*I to reduce viscosity. Conditions for primer extension on Mnase digests were according to Zhu and Thiele (1996). DNA was electrophoretically separated on a 6% polyacrylamide-urea gel. For sequencing analysis a cycle-sequencing kit (Amersham) was used.

RNA analysis

Total RNAs from *S. cerevisiae* were isolated according to Cross and Tinkelenberg (1991). For Northern hybridization analysis 20 µg of total RNAs were separated on a formaldehyde agarose gel and transferred to a positively charged nylon membrane (Biodyne B, PALL) by capillary blotting. Hybridization with specific DNA probes was

performed after ^{32}P-labeling of the respective DNA fragments with the *Prime It II* DNA Labelling Kit from Stratagene. The *HIS7* probe was generated with the oligonucleotides HIS-OL1 (5´gtggtaacctacagtcactaacc3´) and HIS-OL2 (5´ccgatcgatactttatcagcacc3´), and the *ACT* probe with the oligonucleotides ACT-OL1 (5´gctgctttggttattgataacgg3´) and ACT-OL2 (5´cacttgtggtgaacgatagatgg3´). Band intensities were visualized by autoradiography and quantified using a BAS-1500 Phospho-imaging scanner (Fuji).

ß-galactosidase assay

ß-galactosidase activities were determined by using permeabilized yeast cells and the fluorogenic substrate 4-methylumbelliferyl-ß-D-galactoside (MUF) as described (Künzler *et al*., 1993).

Table 2 Yeast strains and plasmids used in this study

Strain	Genotype	Reference
FY1353	*MATα, his3Δ200, ura3-52, leu2Δ1, lys2-173R2*	Sudarsanam *et al*., 2000
RH2561	as FY1353 but with plasmid pRS303 (*HIS3*)	this work
FY1354	*MATα, his3Δ200, ura3-52, leu2Δ1, lys2-173R2, gcn5Δ::HIS3*	Sudarsanam *et al*., 2000
FY1360	*MAT**a**, his3Δ200, ura3-52, leu2Δ1, lys2-173R2, snf2Δ::LEU2*	Sudarsanam *et al*., 2000
RH2563	as FY1360 but with plasmid pRS303 (*HIS3*)	this work
FY1352	*MAT**a**, his3Δ200, ura3-52, leu2Δ1, lys2-173R2, gcn5Δ::HIS3, snf2Δ::LEU2*	Sudarsanam *et al*., 2000
RH1615	*MAT**a**, ura3-52, aro3-2, his7-lacZ*	Springer *et al*., 1996
RH1616	*MAT**a**, ura3-52, aro3-2, his7-lacZ, gcn4-101*	Springer *et al*., 1996
RH2569	*MAT**a**, ura3-52, aro3-2, his7-lacZ, gcn5Δ::loxP*	this work
RH2570	*MAT**a**, ura3-52, aro3-2, his7-lacZ, gcn4-101, gcn5Δ::loxP*	this work
RH1619	*MAT**a**, ura3-52, aro3-2, his7(mut GCRE1)-lacZ, gcn4-101*	Springer *et al*., 1996
RH1622	*MAT**a**, ura3-52, aro3-2, his7(mut GCRE2)-lacZ, gcn4-101*	Springer *et al*., 1996
RH1830	*MAT**a**, ura3-52, aro3-2, his7(mut ABS)-lacZ, gcn4-101*	Springer *et al*., 1997

Plasmid	Description	Reference
pBluescript® II SK (+)	commercial cloning vector with polylinker, *lacZ*	STRATAGENE, CA
pUG6	*loxP-kanMX-loxP* module with *kan*^{R} marker flanked by *TEF2* promoter and terminator, *Amp*^{R}	Güldener *et al*., 1996
pME1768	*loxP-kanMX-loxP* module from pUG6 in pBluescript® II SK (+)	this work
pME2034	774 bp *GCN5* 3`-fragment in pBluescript® II SK (+)	this work
pME2035	862 bp *GCN5* 5`-fragment in pBluescript® II SK (+)	this work
pME2036	1.6 kb *kanMX* fragment from pME1768 in-between *GCN5* 5`- and 3`-fragment	this work
pSH47	*cre* recombinase expression vector	Güldener *et al*., 1996
pRS303	yeast integrative vector, *lacZ*, *HIS3*	Sikorski & Hieter, 1989
p238	YCp50 carrying a *GCN4* allele with mutated uORFs	Mueller & Hinnebusch, 1986

Results

The yeast *HIS7* promoter is covered by a defined nucleosome structure in the absence of Gcn4p activation

The chromatin structure of the basal yeast *HIS7* promoter was investigated by Micrococcus nuclease (Mnase) protection experiments. Crude nuclear extracts from over-night cultures which were grown in the absence of amino acid limitation and expressing low amounts of the transcription factor Gcn4p, were partially digested with Mnase and further treated as previously described (Thoma, 1996). The *HIS7* promoter region carries binding sites for Gcn4p, Bas1/2p and Abf1p which are all hypersensitive to the nuclease (Fig. 14 A, lanes 2+3). In the case of low Gcn4p levels this extended sensitive region is flanked on both sites by protected DNA stretches that correspond in length to positioned nucleosomes. The *ARO4* gene is located upstream of *HIS7* and is transcribed into the same direction (Künzler *et al.*, 1993). One nucleosome separates the *ARO4* 3`-end region and the *HIS7* promoter. The 3`-end region of the *ARO4* gene upstream of the protected region separating the two genes is again hypersensitive (Fig. 14 A lanes 2+3).

Primer extensions with DNA of this Mnase experiment as template revealed the exact borders of the sensitive promoter region with respect to the positions of the flanking nucleosomes. The TATA-element (TA) located 130 base pairs upstream of the translational start codon is part of the unprotected promoter region (Fig. 14 B, lanes 3-5 and 12-13). The transcriptional initiator elements at positions –87, –88 or –95 relative to the start codon (Künzler *et al.*, 1993) lie within the protected region apparently covered by a nucleosome. The upstream border of the hypersensitive region is located only 10 base pairs upstream of GCRE1, one of the two binding sites for the transcriptional activator Gcn4p.

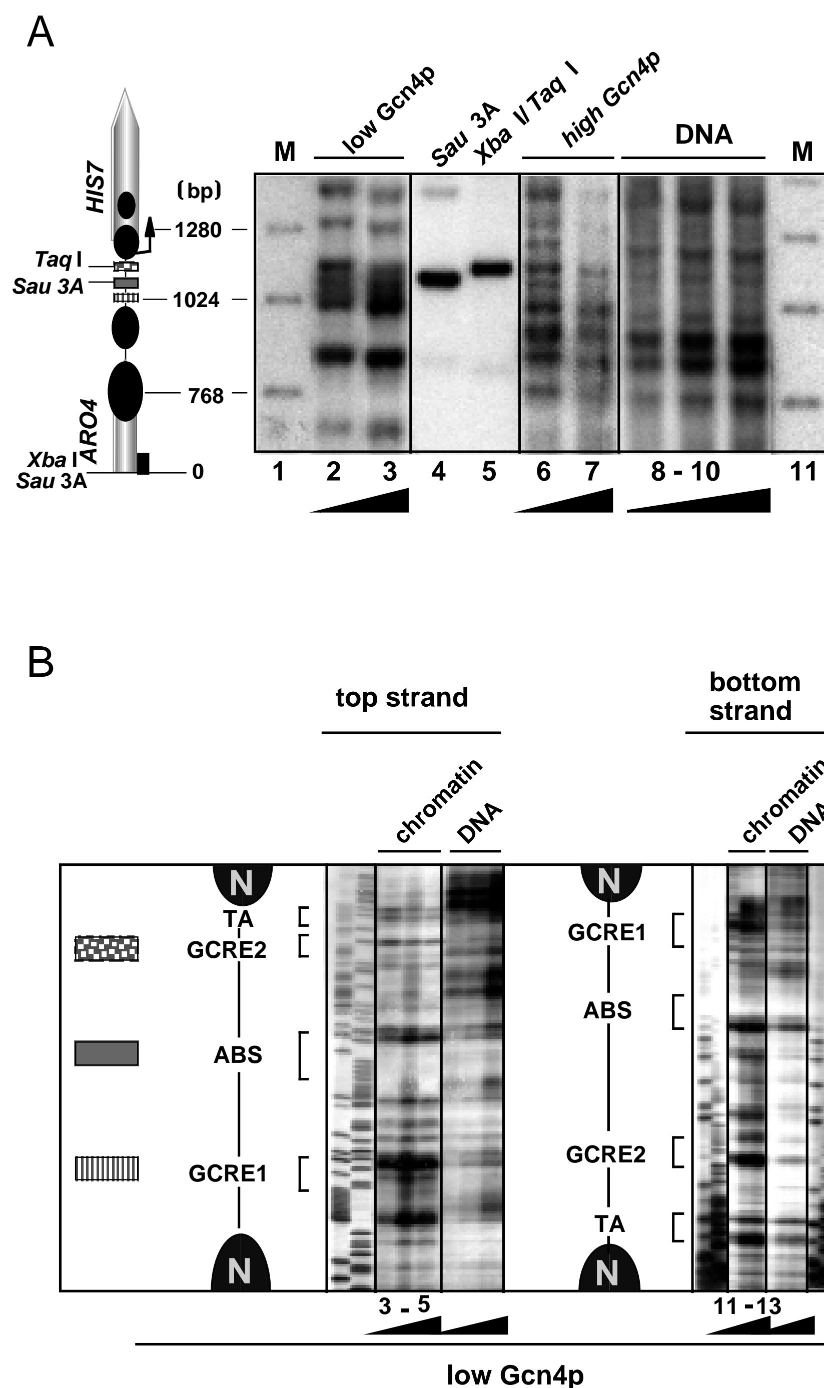

FIGURE 14. Mnase protection experiments of the nucleosome structure of the *ARO4-HIS7* intergenic region. A) Low resolution experiments with chromatin of *S. cerevisiae* strain FY1353 are indicated as wt-*GCN4* expressing low amounts of Gcn4p. A derivative strain expressing high amounts of Gcn4p is FY1353[p238]. Lanes 8-10 show Mnase control digests of the same DNA without nucleosomes. Lanes 1 and 11 display a 256 bp DNA ladder and lanes 4 and 5 are Southern hybridisations that exactly locate GCRE2 (*Xba*I/*Taq*I) and ABS (*Sau*3A) positions. The scheme on the left displays the exact positions of *cis*-elements (small boxes) and relevant restriction sites, respectively. Black ovals represent protected DNA stretches of wt-*GCN4* (lanes 2+3) which correspond in their size imposed positioned nucleosomes. High Gcn4p expression in strain FY1353[p238] removes the positioned nucleosomes. B) Enlargement of the hypersensitive promoter region and its borders of FY1353 expressing low amounts of Gcn4p by high resolution

primer extension experiments. Radioactive oligonucleotides annealing at top and bottom strand, respectively, were used to prime DNA extension in both directions. *cis*-elements (for details see Figure 1) and adjacent protected DNA presumably corresponding to positioned nucleosomes are indicated.

Gcn4p-activation releases a defined chromatin structure at the *HIS7* promoter

The *HIS7* promoter is expressed at a basal level in the absence of amino acid starvation. This basal *HIS7* expression takes place when exogenous purines are not available from the growth medium, and the cell has to synthesize purines *de novo*. Supplementation of the purine adenine to the medium represses the basal *HIS7* transcription. For the basal expression the activity of the adenine-dependent transcription factor Bas1p/Bas2p is required. During amino acid starvation Gcn4p activates the *HIS7* expression eight-fold, whereas high concentration of adenine in the growth medium reduces the expression of the basal promoter to half the value (Springer *et al.*, 1996). We addressed the following questions: (I) How does the binding of transcription factors affect the chromatin structure under basal expression conditions (Bas1p/Bas2p present, low levels of Gcn4p)? (II) What happens to the chromatin structure if the general control system is inactivated (absence of Gcn4p)? (III) Does repression of the basal *HIS7* expression by adenine supplementation alter the chromatin structure? (IV) What happens upon amino acid starvation conditions when *GCN4* expression is strongly increased?

(I) To answer the first question we determined the promoter chromatin structure of the *HIS7* gene in several promoter mutant strains carrying single nucleotide exchanges within the activator binding sites. The strains were cultivated in the absence of amino acid starvation and exogenous adenine, therefore expressing low amounts of Gcn4p but retaining the basal *HIS7* expression by Bas1p/Bas2p. Neither a defective binding site for Bas1/Bas2p (gcre2), previously reported to prevent activator binding and basal *HIS7* expression, nor a simultaneous mutation of GCRE1 and GCRE2, which together mediate the Gcn4p-response (Springer *et al.*, 1996), has changed the chromatin structure of the basal *HIS7* promoter described above (data not shown). This suggests that Gcn4p is not

required for a hypersensitive *HIS7* promoter. A functional binding site for the ubiquitous transcription factor Abf1p (ABS) in-between GCRE1 and GCRE2 supports the basal *HIS7* expression (Springer *et al*., 1997). Two specific single nucleotide exchanges within this *cis*-element drastically reduce binding of Abf1p (Dorsman *et al*., 1990), but neither this mutation alone nor in combination with mutation of GCRE1 or/and GCRE2 had any influence on the promoter chromatin structure (data not shown).

(II) A strain with a *gcn4Δ* background that cannot produce any functional Gcn4 protein displays basal *HIS7* expression in medium without adenine supplementation (Springer *et al*., 1996). The *HIS7* promoter chromatin structure under these circumstances is the same as found for low Gcn4p levels (Fig. 15 B). We therefore conclude that Gcn4p is irrelevant for the basal nucleosomal structure.

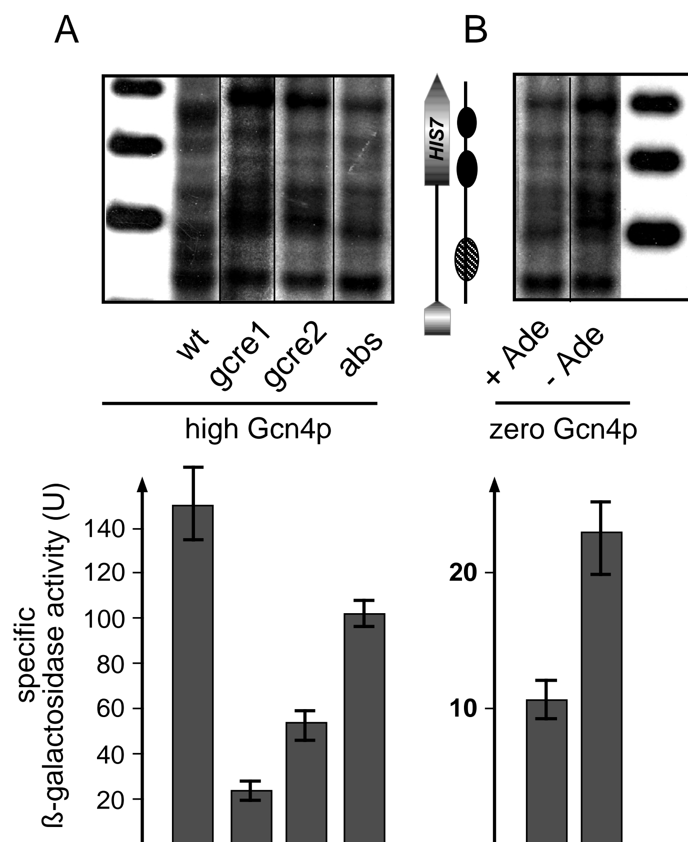

FIGURE 15. Mnase protection experiments of various yeast *HIS7* promoter alleles driving *lacZ* reporter hybrid genes. A Effect of high amounts of Gcn4p within the cell. Yeast strains are wt: RH1616 (*HIS7* wild-type promoter), gcre1: RH1619 (*HIS7* promoter mutant in GCRE1), gcre2: RH1622 (*HIS7* promoter mutant in GCRE2), and abs: RH1830 (*HIS7* promoter mutant in ABS). **B** Effect caused by 0.3 mM adenine on the *HIS7* wild-type promoter (RH1616). *his7-lacZ* expression was quantified as specific ß-galactosidase activities measured as nmol/(h ml $OD_{546})^{-1}$

and graphs are averages of three individual enzyme essays performed with four independent cultures.

(III) Supplementation of the culture medium with adenine suppresses basal *HIS7* expression to a quite low remaining level (Springer *et al.*, 1996). Mnase protection experiments with cells without functional Gcn4p grown in adenine-supplemented medium did not detect any changes of the promoter chromatin structure upon the repressed basal *HIS7* expression (Fig. 15 B). This supports the result from (I) where mutation of the binding site for Bas1p/Bas2p (GCRE2) has not led to changes during basal expression.

(IV) To analyze whether increased Gcn4p levels change the *HIS7* promoter chromatin we performed Mnase protection experiments with cells encoding a constitutively high expressed *GCN4* allele (Müller and Hinnebusch, 1986). In fact, the chromatin structure severely changed upon high levels of the transcriptional activator. The well-defined structure described for the basal expressed gene is replaced by a less ordered nucleosome distribution with hypersensitive sites throughout the entire *ARO4-HIS7* intergenic region (Fig. 14 A, lanes 6+7).

Taken the results from (I) to (III) together, the chromatin of the promoter of the typical house keeping gene *HIS7* has a ‘pre-set’ configuration. Its open and hypersensitive structure is independent from promoter-bound transcription factors Bas1p/Bas2p, Abf1p or low amounts of Gcn4p during basal expression. However, considering the result of (IV), high amounts of Gcn4p present in the cell alter the defined nucleosome distribution severely at that locus.

Both GCREs and the Abf1p-binding site are necessary for chromatin changes at the *HIS7* promoter

As described above, the binding sites for Bas1p/Bas2p (GCRE2), Abf1p (ABS) and Gcn4p (GCRE1+2) of the *HIS7* promoter are not required *per se* for the chromatin structure of the basal transcribed gene. We next analyzed whether the activator binding

sites for Gcn4p (GCREs) are required for the *GCN4*-dependent transition in chromatin structure. Both intact GCREs are prerequisites for the nucleosomal change at the promoter. Mutation of either GCRE1 or GCRE2 fixes the structure to that of the basal expressed gene (Fig. 15 A). Their essential role for the chromatin release correlates with the resulting Gcn4p-driven *HIS7* expression they mediate. Mutation of GCRE1 reduces it to about 25% and mutation of GCRE2 to about 50% relative to the wild-type promoter (Fig. 15 A).

In comparison to the GCRE1 or GCRE2 mutations, a defective ABS-site has a less pronounced effect on Gcn4p-mediated *his7-lacZ* expression. The remaining ß-galactosidase activity of the abs mutant is about 70% of the wild-type promoter (Fig. 15 A). However, this binding site is also essential for the release of the basal chromatin structure. Taken these results together the binding of Gcn4p in concert with Abf1p as auxiliary factor has the potential to change the chromatin structure of the *HIS7* promoter resulting in increased gene expression. In contrast, Bas1p/Bas2p activation by binding to GCRE2 seems to act independently of a nucleosome rearrangement.

Remodeling of the yeast *HIS7* promoter chromatin structure is *SNF2*-dependent

Chromatin remodeling upon transcriptional repression or activation of various genes requires a functional Swi/Snf complex. The chromatin structure of the *HIS7* promoter changes upon high Gcn4p expression but not upon Bas1p/Bas2p-mediated transcription. A role of Swi/Snf on *HIS7* chromatin is therefore more likely for the Gcn4p-mediated chromatin change. By performing Mnase protection experiments we investigated the *HIS7* promoter chromatin of a mutant strain deleted in the *SNF2* gene. We analyzed whether the chromatin structure of the basal transcribed *HIS7* promoter or the Gcn4p-dependent chromatin release is Swi/Snf-dependent.

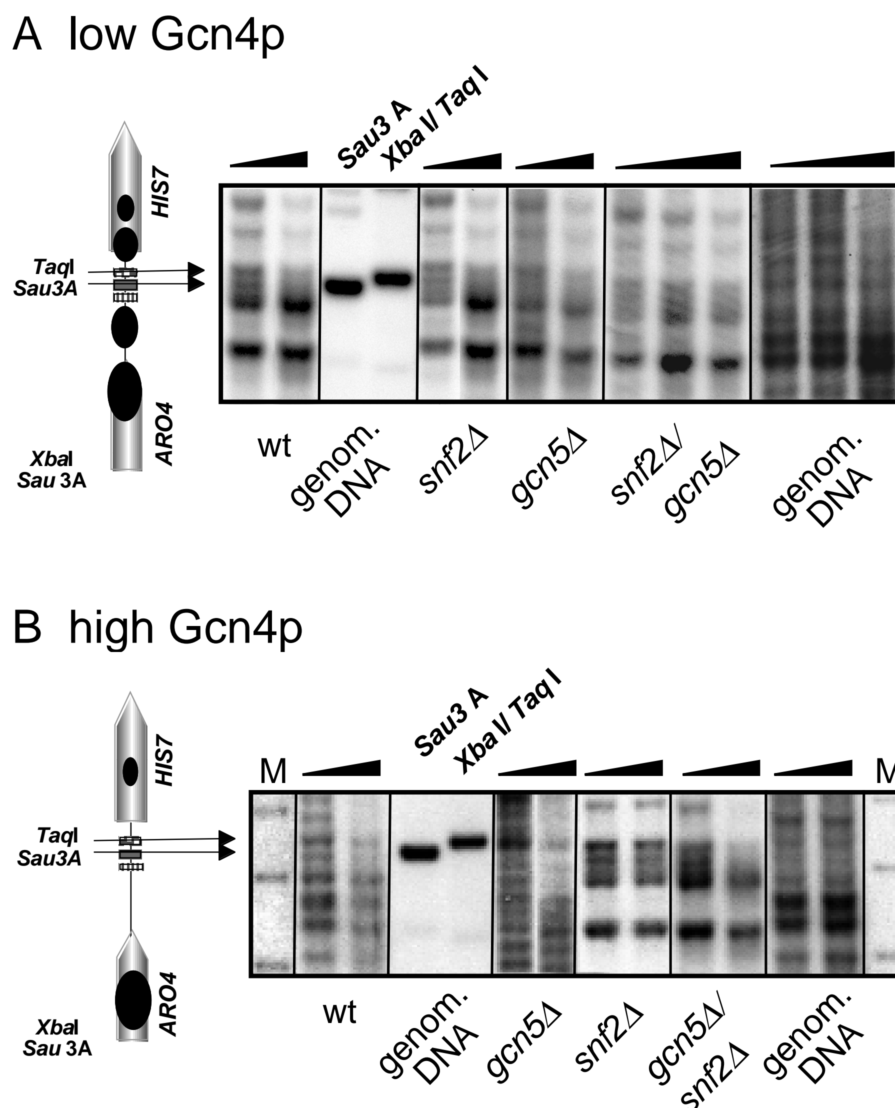

FIGURE 16. Mnase protection experiments of the nucleosome structure of the *ARO4/HIS7* intergenic region of strains defective in nucleosome remodeling and modification. *S.cerevisiae* strains FY1360 (*snf2Δ*), FY1354 (*gcn5Δ*) or FY1350 (*snf2Δ/gcn5Δ*) were analyzed. **A** Experiments performed with strains expressing either low or **B** high amounts of Gcn4p from wt-*GCN4* or the additional ^{C}*GCN4* allele of p238, respectively. Locations of GCRE2 and ABS are shown by Southern hybridisations of chromosomal DNA digested with either *Xba*I/*Taq*I (GCRE2) or *Sau*3A (ABS).

Mnase experiments with a *snf2Δ* mutant strain show a *HIS7* promoter chromatin structure comparable to the wild-type strain grown under basal gene expression conditions (Fig. 16 A). The positions of hypersensitive as well as protected regions within the intergenic region are similar for the *snf2* mutant and wild-type strain. Also the suppression of basal expression by adenine supplementation did not change the chromatin pattern (data not

shown). This suggests that the Swi/Snf complex is not an essential component in arranging and maintaining the defined *HIS7* promoter chromatin structure during its Bas1p/Bas2p-dependent transcription. However, even the basal *HIS7* promoter does not seem to be completely independent of the Swi/Snf complex, because in comparison to the wild-type strain the deletion of *SNF2* increases the basal *HIS7* transcription twofold (Fig. 17 lanes 1, 5). The mechanism by which the Swi/Snf complex might affect *HIS7* expression without affecting the chromatin structure remains to be elucidated.

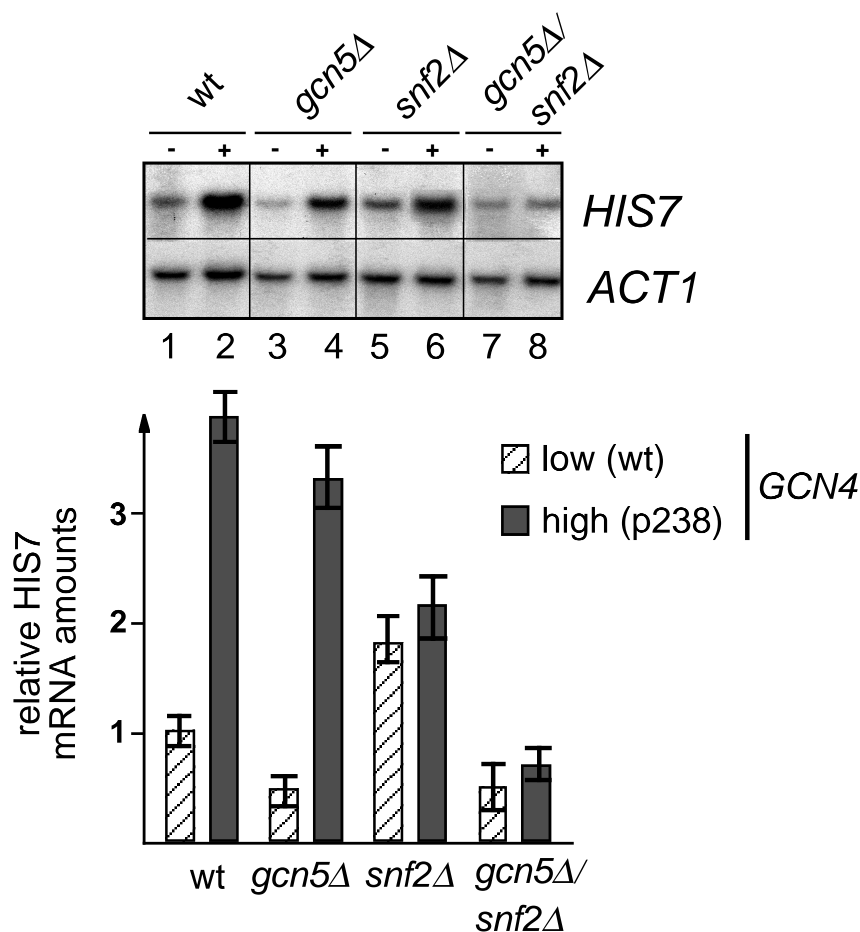

FIGURE 17. *HIS7* mRNA levels in yeast cells defective in components required for nucleosome assembly expressing either low or high amount of Gcn4 protein. Low Gcn4p amounts are expressed from the wild-type *GCN4* gene indicated with (-), whereas high Gcn4p amounts are expressed from plasmid p238 indicated with (+) for strains FY1353 (wt), FY1360 (*snf2Δ*), FY1354 (*gcn5Δ*) and FY1352 (*snf2Δ/gcn5Δ*) . Four independent RNA isolations were hybridized twice in Northern experiments and equalized to *ACT1* mRNA levels, resulting in the average values given in the graph.

The chromatin transition of the *HIS7* promoter during high Gcn4p expression is, however, prevented in a *snf2Δ*-background and chromatin restrained to its defined structure of the basal transcribed promoter (Fig. 16 B). Therefore the Swi/Snf complex is essential for the organization of the nucleosomal change upon Gcn4p-activation. Consistently with this influence on chromatin, Gcn4p-dependent activation of *HIS7* transcription mainly fails in the *snf2Δ* background (Fig. 17 lane 6).

Gcn4p-dependent *HIS7* transcriptional activation functions in the absence of a Gcn5p containing HAT complex

Nucleosomes can be remodeled by e.g. Swi/Snf, but they are also chemical modifiable. Since the *HIS7* promoter is transcribed in a Bas1p/Bas2p-dependent manner at the basal level without remodeling of nucleosomes, we investigated whether such a modification is involved. *GCN5* encodes the histone acetyl transferase activity essential for the function of the HAT complexes SAGA and Ada of *S. cerevisiae*. Acetylation of specific residues of histones within nucleosomes is strongly associated with transcriptional gene activation. In Northern experiments we investigated whether *GCN5* is involved in either activated Gcn4p-dependent *HIS7* transcription or in its basal expression in absence of amino acid starvation with cells synthesize their own adenine. By analyzing the *HIS7* promoter chromatin of a *GCN5* deletion mutant we further addressed the question whether Gcn5p influences the Mnase sensitivity at the promoter-DNA.

In a *gcn5Δ* mutant strain the *HIS7* transcription, measured by Northern hybridisation, can be activated by Gcn4p similarly to the wild-type strain that carries an intact *GCN5* gene (Fig. 17 lane 4). Therefore the Gcn4p-dependent *HIS7* transcription does not require Gcn5p-containing HAT complexes. Accordingly, the *gcn5Δ* mutant strain is still able to release the chromatin structure in the presence of sufficient amounts of Gcn4p (Fig. 16 B).

Whereas Gcn4p-dependent *HIS7* nucleosome structure and gene expression do not require a Gcn5p containing HAT complex, the Bas1p/Bas2p-dependent basal *HIS7*

expression is significantly affected by a gcn5 mutation. The basal *HIS7* mRNA level is diminished to about half the level when *GCN5* is deleted (Fig. 17 lane 3). The distribution of nucleosomes and hypersensitive sites of the basal transcribed gene is independent from functional Gcn5p (Fig. 16 A). However the hypersensitive region that covers the activator binding sites seems to be less sensitive to Mnase in a *gcn5Δ* background than in the wild-type strain. Taken together, Gcn5p contributes to the basal *HIS7* expression during purine limitation conditions but not to the Gcn4p-dependent transcription during amino acid starvation conditions.

Gcn5p is required for the Gcn4p-independent activation of the *HIS7* promoter by adenine limitation

Low amounts of Gcn4p expressed in the cell even under conditions of basal expression. It is known that these low amounts are responsible for various promoters to maintain basal expression levels. To confirm that the Gcn5p-dependency of basal *HIS7* expression is caused by effects on Bas1p/Bas2p-mediated transcription and not on Gcn4p-mediated transcription, we also investigated the role of Gcn5p on *HIS7* expression in the absence of Gcn4p. ß-galactosidase activities of *gcn4Δ* mutant strains with translational *his7-lacZ* fusions instead of the wild-type *HIS7* gene revealed a severe drop in expression from the *HIS7* promoter upon *GCN5* deletion. In the presence of adenine when *HIS7* expression is rather low, deletion of *GCN5* further reduces it to about 30% of that value (Fig. 18 lanes 1, 3). Furthermore any activation of *his7-lacZ* expression upon adenine limitation is prevented in a *gcn5Δ* background (Fig. 18 lane 4). This means that Bas1p/Bas2p requires the histone acetyl transferase activity encoded from *GCN5* to enable increased *HIS7* expression upon adenine limitation.

In summary, the Gcn4p-mediated response on *HIS7* transcription caused by amino acid starvation requires a functional Swi/Snf complex and is also accompanied by changes of the promoter chromatin structure. This response does not depend on functional Gcn5p-containing HAT complexes. In contrast the Bas1p/Bas2p-mediated *HIS7* expression

during purine limitation conditions, that does not cause chromatin remodeling at the promoter, requires the functional *GCN5* gene product but no functional Swi/Snf complex.

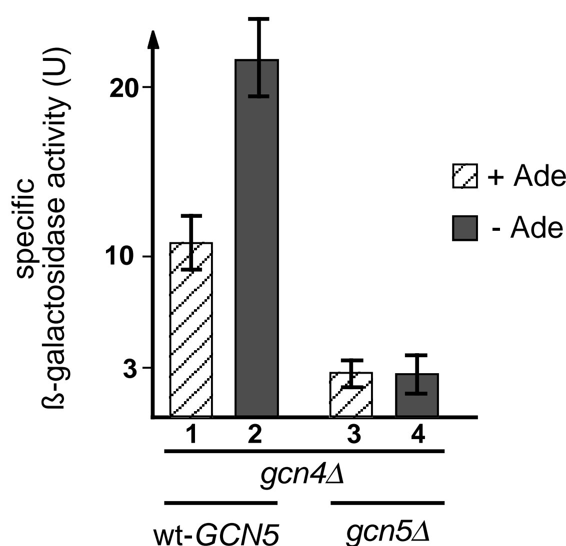

FIGURE 18. *HIS7* promoter expression driving a *lacZ* reporter in yeast cells defective in *GCN4* as well as with defects in chromatin assembly. Effects of external adenine on *his7-lacZ* expression for strains RH1616 (*GCN5*) and RH2570 (*gcn5Δ*) measured as specific ß-galactosidase activity. The graphs give average values of three individual enzyme assays performed with crude extracts from four independent cultures. Strains were cultivated either in adenine-deficient medium (- Ade) or in medium containing 0.3 mM adenine (+ Ade). Specific ß-galactosidase units (U) correspond to as nmol/(h ml $OD_{546})^{-1}$.

Discussion

In this work we show that two eukaryotic transcriptional activators that share one *cis*-element in the promoter of their common target gene respond to different stimuli by using two different nucleosome-associated protein complexes. One of them, the jun-like transcription factor Gcn4p acts together with the multi-protein complex Swi/Snf, corporately remodeling the promoter chromatin structure. The other one, the heterodimeric transcription factor Bas1p/Bas2p, acts together with Gcn5p/HAT complexes without remodeling the nucleosomal distribution (Fig. 19).

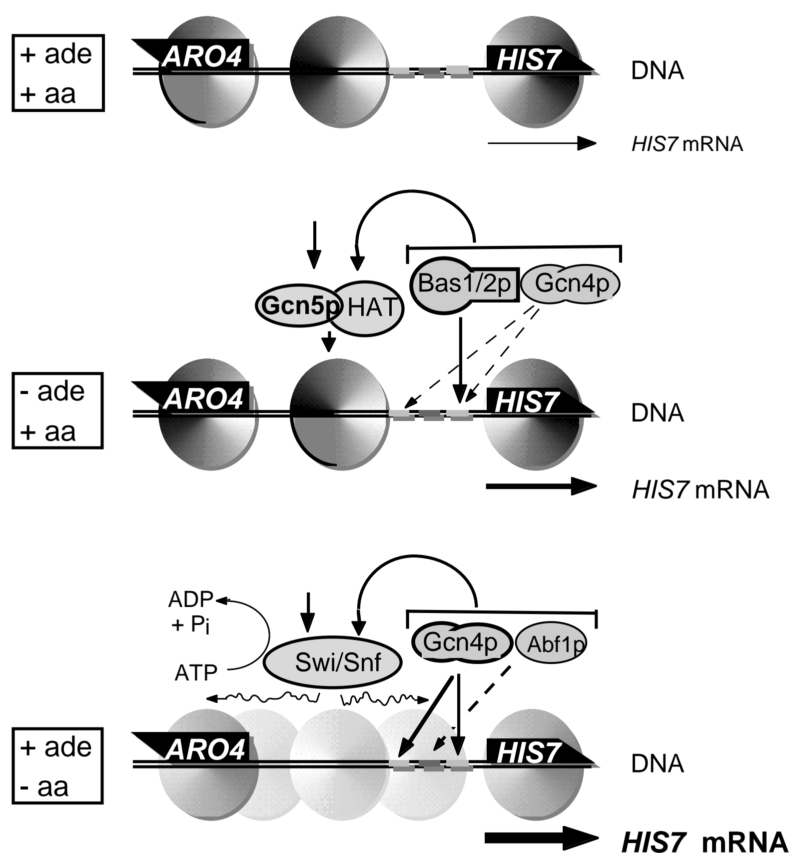

FIGURE 19. Model of the alternative mechanisms of nucleosome-dependent transcriptional activation of yeast *HIS7* initiated by two different transcription factors. + **ade** / + **aa**: Growth under conditions with sufficient amounts of adenine (ade) and amino acids (aa), that is without adenine or amino acid starvation. **- ade / + aa:** Growth without exogenous adenine but in the presence of amino acids (that is with purine starvation). + **ade / - aa:** Growth without exogenous amino acids but in presence of adenine, that is with amino acid starvation. Dark circles represent positioned nucleosomes whereas light circles represent moving nucleosomes.

Activation by Gcn4p but not Bas1p/Bas2p alters a defined chromatin structure at the *HIS7* promoter

The *HIS7* gene is an example for a yeast housekeeping gene that is regulated by to different and independent activation pathways. As consequence of amino acid starvation the transcription factor Gcn4p activates the transcription. In the absence of amino acid starvation, when yeast cells are not supplemented with purines and have to synthesize them *de novo*, the heterodimeric transcription factor Bas1p/Bas2p activates *HIS7* expression to a level which has been termed basal expression (Arndt *et al.*, 1987). Supplementation with adenine represses this basal expression to a lower level which corresponds to the *HIS7* expression of a *bas1* or *bas2* mutant strain (Springer *et al.*, 1996).

Changes in the chromatin structure at promoters during gene activation is a common phenomenon previously reported for numerous genes as e.g. *PHO5*, *PHO8*, SUC2, *CHA1*, *HIS4*, *Gal10*, *CUP1* (Almer *et al.*, 1986; Cavalli and Thoma, 1993; Devlin *et al.*, 1991; Gavin and Simpson, 1997; Gregory *et al.*, 1999; Moreira and Holmberg, 1998a; Shen *et al.*, 2001). Activators that specifically increase gene expression comprise transcription factors of different DNA binding motif classes including the basic-helix-loop-helix activator Pho4p, the acidic-Cys_6-Zn cluster activators Gal4p or Cha4p, and the basic-leucine-zipper Gcn4p.

The yeast *HIS7* gene is an example with a promoter chromatin structure that changes upon the activation by one but not the other physiological operating activator (Fig. 19). Increased Gcn4p levels releases a positioned nucleosome within the *ARO4-HIS7* intergenic region and subsequently increase *HIS7* transcription. Both Gcn4p binding sites, GCRE1 and GCRE2 of the *HIS7* promoter contribute to the change in chromatin structure suggesting that at least two Gcn4p dimer molecules have to bind for this effect. This also supports the previously described synergistic nature of *HIS7* transcription by binding of Gcn4p to two binding sites (Springer *et al.*, 1996). In addition, Abf1p which binds in between the two Gcn4p dimers functions as auxiliary factor, and accordingly an

abolishment of the Abf1p binding site in the *HIS7* promoter fixes the chromatin structure to the basal ordered structure. We assume that the simultaneous presence of the transcription factors causes chromatin remodeling and subsequently *HIS7* activation. Abf1p has been reported to affect the chromatin structure of e.g. *QCR8* (De Winde *et al.*, 1993), the *ARS1* replication site (Tanaka *et al.*, 1994; Venditti *et al.*, 1994), or recently *RPS28A* (Lascaris *et al.*, 2000), by a yet undetermined mechanism.

The *HIS7* gene is furthermore activated by another transcription factor, Bas1p/Bas2p, apparently by another mechanism as nucleosome remodeling was not detectable. This activation depends on the presence or absence of adenine, but the chromatin structure is the same for both conditions (Fig. 19). These findings were confirmed by the complementary *cis*-experiment with a destroyed Bas1p/Bas2p binding site. This binding site largely overlaps with the TATA proximal binding site of Gcn4p, GCRE2. Again, the chromatin structure remains unchanged regardless whether Bas1p/Bas2p can bind the promoter or not.

Gcn4p-dependent *HIS7* chromatin rearrangement demands a functional Swi/Snf complex

Genome-wide expression analysis revealed that about 6% of all yeast genes are affected twofold or more by the inactivation of Swi/Snf. The affected genes are subdivided into two groups, one with reduced amounts of transcript and the other with increased transcript level (Holstege *et al.*, 1998; Sudarsanam *et al.*, 2000). Since Swi/Snf remodels nucleosomes that by themselves repress promoters to which they are bound, remodeling can have two consequences. One is that repressive nucleosomes are removed from the promoter region thereby enforcing gene activity. The other is that nucleosomes are positioned to promoter elements thereby repressing gene activity.

An active role of the Swi/Snf complex in Gcn4p-mediated activation of the yeast *HIS3* gene was previously described (Natarajan *et al.*, 1999). However, a preferential accessibility for Mnase to the *HIS3* promoter was shown to be a general property of the

DNA sequence and not mediated by the Gcn4p-binding site (Mai *et al.*, 2000). Besides, studies have already stated direct interactions between transcription factors and Swi/Snf arguing for recruitment of the remodeling activity to the promoter by binding the DNA-bound activator (Neely *et al.*, 1999; Wallberg *et al.*, 2000; Yudkovsky *et al.*, 1999).
The default nucleosomal structure of the *HIS7* promoter is apparently a 'pre-set' accessible one, so that a total repression of promoter activity by positioned nucleosomes does not occur (Fig. 19). This pre-set structure is obviously compatible with the Bas1p/Bas2p-mediated *HIS7* activation during adenine limitation that does not require further remodeling. Yet this pre-set configuration does not seem to be suitable for high *HIS7* expression during amino acid starvation. For high Gcn4p-mediated *HIS7* expression nucleosomes within the *ARO4-HIS7* intergenic region have to be reorganized. This happens only when Gcn4p appears in greater abundance, and when Abf1p is apparent as an additional factor. For the purpose of the remodeling of nucleosomes a functional Swi/Snf complex is required. Probably as pattern of events the transcription is activated, meaning the increased formation of transcriptional pre-initiation complexes.

The Bas1p/Bas2p-dependent adenine response depends on a functional SAGA/Ada (Gcn5p) complex without changes of the nucleosomal distribution of the *HIS7* promoter

The histone acetyl transferase activity of the SAGA or Ada complexes is encoded by *GCN5* and is necessary for the transcriptional activation of several genes (reviewed in (Berger, 1999)). They include Gcn4p-regulated as well as Gcn4p-independent genes. Previous studies stated Gcn5p-dependence for the Gcn4p-activation of an artificial *PHO5* promoter that also harbors a binding site for Gcn4p, therefore inducible by amino acid starvation. Besides, the same *PHO5* promoter is activated upon phosphate limitation by the transcription factor Pho4p, yet independently of Gcn5p (Syntichaki *et al.*, 2000). Further genes that require a functional *GCN5* gene for the Gcn4p-dependent transcriptional activation are *HIS3*, *TRP3* and *ILV1* (Georgakopoulos and Thireos, 1992).

In contrast, the Gcn4p-dependent activation of the *HIS4* and *ARG4* genes have been shown to be strictly *GCN5*-independent (Georgakopoulos and Thireos, 1992). In common with these latter genes the Gcn4p-response of the *HIS7* promoter does not require *GCN5*. The Gcn4p-binding sites of the *HIS4*, *ARG4* and *HIS7* promoters nearly perfectly match the consensus sequence 5`-TGACTC-3` whereas the *HIS3*, *TRP3* and *ILV1* promoters possess weak Gcn4p-binding sites (Holmberg and Petersen, 1988; Struhl, 1982). Possibly the requirement for Gcn4p increases with decreasing strength of the respective Gcn4p recognition element.

However, since *GCN5* is required for the basal Bas1p/Bas2p dependent *HIS7* transcription, the situation is more complex for this promoter, enabling individual responses to different stimuli by using different mechanisms. Future experiments with antibodies against acetylated histones will show whether the adenine-dependent *HIS7* expression correlates with the promoter-acetylation pattern.

Altogether we demonstrated that the transcriptional regulation of the *HIS7* gene by two independent activation pathways strictly use different chromatin modifying machineries. Gcn4p, together with Abf1p as auxiliary factor, changes the nucleosomal distribution at the *HIS7* promoter upon amino acid starvation. This process requires a functional Swi/Snf complex but no functional SAGA/Ada (Gcn5p) complex. Bas1p/Bas2p-dependent *HIS7* activation, in contrast, requires a functional SAGA/Ada (Gcn5p) complex but is not associated with chromatin remodeling. Astonishingly this is possible although both activators use the same *cis*-element of the *HIS7* promoter.

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Chapter 4

A Positioned Nucleosome Prevents Transcriptional Interference Between the Adjacent *ARO4* and *HIS7* Genes of Yeast

Abstract

The two open reading frames (ORFs) of the *ARO4* and *HIS7* genes of *Saccharomyces cerevisiae* are transcribed into the same direction and are separated by 417 base pairs. Replacement of the *ARO4* promoter by the stronger *ACT1* promoter increases *ARO4* transcription and simultaneously reduces the basal transcription of the downstream *HIS7* gene. This phenomenon is called transcriptional interference. Deletion analyses of the region between the two ORFs revealed that transcriptional interference increases upon the removal of both *ARO4* 3`end and *HIS7* promoter sequences. Even single nucleotide exchanges within a functional Abf1p-binding site of the *HIS7* promoter are sufficient to significantly increase transcriptional interference. The DNA immediately downstream of the *ARO4* ORF is required for efficient mRNA 3' end formation and is hypersensitive to Micrococcus nuclease. In the adjacent region 3' of *ARO4* a positioned nucleosome protects the DNA against the nuclease and separates the *ARO4* terminator from the *HIS7* promoter. Increased *ARO4* transcription driven from the *ACT* promoter causes an additional hypersensitive site within this nucleosomal region presumably due to weakened DNA-histone octamer interactions. Therefore this nucleosome acts as a natural block against reading-through RNA pol II and seems to be adjusted to the normal *ARO4* transcription. Artificially increased *ARO4* transcription presumably causes transcriptional interference by overcoming this nucleosome barrier.

Introduction

Tandemly orientated genes transcribed into the same direction by RNA polymerase II (pol II) are described for different eukaryotic organisms. This arrangement of genes can jeopardize regulated transcription of a cell by a phenomenon called *transcriptional interference*. As consequence of elevated transcription of the upstream gene, transcription of the adjacently following downstream gene might be diminished or ultimately even abolished. Transcriptional interference is favoured by close proximity of genes which are only separated by short intergenic regions between the corresponding open reading frames (ORFs). Whereas transcription in prokaryotes by the RNA polymerase is particularly susceptible to interference because of their densely packed genome (Adhya and Gottesman, 1982; Hausler and Somerville, 1979), the phenomenon is less frequently described in eukaryotes. In HeLa cells two closely spaced α-globin genes in an artificial gene construct were shown to interfere with each other (Proudfoot, 1986). The cryptic promoter within the intron of the *ACT1* gene of *Saccharomyces cerevisiae* is occluded by transcription from the actual *ACT1* promoter at the 5`end of the gene (Irniger *et al*., 1991). Yeast *HIS7* transcription is reduced when the upstream located *ARO4* gene is transcribed from the strong *ACT1* promoter instead of its natural promoter (Springer *et al*., 1997b).

Eukaryotic transcriptional interference is presumably the result of pol II complexes that initiate transcription at the promoter of the upstream gene and subsequently read through the promoter of the downstream gene. Therefore the assembly of functional transcription complexes at the downstream promoter is disturbed resulting in promoter occlusion. The extent of the transcriptional interference by reading-through pol II complexes could be increased when the efficiency of transcription termination of the upstream gene was reduced (Greger and Proudfoot, 1998; Springer *et al*., 1997b). Deletions of *GAL10* poly(A) signals abolish any activity of the downstream *GAL7* gene even when the *GAL7* promoter is intact resulting in a bicistronic read-through transcript. Therefore the *GAL7*

promoter is completely occluded. Polymerase profiles raised in transcription-run-on (TRO) experiments for a strain with a mutated poly(A) site confirmed the accumulation of non-terminated pol II complexes within the *GAL7* promoter (Greger and Proudfoot, 1998). As a consequence of transcriptional interference it was shown that various transcription factors were not any more able to bind to their promoter sites. This was e.g. demonstrated for the tandem HIV-1 promoters integrated into the genome of HeLa cells where promoter occlusion of the downstream promoter correlated with reduced binding of the transcription factor Sp1 (Greger *et al.*, 1998). The Gal4p activator binding to the *GAL7* promoter is also reduced when this promoter is weakened by read-through transcription initiated at the upstream *GAL10* promoter (Greger *et al.*, 2000).

A further aspect to be considered for transcriptional interference is the fact that eukaryotic DNA is packed into histone octamers. The resulting nucleosomes can be positioned stably or more or less moveable due to only weak DNA-histone interactions. For *Drosophila melanogaster* it was shown that reconstituted chromatin with rDNA templates resulted in a positioned upstream nucleosome that is recruited by termination factor TTF-I. This nucleosome can act as barrier to transcriptional interference of the downstream located rRNA genes which are transcribed by the RNA polymerase I complex (Längst *et al.*, 1997).

Up to now there is hardly any knowledge whether there is also a connection between transcriptional interference caused by insufficient termination and chromatin structure for genes. To address this question we constructed various *ARO4-HIS7* alleles of *S. cerevisiae* and analyzed them at the authentic chromosomal locus. Both genes encode amino acid biosynthetic enzymes required for the formation of aromatic amino acids and histidine, respectively. *ARO4* and *HIS7* are transcribed into the same direction and have an intergenic region between the two ORFs consisting of 416 base pairs (bps). We had previously shown that increased *ARO4* transcription as consequence of fusing its ORF to the strong *ACT1* promoter causes significantly reduced expression of the downstream

HIS7 gene (Springer *et al*., 1997b). Here we show for the first time the role of nucleosomes in preventing transcriptional interference of pol II genes.

Experimental Procedures

Yeast strains, growth conditions and plasmids

Yeast strains and their genotypes used in this work are listed in the table 3. For all experiments described here strains were cultivated in minimal vitamin (MV) medium (Miozzari *et al*., 1978) supplemented with the required amino acids according to (Rose *et al*., 1990). To generate functional fusions of the *ACT* promoter with the *ARO4* open reading frame (ORF) at the original *ARO4* locus, a *Sal*I/*Bst*EII 3.1 kb fragment from plasmid pME1429 (Springer *et al*., 1997b) was transformed in the respective strains. The cassette consists of the *ACT* promoter fused to the first half of the *ARO4* ORF, preceded by the divergently orientated *URA3* auxotrophic marker gene that again is preceded by the *ARO4* 5`untranslated region. Transformants that had replaced the wild-type *ARO4* locus by this cassette by homologous recombination were selected by uracil prototrophy and confirmed in Northern hybridisations by increased *ARO4* mRNA levels and by PCR.

RNA analysis

Total RNA from *S. cerevisiae* was isolated according to Cross and Tinkelenberg (1991). For Northern hybridization analysis 20 µg of total RNAs were separated on a formaldehyde agarose gel, transferred to a positively charged nylon membrane (Biodyne B, PALL) by capilar blotting. Hybridization with specific DNA probes was performed after ^{32}P-labelling of the respectice DNA fragments with the *Prime It II* DNA Labelling Kit from Stratagene. One kb PCR fragments generated with the oligonucleotides ARO-OLV19 (5´taccggatccagacgacagagttcttg3´) and ARO-OLV11 (5´cctcaagacgtcttcagtagtttcc caacc3´), or with oligonucleotides HIS-OL1 (5´gtggtaacctacagtcactaacc3´) and HIS-OL2 (5´ccgatcgatactttatcagcacc3´), or with ACT-OL1 (5´gctgctttggttattgataacgg3´) and ACT-

OL2 (5´cacttgtggtgaacgatagatgg3´) served as probes for the *ARO4*, *HIS7* and *ACT* gene, respectively. Band intensities were visualized by autoradiography and quantified using a BAS-1500 Phospho-imaging scanner (Fuji).

Genomic chromatin preparation and nuclease digestions

These methods have been described previously (Thoma, 1996). Biodyne B nylon membranes (Pall, Dreieich, Germany) were used for Southern transfer. Probes were labeled by the random primer method (Feinberg and Vogelstein, 1983).

Indirect end-labeling

Chromosomal DNAs from the nuclease digestion were digested with *Xba*I and *Mlu*I and fractionated on 1.2% agarose gels. The fractionated DNA was blotted on a Hybond-N nylon membrane by the alkaline blotting method and hybridized with a radioactively labelled 250 bp PCR-amplificate, generated with the oligonucleotides HIS7-CHR1 (5´gagattaaagaaattgtcaga3´) and HIS7-CHR2 (5´caagtattgaggagaaatggta3´), annealing just downstream of the *Xba*I site. A DNA ladder consisting of multiples of 256 bp was used for calibration (Thoma *et al*., 1984).

ß-galactosidase assay

ß-galactosidase activities were determined by using permeabilized yeast cells and the fluorogenic substrate 4-methylumbelliferyl-ß-D-galactoside as described previously (Kuenzler *et al*., 1993). The presented values are the means of at least four independent cultures each of them measured three times.

Table 3 Yeast strains used in this study

Strain	Genotype	Reference
RH1381	*MATα aro3-2 gcn4-101 ura3-52*	Paravicini *et al*., 1988
RH1833	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-391/-341)*	Springer *et al*., 1997b
RH1834	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-336/-310)*	Springer *et al*., 1997b
RH1836	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-299/-281)*	Springer *et al*., 1997b
RH1781	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-220/-189)*	Springer *et al*., 1997b
RH2642	*MATα aro3-2 gcn4-101 ura3-52 ARO4::URA3-^{P}ACT-ARO4*	this work
RH2643	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-391/-341) ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2644	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-336/-310) ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2645	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-299/-281) ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2646	*MATa aro3-2 gcn4-101 ura3-52 ΔHIS7(-220/-189) ARO4::URA3-^{P}ACT1-ARO4*	this work
RH1616	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7-lacZ*	Künzler *et al*., 1993
RH1815	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-391/-341)-lacZ*	Springer *et al*., 1997b
RH1816	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-336/-310)-lacZ*	Springer *et al*., 1997b
RH1818	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-299/-281)-lacZ*	Springer *et al*., 1997b
RH1819	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-285/-245)-lacZ*	Springer *et al*., 1997b
RH1822	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-241/-212)-lacZ*	Springer *et al*., 1997b
RH1824	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-220/-189)-lacZ*	Springer *et al*., 1997b
RH1825	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-190/-171)-lacZ*	Springer *et al*., 1997b
RH1826	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-171/-139)-lacZ*	Springer *et al*., 1997b
RH2174	*MATa aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work

Strain	Genotype	Reference
RH2632	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-391/-341)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2633	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-336/-310)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2634	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-299/-281)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2635	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-285/-245)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2636	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-241/-212)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2637	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-220/-189)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2638	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-190/-171)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH2639	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(-171/-139)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work
RH1830	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(mut-ABS)-lacZ*	Springer *et al*., 1997a
RH2640	*MAT**a** aro3-2 gcn4-101 ura3-52 HIS7::^{P}his7(mut-ABS)-lacZ ARO4::URA3-^{P}ACT1-ARO4*	this work

Results

^{P}*ACT*-driven *ARO4* transcription creates a Mnase sensitive site within a nucleosomal DNA that separates the *ARO4* terminator from the *HIS7* promoter

ARO4 and *HIS7* are two adjacent genes transcribed into the same direction. The replacement of the natural promoter of the *ARO4* gene by the stronger *ACT* promoter was previously shown to cause transcriptional interference with the downstream *HIS7* gene (Springer *et al.*, 1997b). This transcriptional interference reduces the *HIS7* expression to about 60 % in comparison to the strain with the wild-type *ARO4* promoter. Since eukaryotic gene expression and its tight regulation in terms of transcriptional initiation and termination processes must take place in the presence of highly ordered chromatin structures, we wanted to know whether the transcriptional interference between the *ARO4* and *HIS7* gene is accompanied by chromatin changes. Therefore we analyzed the chromatin structure of the *ARO4-HIS7* intergenic region in absence and presence of transcriptional interference.

The chromatin structure was investigated by Micrococcus nuclease (Mnase) protection experiments. Crude nuclear extracts from over-night cultures grown in minimal vitamin medium (MV) from strains with the wild-type *ARO4* promoter (RH1381) or the ^{P}*ACT-ARO4* fusion (RH2642), respectively, were partially digested with Mnase and further treated as previously described (Thoma, 1996). In wild-type cells the *ARO4* 3´end region immediately downstream of the ORF is hypersensitive to Mnase (Fig. 20). This short hypersensitive region is followed by a strongly protected region that in length corresponds to a positioned nucleosome. The *HIS7* promoter further downstream is again hypersensitive to Mnase. Although the mutant strain with the ^{P}*ACT-ARO4* fusion gene shows a largely similar chromatin pattern, an additional band within the protected region between the *ARO4* 3´end region and the *HIS7* promoter becomes obvious (Fig. 20, arrow

1). Even if the band is rather weak, this sensitive site is absent in the *ARO4* wild-type strain.

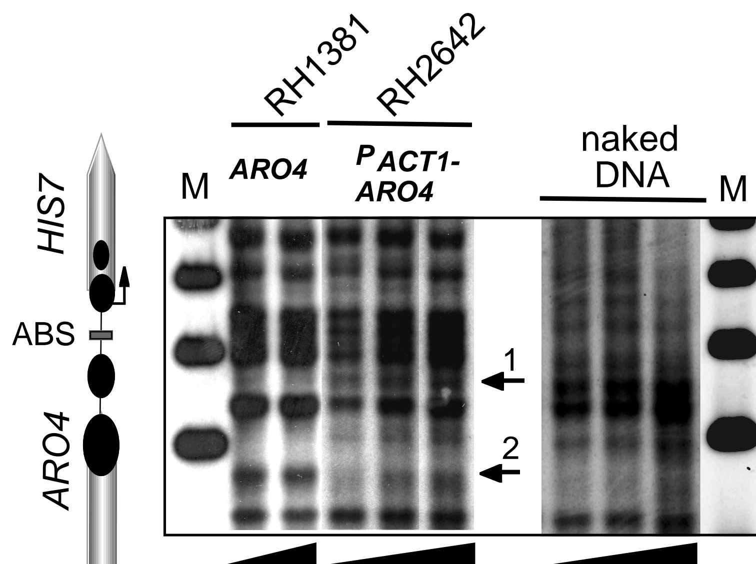

FIGURE 20. Mnase protection experiments of the nucleosome structure of the *ARO4-HIS7* intergenic region. Strain RH1381 possesses the *ARO4* gene with its natural promoter (*ARO4*) whereas strain RH2642 has the *ARO4* promoter replaced by the more efficient *ACT1* promoter (^{P}*ACT1-ARO4)*) instead. Chromatin of both strains and naked control DNA was digested with increasing amounts of Mnase (black ramps). On the left and right outer lanes a size marker of 156 bp-repeats is shown (M). The arrows indicate differences in chromatin structure between both strains. Black ovals in the scheme on the left reflect protected regions, suggested in representing positioned nucleosomes. ABS represents the binding site for the transcription factor Abf1p that was previously shown to bind the *HIS7* promoter thereby supporting basal *HIS7* expression (Springer *et al.*, 1997a).

As consequence of high *ARO4* transcription the transcribing RNA pol II complex therefore presumably weakens the DNA histone interaction at the respective nucleosome thereby creating the hypersensitive site. In addition, a hypersensitive site at the end of the *ARO4* ORF nearly vanishes for the strain with the ^{P}*ACT-ARO4* fusion gene (Fig. 20, arrow 2). Apparently the chromatin structure of the very 3'end of the *ARO4* ORF is also subjected to changes upon strong *ARO4* transcription. These changes of chromatin structures are possibly directly related to termination efficiency and transcriptional interference.

Deletions within the *ARO4* 3`UTR or the *HIS7* promoter increase interference between *ARO4* or *HIS7* transcription

The DNA in-between the two open reading frames of *ARO4* and *HIS7* was shown to possess elements required for efficient 3´ end formation of the *ARO4* mRNA, and others that promote efficient *HIS7* transcription (Springer *et al*., 1997b). Here we have defined DNA regions between these open reading frames which protect the *HIS7* promoter against transcriptional interference caused by high *ARO4* transcription. We established a test-system with the *HIS7* gene replaced by the quantifiable chimeric ^{P}*his7-lacZ* gene, preceded from the *ARO4* gene driven either from its natural promoter (Fig. 21, test system I) or from the *ACT1* promoter (same figure, test system II). To determine important regions in-between both open reading frames that prevent transcriptional interference, specific ß-galactosidase activities for intergenic regions carrying various small deletions were measured. DNA elements that diminish transcriptional interference have been identified by comparison of the ß-galactosidase activities of both test-systems with each deletion construct (Fig. 22). In order to maintain the original chromosomal context the respective test system was established at the authentic *ARO4-HIS7* locus. Deletions were chosen to cover several specific DNA-motifs with different functions including the Zaret/Sherman element (Z/S) required for *ARO4* mRNA 3` end formation, three poly(A) sites (p(A)) defining the actual poly(A) addition sites, C+T and A+T rich regions, the Abf1p-protein binding site (ABS), and both Gcn4p-recognition elements (GCREs) (Fig. 22).

Deletions that cover the Zaret/Sherman element and the first poly(A) site which are elements of *ARO4* mRNA 3´ end formation, and the Abf1p-binding site located within the *HIS7* promoter cause a significant reduction in specific ß-galactosidase activity when measured in test-system II. The 52-base pair deletion within the *ARO4* 3`UTR that eliminates the Zaret/Sherman element does not affect the ^{P}*his7-lacZ* expression when the *ARO4* gene is transcribed from its own promoter in test-system I (strain RH1815 in Fig. 22). The *ACT* promoter of test-system II (strain RH2632), however, causes a severe

reduction of ß-galactosidase activity for this deletion to about 28 % of test-system I. Also the 28-base pair deletion that removes the first polyadenylation site (p(A)) strongly reduces ^{P}*his7-lacZ* expression if present in test-system II (strain RH2633). Only about 22 % activity is left in comparison to test-system I with this deletion (strain RH1816). Deletions of the second and third poly(A) site (I: RH1818; II: RH2634), or C+T rich (I: RH1819; II: RH2635) and A+T rich (I: RH1835; II: RH2638) stretches, or the binding sites for Gcn4p (GCREs; I: RH1822/RH1826; II: RH2636/RH2639) do not increase transcriptional interference. However, the test-system II combined with a deletion of the Abf1p-binding site (ABS) displays a significant loss of specific ß-galactosidase activity, yet keeping in mind that this deletion itself strongly diminishes expression in test-system I.

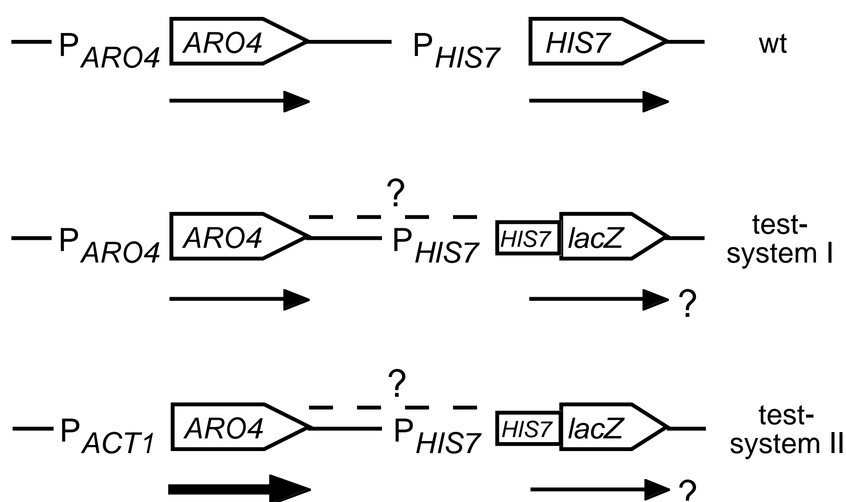

FIGURE 21. Scheme of the test-systems used to determine DNA regions within the *ARO4-HIS7* intergenic region that antagonize transcriptional interference. The top lane shows the wild-type *ARO4-HIS7* locus without any changes. The lane in the middle represents 'test-system I' in which the open reading frame of the *HIS7* gene is replaced by the reporter gene *lacZ*. The 'test-system II' in the lane on the bottom has not only the *lacZ* reporter gene but also an *ARO4* allele in which the natural *ARO4* promoter is replaced by the more efficient *ACT1* promoter. To find out elements that prevent transcriptional interference, both test-systems have been driven with various deletion constructs of the intergenic region, and specific ß-galactosidase activities have been compared with each other (see figure 22).

In summary, the data obtained from this test-system suggest that the Zaret/Sherman element and the first poly(A) site, together responsible for efficient *ARO4* 3´ end formation, are essential elements which inhibit transcriptional interference. In addition, the Abf1p-binding site within the *HIS7* promoter counteracts promoter occlusion. No region has been detected which enhances transcriptional interference since no deletion has resulted in higher ß-galactosidase activities in test-system II when compared with test-system I.

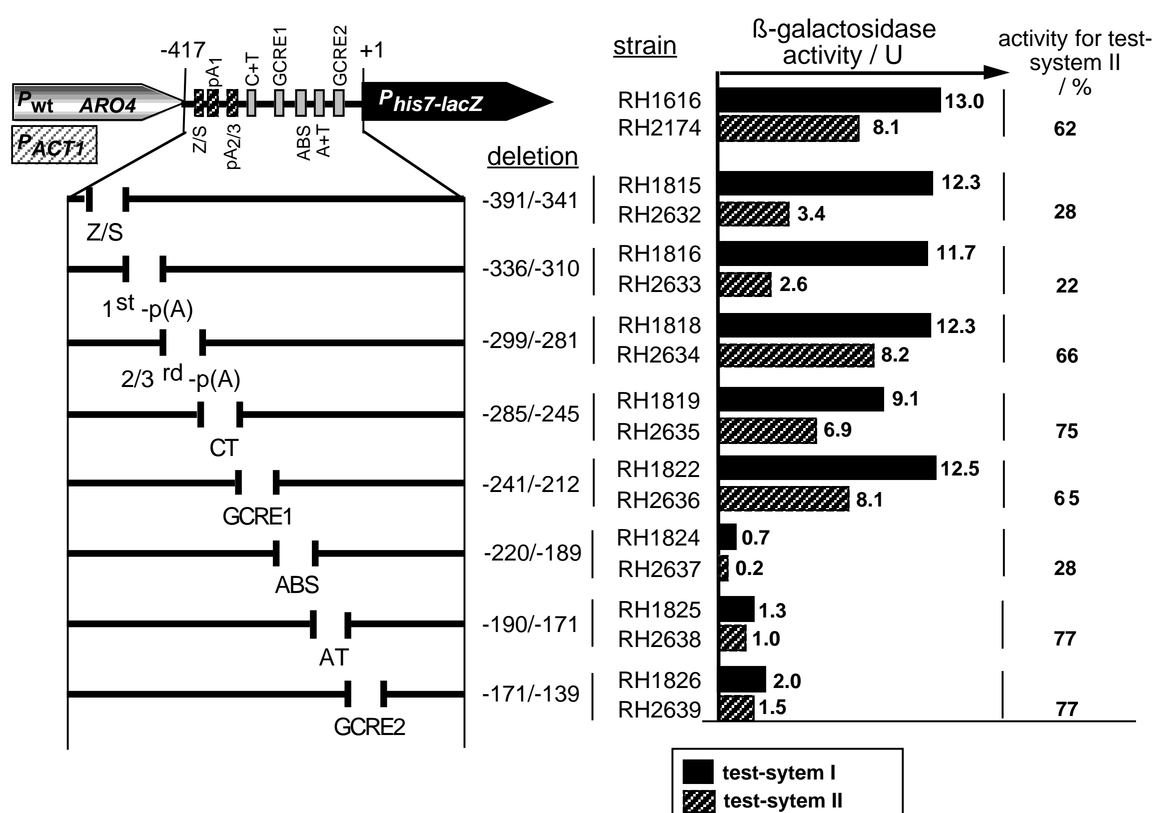

FIGURE 22. Effects of deletions of the *ARO4-HIS7* intergenic region on *his7-lacZ* activity. The strains carry either the *ARO4* gene possessing its natural wild-type promoter (test-system I) or the more efficient *ACT1* promoter (test-system II). ß-galactosidase activities in U measured as nmolMUF/(h ml OD546)$^{-1}$ are indicated in the chart on the right side for strains with various deletions throughout the intergenic region. The scheme on the left side visualizes the exact locations of the deletions according to the translational start side (+1) and the DNA motifs at these positions. Z/S stands for Zaret/Sherman motif necessary for correct *ARO4* 3´ end formation, p(A) for the sites where the pol(A) tail is added to the *ARO4* mRNA 3´ end, CT for a C+T rich element. In the *HIS7* promoter GCRE stands for Gcn4p-recognition element, ABS for Abf1p-binding site, and AT for an A+T-rich sequence. Figures on the outer right headlined as *rest activity* display the percentage of ß-galactosidase activity that is left in ^{P}*ACT1-ARO4* fusion strains relative to its parental strain possessing wild-type *ARO4*.

Transcriptional interference causes histidine auxotrophy for a *HIS7* promoter mutant without Abf1p-binding site by prevention of its transcription

The data of the test-systems that derived from a *lacZ*-reporter gene have suggested that elements required for efficient *ARO4* mRNA 3´ end formation and a *HIS7* promoter element with the Abf1p-binding site are important to prevent transcriptional interference. We investigated whether the increased transcriptional interference of these deletion mutants gives rise to malfunctions in cells that harbor the wild-type *HIS7* gene and synthesize their own histidine. Therefore growth of these wild-type strains was tested on medium without histidine, where cells have to synthesize this amino acid themselves.

When the *ARO4* gene was driven from its own promoter the growth was neither abolished for strains with deletions in the *ARO4* 3´ end region nor for strains with *HIS7* promoter deletions (data not shown). When the *ARO4* gene was driven from the *ACT*-promoter in this *HIS7* background, the strains with deletions in the *ARO4* 3´ end region (ΔZ/S, Δ1st p(A), or Δ2nd/3rd p(A)) grew well without histidine supplementation (growth test in Fig. 23 shows: Δ1st p(A) with the *ARO4* wild-type promoter in strain RH1834, and *ACT1* promoter fused to *ARO4* in strain RH1644). Also the strain RH1781 with the *HIS7* promoter deletion that covers the Abf1p-binding site (ΔABS) and the *ARO4* gene driven from its natural promoter is viable without exogenous histidine. But the same promoter deletion in combination with an *ARO4* gene driven from the *ACT1* promoter causes histidine auxotrophy in strain RH2646 (Fig. 23).

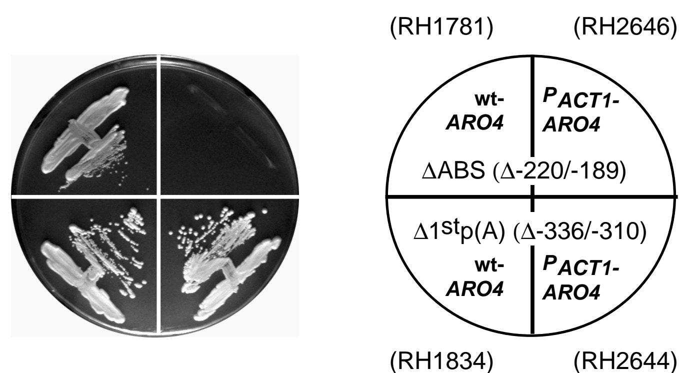

FIGURE 23. Transcriptional interference causes histidine auxotrophy for a *HIS7* promoter deletion that eliminates the Abf1p-binding site. The growth of yeast strains was tested on histidine deficient MV medium. Strains RH1781 and RH1834 possess the *ARO4* gene with its natural promoter and have deletions in either the *HIS7* promoter (ΔABS) or the *ARO4* 3'end region (Δ1ST p(A)). Strains RH2646 and RH2644 have the natural promoter of the *ARO4* gene replaced by the *ACT1* promoter and carry either the *HIS7* promoter deletion ΔABS or the *ARO4* 3´ end deletion Δ1ST p(A).

We compared the effects of the transcriptional interference as obtained by the *his7-lacZ* chimeric genes and the growth tests, with the quantified *HIS7* transcript levels determined by Northern hybridisations (Fig. 24). The fusion of the *ACT1* promoter to the *ARO4* gene increases *ARO4* mRNA levels approximately four-fold (Fig. 24). High *ARO4* transcription derived from the *ACT1* promoter in the background of the wild-type *HIS7* gene and a wild-type *ARO4-HIS7* intergenic region in strain RH2642 has decreased *HIS7* mRNA levels to about 60 % compared to the *ARO4* gene with its natural promoter in strain RH1381. In strain RH2643 the *ARO4* 3`end region with the Zaret/Sherman element as 3´ end formation signal is deleted and the *ARO4* gene driven from the *ACT1* promoter. *HIS7* mRNA levels in this strain were decreased to approximately 40% of strain RH1833 that possesses the natural *ARO4* promoter. The reduction of *HIS7* transcript levels is even more pronounced in strain RH2644 with an intergenic deletion that covers the first site where the poly(A) tail is added to the *ARO4* transcript to about 30% of strain RH1834 with the natural *ARO4* promoter. In contrast the deletion covering the second and third poly(A) addition site does not show any obvious differences in *HIS7* transcripts if compared with the wild-type intergenic region.

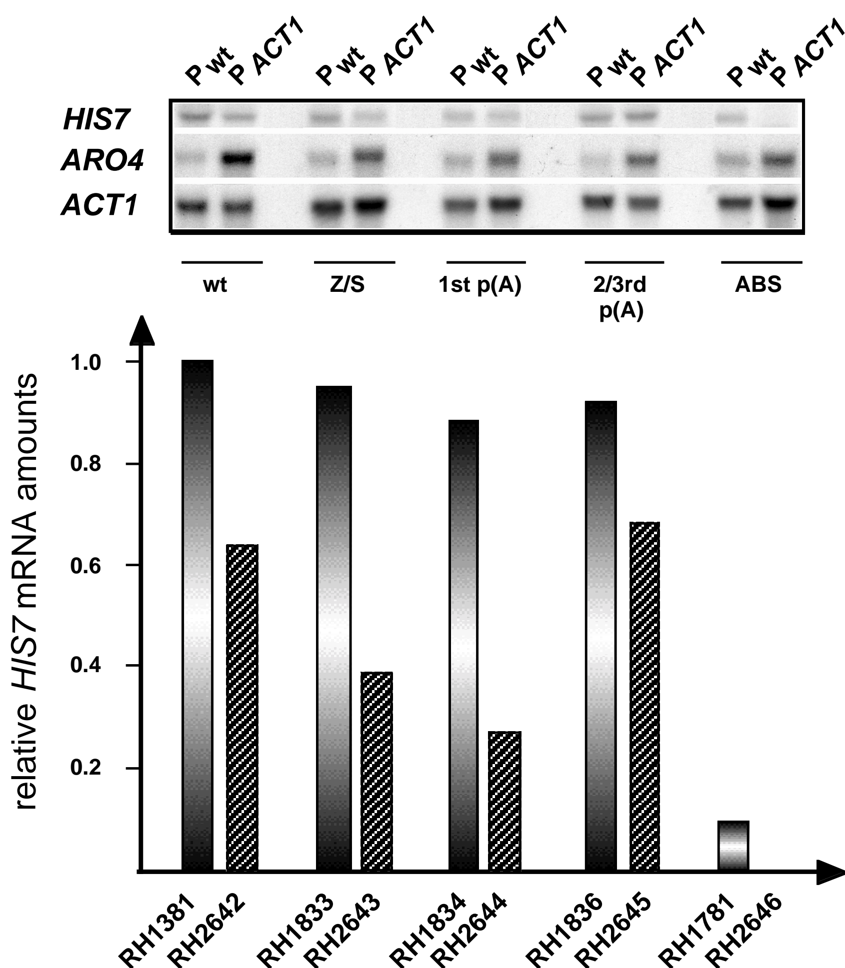

FIGURE 24. Effects of deletions of the *ARO4-HIS7* intergenic region on *HIS7* transcript levels. Northern hybridisations of selected yeast strains with deletions in either the *ARO4* 3`-untranslated region (Z/S, p(A)) or the *HIS7* promoter covering the Abf1p-binding site (ABS). Quantifications were performed by phosphopimager analyses and are presented as averages of at least two hybridisations with total RNAs from three independent cultures. Quantifications of the *ARO4* mRNA amounts revealed a fourfold increase in average if the gene is transcribed from the *ACT1* promoter.

Yeast strain RH1781 with the wild-type *ARO4* gene and a *HIS7* promoter deletion that covers the Abf1p-binding site already displays rather low *HIS7* mRNA levels of less than 20 % if compared with the wild-type promoter. However, in combination with high *ARO4* transcription from the *ACT1* promoter in strain RH2646 no *HIS7* transcripts were detectable any longer. This result confirmed the transcriptional interference as detected before in both the test-system with the *his7-lacZ* reporter gene, and the growth defect on histidine deficient medium.

Taken together, these results imply that *ARO4* 3´ end formation signals as well as *HIS7* promoter elements are required to prevent transcriptional interference. By name these are (I) the *ARO4* mRNA 3´ processing motif that was originally described by Zaret and Sherman for *CYC1* (Zaret and Sherman, 1984), (II) the major site where the *ARO4* mRNA transcript is finally cleaved and the poly(A) tail added, and (III) an element of the *HIS7* promoter that binds the transcription factor Abf1p.

Single nucleotide exchanges within the Abf1p-binding site increase transcriptional interference

The results obtained so far with the deletion constructs suggest that the Abf1p-binding site plays an important role in preventing transcriptional interference at the wild-type *ARO4-HIS7* locus. We investigated whether it has been the broader context of the deleted promoter region or solely the abolished binding of Abf1p itself that antagonizes transcriptional interference. Therefore we investigated the ^{P}*his7-lacZ* expression of a mutant strain with two single nucleotide exchanges within the Abf1p binding site that were previously shown to abolish binding of Abf1p (Dorsman *et al.*, 1990).

In comparison to the strain with the *HIS7* wild-type promoter (RH1616), single nucleotide exchanges within the Abf1p-binding site in strain RH2640 reduce ^{P}*his7-lacZ* expression to about 30 %, both strains expressing wild-type *ARO4*. Transcriptional interference for the binding site mutant strain, induced by the replacement of the *ARO4* promoter against the *ACT1* promoter leading to strain RH2641, is in fact increased in comparison to the wild-type intergenic region. High *ARO4* transcription in combination with the wild-type intergenic region decreases the *his7-lacZ* expression to about 60 % in comparison to strain RH1616 with the natural *ARO4* promoter. Single nucleotide exchanges within the Abf1p-binding site combined with high *ARO4* expression (strain RH2641) cause a reduction to 30 % in comparison to strain RH2640 with low *ARO4* transcription (Fig. 25). This result has demonstrated that it is the binding of Abf1p to its binding site within the *HIS7* promoter, and not a broader promoter context that

antagonizes transcriptional interference at the *ARO4-HIS7* locus. Probably binding of Abf1p to its *cis*-element competes with the transcription of a non-terminated pol II complex, and therefore at least partially blocks transcriptional interference.

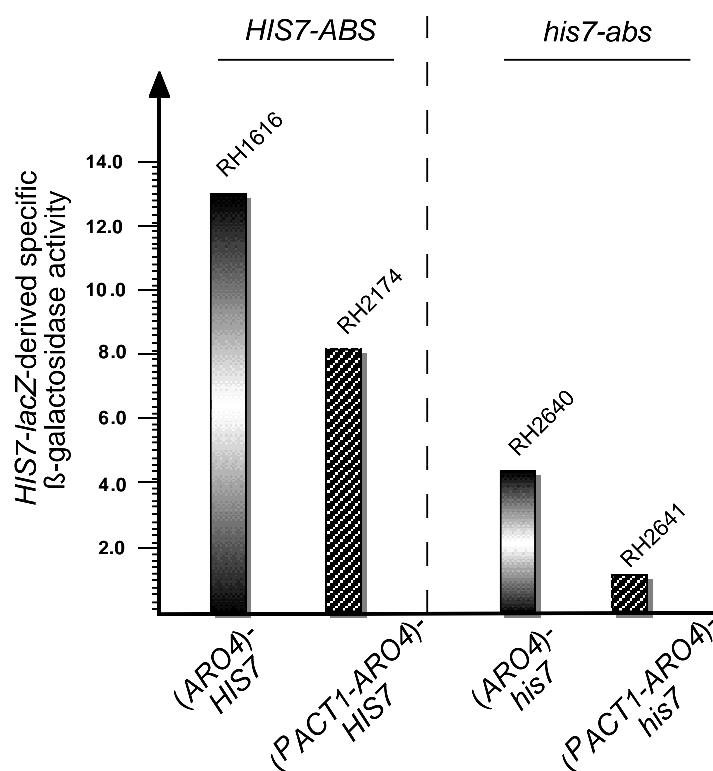

FIGURE 25. Relative ß-galactosidase activities of ^{P}*his7-lacZ* fusion strains with the *ARO4* gene driven either from its own promoter (*ARO4*) or from the more efficient *ACT1* promoter (^{P}*ACT1-ARO4*). Each of both *ARO4* alleles has either been combined with the wild-type *HIS7* promoter (*HIS7*) or with a *HIS7* promoter that had two single nucleotide-exchanges in the Ab1p-binding site (*his7*). This has led to the combinations (I) (*ARO4*)-*HIS7*, (II) (^{P}*ACT1-ARO4*)-*HIS7*, (III) (*ARO4*)-*his7*, and (IV) (^{P}*ACT1-ARO4*)-*his7*. The figures displayed for all strains are averages of total ß-galactosidase activities in U measured as nmolMUF/(h ml OD546)$^{-1}$ of at least four individual cultures.

We have also investigated whether the single nucleotide exchanges within the Abf1p-binding site change the chromatin structure of the intergenic region. However, no changes in comparison to the wild-type intergenic region have been detected (not shown). Therefore we suggest that it is rather the DNA-binding of the protein itself

thereby somehow blocking the transcribing pol II complex, than a reorganization of the nucleosomal structure by Abf1p that subsequently prevents transcriptional interference.

Discussion

All living cells must have developed mechanisms that enable individually regulated expression of adjacent genes that are located in close proximity without disturbing each other. This mainly implies that high expression of one gene as a consequence of e.g. the response to activating environmental stimuli must not interfere with the expression of its neighboring gene that might be lowly expressed under these circumstances. To make individual regulation of two tandemly orientated genes possible, the cell has to cope with numerous mechanistic obstacles.

The transcription of the gene that is transcribed firstly has to be efficiently completed. In eukaryotic cells this process is characterized by the combination of events that generate the mRNA 3` end followed by its polyadenylation, and the actual termination of transcription that is the release of the elongation complex from the intergenic DNA. The efficiency of these processes must be adjusted to the respective expression levels that in turn depend on activating or repressing stimuli regulating the transcription of this gene. For transcription of the second gene an efficient recruitment of the transcriptional pre-initiation complex at the initiation site of its promoter is required. In addition to this recruitment, regulated gene expression requires efficient binding of gene specific transcriptional activators to the promoter upstream of the transcription initiation site. The efficiency of both the 3´ end formation/termination and the initiation of transcription must be adjusted to the 'strength' of the two adjacent genes for different levels of their regulated expression. Otherwise transcriptional interference reduces or even abolishes the expression of the second gene by promoter occlusion. Since eukaryotic DNA is closely associated with histone proteins providing a highly ordered chromatin structure, these processes must take place against the background of nucleosomes that counteract not only the access of DNA binding proteins but also the transcription process itself.

Here we used a test-system for the detection of elements located in-between the ORFs of two closely located genes that antagonizes transcriptional interference. We provided the first gene with a stronger promoter to highly increase its transcription and fused the

second one to a reporter gene. The test-system was integrated at the natural locus of the two genes in the authentic chromosomal context. The chromatin structure at this locus is characterized by a distinct nucleosome that separates two Mnase hypersensitive regions. One of these hypersensitive regions is required for efficient mRNA 3´ end formation of the upstream *ARO4* gene and the other for transcriptional activation of the downstream *HIS7* gene. The defined positioning of the nucleosome between terminator and promoter suggests that it antagonizes transcriptional interference between the two genes. Hence, transcriptional interference generated by high expression of the first gene of the test-system coincides with a diminished DNA-histone octamer interaction at this separating nucleosome. Since there is also an alteration of the nucleosomal structure at the 3´ end of the ORF of the first gene, efficient termination of transcription possibly requires a defined chromatin structure at the very end of an open reading frame. A link between the positioning of an upstream nucleosome, transcriptional initiation at downstream promoters and transcriptional interference was previously reported for genes encoding ribosomal RNA (rDNA) (Längst *et al.*, 1997). In that work it was shown that the positioning of a nucleosome at an upstream terminator element is required to enable transcription from the downstream promoter. To position this nucleosome the DNA-binding termination factor TTF-I, homologous to the yeast Reb1p, was shown to be necessary.

The quantification of reporter gene expression for various ‘intergenic’ deletions within our test-system revealed that there are further elements beside the positioned nucleosome that define the border of two adjacent genes. mRNA 3´ end formation signals like the Zaret/Sherman element as well as the site were the nascent transcript is cleaved and the poly(A) tail added represent borders that belong to the preceding *ARO4* gene. Removing of these elements significantly increase transcriptional interference at that locus. Termination of transcribing RNA pol II and its release from the DNA template was previously shown to be linked to mRNA 3’ processing (Proudfoot, 1989). Destruction of poly(A) signals results in reduced termination events leading to increased transcription

beyond the poly(A) site of a gene and thereby impairing the activity of downstream promoters (Proudfoot, 1989; Springer *et al.*, 1997b).

Another border marked by the downstream *HIS7* gene is the presence of the ubiquitous DNA-binding factor Abf1p at its promoter. Besides its role as activator of *HIS7* transcription it obviously has an additional function in forming a protective barrier against transcription of the upstream *ARO4* gene. Recent publications have reported a molecular explanation for such a link between prevented termination and promoter activity, e.g. for the *GAL10-GAL7* locus in yeast or the tandem HIV-1 promoters integrated in HeLa cells (Greger *et al.*, 2000; Greger *et al.*, 1998). By *in vivo* footprinting it was demonstrated that reduced 3' processing activity of the *GAL10* gene directly weakens binding of the transcription factor Gal4p to the adjacent downstream *GAL7* gene and thereby reducing its transcription. Likewise binding of the transcription factor Sp1 to the downstream promoter of tandemly localized HIV-1 promoters is significantly increased by insertion of an efficient transcriptional termination element upstream of the occluded promoter. A recent report stated that efficient termination enabled by the murine transcript release factor PTRF augments downstream ribosomal gene transcription by enhancing reinitiation at the rDNA promoters (Jansa *et al.*, 2001).

Our data demonstrate a novel function for a positioned nucleosome and Abf1p have because they are not only involved in initiation of *HIS7* transcription but also form a border between the two genes *ARO4* and *HIS7*. They prevent the downstream *HIS7* gene against transcriptional interference and separate the processes of *ARO4* 3´ end formation and initiation of *HIS7* transcription. We suggest that a similar intergenic barrier prevents also transcriptional interference between other tandemly transcribed eukaryotic genes.

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Curriculum vitae

Oliver Valerius, Dipl.-Chem. Univ.

born on August, 5th 1969 in Stadthagen, Germany

Education:

1976 – 1980	**Primary education** in Stadthagen and Fürth
1980 – 1989	**Secondary education** at the Hardenberg-Gymnasium Fürth

Scientific Background:

May 1990 - Aug. 1996	**Study** of Chemistry at the Friedrich-Alexander-University, Erlangen/Nürnberg
Dec. 1995 - Aug. 1996	**Diploma** thesis in Biochemistry: "Untersuchungen zur Transkriptionellen Interferenz am Beispiel der Intergenischen *ARO4-HIS7*-Region der Bäckerhefe *Saccharomyces cerevisiae*"
Nov. 1996 - May 2001	**Scientific assistant** at the Institute of Microbiology & Genetics, Georg-August-University, Göttingen