Multiple Factors Prevent Transcriptional Interference at the Yeast ARO4-HIS7 Locus*

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Eukaryotic transcriptional interference is understood as the result of RNA polymerase 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 to which the reading through of RNA polymerase II complexes occurs critically depends on the efficiency of transcription termination of the upstream gene (3–5). Deletions of GAL10 poly(A) signals abolished any activity of the downstream GAL7 gene, even when the GAL7 promoter was intact, resulting in a bicistronic read-through transcript. Therefore, in the case of GAL7, the promoter was completely occluded. Polymerase profiles raised in transcription run-on experiments for these poly(A) mutant strains confirmed the accumulation of nonterminated polymerase II complexes within the GAL7 promoter (4). As a consequence of transcriptional interference, it was shown that various transcription factors are not able to bind to their promoter sites any more. This was 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 (6). The binding of the Gal4p transcriptional activator to the GAL7 promoter was reduced in a similar fashion by read-through transcription initiated at the upstream GAL10 promoter. Interestingly, Gal4p overexpression can suppress this effect (7).

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Increased transcriptional activity may cause transcriptional interference in organisms with compact genomes such as the yeast *Saccharomyces cerevisiae*. Replacement of the yeast *ARO4* promoter by the stronger *ACT1* promoter increases *ARO4* transcription and simultaneously reduces the basal transcription of the downstream *HIS7* gene. The open reading frames of *ARO4* and *HIS7* are tandemly transcribed and are separated by 416 bp. In wild-type cells, a nuclease-resistant site suggests that the two genes are separated by a single positioned nucleosome. Transcriptional interference correlates with *Micrococcus* nuclease accessibility of this otherwise nuclease-resistant site. Deletion analyses of the region between the two open reading frames revealed that transcriptional interference increases upon removal of either parts of the *ARO4* 3′ end or *HIS7* promoter sequences. The abolition of the Abf1p-binding site within the *HIS7* promoter significantly enhances transcriptional interference, resulting in a histidine auxotrophic strain. Our data suggest that the yeast cell prevents transcriptional interference by the combination of efficient *ARO4* transcription termination, the positioning of a fixed nucleosome, and transcription factor binding to the *HIS7* promoter.

The arrangement of tandemly transcribed RNA polymerase II genes can jeopardize regulated transcription in a cell by a phenomenon called *transcriptional interference*. As consequence of elevated transcription of the upstream gene, transcription of the adjacent downstream gene might be diminished or even abolished. Transcriptional interference is favored by close proximity of genes that are only separated by short intergenic regions between the corresponding open reading frames (ORFs). It was found in HeLa cells that two closely spaced α-globin genes in an artificial gene construct interfere with each other (1). In yeast, the cryptic promoter within the intron of the *ACT1* gene is occluded by transcription from the actual *ACT1* promoter at the 5′ end of the gene (2). We have described previously (3) that *HIS7* transcription is reduced when the upstream-located *ARO4* gene is transcribed from the strong *ACT1* promoter instead of its natural promoter.

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1 The abbreviations used are: ORF, open reading frame; HIV, human immunodeficiency virus; MNase, *Micrococcus* nuclease.

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Northern hybridizations by increased recombination were selected by uracil prototrophy and confirmed in Cross and Tinkelenberg (11). For Northern hybridization analysis, 20 URA3 had replaced the wild-type HIS7 by capillary blotting. Hybridization with specific DNA probes was performed after transfer to a positively charged nylon membrane (Biodyne B; PALL) supplemented with the required amino acids according to Rose described here, strains were cultivated in minimal vitamin medium (9). The genotypes used in this work are listed in Table I. For all experiments ARO4 ACT1 (10). To generate functional fusions of the ARO4 gene by the stronger URA3 auxotrophic marker gene, which is itself preceded by the ARO4 5′-untranslated region for homologous integration. Transformants which had replaced the wild-type ARO4 locus by this cassette by homologous recombination were selected by uracl prototrophy and confirmed in Northern hybridizations by increased ARO4 mRNA levels and by PCR.

RNA Analysis—Total RNA from S. cerevisiae was isolated according to Carninci et al. (11). For Northern hybridization analysis, 0.5 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 32P labeling with the Prime It II DNA Labeling Kit from Stratagene. One-kb PCR fragments generated with the oligonucleotides ARO-OLV19 (5′-taacctgacctacgagcttgct-3′) and ARO-OLV11 (5′-ctccagctgtctctgggtcataaa-3′), HIS-OL1 (5′-ggtagactaaacatccatgtaaacc-3′) and HIS-OL2 (5′-ccagattctcatcatcagctg-3′), and ACT-OL1 (5′-cctgggtttctgctgttgaacg-3′) and ACT-OL2 (5′-cctgggtttctgctgttgaacg-3′) served as probes for the ARO4, HIS7, and ACT1 genes, respectively. In all cases, template was genomic DNA of strain RH1381. Band intensities were visualized by autoradiography and quantified using a BAS-1500 phosphorimaging scanner (Fuji).

Genomic Chromatin Preparation and Nucleosome Digestions—These methods have been described previously (12). Biodyne B nylon membranes were used for Southern transfer. Probes were labeled by the random primer method (13).

Indirect End Labeling—Chromosomal DNA from the nucleosome digestion was digested with XbaI and MluI and fractionated in 1.2% agarose gels. The fractionated DNA was blotted on the nylon membrane by the alkaline blotting method and hybridized with a radioactively labeled 250-bp PCR product generated with oligonucleotides HIS7-CHR1 (5′-gagattccgaacagctgacctg-3′) and HIS7-CHR2 (5′-caatctctgcaagaaagctgacctg-3′), annealing just downstream of the XbaI site. A DNA ladder consisting of multiples of 256 bp was used for size estimation (14). β-Galactosidase Assay—β-Galactosidase activities were determined by using permeabilized yeast cells and the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside as described previously (15).

Yeast cells were cultivated in minimal vitamin medium overnight, diluted to an absorbance of ~0.5 at 546 nm, and cultivated for another 6 h before assay. One unit of β-galactosidase activity is defined as 1 nmol 4-methylumbelliferone h−1 ml−1 l−1. The values presented are the means of at least four independent cultures, each of them measured three times. S.D.s were <20%.

RESULTS

α-Actin-driven ARO4 Transcription Creates a MNase-sensitive Site Within Nucleosomal DNA That Separates the ARO4 Terminator from the HIS7 Promoter—The replacement of the natural promoter of the ARO4 gene by the stronger ACT1 promoter causes transcriptional interference with the downstream HIS7 gene, reducing HIS7 transcription in comparison to wild-type expression (3). Because 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 structure, we wanted to know whether the transcriptional interference between the ARO4 and HIS7 gene is manifested in 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 MNase protection experiments. Crude nuclear extracts from overnight cultures grown in minimal vitamin medium from strains with the wild-type ARO4 promoter (RH1381) or the ACT1-ARO4 fusion (RH2642), respectively, were partially digested with MNase and further treated as described previously (12). In wild-type cells, the ARO4 3′ region immediately downstream of the ORF is sensitive to MNase (Fig. 1). This short sensitive region was followed by a strongly protected region, which corresponds in length to a positioned nucleosome. The HIS7 promoter further downstream was again sensitive to MNase. Although the mutant strain with the ACT1-ARO4 fusion gene showed a largely similar chromatin pattern, an additional band within the pro
detected region between the ARO4 3‘-end region and the HIS7 promoter became obvious (Fig. 1, arrow 1). This sensitive site already appeared at very low MNase concentrations (chromatin digest with 3 units of MNase for 6 min) and got more pronounced at 9 and 18 units of MNase. In contrast, this MNase-sensitive site is faint in the ARO4 wild-type strain, even for the digest with 18 units of MNase.

A feasible consequence of enhanced ARO4 transcription could be that nonterminated, still-transcribing RNA polymerase II complexes weaken the DNA histone interaction at the respective nucleosome, thereby drastically increasing this otherwise very weak MNase-sensitive site. In addition, a sensitive site at the end of the ORF of the wild-type ARO4 gene appears to be less sensitive in the case of the AACT1-ARO4 fusion gene (Fig. 1, 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 may be directly related to the termination efficiency and transcriptional interference.

Specific Deletions within the ARO4 3‘-Untranslated Region or the HIS7 Promoter Increase Interference between ARO4 and HIS7 Transcription—The DNA in between the two ORFs of ARO4 and HIS7 possesses elements required for efficient 3‘-end formation of the ARO4 mRNA and others that promote efficient HIS7 transcription (3). Here we intended to define DNA regions between these ORFs whose loss enhances transcriptional interference caused by increased ARO4 transcription. We established a reporter system with the HIS7 gene replaced by the quantifiable chimeric 3‘his7-lacZ gene, preceded by the ARO4 gene driven from either its natural promoter (Fig. 2, reporter-system I) or the ACT1 promoter (Fig. 2, reporter-system II). To determine regions in between both ORFs that counteract transcriptional interference, specific β-galactosidase activities for various small intergenic deletions were measured. DNA elements that diminish transcriptional interference were identified by comparison of the read-outs of the two reporter systems for each deletion construct (Fig. 3). To maintain the original chromosomal context, the reporter system was established at the authentic ARO4-HIS7 locus, with the separating nucleosome positioned approximately from –235 to –381 relative to the HIS7 ATG start codon. Deletions were chosen to cover several DNA motifs that fulfill different functions, including the Zaret/Sherman element (Z/S) required for ARO4 mRNA 3‘-end formation, three sites defining the actual poly(A) addition sites, C+T- and A+T-rich regions, the Abf1p-protein binding site (ABS), and both Gen4p recognition elements (GCRE1 and GCRE2) (Fig. 3).

When measured in reporter system I, deletions that cover the Zaret/Sherman element or poly(A) sites as elements of ARO4 mRNA 3‘-end formation (RH1815, RH1816, and RH1818) did not affect the 3‘his7-lacZ expression compared with that of the wild-type intergenic region (RH1616). In reporter system II, however, a 52-bp deletion that eliminated the Zaret/Sherman element (RH2632) reduced the specific β-galactosidase activity to about 28% of reporter system I (RH1815). Moreover, a 28-bp deletion that removed the first poly(A) site strongly reduced 3‘his7-lacZ expression if present in reporter system II (RH2633). Only about 22% activity was left in comparison to reporter system I with this deletion (RH1816). Further deletions of the second and third poly(A) site (reporter system I, RH1818; reporter system II, RH2634), C+T-rich (reporter system I, RH1819; reporter system II, RH2635) and A+T-rich (reporter system I, RH1835; reporter system II, RH2638) stretches, or the binding sites for Gcn4p (Gcn4p recognition elements; reporter system I, RH1822/RH1826; reporter system II, RH2636/RH2639) did not increase transcriptional interference. A 28-bp deletion that covered the Abf1p-binding site in reporter system II (RH2637) displayed a severe loss of specific β-galactosidase activity and almost shut off any his7-lacZ expression. In the background of reporter system I, this deletion alone reduced his7-lacZ expression to about one-third of the wild-type promoter.

In summary, the data obtained from our reporter system suggest that Abf1p binding to the HIS7 promoter is an essential element that antagonizes transcriptional interference. Furthermore, the Zaret/Sherman element and the first poly(A) site, which together are responsible for efficient ARO4 3‘-end formation, obviously counteract transcriptional interference. Deletions within these regions enhance transcriptional interference. No deletion has resulted in higher β-galactosidase activities in reporter system II compared with reporter system I, suggesting that there are no cis-elements that support transcriptional interference.

Single Nucleotide Exchanges within the Abf1p-binding Site Increase Transcriptional Interference—The results obtained thus far with the deletion constructs suggested an important
contribution of Abf1p binding in the prevention of transcriptional interference at the wild-type ARO4-HIS7 locus under conditions where ACT1-driven transcription also weakens the DNA-protein interaction of the separating nucleosome. We investigated whether it has been the broader context of the deleted 28-bp promoter region or solely the abolished binding of Abf1p itself that antagonized transcriptional interference. Therefore, we investigated the Phis7-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 (16, 17).

In the background of wild-type ARO4 expression in reporter system I (RH1830), single nucleotide exchanges within the Abf1p-binding site by themselves reduced Phis7-lacZ expression to about 35% of that of wild-type. Integrated in reporter system II (RH2640), these nucleotide exchanges caused a further strong reduction in β-galactosidase activity to about 9% of wild-type Phis7-lacZ expression. This result demonstrated that it was in fact the binding of Abf1p to its binding site within the HIS7 promoter, and not a broader promoter context, that antagonized transcriptional interference at the ARO4-HIS7 locus. It is possible that binding of Abf1p to its cis-element competes with the transcription of a nonterminated polymerase II complex and thus blocks transcriptional interference.

Transcriptional Interference Causes Histidine Auxotrophy for a HIS7 Promoter Mutant without Abf1p-binding Site by Prevention of Its Transcription—The data of the reporter systems that derived from a lacZ reporter gene have shown that the Abf1p-binding site and elements required for efficient ARO4 mRNA 3’-end formation are important to prevent transcriptional interference. We then investigated whether the increased transcriptional interference of these deletion mutants gave rise to malfunctions in cells that harbor the wild-type HIS7 gene. Therefore, the growth rates of these strains were determined in medium without histidine.

When the ARO4 gene was driven from its own promoter, the wild-type’s growth rate of about 0.28 h⁻¹ was not changed in strains with deletions in the ARO4-3’-end region (strains RH1833, RH1834, and RH1836 in Fig. 4A). The growth rate nearly halved with the deletion of the Abf1p-binding site in the HIS7 promoter (0.18 h⁻¹, RH1781). The combination of a deleted first poly(A) site with an induced ARO4 expression (RH2644) also significantly reduced the growth rate in comparison to the wild-type (0.20 h⁻¹). Deletion of the Abf1p-binding site in combination with the ACT1-ARO4 fusion gene (RH2646) was so deleterious for the cell that it resulted in a histidine auxotrophic growth phenotype (Fig. 4, A and B).

We compared the effects of the transcriptional interference as obtained by the his7-lacZ chimeric genes and the growth tests with the quantified HIS7 mRNA steady-state levels determined by Northern hybridizations (Fig. 5). The fusion of the ACT1 promoter to the ARO4 gene increases ARO4 mRNA levels —4-
The growth of yeast strains was tested on minimal binding site.Strains RH1781 and RH1834 possess the ARO4 gene with its natural promoter and carry 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).

Deletion of the Zaret/Sherman element as ARO4 (RH1381). Deletion of the Zaret/Sherman element as ARO4 3’-end formation signal in the background of increased ARO4 transcription (RH2643) reduced the HIS7 mRNA levels to ~40% in comparison to the natural ARO4 promoter (RH1833). When the first ARO4 poly(A) site was deleted, the reduction of HIS7 transcript levels as a consequence of enhanced ARO4 transcription was even more pronounced (RH2644), namely, 30% of the respective strain with wild-type ARO4 expression (RH1834). In contrast, the deletion covering the second and third ARO4 poly(A) addition site did not show obvious differences in HIS7 transcript levels caused by transcriptional interference.

The deletion within the HIS7 promoter that covers the Abf1p-binding site in the wild-type ARO4 background (RH1781) already reduced HIS7 mRNA levels to 40% in comparison to the wild-type HIS7 promoter. However, in combination with high ARO4 transcription from the ACT1 promoter (RH2646), HIS7 transcripts were no longer detectable. This result confirmed the transcriptional interference as detected before in both the reporter system with the his7-lacZ reporter gene and the growth defect on histidine-deficient medium.

We have also investigated whether the single nucleotide exchanges within the Abf1p-binding site alone can change the chromatin structure of the intergenic region without ACT1-driven overexpression of ARO4. However, no changes in comparison to the wild-type intergenic region have been detected (data not shown). Therefore, we suggest that it is the DNA binding of the Abf1 protein itself that somehow blocks the transcriptional properties of the wild-type intergenic region without ACT1.

Taken together, these results imply that binding of the ubiquitous transcription factor Abf1p to the HIS7 promoter counteracts transcriptional interference caused by enhanced ARO4 expression, which is itself accompanied by nucleosomal

**DISCUSSION**

Cells have developed mechanisms that enable individually regulated expression of adjacent genes that are located in close proximity without influencing one another. One essential parameter to prevent read-through transcription is the efficient termination of transcription of the upstream gene. 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 DNA template). To initiate transcription at the downstream promoter, an efficient recruitment of the transcriptional preinitiation complex at the initiation site is necessary. In addition to this recruitment, regulated gene expression requires efficient binding of gene-specific transcriptional activators to the promoter upstream of the transcriptional initiation site. The efficiency of both the 3’-end formation/termination and the initiation of transcription at the downstream gene must be adjusted to the “strength” of the two adjacent genes for their different levels of expression. Otherwise, transcriptional interference reduces or even abolishes the expression of the downstream gene by promoter occlusion. Because the eukaryotic DNA is closely associated with histone proteins, these processes must take place in the context of a highly ordered chromatin structure. Here we show that a nucleosome is strictly localized in such a position between two tandemly arranged yeast genes that it may guard the more downstream gene from transcriptional interference under normal circumstances. This assumption is corroborated through the finding that increased transcription of the more upstream gene weakens this nucleosome.
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 ribosomal DNA promoters (20). Although previous reports demonstrated an influence of Abf1p binding on the local chromatin structure of promoters of the QCR8 and RPS28A genes (21, 22), we could not detect any changes in nucleosome distribution at the ARO4-HIS7 locus upon destruction of the Abf1p-binding site (data not shown). Possibly, in common with other promoters of typical housekeeping genes, the HIS7 promoter has a pre-set accessible chromatin structure that is not directly dependent on the presence or absence of Abf1p.

The different factors that in concert seem to prevent transcriptional interference are outlined in Fig. 6. Because there is also an alteration of the nucleosomal structure at the 3' end of the ARO4 open reading frame upon high ARO4 transcription, efficient termination of transcription might require a defined chromatin structure at the very end of a gene. A link between the positioning of an upstream nucleosome, transcriptional initiation at downstream promoters, and transcriptional interference was not yet described for an RNA polymerase II-transcribed gene. For genes encoding ribosomal RNA, it was shown that the positioning of a nucleosome at an upstream terminator element is required to allow 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 (8).

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Fig. 6. Summary of the ARO4-HIS7 intergenic elements that antagonize transcriptional interference. The ARO4 gene is highly transcribed by RNA polymerase II complexes (pol II) driven from the ACT1 promoter. For reinitiation of a functional RNA polymerase II complex at the initiation site of the HIS7 promoter (I), the transcription of upstream ARO4 has to be efficiently terminated. Elements blocking read-through RNA polymerase II complexes are the Zaret/Sherman element (Z/S) and the major poly(A) site (m(A)) within the ARO4 terminator and the Abf1p binding at the HIS7 promoter. Moreover, the positioned nucleosome seems to be a barrier to transcriptional interference.

We determined additional elements located in between the ORFs of the two yeast genes that contribute to antagonizing transcriptional interference (Fig. 6). Elements were identified that are necessary to separate transcription of the two adjacent genes. mRNA 3'-end formation signals such as the Zaret/Sherman element as well as the site where the nascent transcript is cleaved and the poly(A) tail is added represent borders that belong to the preceding ARO4 gene. The removal of these elements significantly increased transcriptional interference at that locus. Termination of transcription by RNA polymerase II and its release from the DNA template were previously shown to be linked to mRNA 3'-end processing (4). Destruction of poly(A) signals probably results in reduced termination events, leading to increased transcription far beyond the poly(A) site of a gene and thereby impairing the activity of downstream promoters (3, 19). However, future transcription run-on experiments for this locus should confirm transcribing RNA polymerase II complexes driven from the upstream promoter into the ORF of the downstream HIS7 gene.

Another border marked by the downstream HIS7 gene is the presence of the general DNA-binding factor Abf1p in its promoter. Besides its role as an activator of HIS7 transcription, it seems to have the additional function of forming a protective barrier against read-through transcription initiated at the upstream ARO4 gene. This roadblock function of Abf1p is supported by the observation that deletion/mutation of the Abf1p-binding site had different effects in both the wild-type and the pACT1-ARO4 systems on his7-lacZ expression (Fig. 3). Recent investigations focusing on the GAL10-GAL7 locus in yeast or the tandem HIV-1 promoters integrated in HeLa cells also support such a link between termination and promoter activity (6, 7). By in vivo footprinting, it was demonstrated that reduced 3'-end processing activity of the GAL10 gene directly weakens the binding of the transcription factor Gal4p to the adjacent GAL7 promoter and thereby reduces its transcription. In the GAL10-GAL7 system, overexpression of Gal4p seems to counteract some of the transcriptional interference. Because Abf1p is an abundant protein in the yeast cell and it also binds the HIS7 promoter consistently during inactive HIS7 transcription, testing its overexpression in terms of lowering transcriptional interference does not seem promising. The binding of transcription factor Sp1 to the downstream promoter of tandemly
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