The Adjacent Yeast Genes ARO4 and HIS7 Carry No Intergenic Region*

(Received for publication, July 9, 1997, and in revised form, August 11, 1997)

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The region between the open reading frames of the adjacent yeast genes ARO4 and HIS7 consists of 417 base pairs (bp). Termination of ARO4 transcription and initiation of HIS7 transcription has to take place within this interval, because both genes are transcribed into the same direction. We show that the ARO4 terminator and the HIS7 promoter are spatially separated, nonoverlapping units. The ARO4 terminator includes 84 bp of the ARO4 3′-untranslated region with several redundant ARO4 3′ end processing signals. Deletion of the ARO4 terminator does reduce but not completely shut down its expression. The adjacent region of 40 bp is neither required for correct ARO4 3′ end formation nor for HIS7 initiation but contains the nucleotides corresponding to the wild type mRNA 3′ ends. The following 280 bp are required for the HIS7 promoter. Replacement of the housekeeping ARO4 promoter by the stronger ACT1 promoter leads to reduced HIS7 expression due to transcriptional interference. This underlines the compactness of the yeast genome carrying virtually no intergenic regions between adjacent genes.

The sequencing of the genome of the budding yeast Saccharomyces cerevisiae has revealed the remarkable compactness of its genome. This results from the short size of regions between the open reading frames. Open reading frames of divergent promoters on average are only 618 bp apart. Open reading frames of convergent terminators are separated by 326 bp on average. Arrangements with a terminator-promoter combination in yeast the size of the terminator and promoter by the stronger ACT1 promoter leads to reduced HIS7 expression due to transcriptional interference. This underlines the compactness of the yeast genome carrying virtually no intergenic regions between adjacent genes.

The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); MOPS, morpholino-n-propanesulfonic acid.

* This work was supported by Deutsche Forschungsgemeinschaft Grant BR1502/1-2 and by the Fonds der Chemischen Industrie and Volkswagen-Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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tion signals have been analyzed in various test systems. It is hardly known how different mRNA 3' end formation signals affect different promoters in a single test system. Therefore, the aim of this study was to investigate effects on a mRNA 3' end formation signal and a promoter simultaneously.

**The ARO4 gene** encodes the tyrosine-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase catalyzing the first step in the Shikimate pathway (14). Its poly(A) site contains the tripartite TAG...TATGTA...TT motif proposed by Zaret and Sherman (12) and belongs to the class of bidirectionally functional poly(A) sites (10). The HIS7 gene is located just downstream of the ARO4 gene on yeast chromosome II. It encodes the bifunctional glucose amidotransferase/ascorbic acid oxidase catalyzing the fifth and sixth step in the *de novo* histidine biosynthesis (15). Basal transcription of HIS7 requires the global factor Abf1p, and it is activated under conditions of amino acid starvation and adenine starvation conditions by the global factor Abf1p, and it is activated under conditions of amino acid starvation and adenine starvation conditions by the 30% and 30% of the wild type HIS7-lacZ construct pME966 resulting in strain RH1616 (15).

**Construction of the Test Gene**—Plasmid pME800 was constructed on the basis of pSP64 (Promega, Madison, WI) to obtain an integrative vector. Vector pSP64 was modified by cloning the 1.1-kilobase HindIII fragment of pURA3 into the XhoI site, by inserting the 1.1-kilobase BamHI fragment of pME729 (24) into the BamHI site of the polylinker and after cloning the multiple cloning site (double-stranded OLC1-OLCE2) into the ClaI site of the 1.1-kilobase BamHI fragment. The different mutated alleles of the ARO4/HIS7 intergenic region were amplified by using OLCS26 and OLCS27 as primers and the plasmids pME951 to pME956 (3' deletions), pME966 to pME971 (5' deletions) and pME991 to pME995, pME997, pME999 and pME1001 (internal deletions) as templates in a PCR reaction and cloned into the multiple cloning site of plasmid pME800 after restriction with KpnI and BglII.

**Site-directed Mutagenesis of the ARO4/HIS7 Intergenic Region**—Site-directed mutations in the ARO4/HIS7 intergenic region were introduced using the PCR technique (25). Oligonucleotides carrying specific mutations were OLCS36 to OLCS40. These oligonucleotides were used in a PCR reaction together with OLCS27 as second primers and pME800-DNA as template. The final PCR products were cut with KpnI and BglII and cloned into plasmid pME800.

**β-Galactosidase Activity Assay**—β-Galactosidase activities were determined by using permeabilized yeast cells and the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside as described earlier (15). Routinely, yeast cells were cultivated in MV minimal medium overnight, diluted to an optical density of approximately 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 A₅₄₆⁻¹. The given values are the means of at least four independent cultures. The standard errors of the means were less than 20%.

**DAHP Synthase Activity Assay**—3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase activities were determined as described in Taka-hashi and Chan (26). Routinely, yeast was cultivated in MV minimal medium to an optical density of approximately 2 at A₅₄₀, harvested by centrifugation and washed three times with potassium phosphate buffer (50 mM potassium phosphate, pH 7.6, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 1 mM dithiothreitol). The cells were resuspended in 5 ml of potassium phosphate buffer, disrupted in a French press (Aminoce, Silver Spring, MD), and the cell debris was removed (26). The centrifugation was repeated, and the protein concentrate was added to a PD 10 column (Pharmacia Biotech Inc. Uppsala, Sweden). 50 μl of crude cell extract was incubated for 10 min in 50 μl of ethyrosine-4-phosphate (8 mM), 40 μl of phosphoenolpyruvate (10 mM), 50 μl of 0.4 mM potassium phosphate buffer, and 60 μl of H₂O. The enzymatic reaction was stopped by adding 50 μl of trichloroacetic acid (20%). 100 μl of the reaction solution was added to 100 μl of 20 mM Na₂O₃, 0.3% Na₂H₂SO₄ and 0.05% H₂SO₄ (25). This reaction was started by adding 200 μl of NaAsO₂ (2% in 0.5 M HCl). After the solution turned colorless, 800 μl thiobarbituric acid (0.3%) was added, and the mixture was boiled for 10 min. The absorption of the product was measured at 550 nm.

**Isolation of Total RNA from S. cerevisiae**—Yeast cells were grown overnight in a 100-ml culture to an optical density at 546 nm of about 2. The cells were spun at 6000 × g for 5 min at 5°C, the supernatant was aspirated and resuspended in 6 ml of PLE buffer (100 mM PIPES, 100 mM LiCl, 1 mM EDTA, pH 7.4). After centrifugation at 6000 × g for 5 min at 4°C, the cells were resuspended in 300 μl of ice-cold PLE buffer and 100 μl of ice-cold dichloromethane-saturated phenol equilibrated with PLE buffer. Diethylpyrocarbonate (1%, v/v) was added to inactivate RNases. Sterilized glass beads 0.45 mm in diameter were added, and the cells were disrupted by vigorous shaking for six 15-s periods with cooling on ice between. Nucleic acids were extracted once with 1 volume of dichloromethane-saturated phenol equilibrated with PLE buffer, 0.05 g of bencitone, and 1% (w/v) sodium dodecyl sulfate and twice with 1 volume of dichloromethane-saturated phenol equilibrated with PLE buffer. Total RNA was precipitated by 1.5 volumes of ice-cold isopropanol, and the concentration was determined spectrophotometrically. The precipitated RNA was washed twice with 20°C.

**RNA Analysis**—For Northern (RNA) hybridization experiments, approximately 10 μg of total RNA was precipitated, resuspended, and denatured in 30 μl of sample buffer (50%, v/v, deionized formamide, 6% v/v formamide, 1 × loading buffer, 10% [v/v] 10 mM Tris-1 mM EDTA [TE] buffer) for 15 min at 65°C and put on ice. The RNA was separated on a denaturing formaldehyde agarose gel. The 1.4% (v/v) agarose gel
The ARO4 terminator and the HIS7 promoter are nonoverlapping and spatially separated. The DNA region between the ARO4 and the HIS7 open reading frames is shown. The positions of the three mapped ARO4 mRNA 3' ends are indicated by black arrows. The tested deletion constructs of the ARO4/HIS7 intergenic region are shown below. End points of the deletions are indicated by numbers representing the positions relative to the A residue of the ATG start codon of the HIS7 gene. The ARO4-derived enzyme activity was measured as DAHP synthase activity and is shown in shaded boxes, whereas the HIS7-encoded enzyme activity was measured as β-galactosidase activity from corresponding HIS7-lacZ fusions and is indicated by black boxes. Numbers are relative values, with the specific wild type enzyme activity for the ARO4-encoded enzyme DAHP synthase and the wild type activity for the HIS7-lacZ fusion-encoded β-galactosidase as 100%. Each number represents an average value of at least six measurements with a standard deviation of not more than 15%.

RESULTS

The ARO4 Terminator and the HIS7 Promoter Are Nonoverlapping and Spatially Separated Units—The spacing between the open reading frames of the ARO4 gene and the HIS7 gene consists of 417 bp. We wanted to know whether deletions within this region result in interference between ARO4 transcription and the initiation of transcription of the HIS7 promoter. Therefore a deletion analysis of the ARO4/HIS7 intergenic region was performed. ARO4 expression was determined by measuring DAHP synthase activity, which is the gene product. HIS7 transcription was monitored by determining β-galactosidase activities of strains carrying respective translational HIS7-lacZ fusions integrated in single copies at the ARO4/HIS7 locus (Fig. 1). All strains had a ground to avoid interference with the general control of amino acid biosynthesis in yeast.

Deletion of large parts of the ARO4 3'-untranslated region in the yeast strains RH1768 ($\Delta$ -405/-245 relative to the HIS7 AUG start codon) and RH1769 ($\Delta$ -405/-280) (Fig. 1) including the mapped poly(A) sites (14) and the tripartite Zaret/Sherman sequence element (12, 10) reduced ARO4 activity to 37 and 41%, respectively, compared with wild type activity. Smaller deletions of 52 bp in RH1833, 28 bp in RH1834, 12 bp in RH1835, or 20 bp in RH1836 moderately reduced ARO4 expression leading to between 55 and 75% of wild type activity. All these deletions were within the first 140 bp of the ARO4 3'-untranslated region and had no effect on HIS7 expression. The four strains RH1837, RH1839, RH1840, and RH1842 carry various deletions between 13 and 42 bp in length, all located more than 140 bp downstream of the end of the ARO4 open reading frame within the HIS7 promoter. None of these four deletions affected ARO4 expression, but all of them reduced HIS7 expression.

In summary, any deletion within the first 140 bp of the ARO4 3'-untranslated region had a significant effect on ARO4 expression but did not affect HIS7 transcription. By contrast, all deletions within the next 280 bp affected HIS7 transcription, but none of them had any effect on ARO4 expression. These results strongly suggest that the ARO4 termination sequences are located within the first 140 bp of the untranslated region between the ARO4 and the HIS7 genes and do not overlap with the HIS7 promoter. Therefore, the ARO4 termination sequences and the HIS7 promoter sequences are located within spatially clearly separated units.

A Region of Maximal 40 bp between ARO4 and HIS7 Is Not Necessary for Efficient ARO4 mRNA 3' End Formation nor for HIS7 Promoter Activity but Contains the ARO4 Wild type mRNA 3' End Positions—To define whether there is any intergenic spacer region between ARO4 and HIS7, the sequences required for ARO4 mRNA 3' end formation were analyzed more precisely. We tested ARO4 3' end modifications in an artificial test system that we had established earlier (28). The ARO4 polyadenylation element represents the class of yeast 3' processing sites which function in both orientations in an in vivo test system (10). The 3'-untranslated region of the ARO4 gene contains the tripartite sequence motif TAG...TATGTA...TTT, which was proposed to represent a processing consensus element in yeast (Fig. 2) (12). Modifications of the ARO4 3'-
Fig. 2. In vivo test cassette for either wild type or mutant mRNA 3′ processing signals in S. cerevisiae. A, the test cassette consists of the ACT1 promoter fused to the ADH1 terminator. Functional 3′ processing sites were cloned between the ACT1 promoter and the ADH1 terminator and result in short truncated transcripts, whereas nonfunctional sites result in long readthrough transcripts. Because the complete HIS7 promoter is cloned into the test cassette, a short transcript initiated at this promoter and ending in the ADH1 terminator is expected. B, the primary sequence of the ARO4 3′ untranslated region and a part of the open reading frame (in boldface italic type) are shown. The tripartite Zaret/Sherman (ZS) motif TAG...TATGTA...TATT is a putative consensus element and is underlined and in boldface type. The three mapped ARO4 3′ ends are indicated by black arrows. The numbers correspond to the assignment of position +1 to the A nucleotide of the ATG start codon of the HIS7 gene.

untranslated region included 3′ and 5′ end, internal deletions, and specific point mutations inserted into the complete element (Fig. 3). The modified ARO4 3′ end elements were cloned into each cloning site of the test gene consisting of the ACT1 promoter and the ADH1 terminator (Fig. 2) (28). The test gene was integrated into the chromosome at the URA3 locus, thereby avoiding multicopy effects. The effects of all modifications were analyzed at the transcript level by performing Northern blot analysis. Functional 3′ processing elements resulted in short truncated transcripts, whereas nonfunctional elements resulted in long readthrough transcripts as schematically drawn in Fig. 2.

3′ deletion up to position −321 relative to the A residue of the translational start codon ATG of the HIS7 gene (deletion Δ −321/−104 in Fig. 4) resulted in a 3′ processing efficiency (86% truncated transcript) similar to that of the complete wild type ARO4/HIS7 intergenic region (83–86% truncated transcripts). Further deletion to position −337 completely abolished 3′ end formation (deletion Δ −337/−104 in Fig. 4). Therefore the downstream boundary for a completely functional ARO4 3′ processing element in the test system was located in the −337 to −321 region. The mapped 3′ ends (positions −311, −306, and −283) are located downstream of this boundary suggesting that they are not important for the efficiency of mRNA 3′ end formation in the test gene.

5′ deletion of the part containing the ARO4 open reading frame including 12 bp of the 3′-untranslated region had no effect on 3′ end processing (deletion Δ −440/−405 in Fig. 4). In this construct the TAG part of the tripartite TAG...TAT-
Adjustment between Promoter and Terminator Efficiency

FIG. 3. Schematic representation of the mutations constructed in the ARO4/HIS7 intergenic region. The numbers indicated above the constructs represent the boundaries of the deletions relative to the A nucleotide of the HIS7 ATG start codon. The nucleotide exchange in the point mutations are indicated with lowercase boldface letters, whereas a caret indicates deletion of a single nucleotide.

In summary, deletion of the ARO4 terminator has no effect on HIS7 transcription. Overexpression of the ARO4 gene by the ACT1 promoter reduces HIS7 expression by a factor of two. Simultaneous overexpression of ARO4 and deletion of its ter-

genes. Thus, we further investigated the role of the ARO4 terminator for its ability to prevent interference between the transcription of the ARO4 and the HIS7 genes.

Replacement of the ARO4 promoter by the ACT1 promoter increased its expression 4-fold and caused a reduction of HIS7 expression to 50% of the wild type expression (Fig. 5). This effect was even more pronounced using the yeast strain RH1815 carrying a 52-bp deletion within the ARO4 3' end processing signal reducing ARO4 expression to 70%. In this strain HIS7 activity was slightly reduced to 95% compared with wild type activity. Here, replacement of the ARO4 promoter by the strong ACT1 promoter leading to the yeast strain RH2172 reduced HIS7 activity to 30% of wild type activity. These results indicated that expression of the ARO4 gene under the control of the strong ACT1 promoter at its original chromosomal locus interfered with the initiation of transcription at the downstream located HIS7 promoter and therefore caused a reduction of HIS7 expression. This effect is even more pronounced when simultaneously the ARO4 terminator is lacking.

In the ACT1-ARO4 3' end formation test gene where the ACT1 promoter is fused to the ARO4/HIS7 intergenic region with only 90 bp of the open reading frame in between, no transcript initiated at the HIS7 promoter could be detected (Fig. 6). Therefore, we tested whether this is due to the strong initiation at the ACT1 promoter and the incomplete 3' end formation at the ARO4 polyadenylation site in the ACT1-ARO4 hybrid gene. Two constructs served as controls. In the first construct the ACT1 promoter was destroyed by Bal31 digestion. With no transcript initiated at the strong ACT1 promoter, no interference was expected between the ACT1-ARO4 hybrid transcript and the initiation at the HIS7 promoter. Therefore a short transcript initiated at the HIS7 promoter was expected. In the second construct the strong polyadenylation signal of the GCN4 gene (28) was cloned between the ACT1 promoter and the ARO4/HIS7 intergenic region. In this construct the discrepancy between the strong ACT1 promoter and the weak ARO4 terminator should be abolished, and therefore a transcript initiated at the HIS7 promoter was expected.

In a Northern blot experiment with RNA isolated from the yeast strains RH2169 (with inserted GCN4 terminator) and RH2171 (with destroyed ACT1 promoter), a short transcript initiated at the HIS7 promoter could be detected by hybridization with a radiolabeled, 215-bp ADH1 probe. No such transcript was detected using RNA isolated from the yeast strain RH2160 with an intact ACT1 promoter and no inserted GCN4 terminator (Fig. 6).

Hybridization of RNA isolated from the yeast strain RH2169 (with inserted GCN4 terminator) with the radiolabeled 524 bp ACT1 probe led to a great amount of ACT1-GCN4 hybrid transcript. The strong ACT1 promoter directed high levels of initiation of transcription and the downstream inserted strong GCN4 terminator resulted in complete termination of transcription. In the strain RH2171 the ACT1 promoter was completely destroyed, because no transcript could be visualized by hybridization of RNA from this strain with the ACT1 probe. In the strain RH2160 (wild type ARO4/HIS7 intergenic region) both truncated and readthrough transcripts were present, indicating incomplete processing of the ACT1-ARO4 hybrid mRNA. These results demonstrated that expression of the ACT1-ARO4 hybrid mRNA abolished initiation of transcription at the HIS7 promoter located downstream due to transcriptional interference between these two genes.
This study had three major results. (a) We wanted to know whether the authentic 3′ end of a gene is indispensable for its expression at its natural chromosomal locus. We found that we can delete the ARO4 3′ end signal. Therefore, the ARO4 3′ end signal is not essential but required for efficient ARO4 gene expression. (b) We wanted to know whether the ARO4 3′ end formation signals can generally block transcriptional interferences and guarantee efficient H7s expression. We found that 4-fold increased ARO4 expression reduces H7s expression by a factor of two. (c) We wanted to know whether in yeast the terminator and an adjacent promoter are overlapping or whether there is intergenic space between two adjacent genes. Our results suggest two independent nonoverlapping units and no intergenic region between ARO4 and H7s.

Part of our analysis concerns the question of how essential the 3′ end of a gene is for its expression in the natural chromosomal environment. The ARO4 3′ processing signal includes several redundant elements that are located within 84 bp start codon were deleted, and finally the strain RH2172 was constructed by replacement of the ARO4 promoter by the ACT1 promoter in the strain RH1815. The wild type activity was set to 100%. The numbers indicated represent the average value obtained by at least six measurements. The standard deviation did not exceed 15%.

**DISCUSSION**

This study had three major results. (a) We wanted to know whether the authentic 3′ end of a gene is indispensable for its expression at its natural chromosomal locus. We found that we can delete the ARO4 3′ end signal. Therefore, the ARO4 3′ end signal is not essential but required for efficient ARO4 gene expression. (b) We wanted to know whether the ARO4 3′ end formation signals can generally block transcriptional interference and guarantee efficient H7s expression. We found that 4-fold increased ARO4 expression reduces H7s expression by a factor of two. (c) We wanted to know whether in yeast the terminator and an adjacent promoter are overlapping or whether there is intergenic space between two adjacent genes. Our results suggest two independent nonoverlapping units and no intergenic region between ARO4 and H7s.

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**Fig. 5. Effects of the ACT1/ARO4 fusion on H7s expression.** β-Galactosidase activities (black boxes) and DAHP synthase activities (shaded boxes) of the four strains RH1616, RH2174, RH1815, and RH2172 carrying respective H7s/lacZ fusion constructs are shown. The strain RH1616 represents the wild type ARO4/H7s intergenic region, in the strain RH2174 the ARO4 promoter was replaced by the ACT1 promoter, in the strain RH1815 the sequences of the ARO4/H7s intergenic region between positions −392 and −340 relative to the H7s start codon were deleted, and finally the strain RH2172 was constructed by replacement of the ARO4 promoter by the ACT1 promoter in the strain RH1815. The wild type activity was set to 100%. The numbers indicated represent the average value obtained by at least six measurements. The standard deviation did not exceed 15%.

**Fig. 6. Northern experiments with different ACT1-ARO4 hybrid genes.** The strain RH2169 carries the wild type ARO4/H7s intergenic region inserted in the in vivo test cassette. In strain RH2169 the strong 3′ processing signals of the GCN4 gene were cloned between the ACT1 promoter and the ARO4/H7s intergenic region. In strain RH2171 the ACT1 promoter was destroyed by Bal31 digestion. In panel A, the blot was hybridized with a radiolabeled 542-bp fragment of the ACT1 promoter to monitor ACT1/ARO4 hybrid transcripts, whereas in panel B a 215-bp fragment of the ADH1 terminator was used to monitor H7s/ADH1 transcripts.
located HIS7 promoter. Expression of the complete ARO4 gene under the control of the ACT1 promoter resulted in 4-fold increased ARO4 expression, and simultaneous HIS7 expression was reduced by a factor of two. This effect was even more pronounced when parts of the ARO4 poly(A) signal were deleted. In conclusion the 3’ end of a gene is adjusted to its own promoter. Deletion of a poly(A) signal affects the expression of a downstream located gene only if the activity of the upstream promoter is simultaneously increased. The adjustment of the 3’ end formation signal for a mRNA is necessary to prevent transcriptional interference with the adjacent gene. In some further studies, the mechanism should be investigated in more detail, by which transcriptional interference between neighboring genes is prevented.

One remarkable feature of the yeast genome is its compact architecture, resulting from short intergenic regions. Some statistical calculations with the yeast genome revealed an average of 309 bp for a promoter (1). This theoretical value fits well with the observed 280 bp for the HIS7 promoter. The calculated size of an average yeast terminator consists of 163 bp. In this study we mapped the ARO4 poly(A) signals within a region of 84 bp starting 12 bp downstream of the ARO4 stop codon. Adding these 12 bp to the poly(A) signal and taking into account that the actual poly(A) addition sites were mapped within 40 bp downstream of the poly(A) signal, the ARO4 terminator consists of 136 bp. Therefore, the theoretically calculated sizes for yeast promoters and terminators fit well with the concrete situation between the open reading frames of the ARO4 and HIS7 genes. Within the 40 bp between the ARO4 3’ end processing signals and the HIS7 promoter, the actual ARO4 3’ ends are located. In conclusion, there is virtually no intergenic region between the ARO4 and HIS7 genes underlining the compact architecture of the yeast genome.

Acknowledgments—We are grateful to Hans-Ulrich Mösch, Sven Krappmann, and Stefan Irniger for helpful discussions and critical reading of the manuscript.

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