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The yeast transcriptome in aerobic and hypoxic conditions: effects of *hap1*, *rox1*, *rox3* and *srb10* deletions

Manuel Becerra,[1] Luis J. Lombardía-Ferreira,[1] Nicole C. Hauser,[2] Jörg D. Hoheisel,[2] Belén Tizon[1] and M. Esperanza Cerdán[1]

[1]*Dpto. Biología Celular y Molecular, Universidad de La Coruña, F. Ciencias, Campus de La Zapateira s/n 15075, La Coruña, Spain.*

[2]*Functional Genome Analysis Group, Deutsches Krebsforschungszentrum, Heidelberg, Germany.*

Summary

The transcriptome of *Saccharomyces cerevisiae* was screened using the high-density membrane hybridization method, under aerobic and hypoxic conditions, in wild-type and mutant backgrounds obtained by the disruption of the genes encoding the regulatory proteins Hap1, Rox1 and the Srb10 and Rox3 subunits of RNA polymerase II holoenzyme. None of the mutations studied was able to fully overcome the wild-type hypoxic response. Deletion of the *hap1* gene changed the expression profiles of individual open reading frames (ORFs) under both aerobic and hypoxic conditions. Major changes associated with *rox3* deletion were related to the hypoxic activation. Rox3 also caused a repressor effect (oxygen-independent) on a subset of genes related to subtelomeric proteins. With regard to the effect brought about by the deletion of *rox1* and *srb10,* correspondence cluster analysis revealed that the transcriptome profile in aerobic conditions is very similar in the wild-type and both deletion strains. In contrast, however, differences were found during hypoxia between the subgroup formed by wild-type and the Δ*rox*1 deletant compared with the Δ*srb*10 deletant. An analysis of selected ORFs responding to hypoxia, in association with a dependence on the regulatory factors studied, made it possible to identify the clusters that are related to different regulatory circuits.

Accepted 26 September, 2001. *For correspondence. E-mail bmanamrt@udc.es; Tel. (+34) 9 81 167000; Fax (+34) 9 81 167065. Yeast gave us the opportunity to work with Professor Rudi Planta and to enjoy his friendship. To him we dedicate this paper with respect and gratitude.

Introduction

Saccharomyces cerevisiae is a facultative aerobe that can utilize both respiration and fermentation to obtain energy. Under strictly anaerobic conditions, which are difficult to achieve in the laboratory, but also rare as a natural environment for this yeast, cells are completely dependent upon fermentation. They also need sterols and fatty acids given that enzymatic reactions leading to their biosynthesis utilize molecular oxygen as an electron acceptor, and, if oxygen is lacking, they are not functional. This yeast can also adapt to growth under severe oxygen limitation, termed hypoxia or microaerobic conditions, more usual conditions than strict anaerobiosis in natural environments of yeast.

During hypoxia, it is advantageous for the cell to adapt the pattern of gene expression to improve oxygen utilization. Among the genes that are induced during hypoxia are the so-called hypoxic genes whose products use oxygen as a substrate (*ERG11*, *HEM13, OLE1*); isoforms of aerobic genes related to respiration and ATP exchange between cytoplasm and mitochondria (*COX5b*, *CYC7*, *ACC3*); genes related to sterol uptake or biosynthesis (*SUT1*, *HMG2*, *CPR1*) and others whose functions have not yet been well defined (for a review, see Zitomer *et al*., 1997). Hypoxic genes having an aerobic counterpart are essentially inoperative until very low oxygen concentrations (0.5 μM O_2) are reached. In contrast, hypoxic genes that do not have aerobic counterparts are expressed at detectable levels at all oxygen concentrations but their expression is higher when oxygen decreases (Poyton, 1999).

In aerobic conditions, the haem-responsive transcriptional factor Hap1 activates the expression of genes related to respiratory functions by binding to *cis* elements ($CGGN_3TANCGGN_3TA$) present in their promoters (Ha *et al*., 1996). The repressor Rox1, whose gene is transcribed in the presence of oxygen, avoids the expression of the hypoxic genes that are not required. The differential repression of hypoxic genes is the result of a combination of the tightness of Rox1 binding to the *cis* element in the promoters of hypoxic genes (yyYATTGTTct) and the presence or absence of binding sites for Mot3, which enhances Rox1 repression (Kastaniotis and Zitomer, 2000; Kastaniotis *et al*., 2000). When the levels of oxygen decline, so do the levels of Rox1, by a mech-

anism not fully understood and the hypoxic and anaerobic genes are transcribed (Decker *et al*., 1995).

A series of data from our laboratory implicated Srb10 in the expression of the hypoxic gene *CYC7* (unpublished data), and further evidence of this relationship came from genomic analysis (Holstege *et al*., 1998). *SRB10* encodes for a cyclin-dependent protein kinase associated with the RNA polymerase II mediator complex, and it is involved in both the activation of transcription and repression. The phosphorylation of the yeast transcription factor Gal4 at S699 is required for efficient galactose-inducible transcription and it has been reported that this site is a substrate for Srb10 kinase activity (Hirst *et al*., 1999). The involvement of Srb10 in the mechanism of repression caused by the complex Tup1–Ssn6 was also proved *in vivo*, suggesting that one mechanism of repression by Ssn6–Tup1 involves functional interaction with RNA polymerase II (Kuchin and Carlson, 1998).

Rox3 is also implicated in the regulation of the hypoxic gene *CYC7* (Rosemblum-Vos *et al*., 1991) and in the response to stress (Evangelista *et al*., 1996). It has been characterized as a component of the mediator and RNA polymerase II holoenzyme (Gustafsson *et al*., 1997) and interacts with the transcriptional repressor Sfl1, which binds to the *SUC2* promoter (Song and Carlson, 1998)

Previous genomic analyses of the transcriptome of *S. cerevisiae* in aerobic and hypoxic conditions have been reported (ter Linde *et al*., 1999), but no studies regarding the effects of transcriptional regulatory factors during the shift from aerobic to hypoxic conditions have been carried out to genomic scale. Given these precedents, in this research we decided to undertake a genome-wide transcriptional analysis in wild-type and isogenic derivatives, Δ*hap1,* Δ*rox3,* Δ*rox1* and Δ*srb10* mutant strains, grown in aerobic and hypoxic conditions. To control oxygen availability, monitored chemostat cultures were used and levels of glucose were fixed below repressing values to avoid downregulation of respiratory genes.

Results and discussion

Analysis of the S. cerevisiae *transcriptome performed in aerobic and hypoxic conditions in wild-type and mutant backgrounds*

The transcriptome of *S. cerevisiae* was screened using the high-density membrane hybridization method in wild-type and mutant backgrounds obtained by the disruption of the genes encoding the regulatory proteins Hap1, Rox1, Srb10 and Rox3. RNA was extracted from cells cultured in 0.5% glucose in fully aerobic conditions and after 3 h from the shift to hypoxia. Labelled cDNAs obtained from each RNA sample were used to probe the DNA arrays containing the whole set of *S. cerevisiae* ORFs (Hauser *et al*., 1998).

Repeated hybridizations were performed with material of all strains and conditions resulting in at least eight data sets each. Data were analysed by statistical procedures, relative changes were calculated and a measure of significance was determined for each individual data point (Beissbarth *et al*., 2000), the latter indicated by a colour code in the relevant tables. The complete list for all ORFs, as well as the median of normalized signal intensities, can be downloaded from our web pages (http://www.dkfz-heidelberg.de/funct_genome/index.html) and the EUROFAN web page at MIPS (http://mips.gsf.de/proj/eurofan/eurofan_2/b2/index.html).

To confirm the results obtained with the arrays, we compared Northern blot hybridizations, taking advantage of a wide Northern analysis recently carried out in our laboratory using *hap1* and *rox1* mutants. A total number of 203 ORFs of unknown function from chromosomes IV, VII and XIV had been analysed (Lombardía *et al*., 2000). Once several ORFs were discarded (those that produced multiple transcripts or fell below the detection limits), a subset of 147 ORFs was considered in this assessment. Figure 1 shows, that there is a reasonable agreement between the two series of data, even though the strains used in the two studies have different genetic backgrounds.

For data interpretation, the variations were clustered using a bipole algorithm called correspondence analysis (Fellenberg *et al*., 2001). One big advantage of this procedure is the fact that both the experiments and the genes are clustered and presented in one plot. Also, any number of conditions can be clustered in one analysis. In addition, clusters can be queried in an automated process to identify the factor or parameter responsible. Figure 2 shows a typical result of such a clustering, resulting from a comparison of the wild-type, Δ*rox*1 and Δ*srb*10 strains under hypoxic and aerobic conditions.

Among the induced genes in the wild-type strain, there are several hypoxic genes that we may consider to be internal controls, such as *CYC7* or *COX5b*, because their induction in these conditions has also been proved in previous studies by conventional methods using Northern blot, or, by fusion of their promoters to reporter genes. A comparison of the data with those reported by ter Linde *et al*. (1999) shows coincidences but also several discrepancies. The latter can be attributed to (i) variations in the genetic background of the wild-type strains used; (ii) the different time-point selected to proceed with mRNA extraction after induction of hypoxia; and (iii) the noise caused by the many physiological and environmental parameters that are out of the controlled scope in the laboratory. Nevertheless, our data are extremely reliable taking into account the large number of repetitions of each individual measure, the high stringency used during sta-

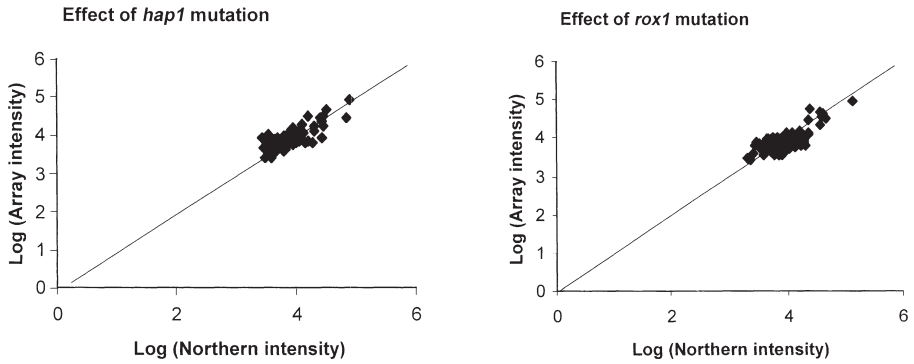


Fig. 1. Comparison of array data and Northern blot data. The whole list of ORFs included in this comparison has been published (Lombardía *et al*., 2000). Data sets from mutant backgrounds obtained by Northern and array systems were normalized in reference to wild-type values.

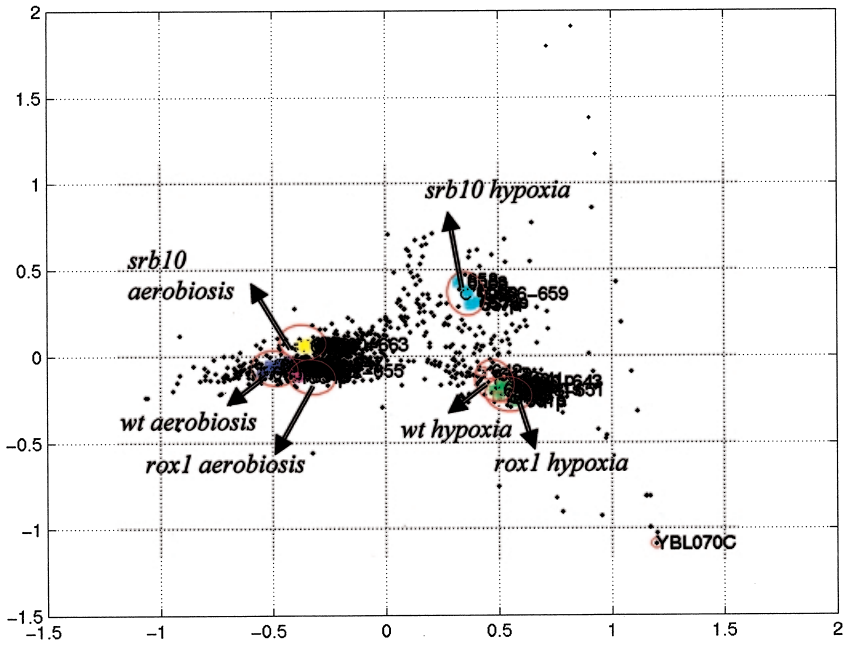


Fig. 2. Correspondence analysis of the normalized data from the *S. cerevisiae* transcriptome in wild-type (wt), Δ*rox1* and Δ*srb10* backgrounds during aerobiosis and hypoxia. The coloured squares represent the different experiments, each repeated eight times. The black dots represent the genes that were significantly differentially transcribed.

tistical processing of data and the high statistical significance obtained.

Given the conditions as outlined here, a summary of the number of genes repressed or activated by hypoxia in the studied strains is depicted in Fig. 3A. For the elaboration of this figure, only the subset of genes of statistical significant variation and regulation ratios (for activation or repression) higher than 2.5 were considered. In all tested genetics backgrounds, the number of genes that are repressed during hypoxia is larger than the corresponding one of activated genes. However, deletion of the regulatory factors causes a more important effect upon hypoxic activation than upon hypoxic repression.

A comparison of the data obtained in aerobiosis and hypoxia reveals that there is a different pattern of expression in the five strains under study (wild-type, Δ*hap*1 Δ*rox*1, Δ*rox*3 and Δ*srb*10). The data were analysed by correspondence cluster analysis (Fellenberg *et al*., 2001) and results are depicted in Fig. 3B. Again, both the experiments at the hypoxic (N_2) and aerobic (O_2) conditions (□ and ○ respectively) and the genes (black dots) are indicated. The majority of genes are located at a central position, indicative of insignificant variations in transcript levels. The more a gene is positioned towards (or even beyond) a cluster of experiments, the stronger were the observed changes in transcript levels. An important conclusion is that none of the single deletions analysed is able to fully overcome the wild-type hypoxic response, although obviously able to modify it. Thus, the hypoxic response has to be considered as a multifactorial event caused by the interplay of several regulatory circuits.

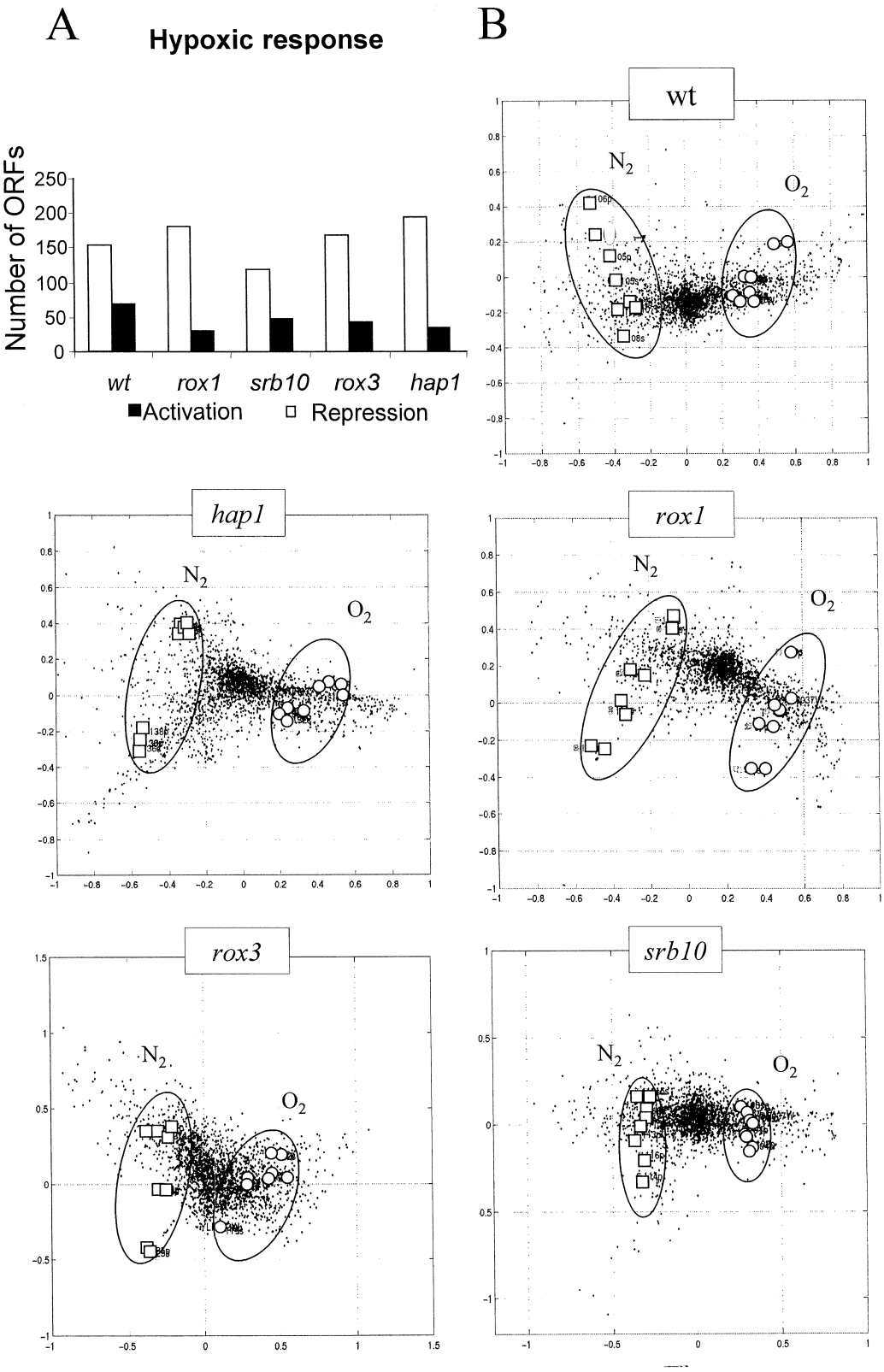
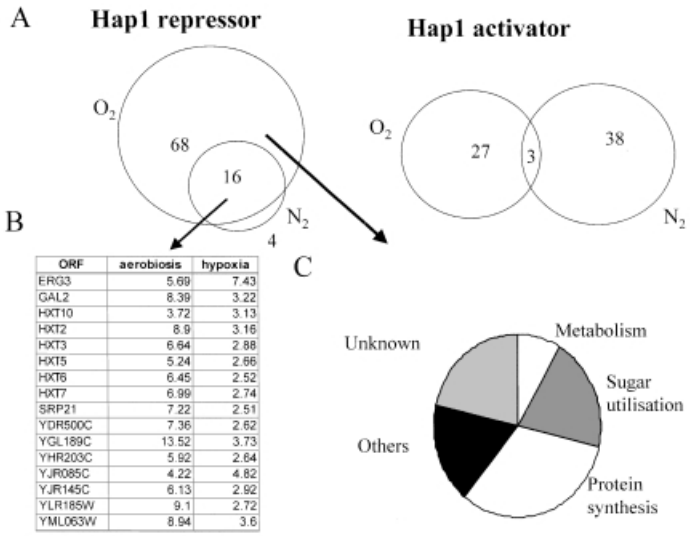


Fig. 3. A. Hypoxic activation and repression in wild-type and mutant backgrounds.
B. Correspondence analyses of the aerobic and hypoxic response in wild-type and mutant backgrounds. □ and ○ represent the individual experiments whereas black dots indicate the position of yeast genes in the plot.



ORF	aerobiosis	hypoxia
ERG3	5.69	7.43
GAL2	8.39	3.22
HXT10	3.72	3.13
HXT2	8.9	3.16
HXT3	6.64	2.88
HXT5	5.24	2.66
HXT6	6.45	2.52
HXT7	6.99	2.74
SRP21	7.22	2.51
YDR500C	7.36	2.62
YGL189C	13.52	3.73
YHR203C	5.92	2.64
YJR085C	4.22	4.82
YJR145C	6.13	2.92
YLR185W	9.1	2.72
YML063W	8.94	3.6

Fig. 4. Aerobic and hypoxic effects of the transcriptional regulator Hap1.
A. Venn diagrams showing the summary of ORFs affected by regulatory effects caused in aerobiosis (O_2) and hypoxia (N_2).
B. Table of repression ratios obtained in aerobiosis and hypoxia for the 16 ORFs that are downregulated in both conditions.
C. Functional distribution of the 68 ORFs repressed in aerobic conditions.

As these data were analysed from the perspective of the ratio of expression under hypoxic conditions to those under aerobic conditions, it is important to take into account that a duplication of aerobic expression or a decrease in hypoxia to half-values, for instance, would equally modify the expression ratio. Therefore, further analyses were carried out comparing the expression ratio of deletants and wild type in each of the conditions tested, and the most relevant results are discussed below.

Aerobic and hypoxic effects of the transcriptional regulator Hap1

Before this study, the number of known genes activated by Hap1 in aerobic conditions was 14. We found 30 new candidates. However, among these genes putatively regulated by Hap1, only four are related to respiratory functions or mitochondrial assembly. These are: *YNL315c* (*ATP11*), coding for a F1-F0 ATPase complex assembly protein; *YIL136w (OM45),* a protein of the outer mitochondrial membrane, and *YPL134c* and *YPR021c,* which are similar to mitochondrial carrier proteins. None of these genes has a canonical consensus sequence for the binding of Hap1, although several CCG triplets can be found, with different interspacing at several points in their promoters.

Although, traditionally, Hap1 was considered as a transcriptional activator during aerobiosis, its plausible role as a transcriptional regulatory factor during anaerobic conditions has also been postulated by other approaches (Chantrel *et al*., 1998). Results from this study show that Hap1 behaves as an activator of a subset of genes during hypoxia. The ORFs activated during hypoxia by Hap1 are distributed into the different functional groups defined at MIPS without any apparent bias (data not shown). A Venn diagram (Fig. 4A) shows that the subset of genes activated by Hap1 during hypoxia is mostly independent of the pool of genes activated during aerobiosis.

The existence of upregulated genes in a *hap1* mutant background during aerobiosis has also been recently probed by Northern analysis (Lombardía *et al*., 2000). A direct repressor effect of Hap1, or regulatory complexes including Hap1 and other proteins, could be postulated to explain these data. Alternatively, the repressor effect of Hap1 could be interpreted by indirect mechanisms caused by the absence of Hap1-dependent activation of one or several repressors. Among the repressors that in our analysis show a high-confidence, Hap1-dependent activation in aerobiosis is Mth1, a transcriptional repressor of hexose transport. It is noteworthy that 11 genes encoding hexose permeases are upregulated in the Δ*hap1* background during aerobiosis, which could be interpreted as a consequence of the release from Mth1 repression in the mutant. Of course, we cannot distinguish whether this general activation of hexose permeases affects each individual gene, or if it is a limitation of the hybridization array technique, which may not discriminate between genes of the same family. Regardless of the number of hexose permeases actually activated, and taking into account that the mutation *Δhap1* turns the cell *petite* and diminishes the respiratory capacity, an increase in hexose import might be necessary to compensate for the energy balance under these circumstances by using fermentative pathways. Interestingly, most of the ORFs that are repressed in a *HAP1* wild-type background, and become activated by its deletion, belong to sugar utilization and protein synthesis pathways (Fig. 4C).

There is a considerable number of genes that are upregulated in a *hap1* mutant background during aerobiosis; however, this effect is not very important in hypoxia.

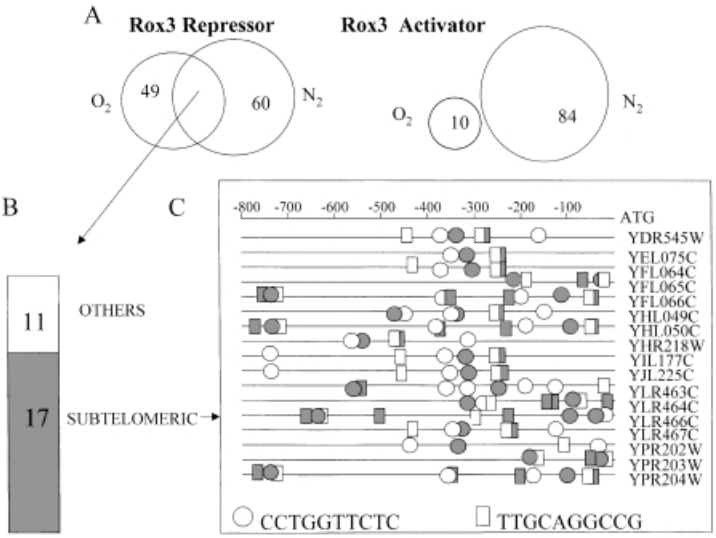


Fig. 5. Aerobic and hypoxic effects of the Rox3 mediator component of RNA Polymerase II. A. Venn diagrams showing the summary of ORFs affected by regulatory effects caused in aerobiosis (O_2) and hypoxia (N_2).
B. Functional distribution of the 28 ORFs that are repressed both in aerobic and hypoxic conditions.
C. Distribution of sequences CCTGGTTCTC and TTGCAGGCCG in the promoters of 17 subtelomeric-like proteins repressed by Rox3; open symbols represent direct strand and solid symbols the reverse one.

Moreover, the gene pool activated during hypoxia is included in the pool of genes activated during aerobiosis (see the Venn diagram from Fig. 4A). After a detailed examination of the 16 genes they have in common (Fig. 4B), the activation ratio observed during aerobiosis was seen to diminish considerably during hypoxia. Therefore, the direct or indirect repressor effect of Hap1 described would appear to be oxygen dependent.

The effect of Rox3 in aerobic and hypoxic conditions

Rox3 exerts an activator effect in hypoxic conditions over a subset of 84 genes that is not observed in aerobiosis (Fig. 5A). Among the hypoxia-activated genes are four that are related to transcription and whose activation could have a multiplicative effect over the others: *YPl037c (EGD1)* is a regulator of Pol II transcribed genes; *YOR210w* is a shared subunit of RNA polymerases I, II and III; *YPR010c (RPA135)* is a RNA polymerase I subunit; and *YMR043w (MCM1)* is a multifunctional regulator.

Rox3 also has a repressor effect upon transcription that is shared in aerobic and hypoxic conditions by a subset of 28 genes of unknown function but strikingly related to subtelomerically encoded proteins (Fig. 5B). *In silico* analysis of the promoters of these ORFs (defined as –800 bp from the ATG) with the RSA tools (embnet.cifn.unam.mx/~jvanheld/rsa-tools/) reveals the existence of two consensus sequences, CCTGGTTCTC and TTGCAGGCCG, which are over-represented in this subset of genes in comparison with their genomic distribution in 5′ regions of the total yeast ORFs. The consensus CCTGGTTCTC and TTGCAGGCG are present (without degeneracy) in 53% and 47%, respectively, of the promoters in the subset, compared with only 0.4% and 0.3% present in the total promoters of the yeast ORFs. Several promoter genes in the subset contain both sequences (Fig. 5C). These sequences are also present in the promoter of the *CAD1* gene, encoding a transcriptional regulator related to multidrug resistance, Cd and Fe chelators and Zn tolerance.

The comparative effect of Rox1 and Srb10 deletion is different in aerobic and hypoxic conditions

A multiple correspondence analysis of the results obtained in the wild-type, Δrox1 and Δsrb10 strains is presented in Fig. 2. In aerobic conditions, the clustering of transcriptomes corresponding to the three strains is very tight. The first conclusion to be drawn is that the general pattern of expression changes very little in the Δ*rox1* deletant, although it has been proved that Rox1 is a major repressor of hypoxic genes during aerobic conditions (Kastaniotis and Zitomer, 2000). Therefore, despite the negative control that this regulator causes over a limited set of genes (the hypoxic genes included in the hypoxic regulon, it has very little influence over the general picture of transcription during aerobiosis. This statement may also be applied to hypoxic conditions.

In hypoxia, two independent clusters can be observed; one includes only the Δ*srb10* deletant and a second one includes both the wild-type and the Δ*rox1* deletant. Therefore, this wide genomic analysis shows that the deletion of *SRB10 has* very different consequences in aerobiosis and during hypoxia, and they are much more pronounced in the latter. Although Srb10 has been involved in the transmission of the signal of specific repressors and activators from the promoters to the general transcriptional machinery (Kuchin and Carlson, 1998; Hirst *et al.*, 1999), this is the first report of its widespread importance in transcription during hypoxia.

One approach to a more concrete analysis of data obtained with the arrays is to consider only the subset of genes that are upregulated during hypoxia, in an attempt to discover new genes related to these conditions, as well as to shed light on the implications of Rox1 and Srb10 in this response. For this study, we have selected only 55 genes that are clearly hypoxic according to the statistical analysis. Table 1 shows only the genes that are overexpressed during hypoxia in a wild-type background, with a ratio (hypoxia:aerobic) over threefold, and with high significance in the statistical analysis (see *Experimental procedures*). Those with minor ratio, or with medium or low significance, can be retrieved from the database but are not considered here. Hypoxic induction is sometimes independent of the genetic background analysed and, at other times, dependent on the functionality of the transcriptional factors selected in a wide interplay of combinations that define clusters. There is no correlation between the clustering of genes according to this dependence and the functional categories (as defined by MIPS) of the ORFs included in each cluster.

Data obtained with the array system reveal that a large fraction of the hypoxic response depends on the Rox1 function. In fact, 50% of the hypoxic overexpression considered in Table 1 is dependent on the aerobic repressor Rox1 (clusters III and IV). Among them are genes that are well-known Rox1-regulated genes, i.e. *CYC*7 (Lowry and Zitomer, 1988) and *COX*b (Lambert *et al*., 1994). Other genes, such as *HEM*13, *ANB*1, *ACC*3, *ERG*11 or *ATF*1, which are regulated by Rox1 (Lowry and Zitomer, 1988; Turi and Loper, 1992; Sabova *et al*., 1993; Amillet *et al*., 1996; Fujiwara *et al*., 1999), do not appear in the table, because the selection of the ratio and statistical significance level has been very restrictive in an attempt to avoid false positives.

It is interesting to note that results from Table 1 (cluster IV) show that 57.2% of the hypoxic genes considered Rox1-dependent genes are also dependent on Srb10, suggesting multifactorial regulation of these genes or that Srb10 could be related to the transmission of repression from the Rox1 repressor to the basal transcriptional machinery. However, our analysis of direct interaction between Rox1 and Srb10 in a two-hybrid analysis (see *Experimental procedures* for experimental approach) showed a negative result (data not shown). A great deal of evidence has been compiled suggesting that the Tup1–Ssn6 complex represses transcription through both nucleosome positioning (Cooper *et al*., 1994) and interaction with the basal transcriptional machinery (Smith and Johson, 2000); indeed, a direct interaction between Tup1 and Srb10 has been proved (Zaman *et al*., 2001). Therefore, it is possible that the interplay between Rox1 and Srb10 is mediated by the general co-repressor complex Tup1–Ssn6, although the implications of other factors, such as Mot3, recently associated with the hypoxic response (Kastaniotis *et al*., 2000), must also to be considered in more detailed studies.

As concluded by the existence of cluster III (Table 1), there is at least a second repressor mechanism, Rox1 dependent but Srb10 independent, operating in the hypoxic response in yeast. Data from our study also indicate that the hypoxic response is independent of the genetic background analysed for 42.8% of the ORFs (clusters I and II), indicating that transcriptional factors other than Rox1 could be implicated in the control of the hypoxic response in a wide range of ORFs. The existence of Rox1-independent hypoxic pathways has been reported by different authors studying genes not listed in Table 1, such as *DAN1* (Sertil *et al*., 1997), *SRP1* (Donzeau *et al*., 1996; Bourdineaud *et al*., 2000), *GPD2* (Ansell *et al*., 1997) or the genes *PAU* (Rachidi *et al*., 2000). This group is probably heterogeneous in relation to the transcription factors and mechanisms implicated and more detailed studies need to be conducted in order to clarify this issue.

Experimental procedures

Strains and culture conditions

The following *Saccharomyces cerevisiae* strains were used: aGH1 (*Mat**a** trp1*–289 *leu2*–3 *leu2*–112 *gal1*Δ152) aGH1-Δ*rox3* (*MAT**a** trp1*–289 *leu2*–3 *leu2*–112 *gal*1Δ152 *rox3::LEU2*) and aGH1–Δ*rox1* (*MAT**a** trp1*–289 *leu2*–3 *leu2*–112 *gal1*Δ152 *rox1::LEU2*) (Balasubramanian *et al*., 1993). Δ*hap1* and Δ*srb10* deletants were obtained for this work from the wild-type aGH1 strain by replacement of the corresponding ORFs with the *kanMX4* module using the method of Wach *et al*. (1994).

The cells were grown in a complete medium (CM) with 5 g l^{-1} of dextrose (0.5%) as carbon source in a Biostat-MD (Braun-Biotech) vessel chemostat. The working volume of the culture was 2 l and temperature was maintained at 30°C. The air flow was 4 l min^{-1} sparred through the culture with an agitation speed of 650 rpm. Dissolved oxygen was measured with a polarographic electrode previously calibrated to 100% values and was kept to this level with a continuous oxygen supply. When cultures reached an absorbency of 0.8 at 600 nm, the oxygen supply was cut off and changed to nitrogen; 200 ml of the culture was taken immediately before the shift and after 3 h of nitrogen supply. As inocula, 100 ml of a liquid precultures on YEPD (1% yeast extract, 2% bactopeptone, 2% dextrose) were used.

RNA isolations

Cells were harvested and immediately frozen in liquid nitrogen, and later disruptied with a Micro-Dismembrator (B. Braun, Biotech International). The resulting powder was mixed with TRIZOL Reagent (Life Technologies) and total RNA was extracted by the method of Chomczynski and Sacchi (1987).

Table 1. ORFs with hypoxic response and their dependence from the regulatory factors Rox1 and Srb10.

ORF	Gene	Function	N_2/O_2	Rox1	Srb10
Cluster I					
YBL078C	AUT7	Autophagy; microtubule-associated protein (putative)	4.3	No	No
YBL101W		unknown	4.2	No	No
YBL101W	ECM21	Cell wall biogenesis	4.1	No	No
YBR214W	SDS24	Unknown; nuclear protein; similar to *Saccharomyces pombe*	3.6	No	No
YCL019W		Unknown	4.7	No	No
YCL020W		Unknown	3.8	No	No
YDL204w		Unknown	10.8	No	No
YDR330W		Unknown; similar to undulin	3	No	No
YEL060c	PRB1	Protein degradation; vacuolar protease B	3.1	No	No
YER024w		Unknown; similar to Yat1p	5.7	No	No
YGL139W		Unknown	3.4	No	No
YGR008C	STF2	ATP synthesis; ATPase stabilizing factor	7.2	No	No
YKL217W	JEN1	lactate transporter	4.6	No	No
YKR049C		Unknown	3.9	No	No
YLL026w	HSP104	Heat shock response/thermotolheat-shock protein	6	No	No
YLR149C		Unknown	9.3	No	No
YML128C		Unknown	13.2	No	No
YMR170C	ALD2	Ethanol utilization; aldehyde dehydrogenase	3	No	No
YMR280C	CAT8	Gluconeogenesis; transcription factor	3.6	No	No
YNL134C		Unknown; similar to *Cochliobolus. carbonum tox*D gene	4.5	No	No
YNL327W	EGT2	Cell cycle; unknown	5.9	No	No
YOR348C	PUT4	Transport; proline and gamma-aminobutyrate permease	4.1	No	No
YOR349W	CIN1	Mitosis, chromosome segregation, unknown	6.2	No	No
YPL223C	GRE1	Unknown; induced by osmotic stress	3.7	No	No
YFL014W	HSP12	Glucose and lipid utilization; heat-shock protein	3.8	No	No
Cluster II					
YAR068W		Unknown; similar to ICWP protein	1133.7	No	Yes
YGR243W		Unknown	3.8	No	Yes
YIL060W		Unknown	3.3	No	Yes
Cluster III					
YBR051W		Unknown	5.1	Yes	No
YDR227W	SIR4	Silencing; nuclear coiled-coil protein	3	Yes	No
YDR366C		Unknown	3.2	Yes	No
YER079w		Unknown	4.4	Yes	No
YER181c		Unknown	3.5	Yes	No
YGL055W	OLE1	Unknown	4.2	Yes	No
YGR060W	ERG25	Sterol metabolism; C-4 sterol methyl oxidase	3.1	Yes	No
YIL111W	COX5B	Oxidative phosphorylation; cytochrome-c oxidase subunit Vb	3.5	Yes	No
YKL187C		Unknown; similar to 4-mycarosyl isovaleryl-Co	4.4	Yes	No
YKR024C	DBP7	Ribosome biogenesis; putative RNA helicase	3.7	Yes	No
YKR025W		Unknown	3.5	Yes	No
YOL106W		Unknown	4.1	Yes	No
Cluster IV					
YCL021W			4.2	Yes	Yes
YGL021W	ALK1	DNA repair (putative)	3541.4	Yes	Yes
YGR265W		Protein synthesis; tRNA synthetase, methiony IYGR265W	3.1	Yes	Yes
YHR145C		Unknown	3.3	Yes	Yes
YOR211C	MGM1	Mitochondrial genome maintenance; dynamin family protein	11.4	Yes	Yes
YPL082C	MOT1	Transcription; putative helicase	6.1	Yes	Yes
YBR208C	DUR1,2	Nitrogen, amino acid, nucleotiurea amidolyase	6.7	Yes	Yes
YCL034W		Unknown	4.6	Yes	Yes
YDL214c		Unknown	14.3	Yes	Yes
YDR254W	CHL4	Mitosis; chromosome segregatiounknown	4.7	Yes	Yes
YDR446W	ECM11	Cell wall biogenesis	4.6	Yes	Yes
YEL039c	CYC7	Unknown	8.3	Yes	Yes
YHR005C	GPA1	Pheromone pathway; alpha subunit of G protein	8.3	Yes	Yes
YHR053C	CUP1–1	Cu^{2+} ion homeostasis; metallothionein	11.6	Yes	Yes
YHR055C	CUP1–2	Cu^{2+} ion homeostasis; metallothionein	11.6	Yes	Yes
YLR327C		Unknown; similar to Stf2p	14.7	Yes	Yes

Probe generation

Probe generation was as described in Hauser *et al*. (1998). Briefly, 60 μg of total RNA was annealed to oligo dT15, and used as a template to synthesize and radiolabel the corresponding first strand cDNA with 50 μCi of [α-^{33}P]-dCTP (Amersham) and SuperScript II (Life Technologies). The reactions were carried out at 43°C for 1 h, after which the RNA was hydrolysed with NaOH at 65°C for 30 min. The probe was purified by isopropanol precipitation and the isotope incorporation was measured to check the efficiency of the reaction.

Filters hybridizations, washing and stripping

Filters were prehybridized for 1 h at 65°C in the hybridization mix: 5x SSC, 5× Denhardt's solution and 0.5% SDS. The probe was then denatured for 5 min at 100°C, cooled quickly on ice and hybridized with the arrays overnight at 65°C. The day after, two washes were carried out at hybridization temperature for 5 and 20 min, respectively, in 2 × SSC, 0.1% SDS. Filter regeneration was done by pouring a boiling solution of 5 mM sodium phosphate (pH 7.5) and 0.1% SDS over the filters before their reuse.

Signal quantification

The filters were exposed for 24 h to a storage phosphor screen and the data were collected using a PhosphorImager Scanning Instrument 425 (Molecular Dynamics). Signal quantification was performed with ARRAY VISION software (Molecular Dynamics), which localizes over each array element a bounding circle fitted to the size of the DNA spot. Local area background was defined by placing manually 10 bounding circles throughout the filter. For each condition, the data from four independent hybridizations were analysed, using two different arrays and RNA samples (a total of eight replica-spots per ORF).

Statistical analysis

Taking into account the numerous intrinsic variations of transcriptional profiling on arrays, a stringent statistical analysis was performed to ensure the significance of the conclusions extracted from the data (Beissbarth *et al*., 2000). New software tools and data warehouse functions (MChips) have been developed (Fellenberg *et al*., 2001) for data assessment, handling analysis and presentation. Signal intensities of repeated hybridizations were normalized and significance levels assessed by two stringency criteria as described by Beissbarth *et al*. (2000). The highly stringent 'min–max separation' is calculated by taking the minimum distance between all data points of the two strains. The less stringent criteria, called 'standard deviation separation', is defined as the difference of the means of the two data sets diminished by one standard deviation. In the tables, a colour code indicates the two stringency measures (http://www.dkfz.de/tbi). According to these criteria, data were classified as being of high, medium or low statistical significance.

Clustering by correspondence analysis

Correspondence analysis is an explorative computational method for the study of associations between variables. Much like principle component analysis, it displays a low-dimensional projection of the data into a plane. It does this simultaneously for two variables, thus revealing associations between them. In the analysis of array-based transcript analysis (Fellenberg *et al*., 2001), the display of genes and experiments proved very valuable for biological data interpretation.

Two-hybrid analysis

The existence of a direct interaction between Rox1 and Srb10 was tested by two-hybrid analysis using the strain H125 (*MATa trp1–901 leu2–3112 ura3.52 his3–200 gal4Δ gal80Δ LYS::gaL1-his3 gal2-ade2 met2::gal7-lacZ*) and vectors pGAD (GAL4 activation domain with the *LEU2* autotrophy marker) and pGBD (GAL4-binding domain with the *trp1* autotrophy marker) described by James *et al*. (1996). The *srb10* gene and the 825 bp fragment, encoding just the amino-terminal domain, were PCR amplified and cloned in-frame in pGAD. *rox1* and *srb11* were cloned in pGBD. The interaction between Srb10 and Srb11 was tested as a previously described positive control. H125 was transformed by the method of Ito *et al*. (1983) and transformants selected in accordance to autotrophy markers. Transformants were then replated on Xgal plates to assess the interaction.

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