The yeast RNA pol II-associated factor Iwr1p is involved in the basal and regulated transcription of specific genes.*

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RNA polymerase II is a multisubunit enzyme that requires many auxiliary factors for its activity. Over the years, these factors have been identified using both biochemical and genetic approaches. Recently, the systematic characterization of protein complexes by TAP and mass spectroscopy has allowed the identification of new components of well-established complexes, including the RNA pol II holoenzyme. Krogan et al. (1) have described in yeast a novel and highly conserved factor, Iwr1p, that physically interacts with most of the RNA pol II subunits. Here we show that Iwr1p genetically interacts with components of the basal transcription machinery and plays a role in both the basal and regulated transcription. We report that the mutation of the IWR1 gene is able to bypass the otherwise essential requirement for the TBP-binding factor NC2, which occurs with different components of the basal transcription machinery, including TFIIA and subunits of the mediator complex. Deletion of the IWR1 gene leads to an altered expression of specific genes, including phosphate-responsive genes and SUC2. Our results show that Iwr1p is a nucleo-cytoplasmic shuttling protein and suggest that Iwr1p acts early in the formation of the pre-initiation complex by mediating the interaction of certain activators with the basal transcription apparatus.

In eukaryotes, RNA polymerase II (RNA pol II) is the enzyme that synthesizes all the mRNA precursors. This enzyme is highly conserved among species and, in yeast, consists of 12 subunits, namely Rpb1 to Rpb12 (2). The RNA pol II holoenzyme activity is regulated by several associated proteins (3-5), and its composition changes with the stage of transcription (5). Therefore, identification of the individual components of the RNA pol II complex is essential for its functional characterization. In recent years, the “tandem affinity purification” (TAP) methodology and mass spectrometry have been used for the systematic genome-wide characterization of protein complexes (1,6). This approach has not only led to the identification of new complexes, but also to the identification of new components of previously well-established complexes. This is the case of Iwr1p (Interacting with RNA pol II; YDL115c). Two works on the proteome-wide purification of yeast protein complexes report a variety of interactions between Iwr1p and the subunits of RNA pol II (1,6). Interactions among Iwr1p and Rpb1p, Rpb2p, Rpb3p, Rpb4p, Rpb5p, Rpb7p and Rpb10p were identified in both analyses. Together, these results suggest that Iwr1p is a component of the RNA pol II holoenzyme complex.

The promoter of the genes transcribed by the RNA pol II contains DNA sequences that are the binding sites of specific transcription factors which regulate their expression in response to environmental and internal changes. Thus, when yeast cells are starved of inorganic phosphate (Pi), Pho4p in cooperation with Pho2p, activates the expression of more than 20 different genes. These include the high-affinity transporter of inorganic orthophosphate PHO84 and the acid phosphatases PHO5, PHO11, and PHO12 (7). Other transcription factors bind to specific sequences in the promoter to repress the transcription of the target genes. This is the case
of Mig1p, which acts as repressor of different genes, including the SUC2 and GAL genes (8).

In addition to the sequence-specific DNA-binding proteins, there is a variety of accessory factors which act in a global manner. They include chromatin-modifying enzymes and proteins that positively or negatively affect the formation of active transcription initiation complexes between RNA polymerase II and general transcription factors (GTFs). Transcriptional repressor NC2 is one example of this second type of regulatory factor. It consists of two subunits (NC2α and NC2β; Bur6p/Ydr1p in yeast) which form a stable complex via histone fold domains (9-11). In yeast, NC2 interacts directly with the TATA-box binding protein (TBP), blocks its association with TFIIA and TFIIIB (11,12), and is able to repress transcription by RNA polymerase II in vitro (13). However, other studies indicate that the NC2 complex activates transcription at some promoters (14,15).

We have previously reported that defects in the transcriptional regulator NC2 can be suppressed by defects in a variety of components of the basal transcription machinery, including the two largest subunits of the RNA pol II, the TFIIH components Tfb1p and Ssl1p, and most of the subunits of the mediator complex (16). Here we report that the deletion of the gene encoding the RNA pol II-associated factor Iwr1p is also able to bypass the essential requirement of the NC2-component Bur6p. Our results show that Iwr1p shuttles between the nucleus and the cytoplasm in a Xpo1p-dependent manner, and that it is able to activate transcription when artificially recruited to a promoter. We report that the deletion of IWR1 positively or negatively affects the basal or induced expression of genes regulated through different pathways. The genetic interactions of the iwr1 mutation with components of the transcription machinery and its effects on the recruitment of the RNA pol II suggest that Iwr1p act early in the gene expression process.

Experimental procedures

Yeast strains and genetic methods- All the strains used in this study are listed in Supplementary Table S1. They were cultured using standard methods. For the growth assays, yeast cultures were diluted to the same OD600 and serial dilutions (1:10) were spotted onto YPD, YPGal or selective plates, and incubated at various temperatures. 5'-FOA containing plates were prepared by adding 1g/L of 5' fluoro-otic acid to synthetic complete medium. Doxycycline was used at a concentration of 10 mg/L. Pi-depleted medium was prepared according to (17). The Pi-free medium was a synthetic complete medium prepared using YNB without phosphate.

Strains containing the P<sub>GAL10</sub>-BUR6 and/or P<sub>GAL10</sub>-YDR1 alleles were constructed by replacing the wild-type promoter by the GAL10 promoter (including 3 copies of the HA-epitope). Deletions of non essential genes were obtained by substituting the coding sequences by the his5 or KAN<sup>R</sup> markers. Two different deletions were constructed for IWR1. In the iwr1-Δ1 allele, the sequence from position +58 (from the ATG) to the STOP codon (position +1130) was removed. In the iwr1-Δ2 allele, the entire ORF was deleted. No differences were observed between both strains in terms of growth or acid phosphatase activity (results not shown). A myc-, HA-, TAP- or GFP-tag was added to the C-terminus of Iwr1p by introducing the transforming cassette into the genomic copy of the IWR1 gene. In all cases, the PCR-based method described in (18) was used. The strain containing the P<sub>tetO</sub>-IWR1 allele was constructed by replacing the wild-type promoter by the tetO promoter using the PCR-based method as described by (19).

Plasmids- To construct plasmid pIWR1-GFP, genomic DNA from the strain containing the IWR1::GFP::Kan<sup>R</sup> allele was digested with XbaI and SphI, and ligated into the YCplac33 vector. Plasmids with the right insert were selected in LB plates containing ampicillin and kanamycin. pIWR1-ΔNES-GFP was constructed by the PCR amplification of pIWR1-GFP with partially overlapping oligonucleotides where the nucleotides encoding the amino acid sequence IIEHIDLAL had been deleted. The PCR product was digested with DpnI and used to transform E. coli. The deletion and absence of further modifications in the resulting plasmid
were checked by sequencing the entire *IWR1* open reading frame. The plasmid used to express the LexA-Iwr1p fusion (pLexA-IWR1) was obtained by the PCR-mediated generation of *Bam*HI and *Sal*I sites immediately upstream and downstream, respectively, of the *IWR1* coding sequence. The resulting fragment was ligated into the *Bam*HI and *Sal*I sites of pBM116 (20). pLexA-IWR1 was introduced into the TAT-7 yeast strain (*lexAop-HIS3*, *lexAop-LacZ*). The Pho4p overexpression plasmid was constructed by the PCR amplification of the genomic *PHO4* locus and by cloning the restriction fragment in the YEplac181 multicopy plasmid.

**Fluorescence microscopy**- Yeast grown in SC at the early exponential phase were used to visualize GFP fusions. Aliquots (1.2 µl) of the cultures were put onto microscope slides and covered with 18 × 18-mm coverslips. Cells were then viewed using a Zeiss Axioskop II fluorescence microscope. Images were scanned with a SPOT digital camera (Diagnostic Instruments Inc.) and processed using the Adobe Photoshop software, v. 8.0.1

**Preparation and Northern analysis of RNA**- For the *SUC2* analysis, yeasts were grown in YPD (with 2% dextrose) at the early exponential phase, and then washed and transferred to YPD containing 0.05% of dextrose at the indicated times. For *GAL1* induction, cells exponentially growing in YPD were washed and transferred to YPGal media and incubated for 4 hours. For *PHO5* and *PHO84* analyses, cells were grown at the exponential phase in Pi-depleted or Pi-free medium supplemented with Pi to 13.4 mM. Then, cells were washed and transferred to Pi-depleted or Pi-free medium containing different Pi concentrations, and were incubated at the indicated times. Alkaline shock was performed according to (21). After these treatments, cells were harvested, washed with water and frozen at −80°C. RNA extraction, analysis and probes have been previously described elsewhere (22).

**β-galactosidase and acid phosphatase assays**- β-galactosidase (23) and acid phosphatase (24) activities were measured as described.

**Chromatin analysis**- Chromatin digestion with *Cla*I restriction nuclease was performed as described (25). The chromatin immunoprecipitation (ChIP) analysis was essentially performed as previously described (26). 8WG16 (anti-Rpb1 antibody) from Neoclone (Madison, WI), 12CA5 (anti-HA antibody) from Roche and 9E10 (anti-myc antibody) from Roche were used. Co-immunoprecipitated DNA was analyzed in triplicate by quantitative PCR using the DNAEngine (BioRad). Oligonucleotides TTGGTCACCTTACTTGGCAAAGG CAT and TGGTAATCTCGAATTTGCTTGCTCT were used to amplify the *PHO5* promoter. Sequences of other oligonucleotides are available upon request.

**Whole-genome transcriptional analysis**- Strains used for the whole genome transcription analysis were grown in YPD at the early exponential phase. cDNA synthesis and labeling, filter hybridization and quantification/normalization of hybridization signals were performed as described (27). RNA was obtained from three independent cultures and analyzed for each strain. Data Excel files will be available at the URL: http://www.uv.es/estruch. Original macroarray data were submitted to the GEO database, where they have been assigned Accession No. GSE17303.

**Western blot analysis**- Protein extracts were prepared by TCA-precipitation of exponentially-growing yeast cells grown in YPGal 2%, and from yeast cells grown in this medium and then transferred to YPD for 5 hours. Bur6-HA and Ydr1-HA were detected with the monoclonal anti-HA antibody (SIGMA) and chemiluminescence visualization (ECL Advanced; Amersham), according to the manufacturer’s instructions.

**RESULTS**

*Mutations in IWR1 suppress the depletion of the transcriptional repressor NC2.* To gain further insight into the function of NC2, we genetically screened for suppressors of the
NC2 defect (16). For this screening, we used a yeast strain in which the genes encoding both NC2 components (YDR1 and BUR6) were placed under the control of the GAL10 promoter. Transposon insertion mutants were generated in this strain using a yeast genomic library which had been mutagenized by the insertion of an mTn3-lacZ/LEU2 transposon (28). Suppressor mutants were selected for their ability to grow on dextrose-containing plates at room temperature, and transposon insertion sites were determined. In this screen, we identified a variety of components of the basal transcription machinery involved in the control of transcription initiation, like the Mediator components NUT2, MED7 and SRB2, the TFIH component TFB1, and the RNA pol II subunit RPB7 (16). Another gene identified in this screen, IWR1, is the subject of the present work.

In our screen, we isolated 5 independent clones where transposon insertions were mapped at different locations in either the IWR1 open reading frame or the 5' flank of this gene, suggesting that the suppression was a result of the complete loss of the Iwr1p function. This point was confirmed by deleting the IWR1 ORF in strains carrying the PGAL10- YDR1 or the PGAL10-BUR6 alleles. As observed in Figure 1A, the deletion of IWR1 partially suppressed the growth defects caused by the depletion of both NC2 components. Since the genetic screen was performed in a strain in which the expressions of both BUR6 and YDR1 genes are under the control of the GAL10 promoter, the ability to grow in media containing dextrose as the sole carbon source may be due to a defective repression of the GAL10 promoter in the iwr1 mutant. To check this possibility, we analyzed the levels of Bur6p and Ydr1p proteins in the iwr1 mutant by making use of the three copies of the HA epitope added to the N-terminal end of the proteins while substituting the BUR6 and YDR1 promoters by GAL10 (18). In the Western analysis shown in Figure 1B, we compared the amounts of Bur6p and Ydr1p (expressed from the GAL10 promoter) in both the iwr1 mutant and the isogenic wild-type strain before and after a 5 hours incubation in dextrose. Deletion of IWR1 reduces the amount of Bur6p-HA and Ydr1p-HA in both galactose and dextrose (Figure 1B), suggesting that this mutation causes defects in the basal and induced expression from the GAL10 promoter. In fact, the Northern blot analysis of GAL10 did not show any constitutive expression in dextrose, but revealed a reduced induction in galactose of this gene in the iwr1 mutant strain (Figure 1C). Therefore, suppression of NC2 depletion by Δiwr1 is not a consequence of the defective repression of the GAL10 promoter in dextrose. Then, we wondered whether the deletion of IWR1 could bypass the absence of the otherwise essential NC2-component Bur6p. For this purpose, we deleted the IWR1 gene from a strain carrying a chromosomal deletion of BUR6 and the wild-type BUR6 gene on an URA3/CEN plasmid. The resulting cells were able to grow on 5'-FOA-containing plates (Figure 1D), indicating that deletion of IWR1 is able to relieve the requirement for the essential function of NC2α, as occurs with mutations in other components of the basal transcription machinery (16,29-31).

Transcriptional defects caused by mutations in IWR1 and BUR6 are partially compensated in the double mutant strain. To assess the effect of the deletion of IWR1 on the gene expression when NC2 was defective, we analyzed the expression of genes whose transcription is affected in NC2 mutants (16,32). The induction of the GAL1 gene by galactose has been reported to be abolished in a bur6-1 mutant strain (32). As Figure 2A indicates, the transcriptional induction of GAL1 was impaired in both the bur6-1 and Δiwr1 mutant strains, but was partially restored in the bur6-1 Δiwr1 double mutant. On the other hand, the depletion of the NC2-component Ydr1p increased the constitutive level of HSP26 (16). As seen in Figure 2B, the bur6-1 mutation also increased the basal expression of the HSP26 gene. A weak constitutive expression of HSP26 was also observed in the Δiwr1 mutant strain. Interestingly, the constitutive expression of HSP26 was not observed in the bur6-1 Δiwr1 double mutant strain (Figure 2B). Therefore, deletion of IWR1 was able to partially compensate some
transcriptional defects caused by mutations in NC2.

**Transcriptional activation by a LexA-Iwr1p fusion.** The nature of the suppressors of NC2 requirement that we and others have identified suggests that Iwr1p could play a positive role in transcription initiation (16,29,33). To investigate this possibility, we expressed a fusion protein which includes the DNA binding domain of LexA and the entire Iwr1p protein (LexA-Iwr1p) in a strain carrying the HIS3 and lacZ reporter genes whose promoters include LexA binding sites. As Figure 3 illustrates, the expression of LexA-Iwr1p has a positive effect on HIS3 and lacZ transcription, as indicated by the ability of the strain carrying the LexA-Iwr1p fusion, but not the LexA binding domain alone, to grow in the presence of 3-aminotriazol (Figure 3A) and by the increased β-galactosidase activity caused by the presence of LexA-Iwr1p (Figure 3B). Therefore, our results show that the binding of Iwr1p to the promoter could have a positive effect on transcription.

*Iwr1p shuttles between the nucleus and cytoplasm in an Xpo1p-dependent manner.* The isolation of *iwr1* mutations as suppressors of the transcriptional repressor NC2, together with the reported physical interactions between Iwr1p and different RNA pol II subunits (1,6), suggest that at least some Iwr1p functions occur in the nucleus. To localize Iwr1p in the cell, we added a GFP tag to the C-terminus of Iwr1p and cloned the gene fusion into a centromeric plasmid. The functionality of the Iwr1p-GFP fusion protein was confirmed by its ability to complement the slow growth phenotype of a Δ*iwr1* mutant strain (results not shown). As Figure 4A depicts, Iwr1p-GFP was distributed throughout the entire cell. Analysis of the Iwr1p amino acid sequence using the NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES/) predicted a possible leucine-rich Nuclear Export Signal (NES) between positions 169 and 177 (IEEHIDLAL), suggesting that Iwr1p could shuttle between the nucleus and cytoplasm in an Xpo1p-dependent manner. To verify this possibility, we examined the localization of Iwr1p in *xpo1-1* cells. Figure 4B illustrates how Iwr1p-GFP was detected mainly in the nucleus following a shift to 37°C, a condition under which the Xpo1-dependent protein export was blocked. No difference in the localization of Iwr1p-GFP was observed between 30°C and 37°C when the fusion protein was expressed in a wild-type strain (results not shown). This result indicates that Iwr1p is a shuttling protein, and suggests the presence of a NES recognized by Xpo1p. Deleting this sequence from Iwr1p-GFP led to the nuclear accumulation of Iwr1p-GFP (Figure 4C), suggesting that this sequence is required for the nuclear export of Iwr1p. Deletion of this sequence only slightly affected the ability of the fusion protein to complement the growth defect caused by the *iwr1* null mutation (results not shown).

**Transcriptional effects of the IWR1 deletion.** We analyzed the genome-wide transcriptional effects caused by the deletion of *IWR1* by comparing the transcriptional profile of a Δ*iwr1* strain with the isogenic wild-type strain when cells were exponentially grown in YPD medium (complete data sets will be available at the URL: http://www.uv.es/estruch). A total of 179 genes showed at least two-fold changes in their expression levels in the Δ*iwr1* strain, as compared to the wild-type control (see Supplementary Table S2). Depletion of *IWR1* resulted in increased transcript levels of 74 genes, whereas a decreased expression was noted for 105 genes. To determine whether the deletion of *IWR1* affects specific metabolic pathways, we analyzed the distribution in functional categories of those genes whose expression levels were altered in the *iwr1* mutant strain (Supplementary Table S3). We noticed that the products of 15 of the 74 genes which were up-regulated in the *iwr1* mutant were localized in the mitochondria membrane. Of these, 10 genes encoded components involved in oxidative phosphorylation, including 7 genes encoding ATP synthase subunits. The possibility of an increased expression of respiratory genes in the *iwr1* mutant resulting in an augmented respiratory activity was analyzed by comparing the oxygen consumption of the *iwr1* mutant with the wild type when cells
were grown in rich glucose-containing medium. Δiwr1 deletion only brought about a modest increase (around 20%) in oxygen consumption (results not shown). The list of genes up-regulated in the iwr1 mutant includes 11 genes whose promoters contain binding sites for the Hap2p/3p/4p/5p complex [GDH1, ATP16, ATP17, COX13, ATP19, ATP15, ATP3, ATP7, QCR7, COX7 and PET9 (SGD; (34)]. Interestingly, HAP4 itself was one of the up-regulated genes in the iwr1 mutant. We also noticed that there were 6 genes whose expression might be regulated by Gcn4p [GDH1, ARG1, HSP78, PCL5, ARG5,6 and TRP5 (SGD, (34,35)]. Among the down-regulated genes in the iwr1 mutant strain, the only significant functional category found corresponded to acid phosphatase activity, and included the PHO3, PHO5, PHO11 and PHO12 genes.

Iwr1p is required for the basal but not the induced transcription of the ARG1 and ATP16 genes. We further studied two well-characterized genes whose expression increased in the iwr1 mutant (Supplementary Table S2). The ARG1 gene, encoding arginosuccinate synthetase, is controlled by two regulatory pathways. It is induced under amino acid starvation through the Gcn4p transcription factor (36) and is repressed by arginine through the ArgR/Mcm1 complex (37,38). The expression of the ARG1 gene can be induced by the addition of sulfometuron-methyl (SM), an inhibitor of isoleucine/valine biosynthesis to the growth medium (39). Figure 5A shows that the basal expression of ARG1 increased in the Δiwr1 mutant. Both the basal and induced expressions of ARG1 were Gcn4p-dependent, but Iwr1p was completely dispensable for the induction of ARG1 by SM (Figure 5A). The deletion of IWR1 moderately increased the basal expression of ARG1 in both the presence and absence of Gcn4p.

The ATP16 gene encodes one of the S. cerevisiae ATP synthase subunits (40). Transcription of genes encoding the electron transport chain components is regulated by the heme-activated protein complex Hap2/3/4/5p by binding to the CCAAT box at the upstream activation sequence (41). The expression of ATP16 was up-regulated in non fermentative carbon sources, like ethanol, in a Hap4p-dependent manner (Figure 5B). As with ARG1, the deletion of IWR1 did not affect the induction of ATP16, but moderately increased its basal expression in both the presence and absence of Hap4p (Figure 5B).

Iwr1p is involved in the expression of the SUC2 and phosphate responsive genes. Many of the aerobic genes up-regulated in the iwr1 mutant (Supplementary Table S2) are subjected to glucose repression. Since our genomic-wide transcription analysis was done with cells exponentially grown in YPD, the increased expression of genes involved in the respiration in this medium could be caused by an impaired catabolite repression in the iwr1 mutant. To check this possibility, we analyzed the expression of SUC2, a well-characterized glucose repressible gene. Figure 6A shows that the iwr1 mutant cells grown in glucose-containing media did not transcribe SUC2, which suggests that the constitutive expression of glucose-repressed genes was not the cause of the increased level of the respiratory gene transcripts observed in the iwr1 mutant. The expression of SUC2 was induced by lowering the amount of glucose in the growing medium. Under such conditions, we observed reduced levels of the SUC2 transcript in the iwr1 mutant strain compared to the wild-type (Figure 6A). We may also note the almost complete absence of SUC2 mRNA after a 60-min incubation under low glucose conditions. We found that the deletion of the MIG1 gene, which encodes a repressor of the SUC2 expression (8), avoided the transient absence of the SUC2 transcript observed after 60 minutes incubation. However, it did not significantly increase the reduced levels of SUC2 in the iwr1 mutant (Figure 6B).

Among the transcripts whose expression was reduced in the iwr1 mutant, we identified several phosphate responsive genes (Supplementary Table S2). These genes are induced when cells are starved for Pi (42), and their expression is regulated by transcription factors Pho4p and Pho2p (43). Figure 6C shows how the Δiwr1 mutation impaired the
induction of the PHO5 gene under low Pi conditions. The defective induction of the PHO5 gene in the iwr1 mutant was not caused by a defective localization of Pho4p since the entrance of this transcription factor into the nucleus under low Pi conditions was not affected by the iwr1 mutation (result not shown). To check whether Iwr1p nucleocytoplasmic shuttling was required for the activation of PHO5 gene, we analyzed the induction of the acid phosphatase by low Pi conditions in a strain carrying a version of Iwr1p from which the NES had been deleted. As observed in Supplementary Figure 1, the induction of acid phosphatase in this strain was similar to that observed in wild type strain. Phosphate responsive genes respond differently to intermediate Pi conditions. Thus, a low expression of PHO5 occurs at intermediate Pi concentrations, whereas PHO84 is significantly induced (44,45). When we analyzed the effect of the IWR1 deletion on the expression of PHO84, we observed increased PHO84 mRNA levels as the Pi concentrations lowered in both the wild-type and the Δiwr1 mutant strains, although the level of transcripts was always lower in the mutant (Figure 6C). The induction of PHO84 observed in the Δiwr1 mutant under low Pi conditions was dependent on Pho4p as no expression was observed in a Δiwr1Δpho4 double mutant strain (Figure 6D). Pho4p has also been found to be involved in the induction of PHO84 by alkaline pH (21). As Figure 6E shows, this induction was also negatively affected by the absence of Iwr1p.

Iwr1p is required for normal chromatin remodeling kinetics at the PHO5 promoter. Among the phosphate responsive genes, PHO5 is a well-studied example of a gene whose promoter undergoes extensive remodeling at the chromatin level during the induction process. Under high Pi conditions, four positioned nucleosomes are associated with the PHO5 promoter region (49,50). These four nucleosomes are evicted during Pi starvation, and the process can be followed by measuring the accessibility of a ClaI restriction site located within nucleosome -2 (Figure 8A). As observed in Figure 8B, while the ClaI site in the wild-type strain was accessible in the phosphate-depleted medium after 4 hours incubation, the level of chromatin opening in the iwr1 at this time was similar to that observed under high phosphate conditions, suggesting that the absence of Iwr1p delays the kinetics of PHO5 induction at the chromatin opening level. Next, we checked the recruitment of the Pho4p activator in a Δiwr1 mutant. Previous reports indicate the requirement of chromatin remodeling for Pho4p binding to the nucleosome-occluded UASp2 under low Pi conditions (17,60,61). Accordingly by chromatin immunoprecipitation (ChIP) analysis, we found that the recruitment of Pho4p upon Pi depletion is delayed when...
Iwr1p is absent (Figure 8C). As expected, the impairment in chromatin remodeling observed in the Δiwr1 mutant also delays the recruitment of Rpb1p to the PHO5 promoter (Figure 8D).

Defects in the induction of SUC2 in the iwr1 mutant are suppressed in the absence of H2A.Z. Synthetic genetic interactions between Δiwr1 and deletions in components of the SWR complex have been identified by synthetic genetic arrays (1). The SWR complex assembles the variant histone H2A.Z/Htz1p into chromatin (51). Since the deletion of both the IWR1 and HTZ1 genes results in synthetic lethality (Figure 9A), we constructed a yeast strain carrying a deletion of HTZ1 and IWR1 under the control of the repressible tetO promoter. As observed, doxycycline addition severely impaired the growth of PtetO-IWR1 Δhtz1 double mutants (Figure 9B). We then analyzed the effect of the Δhtz1 mutation on the defects caused by the depletion of Iwr1p on the transcription of SUC2. As observed in Figure 9C, the induction of SUC2 in the PtetO-IWR1 strain followed the same pattern as that observed in the Δiwr1 strain (Figures 6A and 6B). However, some differences in the transcript levels were noted, which were likely due to the residual expression of IWR1 in the PtetO-IWR1 strain, even in the presence of doxycycline. As with the Δiwr1 strain, an almost complete disappearance of the SUC2 mRNA was noted after an initial induction. Interestingly, the presence of the SUC2 transcript at intermediate induction times was restored by the deletion of HTZ1, and the kinetics of induction in the double mutant PtetO-IWR1 Δhtz1 was similar to that shown by the wild-type strain, although the transcript levels were lower in the double mutant at all the times analyzed (Figure 9C).

Iwr1p was not found preferentially associated with the chromatin of the PHO5, SUC2 and ARG1 promoters. To check whether Iwr1p was recruited together with RNA pol II to the promoter of the genes whose transcription was Iwr1p-dependent, we performed ChIP assays using Iwr1p proteins tagged in different ways (-myc, -HA or -TAP). We measured the amount of Iwr1p present at the PHO5 and SUC2 promoters under repressed (high Pi or high glucose, respectively) and induced (low Pi or low glucose, respectively) conditions. We also analyzed the presence of Iwr1p in the promoter of the ARG1 gene, whose basal expression increased in the Δiwr1 mutant (Figure 5A). The levels of Iwr1p associated with the PHO5 and SUC2 promoters did not vary significantly during their induction, and were similar to the level of Iwr1p found in a non transcribed, intergenic region (Supplementary Figure 2). In addition, no enrichment in the Iwr1p content (in relation to the intergenic region) was observed at the different DNA sequences along the PHO5 locus or in the promoter of the ARG1 gene (Supplementary Figure 2). Therefore, our results suggest that Iwr1p is not associated with the RNA pol II when this enzyme is recruited to the promoter of the active genes.

DISCUSSION

Iwr1p was originally identified as a protein that co-purifies with almost every subunit of RNA pol II (1,6). Iwr1p is highly conserved with a homolog, CG10528, in Drosophila melanogaster, that partly co-localizes with phosphorylated, actively transcribing RNAPII on polytene chromosomes (1). In this report, we present data that further support the relationship between Iwr1p and the basal transcription machinery. We identified IWR1 in a genetic screen for mutations that suppress the requirement for NC2, a general transcriptional repressor that operates by interacting with the core promoter and components of the basal transcription machinery, like TBP (reviewed in (52)). Different suppressors of the NC2 function have been identified (13,16,29,31,33). To date, the common feature of each NC2 suppressor reported is that they are components of the basal transcription machinery which play putative positive roles in transcription initiation. Our finding that deletion of IWR1 can bypass the requirement of the NC2 function (Figure 1) through the compensation of the transcriptional defects (Figure 2) is in agreement with a general role of Iwr1p in transcription initiation. This role is also supported by the ability of Iwr1p to
activate transcription when fused to a heterologous DNA binding domain (Figure 3), which occurs for several subunits of the RNA polymerase II holoenzyme (53,54).

Unlike other general transcription factors, the localization of Iwr1p is not exclusively nuclear. In this work, we demonstrate that Iwr1p shuttles between the nucleus and cytoplasm and can be detected throughout the cell at the steady state (Figure 4). Iwr1p accumulates in the nuclei in spo1-1 mutant cells and contains a leucine-rich sequence (IIEHIDLAL) that likely acts as a nuclear export signal (NES). The capability to shuttle across the nuclear envelope is expected for those proteins that play both nuclear and cytosolic roles. However, a dynamic distribution is not required for those proteins whose function takes place exclusively in the nuclear compartment, as with the typical general transcription factors, suggesting that Iwr1p could have additional functions in cells that require shuttling. Thus, Iwr1p could participate in one or more signal transduction pathways ending in the nucleus. However, even though this possibility may be in agreement with the transcription profile shown by the Δiwr1 mutant, we have not been able to find any condition that modifies the cellular distribution of Iwr1p (results not shown). In addition, we found that shuttling is not required for the induction of the PHO5 gene by low Pi conditions (Supplementary Figure 1).

Although IWR1 is not an essential gene, deletion of IWR1 moderately impairs cell growth under all the tested conditions (Figure 1A; results not shown). Growth impairment is likely related with the defects in the gene transcription shown by the Δiwr1 mutant. We performed whole-genome profiling to further investigate the cellular role of Iwr1p. The most striking finding of this analysis was the increased expression of genes encoding mitochondrial proteins in the Δiwr1 mutant, particularly those involved in oxidative phosphorylation (Supplementary Table S2 and Figure 5). Although most of the genes involved in aerobic respiration are subjected to glucose repression, the transcription profile of the Δiwr1 mutant does not show an upregulation of other glucose repressible genes such as SUC2. An increased transcription of the nuclear genes encoding the components of oxidative phosphorylation has been reported for the mediator component Med5p (Nut1p) (55). Therefore, it is possible that the basic transcription machinery includes several components to coordinate the nuclear gene expression with the respiratory requirements.

In addition to the genes encoding oxidative phosphorylation components, the expression of genes regulated by amino acids (ARG1), carbon source (SUC2 and GAL10) or phosphorus starvation (PHO5 and PHO84) is also altered in the Δiwr1 strain. However, the effect of the deletion of IWR1 on the expression of these genes is very different. Deletion of IWR1 increases the basal expression of ARG1 and ATP16, but not their induction. On the other hand, Iwr1p is involved in the induction of the SUC2, GAL1, PHO5 and PHO84 genes, suggesting that the requirement of Iwr1p is genespecific.

The role of Iwr1p as a target of specific transcription factors could explain the striking kinetics of SUC2 induction observed in the Δiwr1 mutant (Figure 6). Geng and Laurent reported a biphasic pattern for the transcriptional induction of SUC2, the first phase of which is partly mediated by the general stress response transcription factors Msn2p and Msn4p (56). Our results are compatible with an effect of Iwr1p in delaying the second phase of the induction without affecting the early Msn2p/4p-dependent induction. In agreement with this model, we found that induction of HSP26, which depends on Msn2p/4p (57), is not affected by the Δiwr1 mutation (results not shown). Moreover, our results involve Mig1p in the late phase of the SUC2 induction since the transient disappearance of the SUC2 transcript is not observed in the Δiwr1 mig1 double mutant (Figure 6). This suggests that Iwr1p participates in eliminating the repression exerted by Mig1p. The transient disappearance of the SUC2 transcript is also suppressed by the deletion of the HTZ1 gene encoding the histone H2A.Z variant (Figure 9C). Notably, the repression mediated by Mig1p requires the co-repressor Ssn6p-Tup1p (58), and Tup1p has been recently involved in the deposition of histone H2A.Z in the promoter of the GAL1 and SUC2 genes (59). It is, therefore, tempting to speculate that the Mig1p-Ssn6p-Tup1p complex participates in the deposition of H2A.Z in the SUC2 promoter,
which could have a negative effect on the late induction of SUC2, in which Iwr1p is necessary to remove this repression. Future studies examining the presence of all these proteins on the SUC2 promoter at different times during the activation process are required to test this model.

The most striking effect of deleting of IWR1 is observed in the induction of the phosphate responsive genes under Pi starvation conditions. Our results show that the absence of Iwr1p impairs the normal induction of PHO5 and PHO84 (Figures 6 and 7). Different possibilities may be considered to account for the impaired induction of the PHO5 and PHO84 genes in the iwr1 mutant: (i) defects in the PHO signal transduction, e.g., caused by the slower growth rate and, therefore, by the slower exhaustion of intracellular phosphate pools, (ii) a defect in chromatin remodeling, e.g., through the lack of cofactors which are normally recruited via Iwr1p, (iii) a defective recruitment of the transcriptional apparatus to the PHO5 promoter, or (iv) the defective activity of RNA pol II lacking Iwr1p to initiate transcription once bound to the PHO5 sequences. The lack of effect of the iwr1 mutation on the kinetics of the Pho4p nuclear translocation upon Pi depletion (results not shown), or the impairment in the induction of PHO84 by alkaline pH (Figure 6E), does not support the possible delay in the phosphate depletion caused by the slower growth rate of the iwr1 mutant. The response of PHO84 to alkaline pH is also dependent on Pho4p, and exposure to alkali has been suggested to actually generate a situation of phosphate starvation that could be responsible for the induction of some of the PHO genes (21). Under alkaline pH conditions, a significant induction of PHO84 occurs at times as short as 25 minutes as opposed to the reduced growth rate of the iwr1 mutant being the cause of the defective induction of PHO84 and other PHO genes. On the other hand, our results show a delay in the chromatin remodeling of the PHO5 promoter in the absence of Iwr1p (Figure 8B). The failure in chromatin opening is likely to be responsible for the delay in Pho4p and RNA pol II recruitment to the PHO5 promoter observed in the iwr1 mutant (Figure 8C and 8D).

Together, our findings suggest a role of Iwr1p prior to PIC formation that would dependent on the amount of Pho4p recruited to the PHO gene promoter. Thus, the higher requirement of Iwr1p for the induction of PHO5 than for the induction of PHO84 may be explained by the higher accessibility of Pho4p to the PHO84 promoter (44,45). We find that the requirement of Iwr1p for the induction of PHO5 can be partially alleviated by increasing the amount of Pho4p in “reciprocal accordence” with an earlier study in which increased cofactor dependency correlates with decreased Pho4p occupancy at the PHO5 promoter (60). Even though Iwr1p is partially or totally dispensable under full activation conditions (such as Pi-free medium or in the pho80 mutant), it is absolutely essential under intermediate activating conditions (such as the Pi-depleted medium). These findings support the strong influence of Iwr1p on the Pho4p signaling pathway to the PHO5 promoter, and indicate that once the RNA polymerase II lacking Iwr1p is bound to the promoter, it is able to transcribe the PHO5 gene normally.

Despite our many efforts, we have not been able to find any specific recruitment of Iwr1p to the chromatin of its target genes. (Supplementary Figure 2). It is, therefore, possible that the association of Iwr1p with RNA pol II is restricted to the enzyme that is not bound to DNA. Thus, Iwr1p could participate in the regulation of the recruitment RNA pol II to specific promoters, but would leave the enzyme prior to the binding of the enzyme to the DNA. The interaction of Iwr1p with the polymerase that is not bound to chromatin would be in agreement with the fact that only a minor fraction of the total Rpb3p subunit could be recovered when a Iwr1-TAP protein fusion was pulled down using IgG-Sepharose beads (our unpublished results). Further studies will be needed to elucidate the molecular mechanism through which Iwr1p participates in the transcriptional regulation of its target genes.
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Deletion of IWR1 suppresses the depletion of the NC2 components Bur6p and Ydr1p. (A) The IWR1 gene was deleted in strains carrying P_GAL10-BUR6 or P_GAL10-YDR1 alleles. Wild type (FY86) and mutant cells were spotted onto YPGal (galactose) or YPD (dextrose) plates and incubated for 3 days. (B) Suppression of NC2 depletion by Δiwr1 was not due to a defective repression of the P_GAL10-YDR1 and P_GAL10-BUR6 in dextrose. The total protein extracts either obtained from cells exponentially growing in galactose (Gal) or transferred to dextrose for 5 hours (Dext) were analyzed on 8% SDS-PAGE gel. Bur6-HA and Ydr1-HA were detected with mouse monoclonal anti-HA. Ponceau staining of the Western blot is shown as a loading control (C). Effect of the Δiwr1 mutation in the basal and induced levels of the GAL10 gene. Total RNA was prepared from the wild-type or the Δiwr1 mutant strain growing in glucose and after incubation for the indicated times (minutes) in galactose containing medium. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining. (D) Deletion of the IWR1 gene bypasses the requirement of Bur6p. A deletion of IWR1 was introduced in a strain carrying a chromosomal deletion of BUR6 and the wild-type BUR6 gene on an URA3/CEN plasmid. The resulting strain (Δiwr1) and the parental strain (IWR1) were streaked onto a 5'-FOA plate and incubated for 5 days at 30°C.

Figure 2. Deletion of the IWR1 gene alleviates the transcriptional defects caused by mutations in NC2 components. (A) Exponentially growing cells in YPD medium were washed and transferred to YPGal, and were incubated at 30°C for 4 hours. (B) Yeast cells were grown in YPD medium at the early exponential phase. Total RNA was prepared from the indicated strains and analyzed by Northern blot. The RNA pol III transcript SCR1 was used as a loading control.

Figure 3. Iwr1p activates transcription when artificially bound to a yeast promoter. The full-length Iwr1p fused to either the LexA DNA binding domain (LexA-IWR1) or LexA alone (LexA) were expressed in the TAT-7 strain. Transcriptional activation was detected by growth on synthetic complete media lacking histidine and containing the indicated amounts of 3-aminotriazol after 3 days at 30°C (A), or the expression of a P_GAL1-lacZ reporter gene carrying upstream LexA binding sites (B).

Figure 4. Iwr1p-GFP shuttles between the nucleus and cytoplasm in a Xpo1p-dependent manner. (A) Subcellular localization of Iwr1p-GFP. Δiwr1::HIS3 mutant cells expressing a Iwr1p-GFP fusion (see "Experimental Procedures") were grown at the mid-exponential phase in synthetic complete (SC) medium with dextrose as the carbon source at 30°C. (B) xpo1-1 mutant cells expressing Iwr1p-GFP were grown at the mid-exponential phase at 25°C and were heat shocked at 37°C for 30 minutes. (C) Nuclear accumulation of a ΔNES-Iwr1p-GFP. Δiwr1::HIS3 mutant cells expressing Iwr1p-GFP or
Iwr1p-(ΔNES)-GFP (see "Experimental Procedures") were grown in SC medium with dextrose as the carbon source at the mid-exponential phase.

**Figure 5.** Increased basal transcription of the *ARG1* and *ATP16* genes in the Δiwr1 mutant. (A) Yeast strains were cultured in SC medium lacking Ile and Val, and were treated, (+) or not (-), with sulfometuron (SM) for 2 hours to induce Gcn4p synthesis by starvation of Ile/Val, and were then subjected to Northern blot analysis. Two different exposures of the film (long and short) are shown. (B) Yeast strains were grown overnight in YPD (D) or YPEthanol (E). Total RNA was prepared from the indicated strains, and analyzed by Northern blot. The RNA pol III transcript SCR1 was used as a loading control.

**Figure 6.** Defective induction of the *SUC2* and *PHO* genes in the Δiwr1 mutant. (A) and (B) Yeast strains were grown in YPD (2% dextrose) at the early exponential phase, and were then washed and transferred to YPD containing 0.05% dextrose for the indicated times (in minutes). In (B), two different exposures (long and short) of the film are shown. (C) Yeast strains were grown in Pi-depleted medium containing the indicated amounts of KH$_2$PO$_4$. (D) Yeast cells were grown in Pi-depleted medium either without Pi (low phosphate) or containing 13.4 mM of KH$_2$PO$_4$. (E) Yeast cells were grown in YPD at pH 6.4 (n.i) and exposed for the indicated times (in minutes) to pH 7.6. Total RNA was prepared from the indicated strains and analyzed by Northern blot. The RNA pol III transcript SCR1 was used as a loading control.

**Figure 7.** A different requirement of Iwr1p depending on the *PHO5* inducing conditions. (A) Yeast cells were grown in Pi-depleted medium containing 13.4 mM KH$_2$PO$_4$ at the early exponential phase (0 h), and were then washed and transferred to Pi-depleted or Pi-free medium without added Pi. (B) Yeast cells were grown in Pi-depleted medium containing 13.4 mM KH$_2$PO$_4$ to the early exponential phase. (C) The wild-type or Δiwr1 mutant cells transformed with YEplac181 (vector) or YEplac181 carrying the *PHO4* gene (PHO4) were grown in Pi-depleted medium containing 13.4 mM KH$_2$PO$_4$ at the early exponential phase (0 h). They were then washed and transferred to the Pi-depleted medium. Acid phosphatase activity was determined from three independent cultures at the indicated times (in hours).

**Figure 8.** Deletion of Iwr1p delays the chromatin opening and the recruitment of Pho4p and Rpb1p to the *PHO5* promoter. (A) Scheme of the nucleosomal representation of the *PHO5* promoter under repressing conditions (modified from (61)). Chromatin remodeling upon induction makes a ClaI restriction site on nucleosome -2 accessible. (B) Nuclei from the indicated strains grown under repressive conditions (high Pi) or induced in the Pi-deplete medium for 4 h (low Pi) were digested with 100 units of ClaI. The accessibility of the ClaI site was analyzed according to (50). The ClaI cut is denoted by the appearance of the lower band (arrow). (C) ChIP analysis of the Pho4p levels over the *PHO5* UAS region, using a Pho4-HA tagged protein. Yeast cells were grown in Pi-depleted medium containing 13.4 mM KH$_2$PO$_4$ and were transferred to Pi-depleted medium for 4 h. The amount of immunoprecipitation was determined by quantitative PCR. Relative occupancy was determined by the IP ratio in relation to the input divided by the ratio of the IP control (intergenic region) in relation to the input control. (D) ChIP analysis of the Rpb1p levels over the *PHO5* TATA region was performed as described above. All histograms shown present the mean and standard deviation of three independent experiments.

**Figure 9.** Deletion of *HTZ1* partially suppresses the defect in *SUC2* induction shown by the Δiwr1 mutant strain. (A) Synthetic lethality between Δiwr1 and Δhtz1 mutations. The indicated strains carrying the *IWR1* gene in a URA3/CEN plasmids were streaked in SC-ura and 5'-FOA plates and incubated for 3 days at 30ºC. (B) Wild-type and mutant strains were spotted onto YPD plates or YPD.
plates supplemented with 10 mg/L of doxycycline and incubated for 3 days at 30ºC. (C) Yeast strains were grown in YPD (2% dextrose) at the early exponential phase, and were transferred to YPD (2% dextrose) containing 10 mg/L of doxycycline and incubated for 4 hours at 30ºC. Then, cells were washed and transferred to YPD (0.05% dextrose) containing 10 mg/L of doxycycline and incubated for the indicated times (in minutes). Total RNA was prepared from the indicated strains and analyzed by Northern blot. The RNA pol III transcript SCR1 was used as a loading control.
FIGURES

Figure 1
Figure 2
Figure 3

A

B

Graph showing $\alpha$-galactosidase activity with 0 mM AT, 5 mM AT, and 10 mM AT conditions.
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
The yeast RNA pol II-associated factor Iwr1p is involved in the basal and regulated transcription of specific genes
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