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Genetics

The *ORD1* gene encodes a transcription factor involved in oxygen regulation and is identical to *IXR1*, a gene that confers cisplatin sensitivity to *Saccharomyces cerevisiae*

(*COX5b*/hypoxic genes/aerobic repression/high mobility group box)

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ABSTRACT **The yeast *COX5a* and *COX5b* genes encode isoforms of subunit Va of the mitochondrial inner membrane protein complex cytochrome *c* oxidase. These genes have been shown to be inversely regulated at the level of transcription by oxygen, which functions through the metabolic coeffector heme. In earlier studies we identified several regulatory elements that control transcriptional activation and aerobic repression of one of these genes, *COX5b*. Here, we report the isolation of trans-acting mutants that are defective in the aerobic repression of *COX5b* transcription. The mutants fall into two complementation groups. One group specifies *ROX1*, which encodes a product reported to be involved in transcriptional repression. The other group identified the gene we have designated *ORD1*. Mutations in *ORD1* cause overexpression of *COX5b* aerobically but do not affect the expression of the hypoxic genes *CYC7*, *HEM13*, and *ANB1*. *ORD1* mutations also do not affect the expression of the aerobic genes *COX5a*, *CYC1*, *ROX1*, *ROX3*, and *TIF51A*. The yeast genome contains a single *ORD1* gene that resides on chromosome XI. Strains carrying chromosomal deletions of the *ORD1* locus are viable and exhibit phenotypes similar to, but less severe than, that of the original mutant. The nucleotide sequence of *ORD1* revealed that it is identical to *IXR1*, a yeast gene whose product contains two high mobility group boxes, binds to platinated DNA, and confers sensitivity to the antitumor drug cisplatin. Consistent with the latter observations, we found that the ORD1 product could bind to both the upstream region of *COX5b* and to DNA modified with cisplatin.**

Cytochrome *c* oxidase is a heterooligomeric protein complex located in the mitochondrial inner membrane. In the yeast *Saccharomyces cerevisiae*, the cytochrome oxidase complex is composed of 12 nonidentical subunits (1). The genes *COX5a* and *COX5b* specify functionally interchangeable, yet structurally distinct, forms of subunit V of the yeast enzyme (refs. 2, 3; referred to as subunits Va and Vb, respectively). Previous work has shown that the yeast *COX5* genes are inversely regulated at the level of transcription by oxygen, which exerts its effect through a metabolic coeffector, heme (4–7). Specifically, during aerobic growth the presence of oxygen permits heme biosynthesis; heme then interacts with one or more regulatory proteins to activate the transcription of *COX5a* and repress the transcription of *COX5b* (4–7). Under anaerobic or hypoxic conditions (low oxygen tension), when heme is limiting, *COX5a* is not transcribed, whereas transcription of *COX5b* derepresses 5- to 20-fold (4, 5). It has become clear that the *COX5* genes are part of a family of physiologically important yeast genes whose expression is controlled either positively or negatively by oxygen and/or heme. These genes share several common features: most function in oxygen-dependent processes (respiration, sterol synthesis, oxidative damage repair), and several, like the *COX5* genes, exist as pairs inversely regulated by oxygen and heme (8, 9).

Several upstream elements that regulate the expression of the *COX5b* gene have been identified (5). These include two sites of positive control (activation elements or UASs) and three sites of negative control (repression elements or URSs) that mediate aerobic repression. Two of the repression elements contain the consensus sequence ATTGTTCT, which is found upstream of most hypoxic genes and appears to be the binding site for the *ROX1* repressor protein (8, 10). The third repression element contains a 13-bp sequence TCGTTCGTTGCCT, which is also found upstream of several hypoxic genes (5, 10).

In this study we searched for trans-acting factors that are involved in the aerobic repression of *COX5b*. Using a genetic approach, we isolated mutants in which *COX5b* was overexpressed under aerobic growth conditions. The mutants fell into two complementation groups. (*i*) The first corresponds to the previously identified *ROX1* gene. (*ii*) The second defines another gene, which we call *ORD1*. The *ORD1* gene was cloned, and its DNA sequence was determined; it specifies a polypeptide of 67.2 kDa that contains two high mobility group-boxes and several polyglutamine tracts. Surprisingly, we show that *ORD1* is identical to a recently described gene, *IXR1*, which has been shown to confer sensitivity to the antitumor drug cisplatin (11).†

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. The *Saccharomyces cerevisiae* strains constructed in this study were as follows: JM43-GD5a/ord1 (*MATα his4-580 trp1-289 leu2-3, 112 ura3-52 cox5aΔ::URA3 ord1Δ::LEU2*) and JL1-20c (*MATα his4-580 leu2-3, 112 ura3-52 cox5aΔ::hisG ord1-1*). Additional strains used included JM43, JM43-GD5a, JM43-GD5ab, JM6, BMH281, CT39-7B, and CT149-3D, which have been described (2, 3, 6, 7, 12). Yeast strains were grown aerobically at 30°C in either yeast extract/peptone/dextrose (YPD), yeast extract/peptone/glycerol/ethanol (YPGE), or SD medium (supplemented with amino acids as necessary; ref. 13). Respiratory proficiency was tested with YPGE medium, which is nonfermentable.

Genetic Methods and the Isolation of Mutants That Overexpress *COX5b* Aerobically. Strain construction, matings, sporulation, and tetrad analysis were done by using standard yeast genetic techniques (13). Mutants overexpressing

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Abbreviations: UAS, upstream activation site; URS, upstream repression site; HMG, high mobility group.

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†The sequence reported in this paper was previously deposited in the GenBank data base (under *IXR1*; accession no. L16900).

COX5b aerobically were selected by the ability of yeast strain JM43-GD5ab(YCp5b) to grow on YPGE plates (see *Results*).

Approximately 100 spontaneous mutants were selected; these displayed a wide variety of growth rates on YPGE. Mutants displaying the best growth rates on YPGE were subjected to a cis/trans test that consisted of curing the mutant of its plasmid and then retransforming the plasmid-free isolate with a fresh preparation of vector YCp5b. Those mutants retaining the ability to grow on YPGE medium after retransformation were considered to carry trans-acting, chromosomal mutations. Trans-acting mutants were then mated to yeast strain BMH281 to determine whether the mutations they carried were dominant or recessive. Recessive mutations are complemented in the diploid, which results in a respiratory-deficient phenotype observed as the lack of growth on YPGE medium. Five recessive, trans-acting mutants were ultimately chosen for further study. For complementation analysis, appropriately marked derivatives of each mutant were constructed through standard genetic techniques. The complementation test consisted of mating each mutant in pairwise combinations and scoring the diploids for growth on YPGE medium. Mutants that produced diploids capable of growth on this medium were considered to be in the same complementation group.

Cloning the *ORD1* Structural Gene. The *ORD1* gene was cloned by functional complementation of the *ord1-1*-encoded phenotype (in JL1-20c) using a yeast genomic library in the vector YCp50 (14). Ura^+ transformants were first selected on SD (−Ura) medium and then replica-plated onto YPGE medium. Transformants that had lost respiratory competence were screened further by crossing them to the rho^o tester strain JM6 to eliminate strains harboring lesions in mitochondrial DNA. Several rounds of screening yielded a stable rho^+ transformant that failed to grow on YPGE medium. Plasmid DNA was prepared from this strain, amplified in *Escherichia coli* and then used to retransform strain JL1-20c to uracil prototrophy. These transformants failed to grow when patched on YPGE medium.

To delete *ORD1* at its chromosomal locus, we first removed a 1.8-kb *Pst* I–*Eco*RI restriction fragment (a partial digestion product) from the *ORD1* gene. This fragment was replaced with a 2.2-kb *Xho* I–*Sal* I fragment containing the yeast *LEU2* gene. The resulting plasmid (pOrd1ΔPE1.8) was cut with *Bam*HI and *Bgl* II and then transformed into JM43-GD5a. Leu^+ transformants were screened by genomic Southern blot analysis to confirm that the proper integration/replacement had occurred.

DNA Binding Analysis. Binding of Ord1p to *COX5b* DNA was studied by using electrophoretic mobility-shift assays and the probe URS_{5b}(B-S). This probe is a 44-bp *Bam*HI–*Sca* I restriction fragment containing a portion of URS_{5b}, an upstream region of *COX5b* previously shown to mediate the aerobic repression of transcription (5). Ord1p was generated by *in vitro* transcription/translation of the *ORD1* gene (an *Eco*RV fragment) in pBluescript KS(−) using T7 RNA polymerase and rabbit reticulocyte lysate (Amersham).

Miscellaneous Methods. Bacterial DNA preparations, recombinant DNA methods, *E. coli* transformations, and DNA sequence analysis were accomplished by standard techniques (15). Procedures for yeast transformations, nucleic acid preparations, Northern blotting, and Southern blotting have been described (4, 5).

RESULTS

Isolation of Mutants That Overexpress *COX5b* under Aerobic Conditions and Identification of the *ORD1* Gene. Mutants overexpressing *COX5b* aerobically were isolated using the strain JM43-GD5ab which had been transformed with the centromeric plasmid YCp5b. JM43-GD5ab carries chromosomal disruptions of both subunit V genes; because a form of subunit V is essential for cytochrome oxidase activity, which is, in turn, essential for respiration, this strain cannot grow on nonfermentable substrates (4). JM43-GD5ab strains expressing *COX5b* from centromeric or low-copy plasmids, like YCp5b, also fail to grow under these conditions due to the low level of aerobic *COX5b* expression (4). Thus, these strains must overexpress *COX5b* to grow on nonfermentable substrates.

Spontaneous mutants were identified by their ability to grow on YPGE medium. Initially, >100 prospective mutants were characterized. From these, five strains carrying recessive, trans-acting mutations were chosen for further study. Each of these mutants was stable, grew rapidly on YPGE medium, and, from the results of Northern blot analysis, had elevated levels of COX5b mRNA when grown aerobically (data not shown). Each mutant was also analyzed by Southern blotting, which confirmed that centromere function on the plasmid was normal and that the *COX5b* gene had not been amplified (data not shown).

Appropriately marked derivatives of each mutant were mated in pairwise combinations to test for complementation, which was defined as the failure of the resulting diploid strain to grow on YPGE medium. Two complementation groups were identified. Because yeast strains with mutations in either the *ROX1*, *ROX3*, *ROX4* (also called *TUP1* and *AER2*), or the *REO1* genes might also be expected to cause overexpression of *COX5b* (4, 6, 8, 12), we tested whether either of the two complementation groups specified those genes. Centromeric plasmids carrying each respective *ROX* gene were separately transformed into each mutant; transformants were then tested for growth on YPGE medium. Both mutants from complementation group 1 retained the ability to grow on YPGE when transformed with each *ROX* gene, suggesting that complementation had not occurred and that the corresponding mutations were in a gene distinct from either *ROX1*, *ROX3*, or *ROX4*. The defect in each of the three mutants in complementation group 2 was not complemented by either *ROX3* or

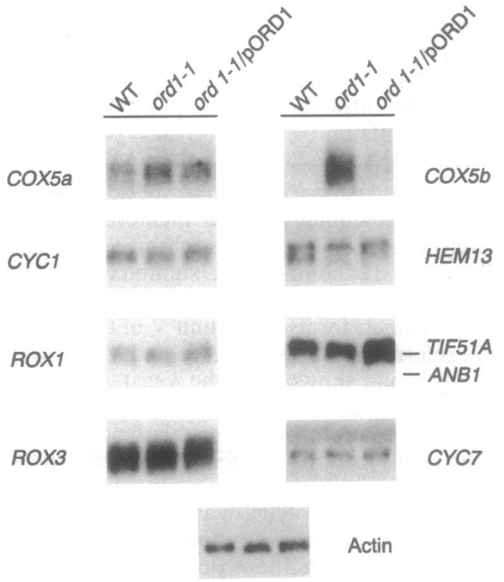


FIG. 1. *ORD1* is a specific regulator of *COX5b*. Total mRNA was prepared from the wild-type strain JM43 (WT), from the *ord1-1* strain JL1-20c (*ord1-1*), or from strain JL1-20c transformed with the *ORD1* plasmid YCpORD(7.5) (ord1-1/pORD1). Thirty micrograms of each RNA was then analyzed by Northern blot hybridization with radiolabeled oligonucleotide probes specific for the genes indicated at left and at right. The same blot was used, after stripping the probe, in each case. The actin probe was used as a control for loading. No ANB1 transcript is observed because the gene is not expressed during aerobic growth.

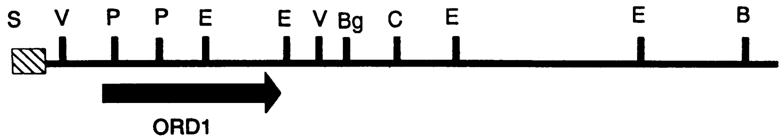
ROX4, but all were complemented by *ROX1*. Thus, this complementation group specifies *ROX1* (this assignment was also confirmed by genetic crosses). To test for allelism with *REO1* (this gene has not been isolated), we crossed each mutant strain to the *reo1* strain CT39-7B (this strain carries a disruption of *COX5a*; the *reo1* mutation causes overexpression of *COX5b*, refs. 6, 12). Diploid strains resulting from a cross between the *reo1* strain and both mutants of complementation group 1 failed to grow on YPGE. Thus, group 1 defines another gene that we have named *ORD1* (for *o*xygen/oxidase *r*egulation *d*efective). Interestingly, complementation was not observed in diploids resulting from crosses between the *reo1* strain and the group 2 (*ROX1*) mutants, suggesting that *REO1* might be identical to *ROX1*. To test this, we transformed the *ROX1* plasmid into the *reo1* strain CT149-3D. We found that the resulting transformants lost the ability to grow on YPGE medium, indicating that the *ROX1* gene complemented the *reo1* mutation. Together, the latter two results strongly suggest that *REO1* and *ROX1* are the same gene.

***ORD1* Is a Specific Regulator of *COX5b*.** Because the upstream elements responsible for aerobic repression of *COX5b* transcription are also found upstream of other hypoxic genes (5, 8), we were interested in determining whether *ord1* mutations (like *rox1* mutations) were pleiotropic. RNA was prepared from the *ord1* strain JL1-20c (the mutant allele is referred to as *ord1-1*) that had been grown aerobically. We then performed a series of Northern blot hybridizations with probes specific for several genes. Confirming what we had observed previously, the *COX5b* transcripts were dramatically overexpressed under aerobic growth conditions in the *ord1-1* strain (Fig. 1). However, expression of the hypoxic genes *ANB1*, *HEM13*, and *CYC7* appeared normal in the mutant, as did the expression of the aerobic genes *COX5a*, *CYC1*, *ROX1*, *ROX3*, and *TIF51A* (Fig. 1, WT and ord1-1).

Yeast strains carrying mutations in *ROX1* overexpress *COX5b* aerobically because the Rox1 repressor is either not made or is not functional (10, 16). Therefore, a possible explanation of the *ord1* phenotype is that the *ORD1* product functions as a positive regulator of *ROX1*. The results of Fig. 1 rule out this possibility because the expression of *ROX1* and *ANB1*, another gene regulated by the *ROX1* product, was normal in the *ord1-1* genetic background. When taken together, the combined results of Fig. 1 suggest that *ORD1* is not a general hypoxic regulator or a regulator of the *ROX1* gene. Thus, with respect to the tested yeast genes known to be regulated by oxygen, *ORD1* appears to be a specific regulator of *COX5b*.

Cloning, Disruption, and DNA Sequence of the *ORD1* Gene. A plasmid carrying the *ORD1* gene was isolated from a YCp50 yeast genomic DNA library by transformation/functional complementation of the *ord1-1* defect. The plasmid YCpOrd1(7.5) contained a 7.5-kb yeast DNA insert. The

A



B

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                                                                        5'  TTCTATCCATTCTGTGATATA CGTACGACGCTAACAGTACCCACAACTGCA   51
     ATGAACACCGGTATCTCGCCCAAACAGGAC GACGCCTCTAACTCCAACTTACTCAACATA GGCCAAGACCATTCGCTGCAGTATCAGGGT CTCGAACACAACGACTCCCAGTACAGAGAT  171
  1  M  N  T  G  I  S  P  K  Q  D  D  A  S  N  S  N  L  L  N  I  G  Q  D  H  S  L  Q  Y  Q  G  L  E  H  N  D  S  Q  Y  R  D

     GCTTCTCACCAGACACCTCACCAATACTTG AACCAGTTTCAAGCCCAGCCTCAACAACAA CAACAGCAGCAGCAGCAGCAACAACAGCAA CAACAACAAGCGCCTTATCAAGGTCACTTC  291
 41  A  S  H  Q  T  P  H  Q  Y  L  N  Q  F  Q  A  Q  P  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  A  P  Y  Q  G  H  F

     CAGCAGTCGCCTCAACAACAACAGCAAAAT GTTTATTATCCACTACCTCCACAATCTTTG ACGCAACCTACTTCGCAGTCGCAACAACAA CAACAGCAATATGCTAATTCGAACTCAAAT  411
 81  Q  Q  S  P  Q  Q  Q  Q  Q  N  V  Y  Y  P  L  P  P  Q  S  L  T  Q  P  T  S  Q  S  Q  Q  Q  Q  Q  Q  Y  A  N  S  N  S  N

     TCAAACAACAATGTTAATGTTAACGCGCTA CCTCAGGATTTCGGTTACATGCAACAAACC GGATCGGGCCAAAACTATCCGACGATCAAT CAACAACAATTTTCCGAGTTTTACAACTCC  531
121  S  N  N  N  V  N  V  N  A  L  P  Q  D  F  G  Y  M  Q  Q  T  G  S  G  Q  N  Y  P  T  I  N  Q  Q  Q  F  S  E  F  Y  N  S

     TTTTTAAGTCATTTAACTCAAAAACAGACA AACCCTTCTGTCACGGGTACAGGCGCGTCT AGTAACAACAACAGTAACAACAACAATGTT AGTAGCGGCAATAACAGCACTAGCAGTAAT  651
161  F  L  S  H  L  T  Q  K  Q  T  N  P  S  V  T  G  T  G  A  S  S  N  N  N  S  N  N  N  N  V  S  S  G  N  N  S  T  S  S  N

     CCTGCCCAGCTGGCAGCCTCCCAATTAAAC CCTGCCACGGCTGCTGCGGCCGCCGCAAAC AATGCTGCTGGCCCGGCTTCGTACTTGTCT CAGCTCCCACAGGTGCAGAGATACTACCCG  771
201  P  A  Q  L  A  A  S  Q  L  N  P  A  T  A  A  A  A  A  A  N  N  A  A  G  P  A  S  Y  L  S  Q  L  P  Q  V  Q  R  Y  Y  P

     AACAACATGAACGCTCTGTCTAGTCTTTTG GACCCTTCCTCTGCAGGAAATGCTGCAGGA AATGCCAACACCGCTACTCATCCTGGTTTG TTACCACCCAATCTGCAACCTCAATTGACT  891
241  N  N  M  N  A  L  S  S  L  L  D  P  S  S  A  G  N  A  A  G  N  A  N  T  A  T  H  P  G  L  L  P  P  N  L  Q  P  Q  L  T

     CACCACCAGCAGCAGATGCAGCAACAGCTG CAATTACAACAACAACAGCAGTTGCAGCAA CAGCAGCAGCTACAACAGCAACACCAGTTG CAACAACAACAACAACTTCAACAACAACAT 1011
281  H  H  Q  Q  Q  M  Q  Q  Q  L  Q  L  Q  Q  Q  Q  Q  L  Q  Q  Q  Q  Q  L  Q  Q  Q  H  Q  L  Q  Q  Q  Q  Q  L  Q  Q  Q  H

     CATCATCTACAACAGCAACAGCAGCAACAA CAGCATCCAGTGGTGAAGAAATTATCTTCC ACTCAAAGCAGAATTGAGAGAAGAAAACAA CTGAAAAAGCAAGGCCCAAAGAGACCTTCT 1131
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     ACTTACAAGAAAAGATTAAAGGAATGGGAA AGTTGTTATCCCGACGAAAATGATCCAAAC GGTAACCCAACCGGTCACTCACATAAGGCC ATGAACATGAATTTGAATATGGACACTAAA 1611
481  T  Y  K  K  R  L  K  E  W  E  S  C  Y  P  D  E  N  D  P  N  G  N  P  T  G  H  S  H  K  A  M  N  M  N  L  N  M  D  T  K

     ATCATGGAGAACCAAGACAGTATCGAGCAC ATAACCGCAAATGCCATCGACTCAGTTACC GGAAGCAACAGTAACAGTACCAACCCAAAT ACGCCCGTTTCTCCTCCGATTTCATTACAG 1731
521  I  M  E  N  Q  D  S  I  E  H  I  T  A  N  A  I  D  S  V  T  G  S  N  S  N  S  T  N  P  N  T  P  V  S  P  P  I  S  L  Q

     CAGCAGCCGCTCCAACAACAACAACAACAG CAGCAACAACAACAACACATGTTATTGGCT GACCCCACTACAAATGGTTCGATCATAAAA AATGAATAACAAATAAACAACTTTAGTTTT 1851
561  Q  Q  P  L  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  H  M  L  L  A  D  P  T  T  N  G  S  I  I  K  N  E  ●

     CCACTGTAACATTATCCCACGCAAACAACG AGAATAAGGAATTCCTTTTTCAACATTTGT TTTATATTGTACTACTCTATTTCCTATTAC TACAAATTTTACTTTATTTA  3'       1961
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FIG. 2. Restriction map and DNA sequence of the *ORD1* gene. (*A*) The 7.5-kb *ord1*-complementing plasmid YCpORD1(7.5) was isolated from a yeast genomic DNA library in the vector YCp50 as described. The complementing region was localized on the plasmid by subcloning fragments into YCp50 and retransforming them into strain JL1-20c. Position of the *ORD1* gene is indicated by the dark arrow. The region shown in the cross-hatched box corresponds to *E. coli* DNA. S, *Sal* I, V, *Eco*RV, P, *Pst* I, E, *Eco*RI, Bg, *Bgl* II, C, *Cla* I, and B, *Bam*HI. (*B*) DNA and predicted amino acid sequence of the *ORD1* gene. Positions of landmark restriction sites and polyglutamine tracts are indicated within the amino acid sequence in boldface type, whereas the position of the two HMG-boxes are underlined. The amino acid sequence differs at a few positions from the *IXR1* sequence recently reported by Brown *et al.* (11), but the two genes appear structurally and functionally identical.

complementing region on the insert was localized to within 4.3 kb (3.8 kb of yeast DNA) by subcloning, transformation, and complementation of the original mutation (Fig. 2*A*).

Evidence that the complementing region contained the *ORD1* gene was first obtained by showing that *ord1* strains transformed with the complementing region no longer overexpressed the COX5b mRNA (Fig. 1, lane 3 of the *COX5b* data). Importantly, the complementing plasmid had no effect on expression of the other genes studied in the experiment of Fig. 1 (Fig. 1, lanes 3), demonstrating further that *ORD1* is a specific regulator of *COX5b*. We next mapped the complementing DNA to the chromosomal *ORD1* locus. The plasmid YIpOrd1SC (which contains the 4.3-kb complementing region in the *URA3* vector YIp5) was constructed, linearized, and transformed into the *ORD1/ura3* strain BMH281. A transformant containing the entire plasmid integrated at the homologous chromosomal (*ORD1*) locus was obtained after screening Ura$^+$ clones by Southern blot analysis. The Ura$^+$/Ord$^+$ transformant was then mated to the *ord1/ura3* strain JL1-20c. Diploids resulting from this cross were sporulated and subjected to tetrad analysis. In all 25 tetrads dissected we observed 2:2 segregation for (*ORD1 URA3*) and (*ord1 ura3*), indicating tight genetic linkage between the *ORD1* locus and the complementing DNA.

A series of Southern blot experiments using genomic DNA prepared from the wild-type yeast strain JM43 was used to confirm that the cloned *ORD1* DNA was colinear with the genomic locus, and second, that ORD1 corresponded to a single nuclear gene (data not shown). Hybridization of an *ORD1*-specific probe to a blot of fractionated yeast chromosomes (17) then allowed us to tentatively assign the *ORD1* locus to chromosome XI. The latter conclusion was confirmed by stripping the blot and rehybridizing it with a probe specific for chromosome XI (18).

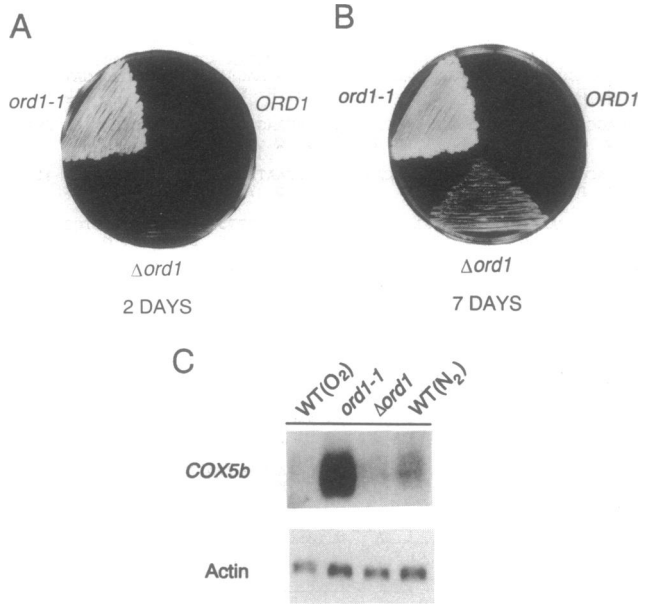

FIG. 3. Yeast strains carrying the *ord1-1* allele display a different phenotype than those carrying *ord1* deletions. (*A*) Growth of yeast strains JL1-20c (*ord1-1*), JM43-GD5a/ord1 (Δ*ord1*), or JM43-GD5a (*ORD1*) on YPGE medium after a 2-day incubation at 30°C. (*B*) Growth of the same strains on YPGE medium after a 7-day incubation at 30°C. The faster-growing colonies can be seen above the background in the Δ*ord1* section. (*C*) Total mRNA was prepared from the wild-type strain JM43 (WT) grown aerobically (O_2) or anerobically (N_2) and from strains JL1-20c (*ord1-1*) and JM43-GD5a/ord1 (Δ*ord1*). Thirty micrograms of the respective RNA was then analyzed by Northern blot hybridization with probes specific for the yeast *COX5b* or actin genes. The amount of COX5b transcript seen in the Δ*ord1* lane is estimated at two to three times that seen in the WT(O_2) lane. The amount of COX5b transcript seen in the WT(N_2) and ord1-1 lanes are estimated at five times and twenty to thirty times that seen in the WT(O_2) lane, respectively.

A null allele of *ORD1* was constructed in strain JM43-GD5a by gene replacement (13). The resulting strain, JM43-GD5a/ord1, contains a deletion of 1.8 kb from the *ORD1* locus (essentially the entire structural gene). This strain grew as well as the wild type when grown aerobically on dextrose medium, indicating that the *ORD1* gene is not essential for viability. As expected, the null strain also grew on YPGE medium. Surprisingly, however, growth on YPGE medium was two to three times slower than that of the original mutant (Fig. 3 *A* and *B*). Consistent with the growth phenotype, the aerobic level of COX5b mRNA in the null strain was elevated only slightly, ≈2- to 3-fold (Fig. 3*C*, lane 3). In contrast, the aerobic level of COX5b mRNA in the original mutant is at least 20 times that of the wild-type strain (Fig. 3*C*, lanes 1 and 2). An intriguing feature of the slower-growing *ord1* null strains is that they give rise to faster-growing derivatives at a relatively high frequency (Fig. 3*B*). Recent results have shown that the fast-growing colonies have COX5b mRNA levels comparable to those of the *ord1-1* mutant, and that the fast-growing phenotype segregates in genetic crosses with the *ORD1* locus (J.R.L., unpublished results).

Approximately 4 kb of genomic DNA containing the *ORD1*-complementing region was sequenced on both strands. The sequence revealed a 592-amino acid open reading frame, encoding a protein with a deduced molecular mass of 67,225 Da (Fig. 2*B*). Interestingly, the predicted protein (Ord1p) appeared identical to a recently described protein named Ixr1 (11). Ixr1 mediates the sensitivity of yeast to the antitumor drug cisplatin (*cis*-diamminedichloroplatinum[II]), a widely used chemotherapeutic agent known to cause intrastrand DNA cross-links and bending (11, 19, 20). Two structural elements of the Ord1/Irx1 protein are noteworthy. (*i*) The protein contains several unusually long stretches of glutamine residues, a feature common among eukaryotic transcription factors and thought to mediate protein–protein interactions (21). (*ii*) The protein also contains two regions with strong homology to a sequence motif referred to as the high mobility group (HMG)-box (residues 356–424 and 429–497; refs. 19 and 22).

Ord1p Binds to Both *COX5b* and Platinated DNA. The phenotype of *ord1* mutants and the predicted sequence of the ORD1 product suggest that it binds DNA and functions as a factor involved in the aerobic repression of *COX5b* transcription. In addition, Brown *et al.* (11) clearly established that the product of the *IXR1* gene binds to DNA modified with cisplatin (11). As a first step toward analyzing the properties of Ord1p, we tested whether the protein, which was generated by *in vitro* transcription followed by translation in rabbit reticulocyte lysates, bound to platinated DNA. The results of that experiment confirmed that Ord1p bound to the same platinated probe as that used in the *IXR1* study (data not shown). Thus, the products of the *ORD1* and *IXR1* genes are functionally identical.

To study the interaction of Ord1p with *COX5b* DNA, we analyzed the DNA-binding properties of the *in vitro* synthesized product using electrophoretic mobility-shift assays and a 44-bp restriction fragment containing URS_{5b} [referred to as URS_{5b}(B-S)]. This portion of the *COX5b* upstream region was chosen because we had previously shown that it mediated the aerobic repression of *COX5b* transcription (5) and because it contained the 8-bp consensus hypoxic operator as well as the 13-bp repression element (5, 8, 10). Fig. 4 (lane 2) shows that Ord1p formed a distinct complex with the *COX5b* DNA. The complex was specific because (*i*) it was formed in the presence of a large excess (≈200-fold) of nonspecific carrier DNA, and (*ii*) its formation could be reduced significantly (up to 70%) by including an excess (25- to 100-fold) of

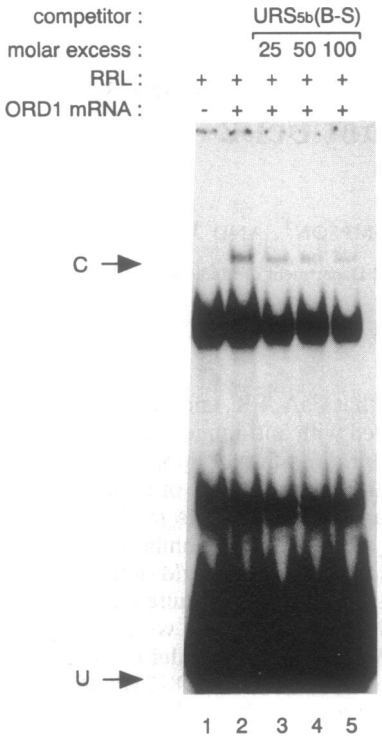

FIG. 4. Ord1p binds to a COX5b URS region. Binding of Ord1p to the radiolabeled COX5b fragment URS_{5b}(B-S) was detected by electrophoretic mobility shift assays. Binding reactions include rabbit reticulocyte lysate (RRL) only or *in vitro*-translated Ord1p (RRL plus ORD1 mRNA). The unlabeled COX_{5b}(B-S) fragment was used at the molar ratios indicated as competitor DNA. Arrows indicate the position of the specific Ord1p-DNA complex (C) and the unbound DNA (U). Reactions contained 0.5 ng of ^{32}P-labeled DNA/4 mM Tris, pH 8.0/4 mM $MgCl_2$/100 mM KCl/12% (vol/vol) glycerol/100 ng of sonicated salmon sperm DNA/1 μl of rabbit reticulocyte lysate (± Ord1p) in a final volume of 15 μl. Mixtures were incubated on ice for 20 min, then analyzed by electrophoresis through a 4.5% polyacrylamide gel at room temperature.

unlabeled specific competitor DNA in the binding reaction (Fig. 4, lanes 3–5). From these results and those described above, we conclude that Ord1p is a DNA-binding protein that can bind to both platinated DNA and a regulatory region of *COX5b* DNA.

DISCUSSION

We describe here the isolation and characterization of mutant strains that fail to properly repress the aerobic transcription of *COX5b*, one of several known hypoxic genes in yeast. The mutants reside in two complementation groups; one specifies *ROX1*, a previously identified repressor of *COX5b* and several other hypoxic genes, whereas the other corresponds to an additional gene, *ORD1*. The observation that the predicted Ord1p/Ixr1 protein contains two HMG-boxes and several extended runs of polyglutamine and our finding that the *in vitro*-translated product also binds DNA strongly suggest that this protein normally functions as a transcription factor in yeast. Whether this protein is involved in controlling the transcription of genes other than *COX5b* remains to be determined. However, it is interesting that Rox1p, a yeast transcription factor involved in *COX5b* regulation, also contains an HMG-box and a polyglutamine tract (16). Recent results have demonstrated that Rox1p is a DNA-binding protein (16) and that Ord1p and Rox1p can bind to the same 44-bp fragment of *COX5b* DNA (Fig. 4; V.W.B. and K. Singh, unpublished results). Yet, it seems likely that Ord1p and Rox1p bind to DNA by functionally different mechanisms. Members of the HMG-box family of proteins can be divided into two general classes based on the nature of their interaction with DNA (19, 22, 23). The first class corresponds to those proteins for which interaction with DNA is structure-specific; they recognize non-B-DNA conformations such as stem-loops, bends, four-way junctions, and cisplatin-modified DNA. These proteins, which often have multiple HMG-boxes, may also facilitate additional bending. In contrast, the second class of HMG-box proteins, exemplified by SRY, exhibit sequence-specific DNA binding (19, 22, 23).

Although the precise means by which Ord1p recognizes *COX5b* DNA has not been established, our current view is that it is a structure-specific DNA-binding protein because it does not appear to recognize either the consensus 8-bp hypoxic operator or the 13-bp repression element (M. R. Hodge, K. Singh, V.W.B., A. Saxena, J.R.L., and M.G.C., unpublished work). In addition, it binds to platinated DNA, which is known to be locally bent (19, 20). Rox1p, on the other hand, is clearly a sequence-specific DNA-binding protein that recognizes the 8-bp consensus operator (16; M. R. Hodge, K. Singh, V.W.B., A. Saxena, J.R.L., and M.G.C., unpublished work). Moreover, Rox1p does not bind platinated DNA (V.W.B., unpublished results, and N. L. Raju and S. J. Lippard, personal communication). We note that these findings, for both Ord1p and Rox1p, are consistent with the notion that Rox1p functions as a general hypoxic regulator, whereas Ord1p acts specifically at *COX5b*.

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1. Taanman, J.-W. & Capaldi, R. A. (1992) *J. Biol. Chem.* **267**, 22481–22485.
2. Cumsky, M. G., Ko, C., Trueblood, C. E. & Poyton, R. O. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2235–2239.
3. Cumsky, M. G., Trueblood, C. E., Ko, C. & Poyton, R. O. (1987) *Mol. Cell. Biol.* **7**, 3511–3519.
4. Hodge, M. R., Kim, G., Singh, K. & Cumsky, M. G. (1989) *Mol. Cell. Biol.* **9**, 1958–1964.
5. Hodge, M. R., Singh, K. S. & Cumsky, M. G. (1990) *Mol. Cell. Biol.* **10**, 5510–5520.
6. Trueblood, C. E., Wright, R. M. & Poyton, R. O. (1988) *Mol. Cell. Biol.* **8**, 4537–4540.
7. Trawick, J. D., Kraut, N., Simon, F. R. & Poyton, R. O. (1992) *Mol. Cell. Biol.* **12**, 2302–2314.
8. Zitomer, R. S. & Lowry, C. V. (1992) *Microbiol. Rev.* **56**, 1–11.
9. Waterland, R. A., Basu, A., Chance, B. & Poyton, R. O. (1991) *J. Biol. Chem.* **266**, 4180–4186.
10. Lowry, C. V., Cerdan, M. E. & Zitomer, R. S. (1990) *Mol. Cell. Biol.* **10**, 5921–5926.
11. Brown, S. J., Kellett, P. J. & Lippard, S. J. (1993) *Science* **261**, 603–605.
12. Trueblood, C. E. & Poyton, R. O. (1988) *Genetics* **120**, 671–680.
13. Rose, M. D., Winston, F. & Hieter, P. (1990) *Methods in Yeast Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
14. Rose, M. D., Novick, P., Thomas, J. H., Botstein, D. & Fink, G. R. (1987) *Gene* **60**, 237–243.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
16. Balasubramanian, B., Lowry, C. V. & Zitomer, R. S. (1993) *Mol. Cell. Biol.* **13**, 6071–6078.
17. Chu, G., Vollrath, D. & Davis, R. W. (1986) *Science* **234**, 1582–1585.
18. Blacketer, M. J., Koehler, C. M., Coats, S. G., Myers, A. M. & Madaule, P. (1993) *Mol. Cell. Biol.* **13**, 5567–5581.
19. Landsman, D. & Bustin, M. (1993) *BioEssays* **15**, 539–546.
20. Bruhn, S. L., Toney, J. H. & Lippard, S. L. (1990) *Prog. Inorg. Chem.* **38**, 477–516.
21. Courey, A. J., Holtzman, D. A., Jackson, S. P. & Tjian, R. (1989) *Cell* **59**, 827–836.
22. Grosschedl, R., Giese, K. & Pagel, J. (1994) *Trends Genet.* **10**, 94–100.
23. Bruhn, S. L., Pil, P. M., Essigmann, J. M., Housman, D. E. & Lippard, S. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2307–2311.