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Regulation of cardiolipin synthase levels in *Saccharomyces cerevisiae*

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Abstract

The *Saccharomyces cerevisiae* cardiolipin (CL) synthase encoded by the *CRD1* gene catalyses the synthesis of CL, which is localized to the inner mitochondrial membrane and plays an important role in mitochondrial function. To investigate how *CRD1* expression is regulated, a *lacZ* reporter gene was placed under control of the *CRD1* promoter and the 5′-untranslated region of its mRNA (P_{CRD1}-*lacZ*). P_{CRD1}-*lacZ* expression was 2.5 times higher in early stationary phase than in logarithmic phase for glucose grown cells. Non-fermentable growth resulted in a two-fold elevation in expression relative to glucose grown cells. A shift from glycerol to glucose rapidly repressed expression, whereas a shift from glucose to glycerol had the opposite effect. The derepression of P_{CRD1}-*lacZ* expression by non-fermentable carbon sources was dependent on mitochondrial respiration. These results support a tight coordination between translation and transcription of the *CRD1* gene, since similar effects by the above factors on *CRD1* mRNA levels have been reported. In glucose-grown cells, P_{CRD1}-*lacZ* expression was repressed 70% in a *pgs1*Δ strain (lacks phosphatidylglycerol and CL) compared with wild-type and *rho*$^{-}$ cells and elevated 2.5-fold in *crd1*Δ cells, which have increased phosphatidylglycerol levels, suggesting a role for phosphatidylglycerol in regulating *CRD1* expression. Addition of inositol to the growth medium had no effect on expression. However, expression was elevated in an *ino4*Δ mutant but not in *ino2*Δ cells, suggesting multiple and separate functions for the inositol-responsive *INO2/INO4* gene products, which normally function as a dimer in regulating gene function.

Keywords

cardiolipin; *Saccharomyces cerevisiae*; mitochondria; *CRD1* gene expression; *β*-galactosidase; inositol; phosphatidylglycerol; diauxic shift

Introduction

Cardiolipin (CL) makes up about 15–20% of mitochondrial phospholipids in eukaryotic cells (Hatch, 1996; Jakovcic *et al.*, 1971). As an anionic phospholipid predominantly found in the mitochondrial inner membrane (Gallet *et al.*, 1997; Hatch, 1996), CL plays an

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essential role in many critical mitochondrial functions, such as solute transport (Battelli *et al.*, 1992; Hoffmann *et al.*, 1994; Mende *et al.*, 1983), protein and phospholipid import (Ardail *et al.*, 1991; Chupin *et al.*, 1995; Eilers *et al.*, 1989; Endo *et al.*, 1989; Shiao *et al.*, 1995), oxidative phosphorylation (Eble *et al.*, 1990; Fry and Green, 1981; Paradies *et al.*, 1997; Petrosillo *et al.*, 2003; Robinson, 1993) and mitochondria-mediated apoptosis (Esposti, 2002; Kirkland *et al.*, 2002; Kriska *et al.*, 2005; McMillin and Dowhan, 2002; Nakagawa, 2004). Much of CL's importance to various mitochondrial processes can be ascribed to its interaction with individual proteins or complexes, which in turn require CL to maintain their structural or functional integrity. For instance, CL is an integral part of the structure of complexes III and IV of the mitochondrial electron transport chain in yeast (Lange *et al.*, 2001) and is required to form a supermolecular complex between these individual complexes that is associated with more efficient growth on non-fermentable carbon sources (Zhang *et al.*, 2002).

In eukaryotes, CL is synthesized from CDP-diacylglycerol (CDP-DAG) through three sequential reactions catalysed by phosphatidylglycerol-P (PG-P) synthase (*PGS1* gene product), PG-P phosphatase and CL synthase (*CRD1* gene product) in the mitochondrial inner membrane; the above nuclear-encoded genes have been characterized (Chang *et al.*, 1998a,b; Jiang *et al.*, 1997; Tuller *et al.*, 1998). Disruption of the *PGS1* gene results in lack of PG and CL as well as growth only on fermentable but not on non-fermentable carbon sources (Chang *et al.*, 1998a), confirming an essential role of PG and/or CL for mitochondrial respiratory function. Compared to *pgs1*Δ cells, null mutants bearing a disruption of the *CRD1* gene have no detectable CL synthesis and are viable on both fermentable and non-fermentable carbon sources, although with a reduced efficiency when using the latter as a carbon source (Chang *et al.*, 1998b; Jiang *et al.*, 1997; Zhang *et al.*, 2002).

Despite the apparent importance of anionic phospholipids in mitochondrial function, regulation of CL synthesis in mitochondrial membranes in response to changes in environment or by factors or mutations affecting mitochondrial functions or general phospholipid biosynthesis has not been completely characterized. Generally, mitochondrial phospholipid biosynthetic activity, as indicated by early genetic and biochemical studies, is subject to regulation by factors affecting mitochondrial development, such as carbon source, growth phase, oxygen, and mutations in mitochondrial DNA (Gaynor *et al.*, 1991), in addition to cross-pathway control by inositol and choline (Greenberg *et al.*, 1988; McGraw and Henry, 1989). Yeast cells grown on non-fermentable carbon sources, entering stationary phase or during aerobic growth have more developed mitochondria and thereby relatively higher CL content in their mitochondrial membranes (Gallet *et al.*, 1997; Gohil *et al.*, 2004; Jakovcic *et al.*, 1971), than cells in early log phase grown on glucose. PG-P synthase activity, the committed step in CL biosynthesis, is regulated in a similar manner by the above factors (Gaynor *et al.*, 1991; Shen and Dowhan, 1998) as well as by inositol or reduced CDP-DAG levels (Shen and Dowhan, 1998). Regulation of PG-P synthase activity by inositol is unique compared with other phospholipid biosynthetic enzymes because an increase in inositol in the media results in a rapid decrease in PG-P synthase activity (Greenberg *et al.*, 1988), which was too fast to be ascribed to repression only in gene

expression and was later found due to inactivation of PG-P synthase by phosphorylation (He and Greenberg, 2004). The PG-P phosphatase does not appear to respond to any of the above regulatory factors (Kelly and Greenberg, 1990). The activity of CL synthase, the final step of the CL synthetic pathway, also appears not to be affected by inositol (Tamai and Greenberg, 1990) but is dependent on mitochondrial respiratory chain function (Gohil *et al.*, 2004) and is regulated by mitochondrial development (Jiang *et al.*, 1999).

In this report, the regulation of *CRD1* expression by factors affecting mitochondrial development, by inositol and by lesions in other structural genes necessary for CL synthesis, was extended to the translation of gene product. The latter was upregulated with increased PG levels and coordinately regulated with *PGS1* gene expression by growth phase, carbon source and mitochondrial respiratory competence, but not by inositol or by the capacity of cells to synthesize CDP-DAG. The magnitude of effects on formation of protein product were in agreement with previously reported effects on mRNA levels, supporting a tight coupling between mRNA levels and final gene product. In addition, evidence is presented for separate and multiple functions of the *INO2* and *INO4* gene products, normally associated with coordinate regulation of gene expression in response to inositol, in regulating *CRD1* expression.

Materials and methods

Materials

All chemicals were reagent grade or better. *o*-Nitrophenyl *β*-D-galactopyranoside (ONPG) was purchased from Sigma. Restriction endonucleases were from Promega. Oligonucleotides were commercially prepared by Genosys Biotechnologies. Polymerase chain reaction (PCR) SuperMix, TRIZOL reagent and 5′ RACE system kit were from GibcoBRL. The TOPO™ TA cloning kit was purchased from Invitrogen. Growth media were products of Bio 101 Inc. Yeast nitrogen base without amino acids was from Difco Laboratories. The BCA kit was from Pierce.

Strains, media and growth conditions

Yeast strains used in this study are listed in Table 1. Cells were pre-cultured at 30 °C in a small volume of complete synthetic medium (unless noted otherwise) (Janitor and Subik, 1993) containing either 2% glucose, 2% galactose, 2% sodium lactate or 3% glycerol, with 0.95% ethanol as the carbon source, for 1 or 2 overnights. Aliquots of the overnight cultures were inoculated into 50 ml of the same medium for continued growth, monitored by absorbance at OD_{600}. Cells were harvested by centrifugation at the indicated time period or desired OD_{600}. Where indicated, 10 μm or 70 μM inositol, with or without 1 mM choline, was added to growth media.

Mapping of the *CRD1* gene transcriptional initiation site

The transcriptional initiation site of the *CRD1* gene was determined using the method of rapid amplification of cDNA 5′ ends (5′ RACE system) (Frohman *et al.*, 1988). Two gene-specific primers, GSP1 (5′-ATCCATAAAATCAGTGATGCT-3′) and GSP2 (5′-AAACAAACCTAATGCTGGGG TCAA-3′), were utilized in this assay. Total RNA was

purified with TRIZOL reagent from yeast cells (Chomczynski, 1993). First strand cDNA was synthesized from total RNA, using the gene-specific primer GSP1. RNase-treated template RNA was used as a control in this step to rule out DNA contamination. After first strand cDNA synthesis, template RNA was removed by treatment with RNase. Synthesized cDNA was then separated, tailed and amplified by nested PCR using a primer against the 5-CAP sequence and gene specific primer GSP2. The PCR product was introduced into the TOPO™ TA cloning vector for subsequent amplification and sequencing.

Plasmid constructions

Plasmid pMA109 (Anderson and Lopes, 1996) that contains the *lacZ* reporter gene and a *URA3* marker was used in this study to generate fusions of the *CRD1* promoter with the *lacZ* gene of *E. coli*. The 5′ promoter region of the *CRD1* gene was amplified from the yeast genome by PCR employing four 5′ primers 5′-AAGGAATTCTGACGAAGGGAGAAGG-3′, 5′-GTCAAGCTTCACTTCACAGTC-ATGTCTTC-3′, 5′-CTCAAGCTTGAAACCATA-TTAAATGTCAA-3′ and 5′-CTTAAGCTTGAGT-ATACAATATTTACAAT-3′ (underlined endonuclease restriction sites were introduced), respectively, with 3′ primer (5′-TAGAATTCCGAAGTAATGC-GGAGC-3′). They were synthesized according to the DNA sequence surrounding the *CRD1* gene (Chang *et al.*, 1998b). The 5′ primers were targeted to the sequence starting from the 338th, 191st, 216th and 96th bp, respectively, upstream of the *CRD1* start codon, and the 3′ primer ends at the 44th bp in the *CRD1* open reading frame (see Figure 1). The PCR-amplified 5′ promoter regions of the *CRD1* gene were then ligated into plasmid pMA109 individually, using restriction sites introduced onto each pair of primers, generating plasmids pSD90, pSD91, pSD92 and pSD93, respectively. The final plasmids include a DNA fragment encoding the first 15 amino acids of the *CRD1* gene product, fused in-frame with the *lacZ* gene. All plasmids were confirmed by sequencing. The plasmid pSD80, in which the *CRD1* promoter was inverted as opposed to plasmid pSD90, was used as a control. Plasmids were introduced into yeast cells by transformation of $CaCl_2$-treated cells (Shen *et al.*, 1996). Transformants were selected by growth in the absence of uracil.

Isolation of *rho*⁻ mutants

Isolation of ethidium bromide-induced *rho*⁻ mutants was performed as described previously (Shen and Dowhan, 1998). *Rho*⁻ mutants were verified by their inability to grow on a non-fermentable carbon source.

Preparation of cell extracts and enzyme assays

Preparation of cell extracts was carried out at 4°C, as previously described (Shen and Dowhan, 1998). In summary, yeast cells were harvested, washed and disrupted using glass beads and a mini bead-beater. The supernatant, after centrifugation at 1500 × *g* for 10 min, was used for *β*-galactosidase activity assays. *β*-galactosidase activities were expressed as Miller units (380 × optical density at 420 nm produced per min per mg of total protein in cell extracts). Protein concentration in each cell extract was determined using a BCA protein assay kit.

DNA gel electrophoresis mobility shift assay

Yeast extracts containing both the soluble cytosolic and nuclear fractions were prepared as reported with modifications (Biswas and Biswas, 1990). Cells grown in 1% yeast extract, 2% peptone and 2% glucose (YPD) were harvested at an OD_{600} of 1.0, washed with and resuspended in 1/30 volume extraction buffer (200 mM Tris–HCl, pH 8.0, 400 mM ammonium sulphate, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride, 1 μg/ml leupepsin and 1 μg/ml pepstatin). Cells were disrupted in a Mini-beadbeater™ and, following incubation at 0°C for 30 min, unlysed cells and cell debris were removed by centrifugation at 10000 × *g* for 1 h at 4°C. Protein in the resulting supernatant was precipitated by the addition of 100% ammonium sulphate in protein buffer (10 mM HEPES, pH 8.0, 5 mM EDTA) to a final concentration of 40%. Following incubation for 30 min at 4 °C with gentle agitation, protein was collected by centrifugation at 25 000 × *g* for 10 min and the pellet was resuspended in protein buffer with 7 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride, 1 μg/ml leupepsin, 1 μg/ml pepstatin and 20% glycerol. The soluble protein extract was then desalted with a PD-10 Sephadex G25M column. The 95 bp DNA template for the gel mobility shift assay was amplified by PCR from the *CRD1* promoter, using primers GEL5 (5′-CACTTCACAGTCATGTCTTCGA-3′) and GEL3 (5′-CCGATTAAGAGGATGCTTCAT-3′). Synthesized DNA was labelled at the 5′ terminus with [γ-P^{32}]ATP by T4 polynucleotide kinase. Binding reactions were carried out in 20 μl binding buffer containing 4 mM Tris-HCl, pH 8.0, 40 mM NaCl, 4 mM $MgCl_2$, 5% glycerol, 0.5 ng radio-labelled DNA probe and 10 μg yeast extract for 25 min at 23 °C. After binding, the reaction mixture was loaded immediately onto a 4% polyacrylamide gel and electrophoresed at 25 mA at room temperature. Following electrophoresis, the gel was dried and exposed to X-ray film overnight.

Results

Transcriptional initiation of CL synthase gene

The transcriptional initiation site for the *CRD1* gene has not been reported. In order to learn the properties and determine an appropriate length of the *CRD1* promoter to be used in this study, we used the 5′ RACE method to map the transcriptional initiation site of the *CRD1* gene. Total cellular RNA was isolated and used to generate a cDNA (Figure 1A) from the *CRD1* mRNA, as described in Materials and methods. The cDNA product was then placed into the TOPO™ TA cloning vector and sequenced. The sequencing data showed that transcription of the *CRD1* gene initiates from either the 63rd or 64th base upstream of the start codon (Figure 1B). However, because the cDNA was capped with polycytidine after reverse transcription, we cannot distinguish between an initiation site at the 63rd base adenosine or the 64th base guanosine. Relative to the transcription start site, a putative TATA box was also predicted, and is indicated in Figure 1B.

Regulation of *CRD1* gene expression by growth phase and carbon source

Growth phase and carbon source can affect CL content as a result of altered mitochondrial development and expression of PG-P synthase activity (Gallet *et al.*, 1997; Jakovcic *et al.*, 1971). Therefore, the expression of the terminal enzyme, CL synthase, in the CL

biosynthetic pathway may also be subject to growth phase and carbon source regulation in coordination with expression of PG-P synthase. Coordinate transcriptional and translational regulation of the CL synthase expression was monitored by β-galactosidase activity, resulting from a *CRD1* gene promoter–*lacZ* reporter gene fusion (plasmid pSD90). P_{CRD1}–*lacZ* gene expression normalized to total protein (Figure 2A) increased with the extent of cell growth, reaching a maximum at early stationary phase and dropping significantly in late stationary phase. Since cellular CL also increases during growth and reaches a maximum in stationary phase (Jakovcic *et al.*, 1971), comparisons were made using mid-log cells in all subsequent studies, unless specified otherwise.

Reporter gene expression in response to carbon sources was tested using two unrelated wild-type yeast strains with different genetic backgrounds (YPH102 and DL1; see Table 1) in order to limit background effects. P_{CRD1}–*lacZ* gene expression was repressed when cells were grown in glucose medium as compared to the expression in non-fermentable media (lactate or glycerol/ethanol as a carbon source) (Figure 2B). Although strain YPH102 exhibited 10–20% higher β-galactosidase activity than strain DL1, the overall dependence of P_{CRD1}–*lacZ* gene expression on a carbon source was the same in both genetic backgrounds. The increase in P_{CRD1}–*lacZ* expression induced by growth on a non-fermentable is in line with the increase in CL content of cells grown on a non-fermentable carbon source (Jakovcic *et al.*, 1971).

Yeast cells grown in glucose have two phases of growth (Gallet *et al.*, 1997), the first of which is characterized by catabolite repression of expression of mitochondrial proteins (Perlman and Mahler, 1974). Before all the glucose in the media is depleted, cells reach stationary phase, where they adaptively increase mitochondrial function in order to metabolize the alcohol accumulated as a byproduct of glycolysis (Hajek and Bedwell, 1994). To investigate the effect of oxidative phosphorylation-dependent growth on P_{CRD1}–*lacZ* expression, a switch of cells from a fermentable to a non-fermentable carbon source (diauxic shift) was carried out (Figure 3, dashed lines). After a shift of mid-log glucose-grown cells to a non-fermentable carbon source, growth was arrested for the first 5 h, during which a slow increase (∼40%) in β-galactosidase activity was observed. The activity increased more rapidly when cell growth resumed after the lag phase and reached a level of 2.3-fold as high as that in cells remaining in glucose, indicating that the metabolism of glycerol/ethanol was important for the rapid derepression of P_{CRD1}–*lacZ* expression. This result also agrees with the 2–2.5-fold increase in β-galactosidase activity for cells grown on a non-fermentable carbon source compared with those grown in glucose.

Next, adaptation of expression of CL synthase to catabolite repression was studied during the reverse process from non-fermentative to fermentative growth (Figure 3, solid lines). A rapid decrease in β-galactosidase activity and little lag in cell growth were observed as soon as the cells were shifted to glucose medium (open square), consistent with catabolite repression by glucose; 4 h after the shift, a steady state was reached of ∼50% of the derepressed level. This is in agreement with Figure 2B, indicating the same level of repression in medium containing glucose as opposed to glycerol/ethanol. A slight increase in β-galactosidase activity was observed after 6 h, when cells began to enter stationary phase, consistent with the observation that *CRD1* gene expression is induced when glucose-grown

cells enter stationary phase (Figure 2A). Treatment of the cells with an inhibitor of nuclear-encoded protein synthesis (10 mM cycloheximide) during the shift from glycerol/ethanol to glucose totally blocked the cell growth, as well as changes in β-galactosidase activity, regardless of the presence of glucose in the media (solid square). These data indicate that the rapid decrease in β-galactosidase in the absence of cycloheximide in the media is due to a dilution effect caused by a rapid cell growth and at the same time significant repression of *Pcrd1-lacZ* expression.

Effect of mitochondrial dysfunction on P_{CRD1} –*lacZ* expression

The above results indicate that carbon source regulation of CL synthase activity might be closely related to mitochondrial biosynthesis. This raises the question of how gene expression would respond in cells with respiratory-deficient mitochondria. This question was addressed using two respiratory deficient strains, *rho*$^-$ (DL1 *rho*) and *pgs1*Δ (YCD4), both derived from the same wild-type parent strain, DL1. *Rho*$^-$ mutants have extensive lesions in mitochondrial DNA and are unable to respire on a non-fermentable carbon source, due to the lack of several mitochondrial-encoded proteins that are critical for oxidative phosphorylation. Similarly, the *pgs1*Δ mutant strain has severe defects in mitochondrial function (Chang *et al.*, 1998a; Janitor and Subik, 1993) and, like *rho*$^-$ cells, cannot grow on non-fermentable carbon sources (Chang *et al.*, 1998a). *Pgs1*Δ cells also exhibit a petite lethal phenotype, initially characterized by incompatibility with extensive mutations in mitochondrial DNA (Janitor and Subik, 1993) but later ascribed to incompatibility with growth on ethidium (usually used to generate petite mutants) because of a defect in cell wall synthesis that is associated with lack of mitochondrial anionic phospholipids (Zhong *et al.*, 2005). In addition, cells carrying this mutation cannot efficiently maintain mitochondrial DNA and eventually become *rho*$^-$ mutants (Zhong *et al.*, 2005). Therefore, the *pgs1*Δ mutation displays a *rho*$^-$ phenotype. Unlike the wild-type respiratory-competent strain DL1, there was no increase in β-galactosidase activity when its *pgs1*Δ or *rho*$^-$ derivatives were shifted from glucose to glycerol/ethanol (Figure 4). Interestingly, in glucose-grown mid-log cells in which mitochondrial function is significantly repressed, expression of P_{CRD1}–lacZ was the same in the *rho*$^+$ and *rho*$^-$ cells but significantly reduced in *pgs1*Δ cells, which, in addition to being respiratory-incompetent, also lack the CL precursor PG.

Expression of P_{CRD1} –*lacZ* in *crd1* Δ and *cds1* Δ mutants

The *crd1* Δ mutant can grow on both fermentable and non-fermentable carbon sources, although it is somewhat compromised when utilizing the latter as substrate (Chang *et al.*, 1998b; Gohil *et al.*, 2004; Jiang *et al.*, 1997; Zhang *et al.*, 2003). In cells lacking the terminal product of the CL biosynthetic pathway, PG accumulates (Chang *et al.*, 1998b). Since experiments with *pgs1*Δ cells suggested a possible role of decreased PG level in downregulating CL synthase expression, the possibility that an elevated level of PG might induce an increased amount of CL synthase expression was investigated. The P_{CRD1}–*lacZ* reporter gene was introduced into a *crd1* Δ mutant strain and cells were cultured in galactose-containing media to prevent glucose repression. The level of β-galactosidase in the *crd1* Δ mutant strain YCD2 was 2.5-fold higher than that in the parental strain YPH98 (Figure 5).

CDP-DAG is an important intermediate for glycerol-phospholipid biosynthesis in yeast, from which three branches of *de novo* phospholipid biosynthetic pathways diverge (Carman and Henry, 1989). Therefore, CDP-DAG is the precursor for the synthesis of several major phospholipids, including phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, PG and CL. The formation of CDP-DAG is catalysed by CDP-DAG synthase encoded by the *CDS1* gene, which occupies a central position in phospholipid metabolism (Shen *et al.*, 1996). Yeast mutants with decreased CDP-DAG synthase activity exhibit a pleiotropic phenotype (Shen and Dowhan, 1997). Reduction of cellular CDP-DAG synthase activity results in elevated inositol 1-P synthase, phosphatidylserine synthase and PG-P synthase levels via transcriptional regulation and a decrease in PI synthase levels through post-translational events (Shen and Dowhan, 1997, 1998). The precise mechanism by which the level of CDP-DAG or the ability of cells to synthesize CDP-DAG affects the levels of these synthases has not been elucidated, but this regulation differs from the regulation of phospholipid metabolism by inositol, in that the former is independent of the *INO2*, *INO4* and *OPI1* regulatory circuit. Since CL synthase functions at the end-point of the CL synthetic pathway downstream of CDP-DAG biosynthesis and directly utilizes a second molecule of CDP-DAG as substrate, we investigated whether CL synthesis is subject to regulation by CDP-DAG levels. As mentioned earlier, the *cds1*Δ mutation is lethal unless complemented by the expression of the human CDP-DAG synthase induced from the P_{GAL1} promoter. Even when grown in galactose-containing medium, the complemented mutant has reduced levels of CDP-DAG synthase and exhibits all of the phenotypes associated with low levels of CDP-DAG synthesis (Shen and Dowhan, 1997). Unlike expression of other phospholipid biosynthetic activities, P_{CRD1}–*lacZ* expression in the *cds1*Δ null mutant strain complemented by the low levels of CDP-DAG synthase (supplied by plasmid phCDS1) was similar to that in the wild-type strain YPH102 with normal levels of CDP-DAG synthase (Figure 5).

Regulation of *CRD1* expression by inositol

Inositol is a major regulator of PC and PI biosynthesis (Carman and Henry, 1989; Greenberg and Lopes, 1996). Inositol also appears to regulate CL biosynthesis by repressing the expression of PG-P synthase activity (Greenberg *et al.*, 1988; Shen and Dowhan, 1998) but not the level of PG-P phosphatase or CL synthase activities (Tamai and Greenberg, 1990). A consensus UAS_{INO} element (untranslated activation sequence responsive to inositol), which could serve as a *cis*-acting site for *trans*-acting factors encoded by the *INO2-INO4-OPI1* regulatory genes in inositol-dependent regulation (Carman and Henry, 1989), is not present near the *CRD1* gene. However, a potential UAS_{INO}-like element is found in the promoter region, which is the same as the consensus UAS_{INO} element in eight of its 10 bases (Figure 1B). P_{CRD1}–*lacZ* expression was examined in the presence of different inositol/choline concentrations in both glucose- and glycerol/ethanol-containing media. Inositol and/or choline had no effect on the fusion gene expression (data not shown), consistent with the previous report of the lack of an effect on CL synthase activity levels (Tamai and Greenberg, 1990). Since the difference between the potential UAS_{INO}-like element and a consensus one lies in the first six bp, which is an E-box motif (Hoshizaki *et al.*, 1990; Nikoloff *et al.*, 1992) critical for the ino2p and ino4p heterodimer binding, it may explain the lack of response of the CL synthase gene to inositol.

Next, we investigated whether fusion gene expression is affected in strains carrying null alleles of the *INO2* or *INO4* regulatory gene. Because these mutants are inositol auxotrophs and cannot grow in inositol-free media, 70 μm inositol was added to the growth media to support cell growth. Compared to the wild-type strain DL1, P_{CRD1}–*lacZ* expression (pSD90) was unaffected in *ino2*Δ and *ino2*Δ/*ino4*Δ strains but was ∼2.5-fold higher in an *ino4*Δ strain (Figure 6A), regardless of the level of inositol and/or choline present in the medium (data not shown). This observation was unexpected, since the *INO2* and *INO4* gene products generally act as a heterodimeric activator (Schwank *et al.*, 1995), and strains null in the *INO2* or *INO4* genes usually express reduced levels of gene products responsible for phospholipid biosynthesis (Bailis *et al.*, 1987; Hirsch and Henry, 1986). To further investigate this effect, a series of deletions in the *CRD1* promoter region were created and the resulting plasmids were transformed into wild-type, *ino2*Δ, *ino4*Δ and *ino2*Δ/*ino4*Δ strains to assess P_{CRD1}–*lacZ* expression. Compared with plasmid pSD90 (full length upstream of the *CRD1* promoter), a four- to five-fold increase in *β*-galactosidase activity was observed in wild-type, *ino2*Δ and *ino2*Δ/*ino4*Δ cells and a more than 20-fold increase in *β*-galactosidase activity was observed in a *ino4*Δ mutant when an 136 bp upstream sequence including the UAS_{INO}-like element was deleted (plasmid pSD91). These data suggested that the 136 bp sequence upstream of the *CRD1* promoter functions as a general repressing element whose deletion enhances *CRD1* expression, particularly in the absence of the *INO4* gene product. To determine whether the UAS_{INO}-like element contributes to this effect, strains carrying plasmid pSD92 (126 bp deletion upstream of the UAS_{INO}-like element) were examined. A slight increase in *β*-galactosidase level was observed in wild-type, *ino2*Δ and *ino2*Δ/*ino4*Δ strains and a small drop was observed in *ino4*Δ cells when compared to plasmid pSD91, but the *ino4*Δ mutant still showed the greatest increase. This result confirmed that the UAS_{INO}-like element plays a limited role in regulating *CRD1* gene expression and the sequence downstream from the UAS_{INO}-like element alone is sufficient for mediating *CRD1* gene expression and responding to regulatory actions by the *INO2/INO4* regulatory genes. When this downstream sequence was deleted (plasmid pSD93), the increased reporter expression in *ino4*Δ cells was abolished. Therefore, the *cis*-acting element responsive in *ino4*Δ cells lies between the UAS_{INO}-like element and the putative TATA site of the *CRD1* gene.

The above results suggest a possible mechanism by which the *cis*-element may play a role in control of *CRD1* gene expression, i.e. by differential binding to transcriptional factors in the wild-type, *ino2*Δ, *ino4*Δ and *ino2*Δ/*ino4*Δ strain backgrounds. To test this hypothesis, gel electrophoresis mobility shift assay (EMSA) was used to determine whether extracts isolated from these strains interact differently with a synthesized 95 bp DNA probe representing the *cis*-element. The result in Figure 6B showed that a few band shifts were detected when using extracts isolated from these strains, two of which appear to exist commonly among all strains except one that occurs predominately in *ino4*Δ cells. Because equal amounts of protein extract and DNA probe were used in this experiment and similar amounts of other band shifts were observed in all strains, the dominant band shift with *ino4*Δ extracts is due to transcriptional factors that can bind to the *cis*-element and exist predominately in the *ino4*Δ background.

Discussion

CL is a phospholipid predominantly located in the mitochondrial inner membrane. Therefore, its levels may be regulated by factors affecting mitochondrial membrane development. This study clearly demonstrated that CL synthesis, as indicated by expression of *lacZ* from *CRD1* promoter–gene fusions, was elevated in cells grown in or shifted to non-fermentable carbon sources and in early stationary phase where alcohol accumulates; normal mitochondrial respiratory function or metabolism of non-fermentable carbon source by mitochondria was a prerequisite for this induction. These results extend similar conclusions reached by monitoring mRNA levels by Northern blot analysis, in that they eliminate translational regulation as a means of controlling CL synthase levels and hence CL content of cells. Such information is relevant in light of the fact that for many genes of *S. cerevisiae* there is little correlation in absolute amounts or in changes in mRNA levels with the amount of the respective protein products, making measurement of mRNA levels only a poor predictor of protein functional level (Gygi *et al.*, 1999). Currently, however, it is not clear how growth phase or carbon sources regulate *CRD1* gene expression. Interestingly, CL, the lipid product of the gene product of *CRD1*, participates in oxidative phosphorylation and is essential for functions of many proteins/complexes in the electron transfer chain located in the inner mitochondrial membrane (Haines and Dencher, 2002; Koshkin and Greenberg, 2000; Rusnak *et al.*, 1997). Therefore, for cells growing in a non-fermentable carbon source, accelerated synthesis of CL would be important for the biogenesis of respiratory-competent mitochondria. The observed lag in growth with increasing β-galactosidase expression during this transition may represent a period when cells are adapting themselves in response to metabolic changes by increasing CL content in the membrane, and may also be related to the need for CL as a structural component of individual components of the electron transport chain (Eble *et al.*, 1990; Fry and Green, 1981; Robinson *et al.*, 1990) or organization of these components into higher-order complexes (Zhang *et al.*, 2002, 2005). These data, taken together with other reports (Jiang *et al.*, 1999), establish unequivocally that factors affecting mitochondrial biogenesis, such as carbon source, growth phase or mitochondrial mutations (discussed below), affect *CRD1* gene expression at the level of transcription and that there appears to be no additional regulation at the translational level as there is for many other genes of yeast.

The lack of derepression of *β*-galactosidase production in respiratory-compromised cells by exposure to a non-fermentable carbon source argues that the presence of respiratory-competent mitochondria, cell growth and/or metabolism of a non-fermentable carbon source, not simply a non-fermentable carbon source, is critical for derepressed *CRD1* gene expression. Similar to data obtained here, it was also reported that *CRD1* mRNA derepression cannot be induced by growth phase in *rho*$^{-}$ cells, in contrast to their isogenic wild-type strains (Jiang *et al.*, 1999), again supporting the existence of functional mitochondria as important to *CRD1* gene expression which may be coordinately regulated with the biogenesis of mitochondria.

Although both *rho*$^{-}$ and *pgs1*Δ cells are respiratory-deficient and are deficient in mitochondrial DNA (Zhong *et al.*, 2005), an additional drop in the expression from the *CRD1* promoter was observed in *pgs1*Δ cells, which cannot be ascribed solely to

mitochondrial dysfunction, but instead could be caused by the lack of PG in the mitochondrial membranes. The lack of the precursor to CL synthesis in mitochondrial membranes may serve as a regulatory signal to repress CL synthase expression, via a mechanism currently not understood. It should be noted that lack of PG also severely represses cytoplasmic translation of *COX4* mRNA without significantly affecting mRNA levels (Ostrander *et al.*, 2001; Su and Dowhan, 2005), resulting in lack of this subunit of the mitochondrial cytochrome *c* oxidase. The increase in P_{CRD1}–*lacZ* gene expression in a *crd1*Δ mutant, which has highly elevated PG levels, also supports a role for PG levels in regulating expression of CL synthase. This result is also in agreement with the report that there was a three-fold increase of a truncated *CRD1* mRNA in *crd1*Δ cells compared with that in wild-type cells, as determined by Northern blotting analysis (Jiang *et al.*, 1999). However, expression of CL synthase was found to be independent of the capacity of cells to synthesize its common substrate and precursor, CDP-DAG.

Current and previous results indicate that both *CRD1* gene transcription and CL synthase activity in yeast are insensitive to cross-pathway control by the inositol regulatory system. However, in this study, *CRD1* gene expression was increased 2.5-fold in *ino4*Δ cells compared with wild-type cells. This result differs from a previous study, which reported there were no difference in *CRD1* mRNA level between wild-type, *ino2*Δ and *ino4*Δ cells (Jiang *et al.*, 1999). The basis for the difference between these two studies is not clear, although the methods of analysis were different. Since the increase in *lacZ* activity occurred only in *ino4*Δ cells but not in wild-type or *ino2*Δ cells, Ino4p may be a negative regulator of *CRD1* gene expression. Additionally, expression of *lacZ* was restored to the wild-type level when *INO2* was deleted in *ino4*Δ cells, suggesting that Ino2p is critical for the elevated expression of the *CRD1* gene. Ino2p and Ino4p are members of the basic helix–loop–helix (bHLH) family of DNA-binding proteins that bind the consensus canonical bHLH site 5′-CANNTG-3′, also known as E-box (Hoshizaki *et al.*, 1990; Nikoloff *et al.*, 1992). Binding of Ino2p/Ino4p to UAS_{INO} is required for activation of many phospholipid synthetic genes (Hirsch and Henry, 1986; Loewy and Henry, 1984). Heterodimerization between Ino2p and Ino4p through their bHLH domains forms a functional heterodimeric activator in which Ino2p provides two separate domains for transactivation, and its dimerization with Ino4p determines their binding specificity for UAS_{INO} (Schwank *et al.*, 1995). Because of the different nature of the *INO2* and *INO4* gene products, it is possible that they may play separate roles in the regulation of phospholipid metabolism. This was first suggested by Morlock *et al.* (1988) in the study of regulation of PA phosphatase by inositol in *S. cerevisiae*. Compared to wild-type cells supplemented with inositol, PA phosphatase activity was reduced in an *ino2*Δ mutant but was not affected in an *ino4*Δ mutant. The *PIS1* gene is another example in which its expression was increased only in an *ino2*Δ but not in an *ino4*Δ cells (Anderson and Lopes, 1996). In addition, the DNA-binding region of Ino2p shares a high homology with the mammalian Myc family of proteins (Nikoloff *et al.*, 1992), which have the ability to form multiple heterodimers with different partners (Amati and Land, 1994). In fact, Ino2p has been implicated in interacting with other factors (Block-Alper *et al.*, 2002). Based on available evidence, the deletion analysis results in Figure 6A may be explained by the following model. In wild-type cells, Ino2p dimerizes preferentially with Ino4p. However, because there is no functional UAS_{INO} sequence in the *CRD1* promoter,

the dimeric activator cannot bind to DNA in the vicinity of the *CRD1* gene and, as a result, *CRD1* gene expression is not affected. In contrast, in the absence of Ino4p, Ino2p may potentially interact with perhaps another bHLH transcriptional factor. Binding to this factor results in specific binding of the dimeric complex to a currently unidentified site between the UAS_{INO}-like element and the TATA site, activating downstream gene transcription. In the absence of Ino2p, such as in *ino2*Δ and *ino2*Δ/*ino4*Δ cells, or in the absence of the *cis*-acting element, the DNA–dimeric activator complex cannot be formed and therefore a high level of transcription cannot be induced. The DNA gel mobility shift assay with the *CRD1* promoter and yeast lysates supports this hypothesis by showing complex formation predominately in *ino4*Δ cells. Our observation coupled with the above results for *PIS* gene expression and PA phosphatase levels suggests a more widespread and new role for *INO2* and/or *INO4* in regulation of phospholipid biosynthesis in yeast.

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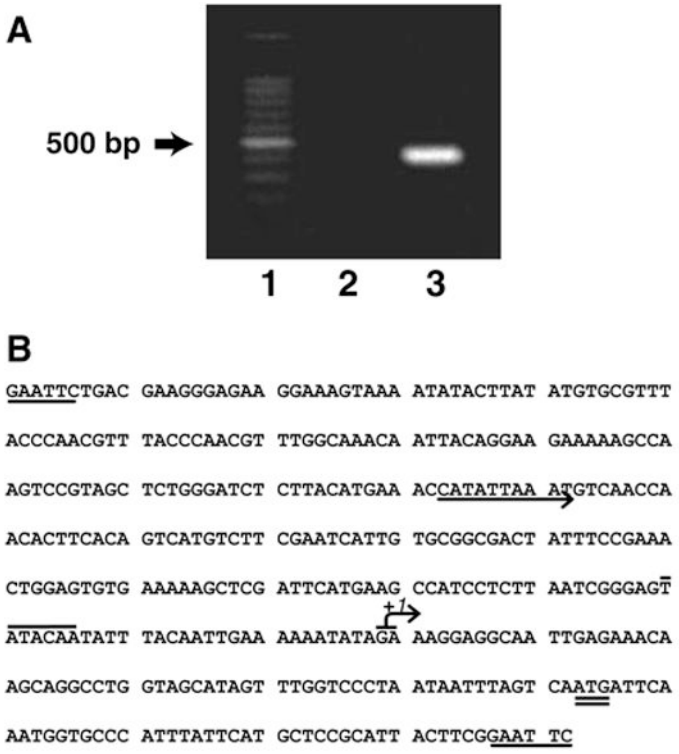

Figure 1.

Mapping of *CRD1* gene transcription initiation site and the *CRD1* promoter sequence. (A) DNA fragment containing the end of the 5′-untranslated region of the *CRD1* gene. Lane 1, Promega 100 bp step marker. Lane 2, sample mRNA treated with RNase prior to the RACE reaction. Lane 3, 5′ RACE product from *CRD1* mRNA. (B) Diagram of *CRD1* promoter. *Eco*RI sites (single underline) used for the construction of gene fusions, transcription initiation start sites (arrow with +*I*), a potential UAS_{INO} (arrow), TATA box of putative promoter (overline) and the putative translation initiation codon (double underline)

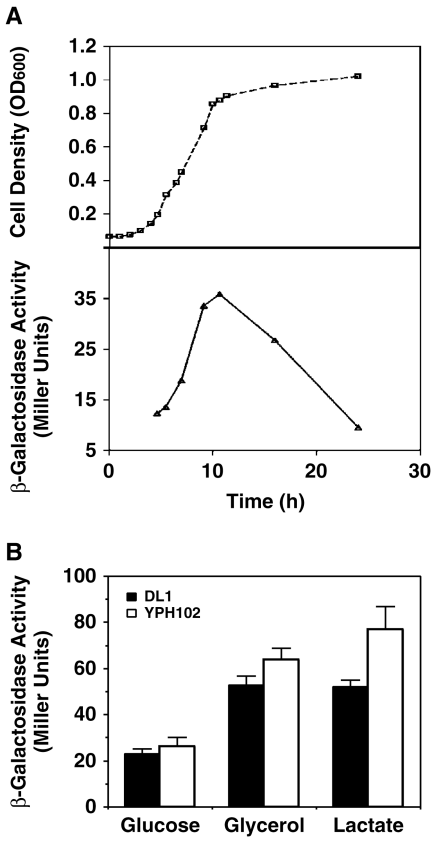

Figure 2.

P_{CRD1} –*lacZ* expression in response to growth phase and carbon source. (A) Wild-type strain DL1 carrying plasmid pSD90 (P_{CRD1} –*lacZ*) was inoculated into inositol-free medium containing 2% glucose. Cell growth was monitored by OD_{600} and *β*-galactosidase activity was determined at the indicated times. (B) Wild-type strains DL1 and YPH102, all bearing plasmid pSD90 (P_{CRD1} –*lacZ*), were grown to mid-log phase in an inositol-free medium containing 2% of different carbon sources. Cells were harvested and *β*-galactosidase activity was determined in whole-cell extracts. Each value represents the mean of at least three experiments

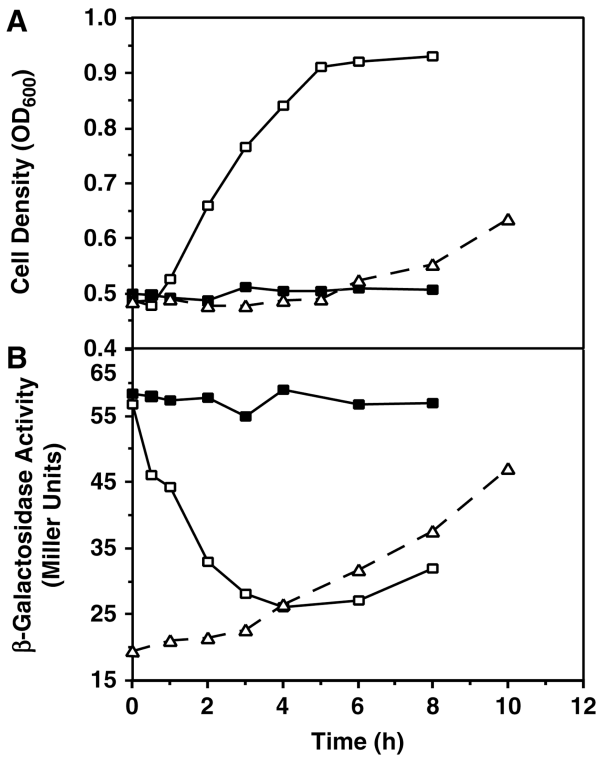

Figure 3.

Adaptation to non-fermentative and fermentative growth during switch of carbon source. Mid-log phase cells of wild-type strain DL1 carrying plasmid pSD90 (P_{CRD1} –*lacZ*) growing in an inositol-free medium with either 2% glucose or 3% glycerol/ethanol were collected, washed with an inositol-free medium without carbon source, and at time zero switched to medium with the opposite carbon source, respectively [either from glucose to glycerol/ethanol (dashed lines) or from glycerol/ethanol to glucose (solid lines)]; in the latter case, the glucose medium was supplemented either with (closed symbols) or without (open symbols) 10 mM cycloheximide. Cells were sampled at the indicated time points. (A) Cell density was measured by OD_{600} and (B) *β*-galactosidase activity was determined in whole cell extracts. Values are representative of three experiments

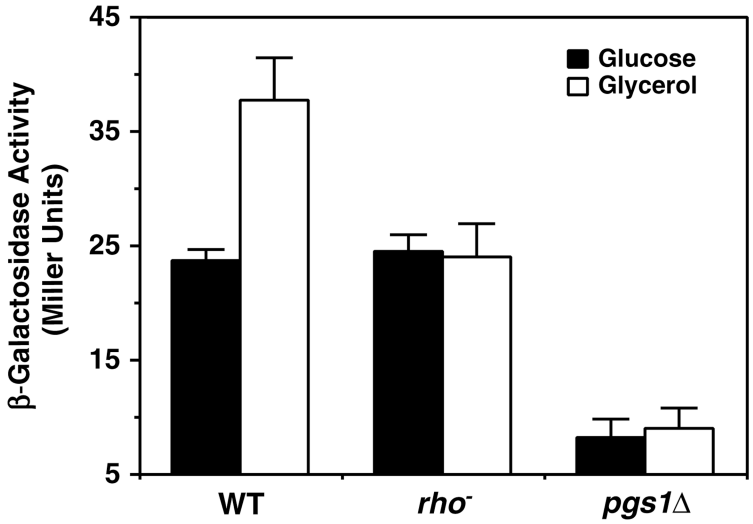

Figure 4.

P_{CRD1} –*lacZ* expression in response to mitochondrial function. The parental strain DL1, a *rho*$^{-}$ mutant and *pgs1A* mutant (YCD4) all bearing plasmid pSD90 (P_{CRD1} –*lacZ*) were grown to mid-log phase in an inositol-free medium containing 2% glucose. Then half of each culture was maintained in glucose medium while the other half was washed and shifted to 3% glycerol/ethanol medium. Cells were harvested after 6 h and *β*-galactosidase activity was determined in whole cell extracts. Each value represents the mean of three experiments

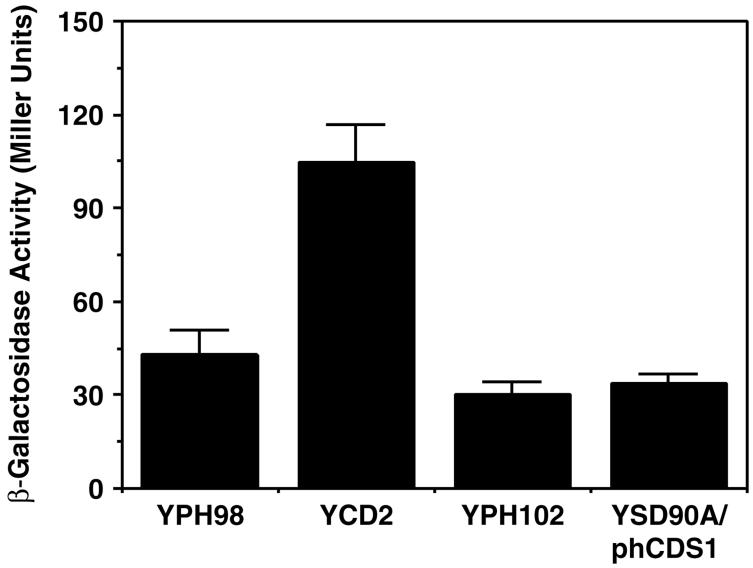

Figure 5.

P_{CRD1}–*lacZ* expression in *crd1*Δ and *cds1*Δ mutant strains. The parental strain (YPH98) and *crd1*Δ mutant (YCD2) with plasmid pSD90 (P_{crd1}–*lacZ*) were grown to mid-log in a minimal medium with 2% galactose. *β*-galactosidase activity of the mutant was compared with that of the wild-type. The *cds1*Δ mutant YSD90A carrying a human cDNA encoding a CDP-DAG synthase (plasmid phCDS) under the control of P_{GAL1} promoter was transformed with plasmid pSD90 and cells were grown to mid-log phase in minimal medium with 2% galactose (to induce the plasmid-borne copy of the h*CDS1* gene). *β*-galactosidase activity was assayed in the whole cell extracts and compared with that of the parental wild-type strain YPH102 (*CDS*) grown in the same medium. Each value represents the mean of three experiments

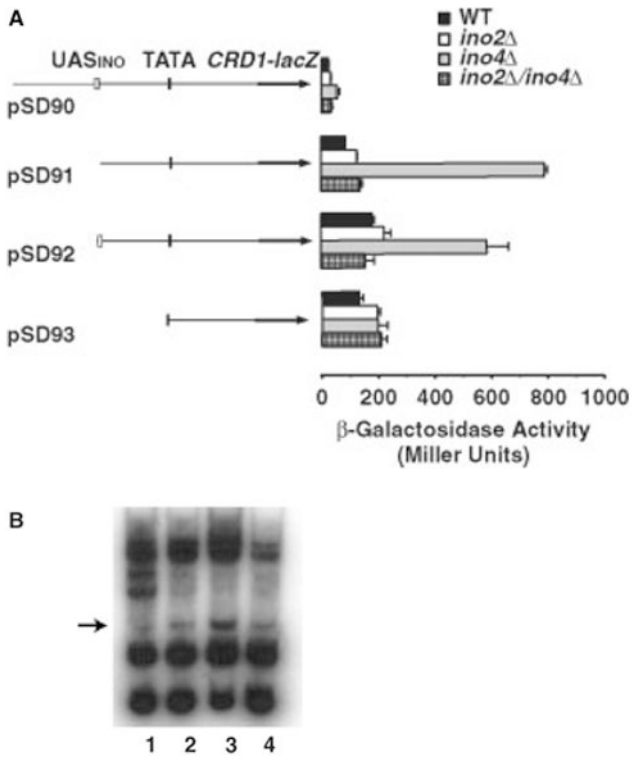

Figure 6.

P_{CRD1}–*lacZ* gene expression in *ino2*Δ and *ino4*Δ mutant strains. (A) Wild-type strain DL1, the *ino2*Δ mutant SH303, the *ino4*Δ mutant SH307 and the *ino2*Δ/*ino4*Δ double mutant SH486, all bearing either plasmid pSD90, pSD91, pSD92 or pSD93, were grown to mid-log phase in a minimal medium containing 2% glucose supplemented with 70 μM inositol. *β*-galactosidase activity was determined in whole cell extracts. Each value represents the mean of three experiments. The diagram illustrates the relevant genetic components carried by each plasmid. (B) DNA gel electrophoresis of mobility shift assay. A 95 bp *CRD1* promoter sequence representing the *cis*-element upstream of the *CRD1* promoter was used as a probe and EMSA was performed using this element and cell extracts isolated from the indicated cells. Lane 1, DL1; lane 2, *ino2*Δ; lane 3, *ino4*Δ; and lane 4, *ino2*Δ/*ino4*Δ

Table 1

Yeast strains and plasmids

Strains or plasmids	Relevant characteristics	Reference or source
Strains		
DL1	*MATa his3-11, 15 leu2-3, 12 ura3-251, 328, 372*	Van Loon *et al.* (1983)
rho$^-$	*MATa rho*$^-$, derivative of DL1	This work
YCD4	*MATa pgs1::HIS3*, derivative of DL1	Chang *et al.* (1998a)
YPH98	*MATa ura3-52 lys2-801 ade2-101 leu2*Δ*1 trp1*Δ1	Sikorski and Hieter (1989)
YCD2	*MATa crd1::TRP1*, derivative of YPH98	Chang *et al.* (1998b)
YPH102	*MATα ura3-52 lys2-801 ade2-101 leu2*Δ*1 his3*Δ200	Sikorski and Hieter (1989)
YSD90A	*MATα cds1::TRP1*, derivative of YPH102	Shen *et al.* (1996)
SH303	*MATa ino2::TRP1 his3 ura3 leu2*	S. A. Henry
SH307	*MATα ino4::LEU2 trp1 his3 ura3*	S. A. Henry
SH486	*his3 leu2 trp1 ura3 ino2*Δ::*TRP1 ino4::LEU2*	S. A. Henry
Plasmids		
pMA109	*lacZ URA3* 2μ and ColE1 origin	Anderson and Lopes (1996)
phCDS1	P_{GAL1} –*hCDS*1 *LEU*2, 2μ and ColE1 origin	Shen and Dowhan (1997)
pSD80	P_{DRC1}–*lacZ*, derivative from pMA109 (inverted promoter)	This work
pSD90	P_{CRD1}–*lacZ*, derivative from pMA109	This work
pSD91	Derivative of pSD90, a 136 bp sequence including the putative UAS_{INO} was deleted from *CRD1* promoter	This work
pSD92	Derivative of pSD90, a 126 bp sequence up to the putative UAS_{INO} was deleted from *CRD1* promoter	This work
pSD93	Derivative of pSD92, a further 107 bp sequence up to the potential TATA box was deleted from *CRD1* promoter	This work