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Research Article

Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast *Pichia pastoris*

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Abstract

Expression of genes involved in methanol metabolism of *Pichia pastoris* is regulated by Mxr1p, a zinc finger transcription factor. In this study, we studied the target gene specificity of Mxr1p by examining its ability to bind to promoters of genes encoding dihydroxyacetone synthase (DHAS) and peroxin 8 (PEX8), since methanol-inducible expression of these genes is abrogated in *mxr1*-null mutant strains of *P. pastoris*. Different regions of DHAS and PEX8 promoter were isolated from *P. pastoris* genomic DNA and their ability to bind to a recombinant Mxr1p protein containing the N-terminal 150 amino acids, including the zinc finger DNA-binding domain, was examined. These studies reveal that Mxr1p specifically binds to promoter regions containing multiple 5'-CYCC-3' sequences, although all DNA sequences containing the 5'-CYCC-3' motif do not qualify as Mxr1p-binding sites. Key DNA-binding determinants are present outside 5'-CYCC-3' motif and Mxr1p preferably binds to DNA sequences containing 5'-CYCCNY-3' than those containing 5'-CYCCNR-3' sequences. This study provides new insights into the molecular determinants of target gene specificity of Mxr1p, and the methodology described here can be used for mapping Mxr1p-binding sites in other methanol-inducible promoters of *P. pastoris*. Copyright © 2010 John Wiley & Sons, Ltd.

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Introduction

Methanol expression regulator 1 (Mxr1p) is a novel zinc finger protein essential for the methanol-inducible expression of genes involved in methanol utilization pathway of *Pichia pastoris* (Lin-Cereghino *et al.*, 2006; Hartner *et al.*, 2008). The amino-terminal zinc finger motif of Mxr1p shares 70% identity with the zinc finger DNA-binding domain of *Saccharomyces cerevisiae* Adr1p (alcohol dehydrogenase II synthesis regulator), a transcription factor regulating the expression of genes involved in the metabolism of glycerol, ethanol and oleic acid (Lin-Cereghino *et al.*, 2006). Mxr1p

is the *P. pastoris* homologue of *Saccharomyces cerevisiae* Adr1p and it was suggested that Mxr1p and Adr1p may bind similar DNA sequences (Lin-Cereghino *et al.*, 2006). The sequence 5'-CYCCRD(N4-36)HYGGRG-3' is the consensus sequence for Adr1p (Shuster *et al.*, 1986; Eisen *et al.*, 1988; Cheng *et al.*, 1994; Cheng and Young, 1995) and DNA sequences similar to *S. cerevisiae* Adr1p-binding sites are present in the promoter of the gene encoding alcohol oxidase I (AOXI), the first enzyme involved in methanol metabolism (Lin-Cereghino *et al.*, 2006; Hartner *et al.*, 2008). Deletion of the CYCC motif of the putative Adr1p-binding site of AOXI promoter was

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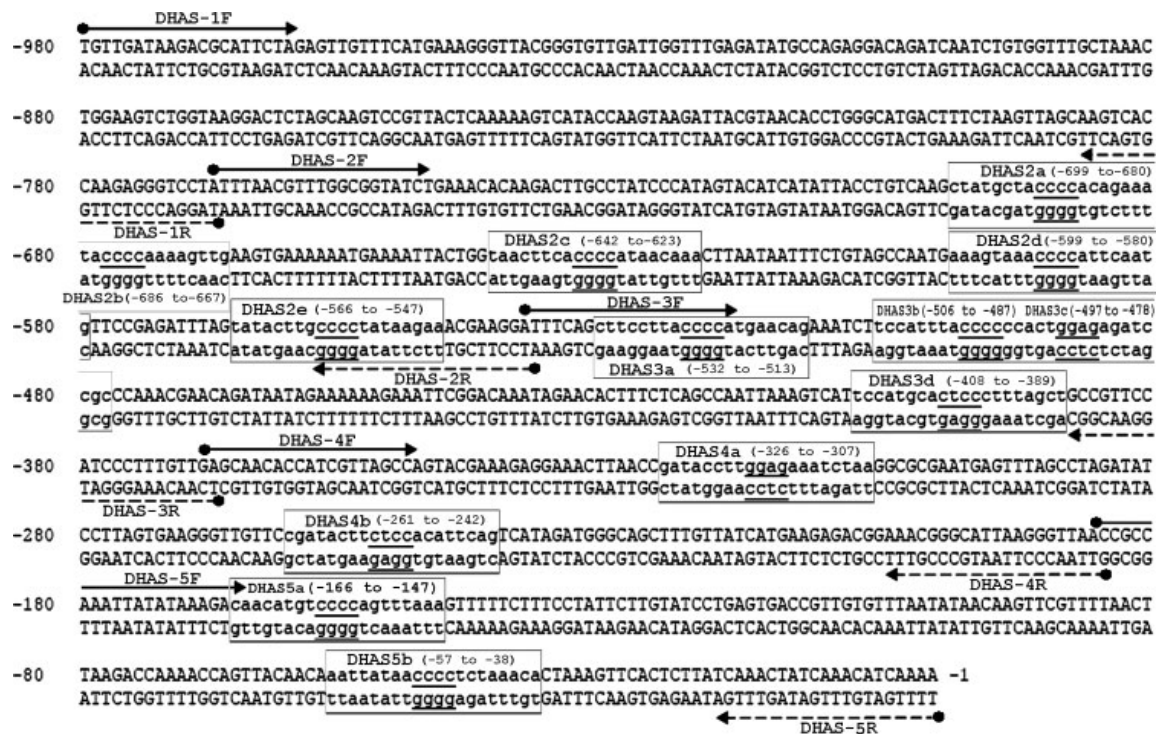


Figure 1. Nucleotide sequence of *DHAS* promoter and strategy for the PCR amplification of promoter DNA fragments. The forward (F) and reverse (R) primers used in PCR reactions to obtain amplicons encompassing five different promoter regions (DHAS1-5) are indicated by unbroken and broken arrows, respectively. Oligonucleotides (20 bp) containing the core 5'-CYCC/GGRG-3' sequence (underlined) used in EMSA studies (Figure 3b) are boxed

shown to result in 70% decrease in *AOXI* gene expression, indicating a key role for this sequence in *AOXI* gene regulation (Hartner *et al.*, 2008). In addition to *AOXI*, expression of a number of other genes involved in the methanol utilization pathway, such as those encoding dihydroxyacetone synthase (*DHAS*), formaldehyde dehydrogenase (*FLD*), peroxins 5, 8, and 14 (*PEX5*, *PEX8*, *PEX14*), is also affected in *mrx1*-null mutant strains (Lin-Cereghino *et al.*, 2006). Whether methanol-inducible expression of these genes is mediated by the direct binding of Mxr1p to CYCC motifs present in their promoters is not known.

When *P. pastoris* cells are grown in methanol, expression of enzymes of methanol metabolism, as well as proteins involved in peroxisome biogenesis (peroxins), is induced (Cregg *et al.*, 1989; Lin-Cereghino *et al.*, 2000; Hartner *et al.*, 2006). During methanol metabolism, methanol is first converted to formaldehyde by *AOX*, which is

further oxidized by *FLD* and formaldehyde dehydrogenase (*FDH*) to generate energy. In another pathway, formaldehyde is condensed with xylulose 5-phosphate by *DHAS* to generate two three-carbon molecules that enter a cyclic pathway leading to the regeneration of xylulose 5-phosphate, as well as synthesis of one net molecule of glyceraldehyde 3-phosphate for every three runs of this cycle. The reactions catalysed by *AOX* and *DHAS* occur in peroxisomes and therefore, peroxisomes are essential for growth of *P. pastoris* in methanol (Cregg *et al.*, 1989; Lin-Cereghino *et al.*, 2000; Hartner *et al.*, 2006). When the carbon source is switched from glucose to methanol, in addition to *AOX*, *FLD* and *DHAS*, expression of a number of *PEX* genes, many of which encode components of peroxisome import machinery, is also induced. In a recent study (Kranthi *et al.*, 2009), we have identified six Mxr1p-binding sites in the *AOXI* promoter, each of which consists of a core 5'-CYCC-3' motif. In this study, we have examined

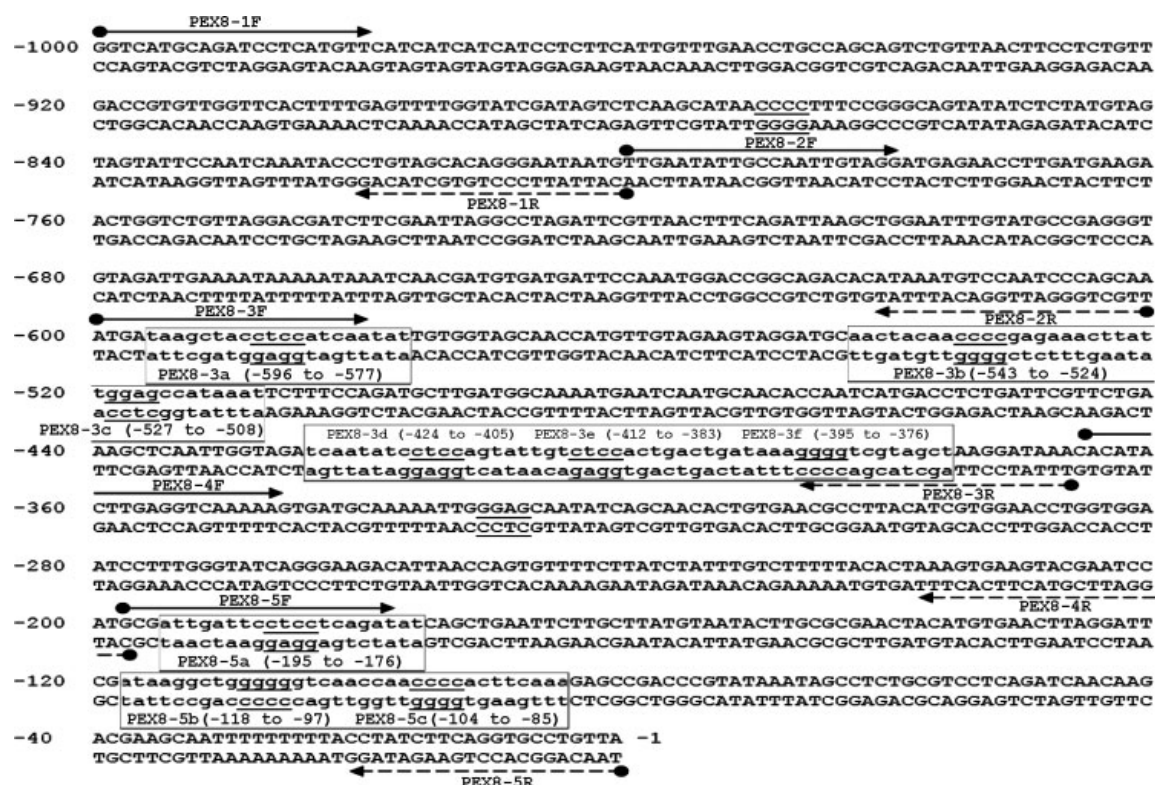
Mxr1p-binding to *Pichia pastoris* DHAS and PEX8 promoters

Figure 2. Nucleotide sequence of PEX8 promoter and strategy for the PCR amplification of promoter DNA fragments. The forward (F) and reverse (R) primers used in PCR reactions to obtain amplicons encompassing five different promoter regions (PEX1-5) are indicated by unbroken and broken arrows, respectively. Oligonucleotides (20 bp) containing the core 5'-CYCC/GGRG-3' sequence (underlined) used in EMSA studies (Figure 3b) are boxed

the promoter sequences of genes encoding DHAS and PEX8 and identified nucleotides outside the core 5'-CYCC-3' motif that are essential for Mxr1p binding. Based on these studies, we have derived a consensus sequence for Mxr1p binding.

Materials and methods

Synthesis of oligonucleotides and preparation of radiolabelled DNA probes

Oligonucleotides were purchased from Sigma-Aldrich Chemicals (Bangalore, India). Promoter regions of DHAS and PEX8 were isolated by PCR amplification of *P. pastoris* genomic DNA, using appropriate primers (Figures 1, 2). ³²P-labelled DNA probes were generated by radiolabelling the 5' ends of double-stranded DNA with (γ -³²P)ATP and polynucleotide kinase, using

standard molecular biology protocols (Sambrook *et al.*, 1989). After radiolabelling, unincorporated radionucleotides were removed by Sephadex G-50 chromatography.

Preparation of recombinant Mxr1p

Overexpression and purification of recombinant Mxr1p has been described recently (Kranthi *et al.*, 2009). This histidine-tagged protein consisting of the first 150 N-terminal amino acids, including the zinc finger DNA-binding domain, is referred to as Mxr1p in this study.

Electrophoretic mobility shift assay (EMSA)

Purified recombinant Mxr1p (10 ng) was incubated with ³²P-labelled DNA probes (20 000–40 000 cpm) in a 30 μ l reaction containing buffer B (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM

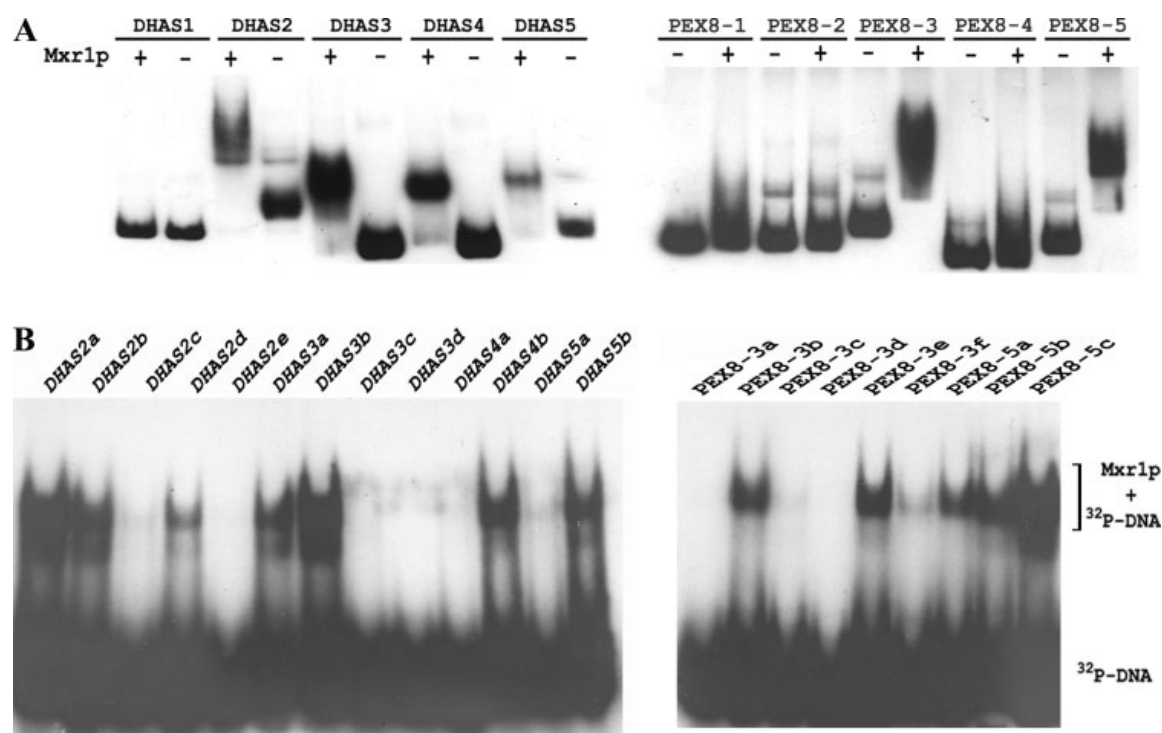


Figure 3. Identification of Mxr1p-binding sites in the promoters of *DHAS* and *PEX8*. (A) Mxr1p-binding to *DHAS* and *PEX8* promoter regions containing varying units of 5'-CYCC-3' motifs. The promoter DNA fragments used in EMSA are described in Figures 1 and 2. (B) Analysis of Mxr1p-binding to *DHAS* and *PEX8* promoter regions containing a single 5'-CYCC-3' motif by EMSA. DNA sequences are described in Figures 1 and 2. DNA-binding studies were carried out with ³²P-labelled DNA probes (30 000 cpm) and 10 ng Mxr1p

DTT, 100 ng poly(dI–dC), 0.05% NP-40 and 6% glycerol) for 30 min at 4 °C. In some experiments, Mxr1p was incubated first with excess unlabelled oligonucleotides for 5 min prior to the addition of ³²P-labelled DNA probes. The reaction mixtures were subjected to electrophoresis at 4 °C on 5% non-denaturing polyacrylamide gel in a buffer containing 7 mM Tris–HCl, pH 7.4, 3 mM boric acid and 1 mM EDTA. The gels were dried and autoradiographed.

Results and discussion

We recently demonstrated that Mxr1p binds at six different regions in the *P. pastoris* *AOXI* promoter, each of which consists of a core 5'-CYCC-3' motif (Kranthi *et al.*, 2009). Mutations within this core motif abolishes Mxr1p binding to DNA and this core motif is essential but not sufficient for Mxr1p binding (Kranthi *et al.*, 2009). We reasoned

that the core 5'-CYCC-3' motif may be a common element of Mxr1p-binding sites of promoters of other genes of methanol utilization pathway as well. To examine this, we selected the promoters of genes encoding *DHAS* and *PEX8*, since methanol-inducible expression of both these genes is abrogated in *mxr1* mutant strains (Lin-Cereghino *et al.*, 2006). The *DHAS* promoter (–980 to –1 bp) and *PEX8* promoter (–1000 to –1 bp) contain 13 and 11 5'-CYCC-3' motifs, respectively (Figures 1, 2). To examine whether DNA sequences containing these motifs bind Mxr1p, specific oligonucleotide primers were designed to amplify different promoter regions of *DHAS* and *PEX8* by PCR from *P. pastoris* genomic DNA (Figures 1, 2) to generate PCR products (DHAS1–DHAS5 and PEX8-1–PEX8-5) containing varying units of 5'-CYCC-3' motifs (Figures 1, 2). These DNA fragments were radiolabelled and their ability to bind to Mxr1p was examined by electrophoretic

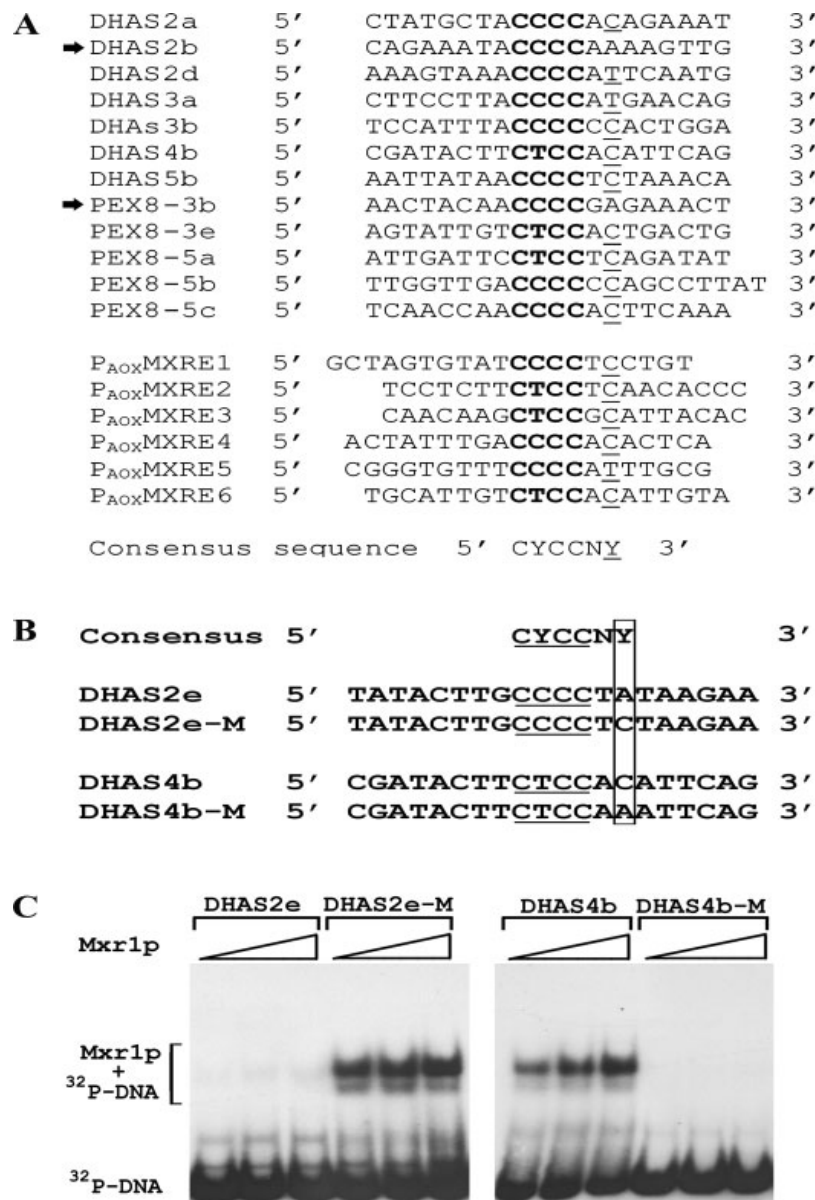
Mxr1p-binding to *Pichia pastoris* DHAS and PEX8 promoters

Figure 4. Identification of a consensus sequence for Mxr1p-binding. (A) DNA sequences in the AOX1, DHAS and PEX8 promoters that bind to Mxr1p are indicated. The core 5'-CYCC-3' motif is shown in bold face. The pyrimidine nucleotide which is conserved in all the DNA sequences except DHAS2b and PEX8-3b (arrows) is underlined. The P_{AOX}MXREs have been described recently by Kranthi *et al.* (2009). (B) Nucleotide sequence of DHAS2e, DHAS2e-M, DHAS4b and DHAS4b-M oligonucleotides. The core 5'-CYCC-3' sequence is underlined. The critical nucleotide outside the core motif vital for Mxr1p-binding is boxed. (C) Demonstration of the importance of the conserved pyrimidine residue outside the core sequence for Mxr1p-binding by EMSA. DNA-binding studies were carried out with ³²P-labelled DNA probes (30 000 cpm) and 10 ng Mxr1p. DHAS2e-M represents the DHAS2e sequence carrying the T → A mutation. DHAS2b-M represents the DHAS2e sequence carrying the C → A mutation



Figure 5. *In silico* analysis of promoters of *FLD1* and *PEX14*. Promoter regions containing the core 5'-CYCC-3' sequence or its complement (underlined) are boxed. Potential Mxr1p-binding sites which conform to the consensus Mxr1p-binding site (5'-CYCCNY-3') are marked by *

mobility shift assay (EMSA). The results indicate that a shift in the mobility of DNA probes due to Mxr1p binding was observed in the cases of *DHAS2*, *DHAS3*, *DHAS4* and *DHAS5* as well as *PEX8-3* and *PEX8-5* probes, which contain at least two 5'-CYCC-3' sequences (Figure 3A). Mobility shift of DNA probes was not observed in case of *DHAS1* and *PEX8-2*, which lack 5'-CYCC-3' motifs, as well as *PEX8-1* and *PEX8-4*, containing only one 5'-CYCC-3' motif (Figure 3A). These results are consistent with our earlier studies on the *AOX1* promoter, in which the 5'-CYCC-3' motif was shown to be an essential component of an Mxr1p-binding site (Kranthi et al., 2009). To further localize Mxr1p-binding sites within these promoter regions, oligonucleotides of ~20 bp length containing 5'-CYCC-3' sequences were

synthesized (Figure 1, 2) and their ability to bind to Mxr1p was evaluated by EMSA. The results presented in Figure 3B indicate that Mxr1p does not bind to all oligonucleotides containing the 5'-CYCC-3' motif, indicating that, in addition to the 5'-CYCC-3' motif, additional sequences flanking this core motif also play a key role in Mxr1p binding.

In a recent study based on the analysis of Mxr1p-binding to *AOX1* promoter sequences, we had identified the 5'-CYCC-3' motif as the consensus sequence for Mxr1p binding (Kranthi et al., 2009). In this study, we examined whether the *DHAS* and *PEX8* promoter sequences which bind Mxr1p conform to this consensus sequence. The results presented in Figure 4A indicate that all the DNA sequences that bind Mxr1p, except

DHAS2b and PEX8-3b, conform to the sequence 5'-CYCCNC-3' or 5'-CYCCNT-3', suggesting that the consensus sequence for Mxr1p binding is 5'-CYCCNY-3'. This was confirmed by modifying the non-binding sequence 5'-CCCCTA-3' in DHAS2e to 5'-CCCCTC-3' (DHAS2e-M) (Figure 4B). In an EMSA, Mxr1p-binding was observed only with DHAS2e-M but not DHAS2e (Figure 4C), indicating that DNA sequences containing the 5'-CYCCNY-3' motif are ideal for Mxr1p binding. Further, Mxr1p binding to DHAS4b is abolished when the sequence 5'-CTCCAC-3' is modified to 5'-CTCCAA-3'. Thus, the inability of Mxr1p to bind to PEX8-4 can now be explained based on the fact that it contains the 5'-CTCCCA-3' rather than the 5'-CTCCCY-3' motif. However, DHAS2c, DHAS3d and PEX8-3a as well as PEX8-1 are exceptions, as they do not bind Mxr1p despite containing the 5'-CYCCNY-3' motif, suggesting that DNA elements other than those identified in this study may also play a role in Mxr1p-binding to DNA. The strategy for identification of Mxr1p-binding sites described in this study is simple and versatile and can be used for the identification of Mxr1p-binding sites in any promoter of interest. Based on this study, we have carried out an *in silico* analysis of promoters of *FLD1* and *PEX14* genes and identified potential Mxr1p-binding sites within 1.0 kb upstream region (Figure 5). Promoter constructs carrying specific mutations in each of these Mxr1p-binding sites can be examined for their ability to activate reporter gene expression, and such studies can pave way for the identification of Mxr1p-binding sites that function as methanol response elements *in vivo*.

Acknowledgements

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