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Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the *MNN4* gene in *Saccharomyces cerevisiae*

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Abstract Mannosylphosphorylation is a major oligosaccharide modification that provides negative charge in the *Saccharomyces cerevisiae* cell wall. Although two genes, *MNN6* and *MNN4*, which encode a mannosylphosphate transferase and its putative positive regulator, respectively, are involved in this modification, the amount of Mnn4p has been found to be a limiting factor for mannosylphosphorylation. The level of mannosylphosphorylation increased at late-logarithmic and stationary phases of cell growth, and this was correlated to the transcriptional enhancement of *MNN4*. We also find that mannosylphosphate transfer to mannan is negatively regulated by the protein kinase A pathway, while the presence of 0.5 M potassium chloride enhanced *MNN4* transcription. This type of transcriptional regulation is observed in many stress response genes, implying that mannosylphosphate transfer is involved in the cellular response to a variety of stresses.

Key words: Mannosylphosphate; Gene regulation; Glycosylation; Stress response; *Saccharomyces cerevisiae*

1. Introduction

The cell wall is dynamic supramolecule that is essential for the cell integrity and the maintenance of cell shape, and its composition and architecture vary greatly depending on the environment and cell growth. In yeast *Saccharomyces cerevisiae* biosynthesis of the cell wall has been well studied [1]. There is a unique oligosaccharide modification, mannosylphosphorylation, in the cell wall mannoproteins of *S. cerevisiae* [2–4]. The mannosylphosphate moiety is transferred from GDP-mannose to *N*-linked oligosaccharides in the Golgi apparatus [5]. This modification gives a net negative charge to the yeast cell surface, although its biological function has yet to be determined.

We previously cloned two genes involved in mannosylphosphorylation, *MNN4* and *MNN6*, which are predicted to encode type II membrane proteins [6,7]. Mnn6p shows amino acid sequence similarity to α1,2-mannosyltransferase, Kre2p [8] and functions as a mannosylphosphate transferase [7], while Mnn4p is likely to function as a positive regulator, because the extent of mannosylphosphorylation catalyzed by Mnn6p is dependent on the positive effect of Mnn4p function [6].

We have found that mannosylphosphate transfer to cell wall mannans occurs at stationary phase, but not at the early stages of growth. Here, we report that the level of *MNN4* transcription is correlated with the extent of mannosylphosphorylation and that its transcription is enhanced by low intracellular cAMP level and by high extracellular osmolarity, in a similar fashion to the transcription of stress response genes [9]. This is a novel type of regulation for oligosaccharide modification, and suggests a close relationship between mannosylphosphorylation and stress response in *S. cerevisiae*.

2. Materials and methods

2.1. Strains, plasmids and media

All yeast strains used in this study were derived from G2-9/G2-10 background [10]. Wild-type cells, G2-9 (*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2*) and G2-10 (*MATα ura3 lys2 ade2 trp1 his3 leu2*), were provided by Dr. C.B. Hirschberg (University of Massachusetts Medical School). TOY2 strain was constructed by replacing the *MNN4 Pst*I-*Eco*T22I fragment with the *LYS2* gene in G2-10 [6]. XW13 strain was constructed by replacing *MNN6 Bgl*II-*Bcl*I fragment with the *ADE2* gene in G2-10 [7]. All gene replacements were confirmed by PCR [11] or Southern hybridization [12].

YEp24-PDE2 was provided by Dr. A. Toh-e (University of Tokyo). YEpT-TPK1 was given by Dr. T. Morishita (Osaka University) [13]. pCL-MNN4 and pEL-MNN4 were constructed by inserting the *MNN4 Nru*I-*Ehe*I fragment (4 kb) into pRS315 [14] and YEp351 [15], respectively. pRS-MNN6 and YEp-MNN6 were similarly constructed by inserting the *MNN6 Hpa*I-*Nru*I fragment (2.7 kb) into pRS316 [14] and YEp352 [15], respectively.

YPD and SD media [16] were used to cultivate yeast strains. Because the extent of mannosylphosphorylation was affected by the presence of adenine as a nutritional supplement (data not shown), adenine sulfate was omitted from the culture medium throughout this work. Yeast transformation was carried out by the lithium-acetate method [16].

2.2. Alcian blue staining

Standard method for alcian blue staining of yeast cells was as described by Friis and Ottolenghi [17]. Visual evaluation of cell staining was as previously described [6]. We have also developed a new method for the quantitative analysis of alcian blue binding to the specific amount of cells (A_{600} = 1.0) by measuring unbound alcian blue amount in the supernatant of binding assay, because alcian blue solution has an absorbance at 600 nm and the OD_{600} value is found to correlate with alcian blue concentration (61.3 μg alcian blue/OD_{600}). Culture was diluted to the OD_{600} of approximately 0.5 (dilution ratio is shown by d) and measured its cell density (OD_{600} c.c.). Cells of 1 ml culture were harvested and washed with 0.02 N HCl. Washed cells were suspended in 1 ml of 100 μg/ml alcian blue solution. After settling for 10 min, cells were collected by centrifugation at 25°C for 5 min at a maximum speed (15 000 rpm). The OD_{600} of the supernatant (OD_{600} sup) was measured, then the difference between original solution (OD_{600} ori) and supernatant (OD_{600} sup) was calculated according to the following formula:

$$T\ (\mu g) = 61.3 \times (OD_{600}\ \text{ori} - OD_{600}\ \text{sup}),$$

where T is a total amount of adsorbed alcian blue and alcian blue binding is represented by the following:

$$\text{Alcian blue binding}\ (\mu g/OD_{600}) = T \times d/OD_{600}\ \text{c.c.},$$

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Abbreviations: PKA, protein kinase A

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where d is a dilution ratio to measure diluted cell density (OD_{600} c.c.).

2.3. Northern blotting

Total RNA was extracted as described by McKinney et al. [18]. Other steps for Northern blot analyses were carried out according to Horecka and Sprague [19] and to Boehringer Mannheim's instructions for their DIG system. DIG-labeled RNA probes were generated using a DIG-RNA labeling kit (Boehringer Mannheim) and detected on blots using CDP-star (TROPIX).

3. Results and discussion

3.1. Mannosylphosphorylation occurs at stationary phase

To investigate a possible relationship between cell growth and mannosylphosphate transfer to cell wall mannans, we harvested yeast cells at various stages of cultivation and stained them with alcian blue dye. It was already reported that the extent of alcian blue staining of yeast cells correlated with the amounts of phosphate in the cell wall [17]. Overnight cultures of several strains were transferred into the fresh SD medium as a 1% inoculum and then cultivated at 25°C. Cell concentration was measured at 3 h intervals by photoelectric colorimeter (Klett-Summerson model 800-3, Klett MFG Co., USA). Cells from 1 ml culture were collected by centrifugation for alcian blue staining. The extent of mannosylphosphorylation was scored by the blue color intensity of stained cells (Fig. 1B). The wild-type strain (G2-10) showed no staining during the logarithmic growth phase (before 45 h) then increased its staining during the stationary phase (65 h to 96 h, Fig. 1A and B).

It was reported that the total glucan and mannan contents of the cell wall were little affected by growth rate, and that the phosphorus and protein content of cell wall increased as the growth rate decreased [20]. Therefore it is likely that a sufficient amount of the oligosaccharides of cell wall mannoproteins is produced at the earlier stages of growth to receive mannosylphosphate transfer. Taken together our results and data from previous reports indicate that cells in the early growth phase contain little mannosylphosphate in their *N*-linked oligosaccharides, but this amount is gradually increased as a result of mannosylphosphate transfer to the cell wall mannans during the late-logarithmic and stationary phases of cell growth.

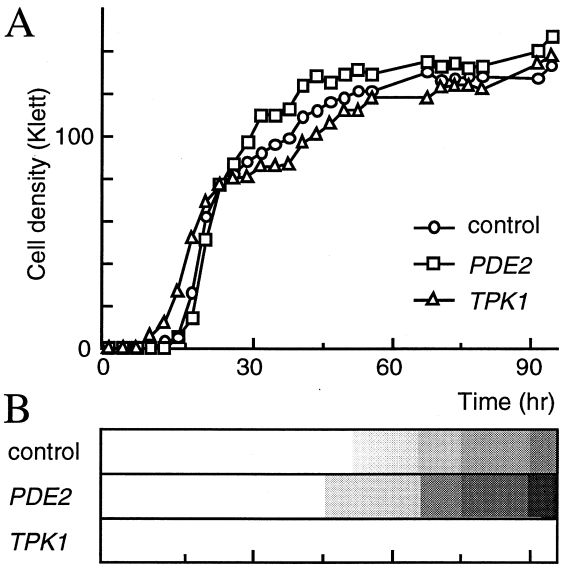

Fig. 1. Mannosylphosphorylation occurs at stationary phase and is affected by the cAMP pathway. A: Growth curves of yeast strains grown in SD medium at 25°C. Control, G2-10 cells containing vector only (circle); *PDE2*, G2-10 cells expressing *PDE2* (square); *TPK1*, G2-10 cells expressing *TPK1* (triangle). B: Alcian blue staining of cells from the experiment in A. The blue color intensity of stained cells is represented by a gray scale based on visual observation as described previously [6].

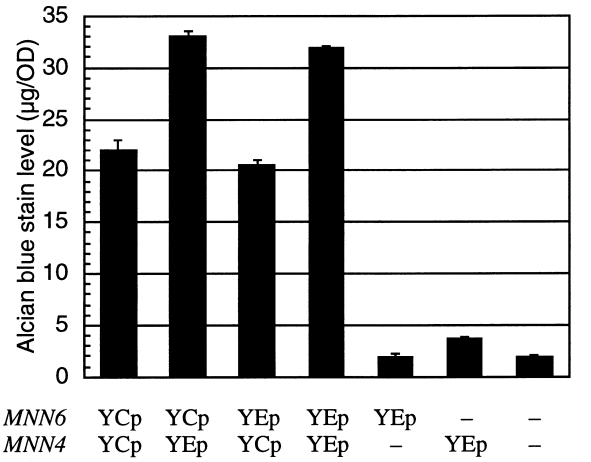

Fig. 2. The relationship between *MNN4* and *MNN6* with respect to alcian blue binding. TO29-1D cells (*Δmnn1 Δmnn4 Δmnn6*) harboring different combinations of single-copy and multi-copy *MNN6* and *MNN4* genes were grown in SD-uracil-leucine medium at 30°C for 24 h and then assayed for alcian blue binding. YCp and YEp indicate single-copy and multi-copy plasmids, respectively. Control multi-copy plasmid without *MNN4* or *MNN6* (YEp352 or YEp351) is shown by '–'. Alcian blue binding (μg/OD) shown by bar was scored based on the amount of dye binding to the cells according to the methods described in Section 2.

3.2. Kinase A related genes affect the level of mannosylphosphorylation

It is well known that intracellular cAMP level decreases at the stationary phase [21]. We have investigated whether genes involved in the cAMP pathway may affect the level of mannosylphosphorylation. Either *PDE2* (encoding high affinity phosphodiesterase) [22] or *TPK1* (encoding PKA catalytic subunit) [23] carried on high copy plasmids YEp24-PDE2 and YEpT-TPK1, respectively, was transformed into strain G2-10. Strains were then cultivated at 25°C and assayed for culture density and alcian blue binding. These strains did not show any significant differences in cell growth relative to the control strain (Fig. 1A). Mannosylphosphorylation was observed much earlier at 45 h in *PDE2* expressing cells (Fig. 1B), while *TPK1* expressing cells were not stained at all within the cultivation period of this experiment. These results suggest that low levels of intracellular cAMP may enhance mannosylphosphate transfer.

3.3. Mnn4p is a limiting factor for mannosylphosphorylation

mnn4 and *mnn6* mutants are known to be defective for mannosylphosphorylation [5,24]. We have cloned the corresponding genes and reported that Mnn6p is a mannosylphosphate transferase and requires Mnn4p for its function [6,7]. To investigate further a functional relationship between Mnn6p and Mnn4p and to test whether one or both of these

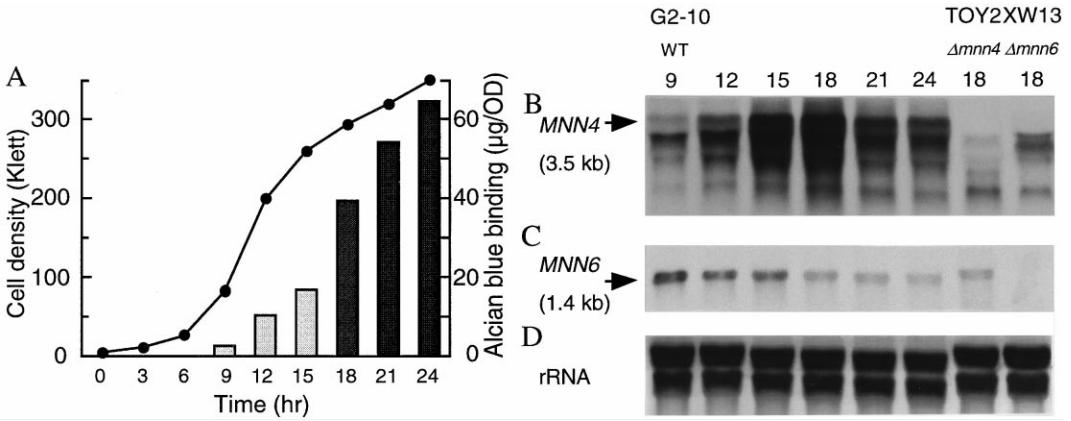

Fig. 3. Northern blot analyses of *MNN4* and *MNN6*. A: Growth curve and alcian blue binding of G2-10 cells in YPD medium at 30°C. Cell density (Klett) is represented by a curved line. Alcian blue binding shown by bar is determined by the same way as shown in Fig. 2. Visual score of staining is also indicated as gray scale in the bars. B–D: Northern blot analyses. Total RNA samples (10 μg) from G2-10 cells grown at 9–24 h and those from Δ*mnn4* cells (TOY2 strain) and from Δ*mnn6* cells (XW13 strain) were used for this experiment. DIG-labeled riboprobes for *MNN4* (*Bgl*II-*Xba*I 0.9 kb) and *MNN6* (*Xba*I-*Cla*I 0.5 kb) were used for hybridization. B: Probed with *MNN4* (2 h exposure). C: Probed with *MNN6* (10 min exposure). D: Methylene blue staining of rRNA (25 s and 18 s) as a control of loaded total RNA. Multiple lower bands observed for *MNN4* in panel B were derived mainly from partial degradation of the *MNN4* transcript, since they were mostly decreased in the Δ*mnn4* strain; the lowest band (about 0.5 kb) was non-specific since its amount was not changed in Δ*mnn4* strain.

genes was limiting for mannosylphosphate transfer, single-copy and multi-copy plasmids harboring *MNN6* and *MNN4*, were transformed into a Δ*mnn1* Δ*mnn4* Δ*mnn6* triple disruptant strain. Every transformant showed no significant differences in cell growth measured by the A_{600} after 24 h of culture. As shown by a quantitative alcian blue dye binding assay, mannosylphosphorylation required the co-existence of Mnn6p and Mnn4p (Fig. 2). Interestingly, in the presence of single-copy *MNN4*, the level of mannosylphosphorylation was not enhanced by the co-existence of multi-copy *MNN6* over the level conferred by single-copy *MNN6*, suggesting that Mnn6p is not limiting for mannosylphosphorylation. In contrast, multi-copy *MNN4* enhanced mannosylphosphate transfer relative to single-copy *MNN4*, and this enhancement was found to be the same whether cells carried either single-copy or multi-copy *MNN6*. Although we did not measure the correct copy number of *MNN4*, these data suggest that Mnn4p is the limiting factor relative to Mnn6p in this system. Mnn6p is a member of the Kre2p/Mnt1p mannosyltransferase family [7,25], but a requirement of another gene product, namely Mnn4p, for the sugar transfer is a unique feature of Mnn6p in this family, suggesting some specialized function of the Mnn6p in the mannosylphosphate transfer to cell wall mannans.

3.4. *MNN4* transcript increases at stationary phase

To investigate whether mannosylphosphorylation is regulated at the transcriptional level, we have carried out Northern analyses of *MNN4* and *MNN6* transcripts. Total RNA samples were prepared from cells grown for 9–24 h as described in Section 2 (Fig. 3A). The level of *MNN4* transcription was very low at 9–12 h but it increased at 15–18 h, the period where the mannosylphosphorylation occurred strongly (18 h, Fig. 3B). In contrast, *MNN6* transcription was fairly constant during the early phase of cell growth (9–15 h) and then decreased slowly at the late-logarithmic and stationary phases (18–24 h, Fig. 3C). These results indicate that mannosylphosphate transfer occurs at the late stage of cell growth at least due to the enhancement of *MNN4* gene expression, although a possibility of further translational and/or post-translational regulation was not completely excluded. This type of transcriptional regulation is similar to that of various stress response genes containing a stress response element (STRE) in their upstream region [9], suggesting the possibility of some relationship between cellular stress response and mannosylphosphate transfer to cell wall mannans. Indeed, the upstream region of *MNN4* gene contains a STRE core consensus sequence (CCCCT) (Fig. 4). An additional STRE like sequence (CCCTCT) was also located around the −270 position from the start codon (ATG). These sequences might function as a *cis*-acting element for the *MNN4* transcription we observed at stationary phase. A poly-A-rich stretch was also observed between −233 and −194 nucleotides, which is known to act as an element for constitutive transcriptional enhancement due to the poor chromatin formation in this region [26].

3.5. Osmotic stress enhances mannosylphosphorylation

Since *mnn4-1* original mutant was known to change its dominant-recessive phenotype in the presence of either 0.5

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TCGCGATGCA ATGGGCTTGG TATTCCGTGG AAGAAAATGA AAACCGGCTG CAAAGAATCC -477
TCCGAGAAAA TTTGATGCGG TGCACGGATT TCAGCACGGG TTTCAGCACG GATTTTATTT -417
CTTTGCACCC CGCAAATTTC TGGGCGATGA TAAACATACC GCATCTAAAA CCGTATACAG -357
ACTACGGCAC AGAAGCCAAG ACTAACAAAA AAAAAAAAAA AAAAAAAAAA GAAAAAAGAA -197
AAATGTCACG TGTTATTCCT TGTGCCCTCT GACCTACGTA GTTACTTGCG TGGCTTCAAA -237
TTTGATATCA GCTCTCGAGA CGTCCTGATC TCTATACCTT ATTGCTTGCT TTAGGTTGAG -177
GCAATAAAGA AATAATTACA TCTTTGGTTC TTTCCCCCTT TTTATATATC TGCAACAATA -117
TTAAACAATT AAATTAAAGA AGCACGTTTC CGTTTTTTAT CATATACCAT TCCCCTAACC -57
GCCGAACTCA CCGCATCACA ACGTCACTAT TCCTTCACAC AAATAAACTA ATTAGTTATG +3
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Fig. 4. Upstream and promoter sequence of the *MNN4* gene. Translation initiation codon (ATG) is boxed. The STRE core consensus sequence (CCCCT) and resembling sequence (CCCTCT) are indicated by an open box and a single underline, respectively. The poly-A stretch [26] is indicated by a dotted line. The sequence shown here is part of entire *MNN4* gene sequence; accession number (EMBL/GenBank/DDBJ) is D83006.

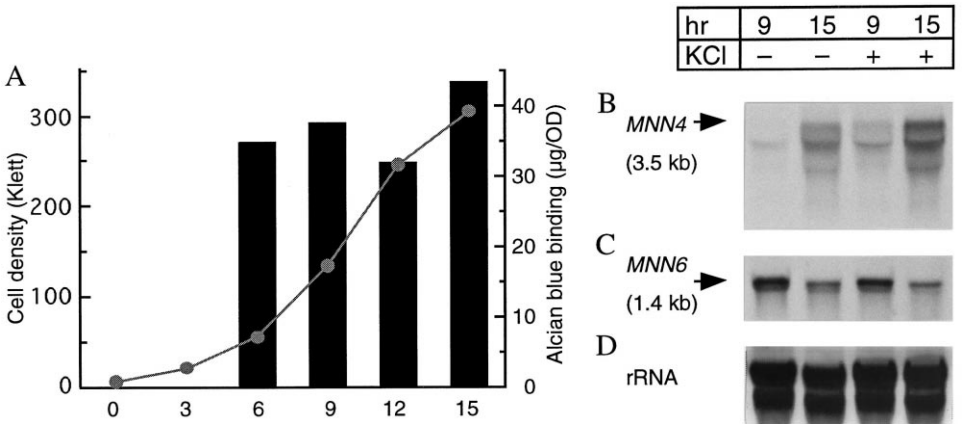

Fig. 5. Osmotic stress enhances *MNN4* transcription and mannosylphosphorylation. A: Growth curve and alcian blue binding of G2-10 cells grown in YPD plus 0.5 M KCl at 30°C. Cell density and alcian blue binding were performed as in Fig. 3A. B–D: Northern blot analyses of RNA samples (10 μg) from cells grown at 9 and 15 h in YPD medium with or without 0.5 M KCl. B: Probed with *MNN4* (30 min exposure). C: Probed with *MNN6* (10 min exposure). D: Methylene blue staining of rRNA (25 s and 18 s) as a control of loaded total RNA.

M KCl or 1 M sorbitol [4], high osmolarity condition was used to investigate further an osmotic stress response of mannosylphosphorylation. In the presence of 0.5 M KCl in YPD medium, mannosylphosphorylation was observed at the earlier stage of cell growth (6–9 h, Fig. 5A). In a Northern blot experiment using G2-10 cells, *MNN4* mRNA levels of cells grown at 9 h in YPD plus 0.5 M KCl medium was significantly higher than that of cells grown at 9 h in YPD medium alone (Fig. 5B). The level of *MNN6* transcription was not affected by the presence or absence of 0.5 M KCl (Fig. 5C).

3.6. Conclusion

The regulation of mannosylphosphate transfer to mannan is found to be different from the regulation of other neutral mannan or glucan syntheses, which are known to be regulated transcriptionally in a cell cycle dependent manner [27–30]. We have found that *MNN4* transcription is regulated in a manner similar to the STRE-mediated transcriptional regulation observed in many stress genes [9]. Mannosylphosphate transfer is a unique modification that gives a net negative charge to the yeast cell wall. Since the mannosylphosphate transfer is enhanced by a high osmolarity stress, one of its possible functions is to make a hydration shell in the cell surface to protect cells or certain proteins from a high salt stress.

It is known that the cells at stationary phase are more resistant than cells at logarithmic phase to the cell wall digesting enzyme, Zymolyase [31], and that the changes in resistance to Zymolyase is mainly caused by the changes in oligosaccharide modifications [32]. However, it is still unclear whether mannosylphosphorylation itself may participate in the cell wall integrity, in terms of Zymolyase sensitivity, because mannosylphosphorylation is also accompanied with changes of protein components in the cell wall mannoproteins during the cell cultivation. It will be interesting to investigate the possibility that the mannosylphosphate content in the oligosaccharides may affect the covalent crosslinking between glucan and mannoproteins to make a more rigid structure of the cell wall at stationary phase.

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