Schizosaccharomyces pombe UDP-galactose transporter: identification of its functional form through cDNA cloning and expression in mammalian cells

Hiroaki Segawa[a], Nobuhiro Ishida[a], Kaoru Takegawa[b], Masao Kawakita[a,*]

[a] *Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), 18-22, Honkomagome 3-chome, Bunkyo-ku, Tokyo 113-8613, Japan*
[b] *Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan*

Received 19 March 1999; received in revised form 20 April 1999

Abstract The *Schizosaccharomyces pombe* UDP-galactose transporter cDNA (SpUGT cDNA), encoding the product of the *gms1*$^+$ gene which consists of two exon sequences separated by a 173-bp intron, was cloned by RT-PCR. Its product, a hydrophobic protein of 353 amino acid residues resembling its human counterpart, was expressed in the Golgi membranes of UDP-galactose transporter-deficient Lec8 cells, and complemented the genetic defect of the mutant cells. This indicated that SpUGT cDNA encodes the functional *S. pombe* UDP-galactose transporter. The product of an ORF found in the second exon, which was previously assumed to be the *S. pombe* UDP-galactose transporter, thus represents an inactive, truncated form of the SpUGT protein.

Key words: UDP-galactose transporter; Galactomannan synthesis (*gms*) gene; Reverse transcription polymerase chain reaction; *Griffonia simplicifolia* lectin II; Lec8 cell

1. Introduction

Nucleotide-sugar transporters are localized in the membranes of the Golgi apparatus, and provide various glycosyl transferases in the Golgi lumen with appropriate substrates for their reactions (for recent reviews see [1,2]). Recently, several nucleotide-sugar transporter cDNAs have been cloned. These include the human UDP-galactose (UDP-Gal) transporter [3,4], the human, murine and hamster CMP-sialic acid transporters [4–6], the human, canine and yeast UDP-*N*-acetylglucosamine transporters [7–9], and the yeast and protozoan GDP-mannose transporters [10,11]. These cDNAs encode highly hydrophobic proteins with multiple putative transmembrane helices. Several of these were in fact shown to be expressed in the Golgi membranes, where they were active in transporting specific substrates [7,10–14].

Recently, the *gms1*$^+$ gene of *Schizosaccharomyces pombe* has been reported to be the fission yeast UDP-Gal transporter gene, and a 1.4-kbp DNA fragment containing an open reading frame (ORF) encoding a putative membrane protein has been presumed to define the coding region of the fission yeast UDP-Gal transporter [15]. Its expected amino acid sequence is in fact similar to that of the human UDP-Gal transporter (hUGT) throughout the overlapping region. However, the predicted protein is considerably shorter than the hUGT protein, and lacks the characteristic N-terminal portion of hUGT, which contains putative transmembrane helix 1 (TM1) located next to the N-terminal loop. Moreover, the minimal DNA fragment covering the entire ORF did not correct the genetic defect of the *gms1* mutant, although longer DNA fragments did [15]. This led us to presume that a missing exon might be present in the *S. pombe* genome upstream of that ORF.

In this report, we describe the isolation and characterization of a cDNA encoding the functional *S. pombe* UDP-Gal transporter (SpUGT), that is gms1$^+$p. In this cDNA, an hUGT-TM1-homologous stretch located some 200 bp upstream of the afore-mentioned ORF, which is designated below SpUGT(ΔTM1) ORF, was joined in-frame to it to form a longer ORF. Upon introduction into UDP-Gal transporter-deficient CHO Lec8 cells, the SpUGT cDNA, but not the SpUGT(ΔTM1) cDNA, corrected the mutant phenotype.

2. Materials and methods

2.1. Strains and cell culture

S. pombe strain TY741 (*h*$^-$ *ade6-M216 leu1 ure4-D18*) was obtained from Dr. M. Yamamoto (University of Tokyo, Japan) and grown in YPD medium. Lec8 (ATCC CRL1737) was maintained in minimum essential medium α (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum.

2.2. Antibodies and Griffonia simplicifolia lectin II (GS-II)

These were purchased from the following sources: polyclonal rabbit anti-HA antibody, Medical and Biological Laboratories, Nagoya, Japan; rat anti-HA monoclonal antibody (3F10), Roche Diagnostics, Basel, Switzerland; horseradish peroxidase-conjugated anti-rat IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA; fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, ICN Pharmaceuticals Inc.-Cappel Products, OH, USA; Alexa 594-conjugated goat anti-rat IgG, Molecular Probes, Eugene, OR, USA; FITC-conjugated GS-II, EY laboratories, CA, USA.

2.3. Oligonucleotides

NI412 (5′-GAATTCAGTAATGAGAAAATGGCTGTC) and NI371 (5′-GGCGGCCGCTTAATGCTTATGATCAACGTCCTT) were designed as putative *S. pombe* UDP-Gal transporter upstream and downstream primers, respectively. NI370 (5′-CCGAATTCAACATGCCTGGATATGATGATAAAC) was designed as a SpUGT(ΔTM1) ORF upstream primer. An influenza virus hemagglutinin (HA)-tag-containing primer, NI436 (5′-GGCGGCCGCTTAGGCGTAGTCAGGGACGTCGTAAGGGTAATGCTTATGATCAACGTCCTT), was used to introduce an HA tag into the SpUGT ORF and SpUGT(ΔTM1) ORF products. NI412 and NI370 each contain an *Eco*RI restriction site, and NI371 and NI436 each contain a *Not*I restriction site (underlined in the nucleotide sequences above).

2.4. RT-PCR

The poly(A)$^+$ RNA was prepared from transfected Lec8 cells using

*Corresponding author. Fax: (81) (3) 3823-2965.
E-mail: kawakita@rinshoken.or.jp

PII: S0014-5793(99)00596-7

a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). Total RNA was prepared from *S. pombe* strain TY741 using Isogen (Nippon Gene, Tokyo, Japan). The RNA was treated with DNase I prior to use for RT-PCR, which was carried out using a Titan RT-PCR kit (Roche Diagnostics).

2.5. DNA sequencing

The nucleotide sequences of both strands of PCR products were determined by the dideoxy chain termination method [16] using a Thermo Sequenase dye terminator cycle sequencing pre-mix kit II (Amersham Pharmacia Biotech) with an ABI Prism 377 sequencer (Perkin-Elmer Applied Biosystems). At least three independent clones from a PCR product were sequenced in order to exclude errors introduced by PCR.

2.6. Western blot analysis

Western blot analysis was performed as previously described [13]. The HA-tagged proteins were detected with anti-HA monoclonal antibody 3F10.

2.7. Lectin staining and indirect immunofluorescence staining

Cells were transfected with appropriate expression vectors using LipofectAMINE reagent (Life Technologies), following the manufacturer's instructions. Twenty-four hours after transfection, the cells were transferred onto a coverslip, grown for another 24 h at 37°C under 5% CO_2, washed once with phosphate-buffered saline (PBS), and then fixed with 3.7% formaldehyde-sodium phosphate buffer at room temperature for 30 min. The fixed cells were washed twice with PBS, and then permeabilized with PBS-0.1% Triton X-100 at room temperature for 30 min. The cells were treated with FITC-conjugated-GS-II (1:50) at 37°C for 30 min, washed twice with PBS, further treated with the anti-HA antibody (1:100) at 37°C for 1 h, and then washed twice with PBS. Finally, the cells were incubated with anti-rat IgG-Alexa 594 (1:100) at 37°C for 1 h, washed twice with PBS and once with water, and then mounted with Permafluor (Immunotech, Marseille, France). Fluorescence labeling was visualized under a laser scanning confocal microscope (Carl Zeiss LSM510).

3. Results

3.1. Cloning of S. pombe UDP-Gal transporter cDNA

An ORF found in the *gms1*$^+$ locus, which we call SpUGT(ΔTM1) ORF here, was previously proposed to be the coding region of the *S. pombe* UDP-Gal transporter [15]. However, the minimal DNA fragment covering it did not complement the defect of the *gms1* mutant (see Fig. 1A in [15]). We therefore presumed that the ORF might represent an N-terminally truncated form of the *S. pombe* UDP-Gal transporter. Interestingly, the *S. pombe* genomic DNA sequence determined by Lyne et al. at Sanger Center (GenBank/EMBL/DDBJ accession number AL022598) suggested the occurrence of a putative exon upstream of that ORF. The exon exhibited significant similarity to the N-terminal portion of hUGT. Since the nucleotide sequence of the putative intron between the new exon and the exon containing the entire SpUGT(ΔTM1) ORF fulfilled the GT-AG rule [17], we tested whether this putative intron could be recognized and excised in mammalian cells.

A 1.2-kbp fragment was isolated from *S. pombe* genomic DNA by PCR using NI412 and NI371. The fragment contained the SpUGT(ΔTM1) ORF and a 314-bp extension into its upstream region that include the putative exon and intron sequences, and was designated the *UGT* gene fragment. The

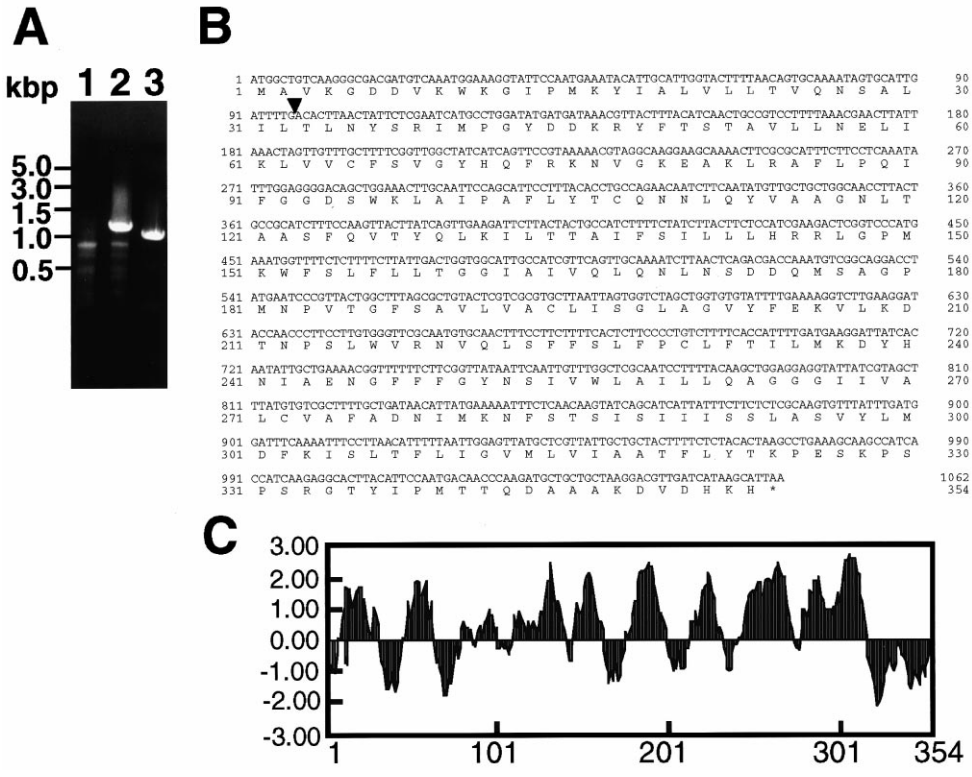

Fig. 1. Cloning of *S. pombe* UDP-Gal transporter. A: Agarose gel electrophoresis of RT-PCR products. RT-PCR was carried out with poly-(A)$^+$ RNA prepared from pMKIT-neo-transfected (vector control) (lane 1), or pMKIT-neo-*UGT*-transfected (lane 2) Lec8 cells, and total RNA prepared from wild-type *S. pombe* cells (lane 3) as templates. B: Nucleotide and deduced amino acid sequences of *S. pombe* UDP-Gal transporter. DDBJ/EMBL/GenBank accession number of the nucleotide sequence is AB023425. The arrowhead indicates the junction of the first and the second exons. C: Kyte-Doolittle hydrophobicity plot [21] of *S. pombe* UDP-Gal transporter. The plot was calculated with a window size of 12 amino acids.

expression vector pMKIT-neo-*UGT* was prepared by insertion of this fragment, and was introduced into UDP-Gal transporter-deficient Lec8 cells. Poly(A)$^+$ RNA was then prepared from the transfected cells. We also extracted total RNA from wild-type *S. pombe* cells to see if the putative intron between the two hUGT-homologous sequences was spliced out in the yeast cells. RT-PCR was carried out using NI412 and NI371 as primers.

As shown in Fig. 1A, specific PCR products were detected using poly(A)$^+$ RNA from Lec8 cells transfected with pMKIT-neo-*UGT* (lane 2), and total RNA from the wild-type *S. pombe* cells (lane 3) as templates. In lane 2, a 1.2-kbp band (the size corresponding to the unspliced transcript) was detected. In contrast, RT-PCR products from *S. pombe* total RNA gave a 1.0-kbp band (lane 3). The size of this band was that expected for the product obtained after the excision of the putative intron. The 1.0-kbp RT-PCR product was cloned into pMKIT-neo between the *Eco*RI and *Not*I sites, and the nucleotide sequence of the insert was determined. The sequence shown in Fig. 1B indicates that the putative intron was precisely spliced out, and the hUGT-homologous upstream stretch was joined in-frame to the SpUGT(ΔTM1) ORF to form a novel ORF (SpUGT ORF) encoding 353 amino acids. These results indicated that SpUGT ORF was composed of two exon sequences. The yeast splicing signal was not recognized in mammalian cells. The protein encoded by this ORF is very hydrophobic, and has multiple putative transmembrane domains. Its hydrophobicity profile (Fig. 1C) is very similar to those of other nucleotide-sugar transporters.

3.2. Expression of *S. pombe* UDP-Gal transporter in Lec8 cells

We examined whether the products encoded by SpUGT and SpUGT(ΔTM1) ORFs were expressed in Lec8 cells. To facilitate the detection of the products, an HA tag was introduced by PCR at the C-terminal portions of SpUGT and SpUGT(ΔTM1) using NI412 and NI436, and NI370 and NI436 as primers, respectively. These DNA fragments were inserted into pMKIT-neo expression vector, and introduced into Lec8 cells.

As shown in Fig. 2A, both the HA-tagged SpUGT(ΔTM1) (lane 2) and the HA-tagged SpUGT (lane 3) proteins were detected in the lysates of transfected Lec8 cells probed with the anti-HA monoclonal antibody. The apparent molecular masses of the proteins were 33 and 36 kDa, respectively. This is consistent with the fact that the SpUGT ORF encodes a protein that is longer by 39 amino acids than the SpUGT(ΔTM1) product. The amount of the SpUGT ORF product expressed in Lec8 cells was about 5-fold more than that of the SpUGT(ΔTM1) ORF product.

The intracellular localization of these proteins and their ability to complement the UDP-Gal transport deficiency of Lec8 cells were examined in cells transiently expressing the ORF products. Cells transfected with an appropriate expression plasmid were doubly labeled with the anti-HA monoclonal antibody and FITC-conjugated GS-II lectin (FITC-GS-II). Lec8 cells efficiently bind FITC-GS-II, which recognizes the terminal GlcNAc residues on cell-surface glycoconjugates (data not shown, cf. [18]). If an HA-tagged, functional UDP-Gal transporter is expressed in Lec8 cells, the cells will be labeled with the anti-HA antibody, but not with FITC-GS-II. On the other hand, if the product of the HA-tagged cDNA is inactive in UDP-Gal transport, then the transfected cells will be labeled with both the anti-HA antibody and FITC-GS-II.

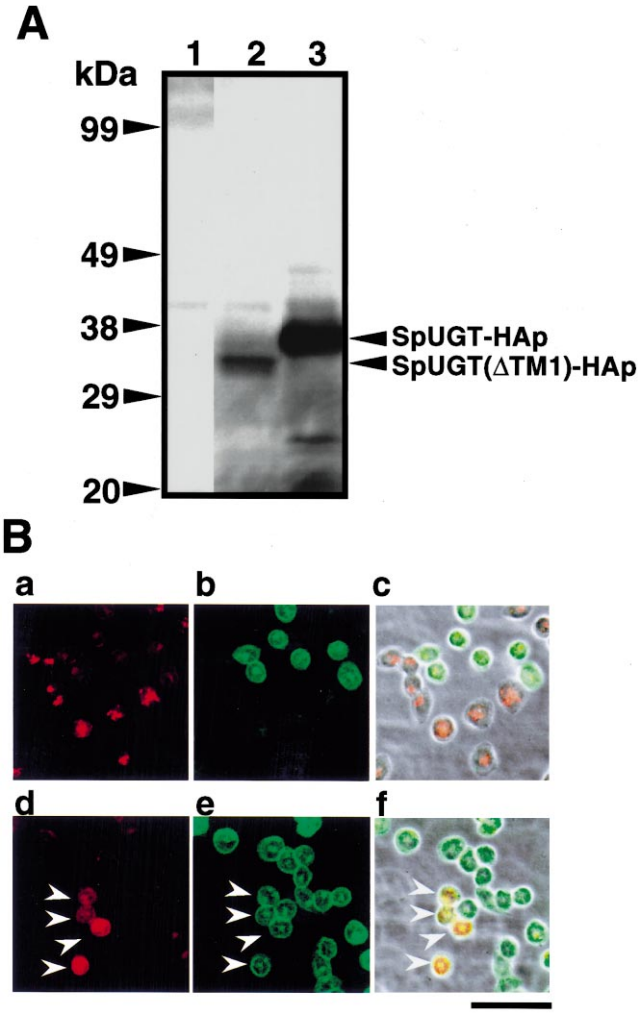

Fig. 2. Expression of *S. pombe* UDP-Gal transporter in mammalian cells. A: Western blot analysis. Lec8 cells were transfected with pMKIT-neo (lane 1), pMKIT-neo-SpUGT(ΔTM1)-HA (lane 2) or pMKIT-neo-SpUGT-HA (lane 3). Forty-eight hours after transfection, the cell lysates were prepared, and Western blot analysis was carried out. B: Analysis of the SpUGT ORF and SpUGT(ΔTM1) ORF products and binding of GS-II to the transfected Lec8 cells. Lec8 cells were transfected with pMKIT-neo-SpUGT-HA (a–c) or with pMKIT-neo-SpUGT(ΔTM1)-HA (d–f). Forty-eight hours after transfection, cells were examined for the binding of FITC-GS-II (b and e) and anti-HA monoclonal antibody 3F10 (a and d). The bound antibody was visualized with Alexa 594-conjugated anti-rat IgG. The fluorescence images were merged with the phase contrast images (c and f). The arrowheads in d–f indicate the cells expressing the HA-tagged SpUGT(ΔTM1) ORF product. Bar, 50 μm.

Panels a–c in Fig. 2B show that cells expressing the HA-tagged SpUGT ORF product, which was localized in the Golgi region, were not labeled with FITC-GS-II. The efficiency of transfection in this experiment was about 50%. On the other hand, FITC-GS-II was bound to cells expressing the HA-tagged SpUGT(ΔTM1) ORF product (Fig. 2B, d and e). This clearly indicates that the SpUGT ORF product, but not the SpUGT(ΔTM1) ORF product, was the functional UDP-Gal transporter of *S. pombe*, that is gms1$^+$p. The HA-tagged SpUGT(ΔTM1) ORF product was diffusely distributed, suggesting its occurrence in the ER (Fig. 2B, f). About 10% of the

total population of cells expressed the truncated protein. These results suggest that the N-terminal portion of SpUGT is important for its targeting to the Golgi apparatus as well as for its stability in the cell.

4. Discussion

We have isolated a cDNA clone that encodes the functional UDP-Gal transporter of *S. pombe*. In this cDNA clone, the ORF designated here SpUGT(ΔTM1) ORF and previously assumed to encode the transporter, is preceded by an upstream sequence joined in-frame to it to form an N-terminal extension of the coding region. The novel ORF encodes a hydrophobic protein of 353 amino acid residues that is similar to the human UDP-Gal transporter. The DNA sequence of the SpUGT ORF exactly matched the putative ORF in the *S. pombe* genome proposed recently by Lyne et al., which was predicted using the Genefinder program in PomBase with the aid of the Sp3splice program [19] to predict the branch-acceptor sites.

Both the SpUGT ORF and SpUGT(ΔTM1) ORF products were expressed heterologously in UDP-Gal transporter-deficient Lec8 cells, but only the former was able to complement the genetic defect of the mutant cells. This clearly indicates that SpUGT represents the genuine *S. pombe* UDP-Gal transporter and is, by definition, gms1$^+$p. Comparisons of amino acid sequences between *S. pombe* and mammalian UDP-Gal transporters revealed 40% and 43% identity with human [3] and murine (N. Ishida, unpublished data) UDP-Gal transporters, respectively. The functional implications of this identity and non-identity remain to be elucidated in future studies. The quick and convenient procedure for the assessment of the UGT activity devised in the present study would be useful in such studies directed toward the understanding of the structural basis of the UGT function. Mutants with attractive features may be selected by this procedure for isolation of stable transformants, which in turn may be utilized for further biochemical studies using Golgi-enriched membrane vesicles.

Functional expression of mammalian UDP-Gal, UDP-*N*-acetylglucosamine and CMP-sialic acid transporters in budding yeast cells has been reported [7,12,14,20]. We showed here that the HA-tagged SpUGT protein (gms1$^+$p) was successfully expressed in Lec8 cells, and was localized in the Golgi apparatus. To our knowledge, this is the first example of correctly targeting a multiple-membrane-spanning yeast Golgi protein to the mammalian Golgi apparatus. Essential features specifying the localization of Golgi membrane-transporter proteins are likely common to higher eukaryotes and yeasts. Systematic alteration of amino acids conserved between the fission yeast and mammalian UDP-Gal transporters will provide information about the molecular basis of the proper targeting and functioning of the transporter.

SpUGT(ΔTM1) protein lacks the first putative transmembrane helix of the *S. pombe* UDP-Gal transporter. It was expressed in Lec8 cells, but was inactive, and showed an abnormal intracellular distribution. This is consistent with our preliminary results indicating that the integrity of the transmembrane helices is important for the proper targeting of hUGT protein to the Golgi membranes as well as for its stability (K. Aoki, unpublished observation). Similarly, a chimeric protein in which the N-terminal portion of hUGT was replaced by the corresponding region of CMP-sialic acid transporter failed to be targeted to the Golgi apparatus (K. Aoki et al., manuscript in preparation). Targeting signals and sorting mechanisms of multiple-membrane-spanning Golgi proteins have remained quite obscure until now, and must be different from those of the type II transmembrane proteins analyzed so far in considerable detail. Chimeric molecules between human UDP-Gal transporter and *S. pombe* UDP-Gal transporter will serve as useful models for analyzing these challenging issues.

Acknowledgements: We are grateful to Dr. K. Maruyama for providing us with pMKIT-neo plasmid. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Area No. 10178220 from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from Kirin Brewery Co., Japan.

References

[1] Hirschberg, C.B., Robbins, P.W. and Abeijon, C. (1998) Annu. Rev. Biochem. 67, 49–69.
[2] Kawakita, M., Ishida, N., Miura, N., Sun-Wada, G.-H. and Yoshioka, S. (1998) J. Biochem. 123, 777–785.
[3] Miura, N., Ishida, N., Hoshino, M., Yamauchi, M., Hara, T., Ayusawa, D. and Kawakita, M. (1996) J. Biochem. 120, 236–241.
[4] Ishida, N., Miura, N., Yoshioka, S. and Kawakita, M. (1996) J. Biochem. 120, 1074–1078.
[5] Eckhardt, M., Mühlenhoff, V., Bethe, A. and Gerardy-Schahn, R. (1996) Proc. Natl. Acad. Sci. USA 93, 7572–7576.
[6] Eckhardt, M. and Gerardy-Schahn, R. (1997) Eur. J. Biochem. 248, 187–192.
[7] Ishida, N., Yoshioka, S., Chiba, Y., Takeuchi, M. and Kawakita, M. (1999) J. Biochem. (in press).
[8] Guillen, E., Abeijon, C. and Hirschberg, C.B. (1998) Proc. Natl. Acad. Sci. USA 95, 7888–7892.
[9] Abeijon, C., Robbins, P.W. and Hirschberg, C.B. (1996) Proc. Natl. Acad. Sci. USA 93, 5963–5968.
[10] Dean, N., Zhang, Y.B. and Poster, J.B. (1997) J. Biol. Chem. 272, 31908–31914.
[11] Ma, D.Q., Russell, D.G., Beverley, S.M. and Turco, S.J. (1997) J. Biol. Chem. 272, 3799–3805.
[12] Berninsone, P., Eckhardt, M., Gerardy-Schahn, R. and Hirschberg, C.B. (1997) J. Biol. Chem. 272, 12616–12619.
[13] Yoshioka, S., Sun-Wada, G.-H., Ishida, N. and Kawakita, M. (1997) J. Biochem. 122, 691–695.
[14] Sun-Wada, G.-H., Yoshioka, S., Ishida, N. and Kawakita, M. (1998) J. Biochem. 123, 912–917.
[15] Tabuchi, M., Tanaka, N., Iwahara, S. and Takegawa, K. (1997) Biochem. Biophys. Res. Commun. 232, 121–125.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. and Chambon, P. (1978) Proc. Natl. Acad. Sci. USA 75, 4853–4857.
[18] Ishida, N., Ito, M., Yoshioka, S., Sun-Wada, G.-H. and Kawakita, M. (1998) J. Biochem. 124, 171–178.
[19] Chen, T. and Zhang, M.Q. (1998) Yeast 14, 701–710.
[20] Kainuma, M., Ishida, N., Yoko-o, T., Yoshioka, S., Takeuchi, M., Kawakita, M. and Jigami, Y. (1999) Glycobiology 9, 133–141.
[21] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.