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Full Length Research Paper

A study of how a Chinese Hamster Ovary cell line can translate a functional recombinant Dsn1p from the yeast

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Dsn1p is a component of the mind complex (Mtw1p Including Nnf1p-Nsl1p-Dsn1p) that is essential for the segregation of chromosome in yeast cells. This protein assists the joining of kinetochore subunits contacting DNA to those contacting microtubules in yeast cells. Null mutants of this protein are not viable while the over-expression of the Dsn1 protein in yeast cells results in nuclear anomalies and growth defects. In this paper, we show that tetrad analysis indicates haplo-insufficiency and segregational errors in yeast diploid single deletants. Expression of Dsn1p in CHO has been achieved using the pcDNA 3.1/HIS A expression vector. Analysis by DNA sequencing showed no changes in the DSN1 DNA sequence. We also showed that nuclear fragmentation and cellular morphological changes takes place in CHO cell line that were successfully transfected with the pcDNA 3.1/HIS A expression vector containing the Dsn1p.

Key words: *Saccharomyces cerevisiae*, kinetochore, Dsn1p, Chinese Hamster Ovary (CHO).

INTRODUCTION

DSN1, dosage suppressor gene for the *NNF1* (need for nuclear function-1), gene is a member of the MIND complex that form part of the gigantic kinetochores complexes with an estimated size of 5MDa (Demarcq et al., 1994). The kinetochores function in ensuring that the microtubules of the spindle can properly attach to the centromere of the chromosomes by acting like a bridge between the two structures. This, in turn will enable the accurate segregation of the sister chromatids during cell division (Wulf et al., 2003).

In our previous reports, we showed that it was necessary for the Dsn1p to maintain its stoichiometry in the *Saccharomyces cerevisiae*. A variation in the gene dosage can cause nuclear anomalies and growth retardation (Yiap et al., 2004; Yiap et al., 2004b). Dosage sensitivity refers to the effect of a gene and its gene products are dependence of its gene copy number and hence it's translational level (Sambrook et al., 1992). This phenomenon is built on the basis of "Balance Hypothesis," which states that the imbalance in the subunits of a protein-protein complex can be detrimental (Lock and Ross, 1990).

Currently there is little information on the presence of a nuclear homologue in other eukaryotic cells with similar function as Dsn1p in the budding yeast. It is the objective of this study to determine if Chinese Hamster Ovary (CHO) cell line can successfully translate a functional recombinant Dsn1p from the yeast.

MATERIALS AND METHODS

Polymerase chain reaction (PCR)

PCR amplifications of the genes were performed in 20 ∝l reaction volumes in the presence of 1X *Pfu* PCR buffer with 1.5 mM $MgCl_2$

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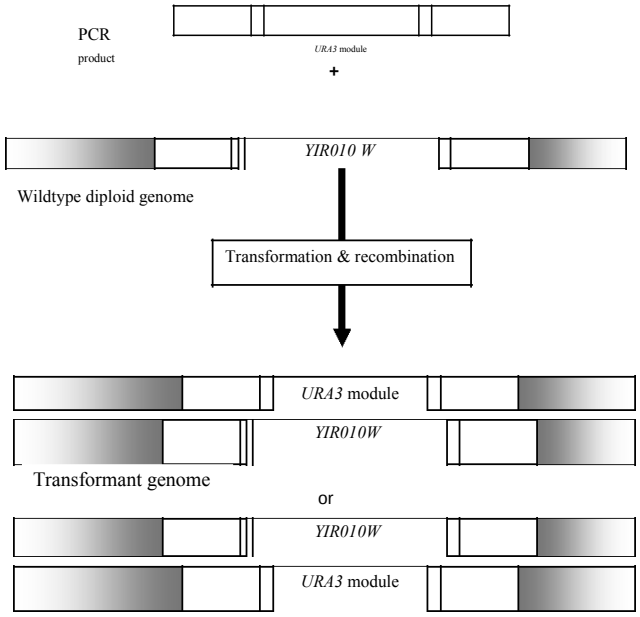


Figure 1. Diagrammatic representation of homologous recombination of the deletion construct (*URA3* module) replacing one copy of the genes (*YIR010W*) in a diploid genome upon transformation into the cell.

(Fermentas), 2.5 U of *Pfu* DNA polymerase (Fermentas), 1.5 mM NH_2SO_4, 1 mM of each dNTPs (Fermentas), 0.5 ∝M of each primers and 100 ng of DNA as template. The Hot-Start method was employed and 35 cycles of amplification were performed in the following condition (unless otherwise stated):5 min at 95°C, 1 min at 95°C, 1 min at 55 - 65°C, 3 min at 72°C and additional final extension at 72°C for 10 min. The PCR reaction was done in the MasterCycler thermal cycler machine (Eppendorf).

Transformation of *S. cerevisiae*

A modified method was used for routine transformation (Lock and Ross, 1990). A single colony of yeast cell was inoculated into 5 ml of YPD liquid medium and incubated overnight with vigorous shaking. Fresh culture was inoculated in new YPD broth for several division of growth until the cell titre reached 2 x 10^7 cells/ml. Cells were harvested by centrifugation at 3000 g for 5 min, washed with sterile distilled water and re-suspended in 100 mM lithium acetate/TE solution to a final cell concentration of 2x 10^9 cells/ml. This was incubated at 30°C for an hour. Cells were then pelleted and re-suspended in 1 ml lithium acetate, 20% (v/v) glycerol before being stored at -80°C in 50 µl aliquots.

For each transformation, each aliquot was mixed with the following recipe: 240 µl polyethyleneglycol 50% (w/v), 36 µl 1M lithium acetate pH 7.5, 36 µl 1M Tris – EDTA pH 7.5, 10 µl single stranded carrier DNA (boiled) (10 µg/ml), 5 – 10 µl transforming DNA (up to 5 µg) and top up to 360 µl with sterile distilled water.

This mixture was incubated at 30°C for an hour with occasional gentle tapping of tube to prevent clumping of cells. Then 36 µl of DMSO was added and mixed by inversion. The yeast cells were heat shocked at 42°C for 30 min. The cells were then collected by centrifugation and transferred to YPD broth for culturing at 30°C for 2 – 3 h with vigorous shaking. They were then plated on selective medium for 3 – 4 days incubation at 30°C before the appearance of transformants.

Sporulation and tetrad dissection

All diploids were first cultured in presporulation medium at 30°C for 2 days before being transferred to sporulation medium and incubated for 3 – 4 days at 30°C.

A loopful of cells and ascus mixture was transferred into sterile distilled water containing 0.3 mg/ml Zymolyase (20T) and 0.5% (w/v) β -Mercaptoethanol (BME). This was incubated at 35°C for a few minutes until the ascus wall has been visibly digested. The mixture was then spread on YPDA for tetrad dissection using Singer MSM System series 200 micromanipulator according to manufacturer's instructions (Demarcq et al., 1994).

Microscopy and DAPI staining

Bright field microscopic analyses of the cells were carried out using the Nikon T1-SM inverted microscope. Canon PowerShot S40 4.0MP PC1016 digital camera was used to take digital photographs of the cell cultures. All fluorescence microscopic studies were performed under the Leica DMLS fluorescence microscope using System A filter (UV excitation, BP=340-380, DM=400, LP= 425 nm). Image acquisition was completed using Evolution^{TM} MP Color 5.1MP digital camera by Media Cybernatics and processed by the IMAGE PRO EXPRESS software.

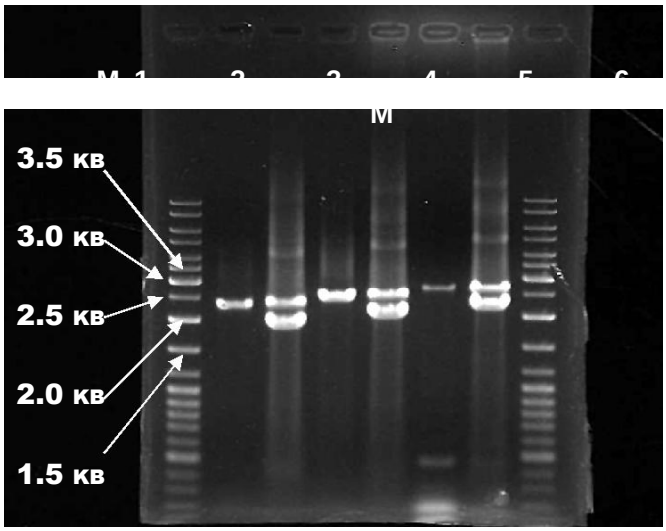
Sample preparation for DAPI staining was achieved through culturing the CHO cells on sterile glass cover slips. Ten-µl of trypsinized CHO (10^6 cells) was mixed with 90 µl DMEM + 10% FBS in a sterile 1.5 ml tube. 20 µl of the mixture was dropped onto the cover slip that was inside a sterile Petri dish and incubated at 37°C with 5% CO_2 for 4 h to achieve cell adherence (Bala'zs et al., 2003). Following this, 5 ml of DMEM supplemented with 10% FBS was added to the Petri dish before it was returned to the incubator and cultured until 80% of cell confluency was attained. A solution of the 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (ext= 358, em=461 nm) (Research Organics, USA) was prepared by dissolving this chemical in DMEM supplemented with 10% FBS to a final concentration of 1 µg/ml. For the purpose of DAPI staining, the medium covering the cover slips with adhering CHO cells was removed from the Petri dish. Later 20 µl of the DAPI solution was dropped onto the cover slip prior to returning to the incubation at 37°C with 5% CO_2 for 30 min (Mosser and Massie, 1994). The cover slip was then inverted and put onto a sterile glass slide for microscopic viewing.

RESULTS

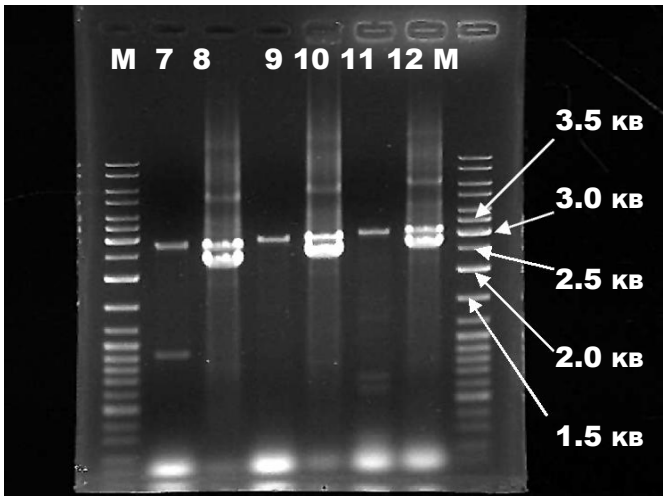
Gene deletion of DSN1 from *S. cerevisiae*

Gene deletion was achieved via homologous recombination of PCR product of deletion cassette. Figure 1 illustrated the final products of the process of transformation and recombination. The deletion cassette could replace either one of the DSN1 gene on the pair of chromosomes IX in the diploid yeast. Successful deletants were obtained through selection with a uracil dropout minimal synthetic medium as they would confer the ability to produce this nucleotide from the additional gene on the deletion cassette.

Second stage confirmation of gene deletion was done by using PCR amplification of the deletion cassette. A series of forward and reverse primers were utilized to cover the region within and flanking the deletion sites. As shown in Figure 2, all the odd-numbered lanes were PCR



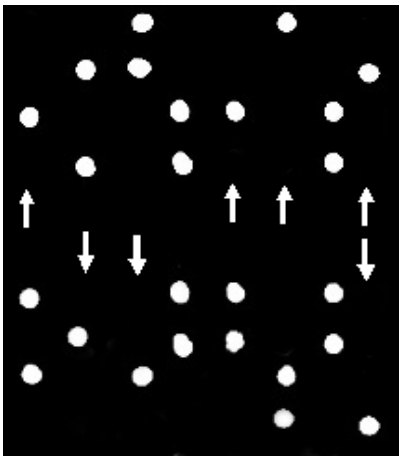
[a]



[b]

Figure 2. PCR confirmation of deletion. (a) and (b) Gel photos showing a double bands PCR products in diploid heterozygous single deletants (lanes 2, 4, 6) besides single banded PCR product in non-deletant wildtype control (lanes 1, 3, 5) using 6 primer pairs that produced an increasing molecular weight PCR products. There was an obvious increment of molecular weight of PCR products produced by these different primers as shown in lanes 2, 4, 6. M denotes molecular weight marker of GeneRuler® LadderMix.

products from the non-deletant that exhibited the original size of the DSN1 gene (1.7K bp). On the other hand, the deletant in the even-numbered lanes carried an extra band of a size corresponding to that of the URA3 gene. The increase in the PCR products size of subsequent lanes after lane 1 and 2 was due to the fact that the primers pairs used were covering regions wider that the one earlier.



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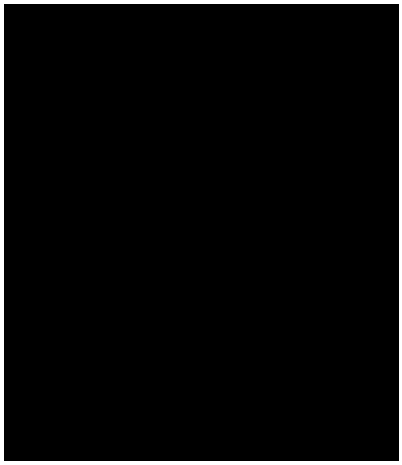


[b]

Figure 3. Tetrad dissection of yir010w deletant. (a) Tetrad dissection plate demonstrating a 2:2 (vital: non-vital) segregation of yir010w deletant's tetrad on YPD rich medium. Lanes indicated by white arrow show single or no colony possibly due to cell replication defect. (b) Replica plated tetrad on selective medium (SD Uracil dropout) showing no growth indicating the survivors in the rich medium were non-deletant wildtype haploid cells.

Tetrad analysis of the diploid deletants

Upon culturing in sporulation medium, the diploid yeast cells would be induced to form spores that contain four haploid constituents known as the tetrad. As the haploid tetrad was formed through meiotic division of the diploid yeast cell, they contained one set of the homologous pair of chromosomes. Figure 3 shows the separation of each tetrad in different columns on either rich medium or uracil dropout minimal synthetic medium.

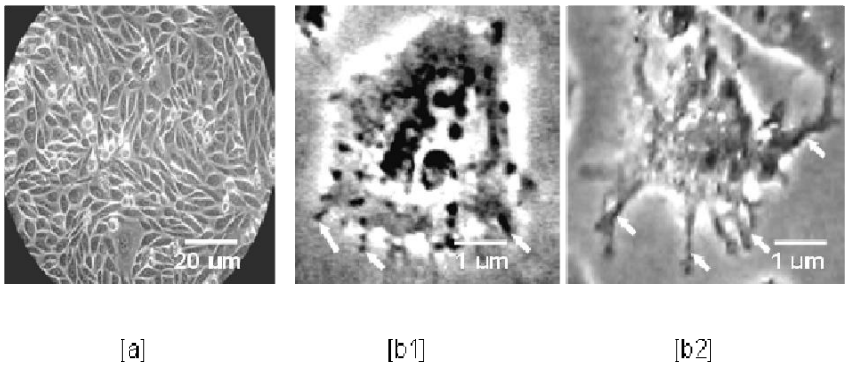


Figure 4. Cellular transformation of CHO cells due to the expression of Dsn1p. (a) Cellular morphology of normal CHO without the Dsn1p. (b) The expression of Dsn1p caused malignant-liked transformation in the CHO that show surface projections (arrows) and having rounded shape as compared to the spindle shaped normal control CHO.

In Figure 3a, there were only two colonies growing out of the four tetrads in most of the columns. Additionally, there were a few columns, as indicated by the arrows, showing only a single colony of haploid yeast cells managed to survive. When these colonies on the rich medium agar plate were replica plated onto the uracil dropout minimal synthetic medium, no survival was observed.

Cellular morphological changes in CHO expressing Dsn1p

Upon transfection with the pcDNA3.1 HIS A carrying the *DSN1* gene into the CHO cells, there was no significant change in the cellular morphology observed. The transfected cells size, shape and growth were similar to that of the control cells either without any expression vector or with just the null vector of pcDNA3.1 HIS A as shown in Figure 4a. The cells were spindle in shape and maintain their adherent ability to the culture surface and to themselves.

Four week post-transfection, the CHO expressing recombinant Dsn1p start to grow sluggishly and lost its clonogenecity (data not shown). Subsequent sub-culturing of the cells led to worsening of the growth pattern. Cellular morphological changes accompanied the declining growth rate. The cells would first become rounded up and shrank in size. This was followed suit by the malignant-liked transformation in the CHO that showed surface projections as shown in Figures 4b1 and 3b2.

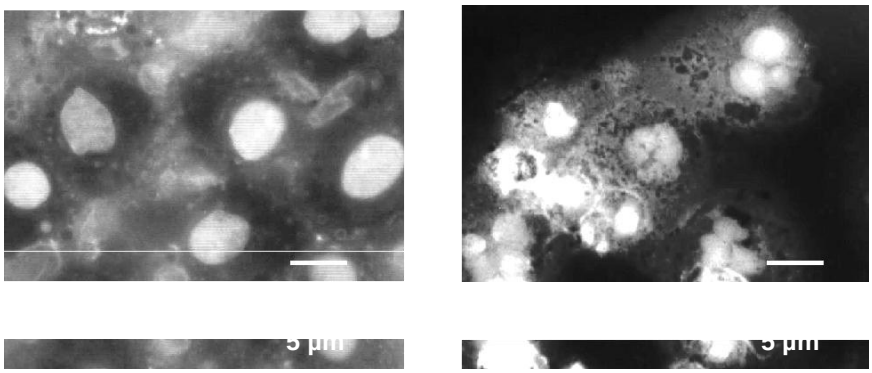
Dsn1p expression caused nuclear fragmentation in the CHO

Under normal growing condition, the control CHO either without any expression vector or with just the null vector of pcDNA3.1 HIS A would contain less compact and rounded nuclei as depicted by Figure 5a through the DAPI nuclear staining. Conversely, Figure 5b illustrated the more intensely stained nuclei of the CHO expressing Dsn1p exhibiting condensation of the nucleus. These cells were having nuclear fragmentation with the initial breakdown to larger pieces followed by smaller ones.

DISCUSSION

The purpose of carrying out the gene deletion was to detect possible changes to the yeast cells so as to indirectly elucidate its function (Euskirchen 2002; Pan et al., 2000). Replacement of the *DSN1* gene with the gene deletion cassette was done through homologous recombination which happens at a very high frequency in the yeast. Two stages of confirmation of the deletion were executed. First was the selection of transformants from those non-transformed cells using a uracil dropout minimal synthetic medium. Due to the fact that the deletion construct was made of uracil producing gene, this selection medium would be specific in obtaining the correct transformant. In the subsequent confirmation, a series of forward and reverse primers were employed to cover a region of 500 bp both upstream and downstream of the recombination sites. This was to ensure that the recombination process did not accidentally disrupt any possible genes upstream or downstream of it.

Tetrad analysis of the diploid deletants was done to ascertain the essentiality of the gene on cell survival and if the meiotic process was intact (Dunkern and Kaina, 2002). Each haploid tetrad contained a set of the sixteen chromosomes after the meiotic division that led to them. Therefore, we could expect half of the tetrad to carry the normal *DSN1* gene in their chromosomes IX and the other half to be consisted of the deletion cassette at the *DSN1* gene loci. If a gene is non-essential for cell survival, all the tetrad would be able to survive on the rich



[a] [b]

Figure 5. Nuclear staining of CHO cells with DAPI. (a) Normal control CHO cells show well defined, rounded and intact nuclei. (b) Nuclear fragmentation can be observed in the CHO expressing Dsn1p.

medium. In the contrary, we would expect a 2:2 ratio of viable to non-viable tetrad for the deletants. Those survivals were non-deletants having the intact and functional *DSN1* gene. These were confirmed when they were unable to survive on the uracil dropout minimal synthetic selective medium as they did not possess the ability to produce the uracil (McAinsh et al., 2003).

The rare phenotype of single or no zygote survival from the four tetrads could be caused by the defect during the meiosis, whereby the chromosomal segregation was not equal in both zygotes. This was a sign of haploinsufficiency by the gene *YIR010W*, whereby single copy of it was unable to function optimally in proper segregation of the chromosomes (Johnson et al., 1999). Mating test on the single survival zygotes confirmed that those were haploid with diploid mating characteristics, possibly due to extra set of chromosomes (Luykx, 1965). As reported, reduced spore viability and disome (or diploid progeny) appeared to be part of the *YIR010W* mutant phenotypes (Lock and Stribinskiene, 1996). The disomy or diploidization that occurred implied that Dsn1p was required in a precise stoichiometry for chromosomal segregation (Wahl et al., 1996).

In order to see if the *DSN1* gene from the yeast *S. cerevisiae* in functional in other higher eukaryotic cell, we have expressed the Dsn1p in the Chinese Hamster Ovary cells (Mercille and Massie, 1994; Rusinol et al., 2000). There were no observable changes to the cells within four weeks post transfection. Beyond that time frame, the CHO expressing Dsn1p exhibited morphologi-cal changes of having protrusions on the cell surface. This malignant-liked transformation might be due to the effect of Dsn1p on the nuclear segregation process as it was in the yeast cell (Measday et al., 2002). It became apparent when nuclear fragmentation was observed in these CHO expressing Dsn1p, thereby it was clear that Dsn1p expression has led to cellular morphological changes and subsequently becoming detrimental to cell survival and growth (Yiap et al., 2004b; Goswami et al., 1999; Ink et al., 1995; Jeffrey et al., 2002).

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