Studies on the Functions of DHH1,an RNA Helicase-Encoding Gene,and its Interplay with Genes Involved in the Cellular Morphology in Saccharomyces cerevisiae

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博士論文

Studies on the Functions of *DHH1*, an RNA Helicase-Encoding Gene, and its Interplay with Genes Involved in the Cellular Morphology in *Saccharomyces cerevisiae*

平成10年１月

神戸大学大学院自然科学研究科

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出芽酵母のRNAヘリカーゼをコードする*DHH1*遺伝子の機能ならびに細胞形態にかかわる遺伝子との相互作用の研究

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A dissertation for partial fulfilment of a Doctoral Degree at the Graduate School of Science and Technology, Kobe University.

January, 1998

Hisao Moriya

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January, 1998

Hisao Moriya

SUMMARY

DEAD-box proteins are known to be involved in many processes of RNA metabolism with their ATP-dependent RNA helicase activities. In the yeast *Saccharomyces cerevisiae*, one of the most advanced organisms in the study of molecular genetics, a total of twenty-seven DEAD-box protein genes have been identified the products of which play their roles in the process of splicing, translation, ribosome assembly and so on. Thirteen of the DEAD-box proteins have already been functionally characterized, while the remaining fourteen genes including *DHH1* are to be characterized further. *DHH1* encodes a protein that is one of the most highly conserved DEAD-box proteins among eukaryotes and its homologues are reported to function in the development and maintenance of the germ-line cells in *Xenopus* and *Drosophila*, and in carcinogenesis in human and mouse. However, the function of *DHH1* in yeast has not been well documented yet. In this thesis work, I performed several categories of experiments to establish its function. It has previously been reported that the disruption of *DHH1* causes slow growth. I confirmed this and found in addition: 1) that sporulation was defective in a Δ*dhh1* homozygous diploid, and 2) that the cell viability was distinctly reduced. Furthermore, with a Δ*dhh1* derivative of strain W303, I observed that *DHH1* disruption caused: 1) SDS hypersensitivity, 2) pronounced slow growth on synthetic media, and 3) temperature sensitive slow growth.

To identify the target RNA molecules of Dhh1p *in vivo*, I performed an experiment which I named the 'RcDNA sequencing' procedure. In this procedure, about thirty clones of cDNA converted from the RNAs associated with Dhh1p *in vivo* were analyzed. Most of them were originated from stable RNA species, but mRNAs of *GLK1*, *TDH1* and other genes were also identified. Therefore, I concluded that Dhh1p binds to RNA, but not in a specific manner. The subsequent Northern blot analysis indicated, however, that the level of mRNAs the fragment of which had been found to be associated with Dhh1p were significantly different between the wild type and a *DHH1* disruptant. Upon reporter assay, I found that the expression from a *TDH1* promoter in the *DHH1* disruptant was significantly reduced in comparison with the wild type, while the expression from an *ACT1* promoter was not so much altered. I also found that Dhh1p showed a transcription stimulating activity when fused

to the Gal4p DNA binding (DB) domain. By truncating the DB-Dhh1 protein, the C-terminal PQ-rich region was found to be essential for the Dhh1p function and to possess the transcription stimulating activity. These data suggest that Dhh1p has a role in the RNA metabolism during transcription.

To understand the genetical interactions of *DHH1*, I screened mutants exhibiting synthetic lethality with a *DHH1* deletion, using the *ADE2/ADE3* colony sectoring assay. I analyzed about fifty of them and named them *ddg* (*DHH1* dependent growth) mutants. Seven of the nine mutants that were characterized further showed abnormal cellular morphology and suffered from cell wall fragility, which indicated that *DHH1* functions on the morphology determining genes. Using a single copy yeast genomic DNA library, I identified that the *SSD1* gene, which was thought to be involved in RNA 3'-end processing, was capable of complementing the *ddg* phenotype of a mutant termed H104. From several lines of evidence, I concluded that the parental strain used for the screening of the *ddg* mutants harbored an inactivated *SSD1*. Mutant H104 was found to have an additional mutation in *CDC28*. From the phenotypic characterization of an *SSD1 DHH1* double disruptant along with *ddg* mutants, I found that *SSD1* and *DHH1* function cooperatively in many cellular processes such as cellular morphology, cell wall integrity, and nucleotide and nutrient metabolisms. Perhaps, they achieve these functions through the metabolisms of related RNA molecules.

Through the analysis of mutant H106, another gene termed *ELM1*, was identified as a suppressor of the *ddg* phenotype. A mutation in *ELM1* was previously reported to cause an elongated cellular morphology. A triple mutant, *ssd1 Δelm1 Δdhh1*, was found to be very sick and grow only on plates with an osmotic stabilizer. It showed defects in cytokinesis and mitosis in addition to cell wall fragility. The product of *ELM1* fused to GFP was found to be localized at the bud neck and its synthesis seemed to be regulated during the cell cycle. From these and other results, I concluded that Elm1p functions in cytokinesis without a direct interaction with Dhh1p.

With the *ddg* mutants thus isolated starting with the *dhh1 ssd1-d* strain as described in this thesis, many morphology determining genes the individual mutations in which are not lethal can be identified and characterized.

INTRODUCTION

My personal interests in biology

Life is one of the most mysterious things to me even now. I have been interested in various phenomena produced by organisms such as evolution, cell cycle, development, morphological diversity, behavior and so on. Of them, the most interesting thing to me is to view and describe life as an organization of molecular mechanisms at the whole cell level and to know what is the status of a thing to be 'living'. I think that being 'living' can be defined by the existence of maintenance and self-control of the cellular activities in addition to the self-replication of the genetic materials. Living things are regulated by a large set of genetic materials termed genes. Although each organism has its own gene set, it has widely been understood that there are many molecular mechanisms that are common to the life of almost all living organisms. To study the fundamental matters of life in molecular and genetical details, complicated multicellular organisms are obviously not very suitable, because they usually have a long life span and contain too many genes. Therefore, I chose a single cellular organism, the yeast *Saccharomyces cerevisiae*, as the target of my study for a doctor's degree.

Yeast *Saccharomyces cerevisiae*

As an organism, *S. cerevisiae* has been giving various profits to human beings such as to make us possible to produce breads for food and alcohols for a joy by its fermentation activity. This single cellular organism is of round cell shape and proliferates by budding. It is a typical eukaryote harboring a nucleus, intra-cellular membrane structures such as endoplasmic reticulum, golgi body and vacuole, and organelles such as mitochondrion and peroxisome. Because of its easiness of manipulation, it is now the best studied eukaryotic organism in biochemistry, molecular and cellular biology, and genetics. Needless to say, *S. cerevisiae* is the best suited organism for most advanced genetic analysis for the study of interactions of genes. In addition to its natural advantage for genetic studies (i.e., easiness to convert between the diploid and haploid states), many techniques in molecular genetics have been developed with *S. cerevisiae*. The high efficiency of transformation and homologous

recombination makes it possible to introduce DNA of exogenous origins and to perform targeted disruption of a gene. Using these techniques, we can study interactions among genes by such methods as the two-hybrid system, genetic suppression, synthetic lethal interaction, unlinked non-complementation and so on. Many discoveries in the metabolic pathways, protein synthesis, DNA replication, RNA metabolism, organellar synthesis, cell cycle and cell wall and membrane synthesis have been made with this organism. They are of course mostly common to higher eukaryotes such as plants and animals as well. Thus, *S. cerevisiae* is undoubtedly an important model organism for the studies of life.

In 1996, the total genomic DNA sequence of *S. cerevisiae* was determined as a first eukaryote, and consequently, the analysis of *S. cerevisiae* has entered a new era: various functional analyses can now be made at the whole genome scale. *S. cerevisiae* has the genomic DNA of about 12 Mb in size and is estimated to contain 6,000 active genes. An immediate advantage of the availability of the total genomic sequence data will be of course, the easiness of the analysis of nucleotide and derived amino acid sequence data in various ways. If one determines the amino acid sequcences of the peptides derived from a protein for a few amino acid long stretch, then one can identify the gene encoding it by matching the fragment sequence with the amino acid sequence data predicted from the genomic DNA sequence. Similarly, if one isolates a gene by functional cloning, its total sequence can be readily obtained by sequencing less than 100 bp from both ends of the gene. Further information concerning the cloned gene can be obtained from the yeast genome database pages on the world wide web such as MIPS, YPD and SGD. Also, a gene/ORF which one wants to study can easily be cloned by polymerase chain reaction (PCR) using primers specific to that gene/ORF in question by surveying and extracting the primer sequence designed from the genomic sequence data.

Another advantage will be the genome-wide study of proteins constituting a family. Many proteins are known to form a family which consists of functionally and structurally related proteins. Many nucleotide-binding proteins including protein kinases, protein phosphatases, GTP-binding proteins, DNA and RNA helicase are such examples. If the total genomic sequence data are available, the data for proteins which make a family can be obtained, and using various potent molecular genetic techniques currently available, one can study all the member proteins belonging to the family. A third advantage will be that studies

on the functions of, for example, genes which cause diseases in human can be carried out. If a gene which is involved in a human disease through a fundamental cellular process is cloned, and if there is its homologue in the *S. cerevisiae* genome, then we can study the function of the *S. cerevisiae* gene and infer the consequence of its defect in human.

In addition to the powerful molecular genetic techniques applicable to the analysis of *S. cerevisiae* cells, advanced knowledges concerning the biochemistry and physiology of yeast cells along with the fact that it has many features common to higher eukaryotes has made *S. cerevisiae* one of the most important model organisms. A next step after the determination of the total genomic sequence will thus be functional characterization of individual genes/ORFs and determination of interactions among them.

RNA metabolism in the cell

RNA is one of the essential informational macromolecules in the cell and its metabolism is a pivotal process in the cellular activity. Highly established databases such as MIPS, SGD and YPD have been developed to date. Using these databases, we can obtain up-to-date knowledge of cellular pathways from the information concerning the genes that are involved in them. Since genes function as fundamental agents for the cell to achieve various metabolic pathways, the understanding of the genes involved in the pathways will lead to the understanding of the pathways themselves. In the MIPS Yeast Functional Catalogue, genes involved in 'RNA metabolisms' are categorized in 'TRANSCRIPTION' and 'PROTEIN SYNTHESIS' (the details are shown in Table 1) and about 1,000 genes have been assigned to be involved in these processes. 'RNA metabolisms' has been defined to contain ribosome assembly, tRNA processing, transcription, mRNA splicing, mRNA transport, translation and mRNA degradation. Recently, a family of proteins collectively termed DEAD-box proteins have been identified to play important roles in these processes.

DEAD-box proteins in *S. cerevisiae*

RNA helicases have an ATP-dependent RNA unwinding activity and contain conserved amino acid sequences, one of which is the sequence containing D, E, A and D. Therefore, they are termed DEAD-box proteins. Many genes encoding DEAD-box proteins have been described in a variety of organisms (for review, see Schmid and Linder, 1992). In *S. cerevisiae*,

Table 1. Classification of genes involved in RNA metabolisms. The table is copied from MIPS Yeast Functional Catalogue and formatted[a].

Functional Categoly	Number of genes
TRANSCRIPTION	
rRNA synthesis	38
rRNA processing	40
other rRNA-transcription activities	2
tRNA synthesis	23
tRNA processing	31
tRNA modification	10
other tRNA-transcription activities	4
mRNA synthesis	
general transcription activities	59
transcriptional control	289
chromatin modification	23
mRNA processing	
splicing	80
5'-end, 3'-end processing and mRNA degradation	33
other mRNA-transcription activities	13
RNA transport	26
other transcription activities	59
PROTEIN SYNTHESIS	
ribosomal proteins	207
translation (initiation, elongation and termination)	61
translational control	27
tRNA-synthetases	37
other protein-synthesis activities	11

a) Taken from "Yeast Functional Catalogue" (Last update: Thu Nov 13 11:18:20 MET 1997)

twenty-seven DEAD-box proteins are known to be encoded in its genome (Table 2 MIPS, SGD and YPD). They are involved in the processes of RNA metabolism such as translational initiation (*TIF1*, *TIF2*, and *DED1*), ribosomal RNA maturation and ribosome assembly (*SPB4*, *RRP3*, etc), splicing (*PRP5*, *PRP8* and *PRP28*), and so on. Mutations in most of the DEAD-box genes result in growth defects, indicating that DEAD-box proteins are generally essential and play important roles in *S. cerevisiae* through RNA metabolism. As shown in Table 2, thirteen of the 27 DEAD-box proteins have not been functionally characterized yet. Since each of the DEAD-box proteins has its own target RNA, the functional analysis of the unknown DEAD-box proteins may lead to discoveries of previously unknown processes of RNA metabolism, and will eventually lead to the description of the entire picture of RNA metabolism in the cell.

DHH1 is one of the most highly conserved DEAD-box protein genes among eukaryotes (Strahl-Bolsinger and Tanner, 1993) and its homologues have been identified in *Schizosaccharomyces pombe* (Maekawa et al., 1994), *Drosophila melanogaster* (De Valoir et al., 1991), *Xenopus leaves* (Ladomery et al., 1997), mouse and human (Lu et al., 1992, Akao et al., 1995, Seto et al., 1995) as shown in Fig. 1. The *DHH1* homologues in some of these organisms seem to play a role in the organization and maintenance of germ line cells, while in others the gene is thought to function as a proto-oncogene. Despite all these, the precise function of *DHH1* is not fully established yet. In this study, I tried to understand the function of *DHH1* in the cellular RNA metabolism. First, I used molecular biological techniques to establish the function of Dhh1p, the protein product of the *DHH1* gene. Second, I used more advanced molecular genetic techniques to isolate and study the synthetic lethal mutations with *DHH1* deletion to identify the genes playing roles in accordance with *DHH1*, and discovered that *DHH1* functions in many cellular processes and cell morphogenesis as will be described below.

Table 2. DEAD-box genes in *S. cerevisiae* genome

ORF	Gene	Function	Gene disruption
YBR142w	*MAK5*	60S ribosome assembly	lethal
YBR237w	*PRP5*	pre-mRNA splicing	lethal
YDL160c	*DHH1*	*ste13* homologue	slow growth
YDR194c	*MSS116*	mitcondoria splicing	slow growth
YDR243c	*PRP28*	pre-mRNA splicing	lethal
YDR021w	*FAL1*	18SrRNA maturatiom	lethal
YDL031w			
YDL084w			
YFL002c	*SPB4*	25SrRNA maturation	lethal
YGL064c			
YGL078c	*DBP3*	25SrRNA maturation	lethal
YGL171w	*ROK1*	18SrRNA maturation	lethal
YHR065c	*RRP3*	18SrRNA maturation	lethal
YHR165c	*PRP8*	pre-mRNA processing	lethal
YHR169w	*DBP8*		lethal
YJL033w	*DBP4*	18SrRNA maturation	lethal
YJL138c	*TIF2*	translation initiation	viable[a)]
YKR024c	*DBP7*		slow growth
YKR059w	*TIF1*	translation initiation	viable[a)]
YLL008w	*DRS1*	25SrRNA maturation	lethal
YLR276c	*DBP9*		lethal
YMR290c			
YNL112w	*DBP2*	p68 homologue	slow growth
YNR038w	*DBP6*		lethal
YOR046c	*DBP5*		lethal
YOR204w	*DED1*	translation initiation	lethal
YPL119c	*DBP1*	PL10 homologue	viable

[a)] Double disruption of *TIF1* and *TIF2* is lethal.

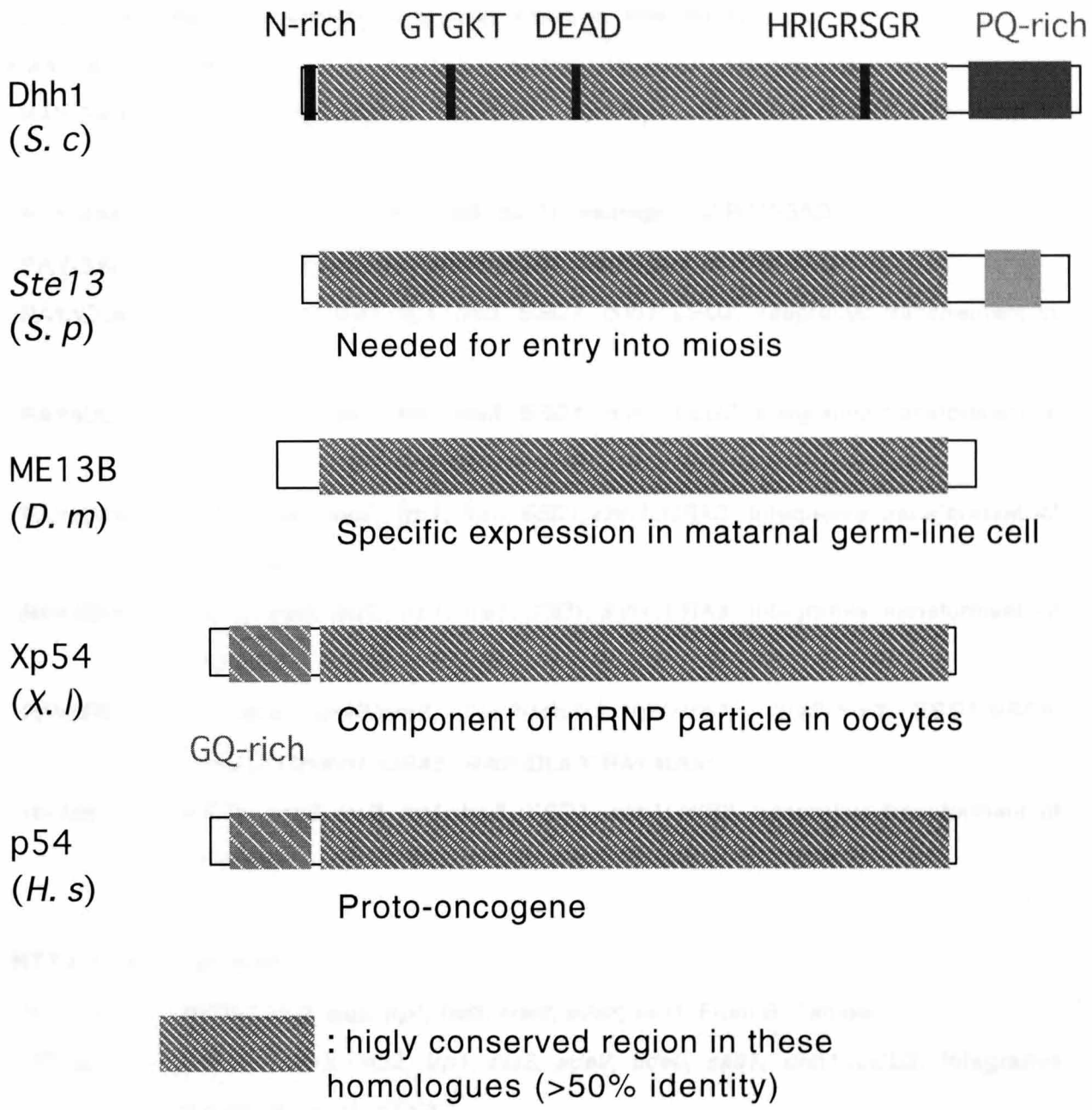


Fig. 1. DEAD-box proteins which is highly homologous to Dhh1p. Abbreviated organism names are: *S. c.*, *Saccharomyces cerevisiae. S. p.*, *Schizosaccaromyces pombe. D. m.*, *Drosophila melanogaster. X. l.*, *Xenopus laevis. H. s.*, *Homo sapiens.*

MATERIALS AND METHODS

1. *Saccharomyces cerevisiae* strains used in this study

RAY-3A background

RAY-3AD	*MAT**a**/α, ura3/ura3, leu2/leu2, trp1/trp1, his3/his3, SSD1/SSD1.* From A. Toh-e
RAY-3A**a**	*MAT**a**, ura3, leu2, trp1, his3, SSD1.* Segregant of RAY-3AD
RAY-3Aα	*MATα, ura3, leu2, trp1, his3, SSD1.* Segregant of RAY-3AD
RAYΔDL**a**	*MAT**a**, ura3, leu2, trp1, his3, SSD1, dhh1::LEU2.* Integrative transformant of RAY-3Aa
RAYΔDLα	*MATα, ura3, leu2, trp1, his3, SSD1, dhh1::LEU2.* Integrative transformant of RAY-3Aα
RAYΔDU**a**	*MAT**a**, ura3, leu2, trp1, his3, SSD1, dhh1::URA3.* Integrative transformant of RAY-3Aa
RAYΔDUα	*MATα, ura3, leu2, trp1, his3, SSD1, dhh1::URA3.* Integrative transformant of RAY-3Aα
RAYΔDD	*MAT**a**/α, ura3/ura3, leu2/leu2, trp1/trp1, his3/his3, SSD1/SSD1, dhh1::LEU2/dhh1::URA3.* RAYΔDLa X RAYΔDUα
RAYΔE	*MATα, ura3, leu2, trp1, his3, SSD1, elm1::HIS3.* Integrative transformant of RAY-3Aα

HTY2-1 background

HTY2-1	*MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1,* From S. Tanaka
HTYΔD	*MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2.* Integrative transformant of HTY2-1

HTY2-1/RAY-3A background (obtained as described below)

HTYΔDp	[*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2.*
H101	[*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1,*

dhh1::LEU2, ddg1. Mutagenesis of HTYΔDp

H102 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, ddg2.* Mutagenesis of HTYΔDp

H103 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, erg10?.* Mutagenesis of HTYΔDp

H104 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, cdc28.* Mutagenesis of HTYΔDp

H105 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, ddg5.* Mutagenesis of HTYΔDp

H107 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, elm1-7.* Mutagenesis of HTYΔDp

H108 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, ddg8.* Mutagenesis of HTYΔDp

H109 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, ddg9.* Mutagenesis of HTYΔDp

H110 [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, ddg10.* Mutagenesis of HTYΔDp

HTY2ΔSp [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, dhh1::LEU2, ssd1::HIS3.* Integrative transformant HTYΔDp

HTY2ΔEp [*ADE3, URA3, DHH1*] *MATα, ura3, leu2, trp1, his3, ade2, ade3, ssd1, dhh1::LEU2, elm1::HIS3.* Integrative transformant HTYΔDp

W303-1B background

W303-1B *MATα, ura3, leu2, trp1, his3, ade2.* From A. Toh-e

W303ΔD *MATα, ura3, leu2, trp1, his3, ade2, dhh1::LEU2.* Integrative transformant of W303-1B

W303ΔE *MATα, ura3, leu2, trp1, his3, ade2, elm1::HIS3.* Integrative transformant of W303-1B

W303ΔDE *MATα, ura3, leu2, trp1, his3, ade2, dhh1::LEU2, elm1::HIS3.* Integrative transformant of W303-1B

Other strains

Y190	*MATa*, *ura3*, *leu2*, *trp1*, *his3*, *ade2*, *lys2*, *gal4*, *gal80*, *cyh^{r}2*, *LYS2::P_{GAL}:HIS3*, *URA3::P_{GAL1}:lacZ.* (Clontech)
K1169	*MATa*, *gal2*, *ura3*, *bar1::URA3* (Nasmyth et al., 1990)

2. Plasmid used in this study

Plasmids for general purpose

pUC19	amp^r.
pUC118	amp^r.
pUC119	amp^r.
pBluescript	amp^r. (Stratagene)
pT7-Blue	amp^r. (Novagen)
YEp358R	*URA3*, *2μARS*, *lacZ*, amp^r. (Myers et al., 1984)
pRS314x	*TRP1*, *CEN6-ARSH4*, amp^r. Made by inserting a 0.5 kb *Pvu*II fragment containing multi-cloning site of pUC119 into the *Pvu*II site of pRS314 . (Sikorski and Hieter, 1989).
pASΔNS	*TRP1*, *2μARS*, amp^r. Made by removing 2.7 kb *Nco*I/*Sac*I fragment of pAS2-1.
pUCcgHIS3	*cgHIS3*, amp^r. pUC19 containing 2.7 kb *Candida glabrata HIS3* gene. (Kitada et al., 1993)
pUCURA3	*URA3*, amp^r. Made by inserting 1.5 kb *Hpa*I/*Pvu*II fragment containing *URA3* gene from YEp358R into the *Sma*I site of pUC119.
pUCLEU2	*LEU2*., amp^r. 1.2 kb *LEU2* fragment is inserted at *Bam*HI site of pUC118.
pGAL1	P_{GAL1}, amp^r. Made by inserting a 0.7 kb flushed PCR fragment of *GAL1* promoter amplified from genomic DNA of RAY-3AD using pGAL1-a and PGAL1-s, into the *Hinc*II site of pUC119.
YEpGAL1	*URA3*, *2μARS*, P_{GAL1}:*lacZ.*, amp^r. Made by inserting a 0.7 kb *Bam*HI/*Hind*III fragment containing *GAL1* promoter from pGAL1, into the *Bam*HI/*Hind*III site of YEp358R.

Plasmids for synthetic lethal mutant screening

YEpADE3 *URA3*, *ADE3*, *2μARS*, amp^r. YEp352 containing 5.4 Kb DNA fragment of *ADE3*. From S. Tanaka

YEpA3D1 *URA3*, *ADE3*, *DHH1*, *2μARS*, amp^r. Made by inserting a 2.5 kb fragment of *DHH1* ORF which was amplified by PCR using primer A and B from pUCfDHH1 and digested by *Sac*I and *Xma*I, into the *Sac*I/*Xma*I site of YEpADE3.

Plasmids for *DHH1* gene manipulation

pUCDHH1 *DHH1*, amp^r. Made by inserting a 1.6 kb fragment of *DHH1* ORF which was amplified by PCR from genomic DNA of RAY-3AD using primer DHH1-a and DHH1-b and digested by *Sac*I and *Sma*I, into the *Sac*I/*Sma*I site of pUC119.

pUCfDHH1 *DHH1*, amp^r. Made by inserting a 2.5 kb flushed PCR fragment of *DHH1* amplified from genomic DNA of RAY-3AD using primer DHH1-5' and DHH1-3', into the *Hinc*II site of pUC118.

YEpDHH1 *TRP1*, *2μARS*, *DHH1*, amp^r. Inserting a 2.5 kb fragment of *DHH1* ORF which was amplified by PCR from pUCfDHH1 using primer A and B and digested by *Sac*I/*Xma*I, into the *Sac*I/*Xma*I site of pASΔNS.

YCpDHH1 *TRP1*, *CEN6-ARSH4*, *DHH1*, amp^r. Isolated from a pRS314x-based genomic library on the basis of its ability to complement the FOA$^-$ phenotype of *ddg* strains (H104, H105 and H108), with a 4.9 kb DNA fragment containing *DHH1*.

YCpDHH1$_{\Delta PQ}$ *TRP1*, *CEN6-ARSH4*, *DHH1*ΔPQ, amp^r. This plasmid was made by multiple step. A 0.8 kb *Kpn*I fragment of *DHH1* lacking N- and C-terminal region was inserted into the *Kpn*I site of pBluescript to yield pBSK-DH-K. A 1.0 kb *Xho*I/*Bam*HI fragment containing a Gal4p activation domain and a *ADH1* terminator from pPC86 (our laboratory stock) into the *Xho*I/*Bam*HI site of pBSK-DH-K to yield pΔQAD. A *Xho*I/*Sal*I fragment containing Gal4p activation domain of pΔQAD was removed by *Sal*I/*Xho*I digestion and self-ligation to yield pΔQADsx. A 1.1 kb *Bgl*II/*Sac*I fragment of pΔQADsx was inserted into the

*Bgl*II/*Sac*I site of YCpDHH1

pUCΔdhh1 ::URA3 *dhh1::URA3*, amp^r. Made by inserting a 1.5 kb *Kpn*I fragment of *URA3* gene between two *Kpn*I sites of pUCDHH1.

pUCΔdhh1::LEU2 *dhh1::LEU2*, amp^r. Made by inserting a 1.2 kb *Bam*HI fragment of *LEU2* gene between *Bcl*I and *Bgl*II sites of pUCDHH1

Plasmids for the expression of GST-fusion protein

pT7-GST GST, amp^r. Made by inserting a 0.7 kb PCR fragment of GST amplified from pGST-hrpA (Moriya, 1995) using GST-s and PE1 (Moriya, 1995), into pT7-blue.

pUC-PDHH1 P_{DHH1}, amp^r. Made by inserting a 600 bp PCR fragment containing a *DHH1* promoter amplified from RAY-3AD genomic DNA using PDHH1a and fDHH1-5', into the *Hinc*II site of pUC118.

YEpGG *URA3*, *2μARS*, P_{GAL1}:GST, amp^r. Made by inserting a 0.6 kb *Bam*HI/*Xho*I fragment containing GST of pT7-GST into the *Bam*HI/*Xho*I site of YEp358R.

YEpGGDH *URA3*, *2μARS*, P_{GAL1}:GST-*DHH1*, amp^r. Made by inserting a 1.2 kb fragment containing *DHH1* which was amplified from RAY-3AD genomic DNA using DHH1-a and DHH1-b, then digested with *Sac*I and *Sma*I, into the *Sac*I/*Sma*I site of YEpGG.

YEpGGDH3 *URA3*, *2μARS*, P_{GAL1}:GST-*DHH1*-T_{DHH1}, amp^r. Made by inserting a 1.0 kb *Eco*RI/*Sac*I fragment containing 3' region of *DHH1* from pUCfDHH1, into the *Eco*RI/*Sac*I site of YEpGGDH.

YEpDGDH3 *URA3*, *2μARS*, P_{DHH1}:GST-*DHH1*-T_{DHH1}, amp^r. Made by inserting a 0.6 kb *Pst*I/*Xho*I fragment containing the *DHH1* promoter, into the *Pst*I/*Xho*I site of YEpGGDH3.

Plasmid listed below are used for the control experiments of GST-Dhh1p expression (data not shown).

YEpGG(B) *URA3*, *2μARS*, P_{GAL1}:GST-*lacZ*, amp^r. YEpGG was digested with *Bam*HI, after

filled-in the end, re-circularized.

YEpDG(Z) *URA3*, *2μARS*, P_{DHH1}:GST-*lacZ*, amp^r. Made by inserting a 0.6 kb *Pst*I/*Xho*I fragment containing the *DHH1* promoter, into the *Pst*I/*Xho*I site of YEpGG(B)

YEpGGDH-L *LEU2*, *2μARS*, P_{GAL1}:GST-*DHH1*-T_{ADH1}, amp^r. Made by inserting a 3.5 kb *Hpa*I/*Sac*I fragment containing T_{ADH1} and *LEU2* from pACT2, into the *Hpa*I/*Sac*I site of YEpGGDH.

YEpGG-L *LEU2*, *2μARS*, P_{GAL1}:GST-T_{ADH1}, amp^r. Made by removing a 1.2 kb *Sac*I/*Sma*I fragment containing *DHH1* from YEpGGDH-L.

Plasmids for the preparation of radio-labeled DNA probes for Northern blot analysis

pD101 *TDH1* fragment (65 bp), amp^r. Obtained from a RcDNA sequencing.

pD502 *GLK1* fragment (200 bp), amp^r. Obtained from a RcDNA sequencing.

pD602 *YHR110c* fragment (130 bp), ampr. Obtained from a RcDNA sequencing.

Plasmids for *lacZ* reporter assay

YEpACT1 *URA3*, *2μARS*, P_{ACT1}:*lacZ*., amp^r. Made by inserting a 1 kb *Bam*HI/*Bcl*I fragment containing an *ACT1* promoter of pYA301 (Gallwitz and Seidel, 1980) into the *Bam*HI site of YEp358R.

YEpTDH1 *URA3*, *2μARS*, P_{TDH1}:*lacZ*., amp^r. Made by inserting a 0.8 kb fragment containing a *TDH1* promoter which was amplified by PCR from genomic DNA of RAY-3AD using primer GAP3-a and GAP3-s and digested by *Bam*HI and *Bcl*I, into the *Bam*HI site of YEp358R.

YEpTDH1$_{-100}$ *URA3*, *2μARS*, $P_{TDH1\text{-}100}$:*lacZ*., amp^r. Made by removing a 0.5 kb *Sph*I fragment from YEpTDH1.

Plasmids for one-hybrid and two-hybrid assay (Fig. 7)

Gal4 DNA-binding domain (DB) hybrid

pAS2-1 *TRP1*, *2μARS*, *GAL4* $_{(1\text{-}147)}$ DNA-BD, *CYH*^{s}*2*, amp^{r}. (Clontech)

pSD1(FL) *TRP1*, *2μARS*, *GAL4* $_{BD}$-*DHH1*, *CYH*^{s}*2*, amp^{r}. Made by inserting a 2.0 kb *Xma*I/*Sal*I fragment containing *DHH1* ORF from pCD1 into the *Xma*I/*Sal*I site of pAS2-1.

pSD1-N *TRP1*, *2μARS*, *GAL4* $_{BD}$-*DHH1N*, *CYH*^{s}*2*, amp^{r}. Made by removing a 500 bp *Cla*I/*Sal*I fragment from pSD1.

pSD1-C *TRP1*, *2μARS*, *GAL4* $_{BD}$-*DHH1C*, *CYH*^{s}*2*, amp^{r}. Made by removing a 700 bp *Eco*RI fragment from pSD1.

pSD1-R *TRP1*, *2μARS*, *GAL4* $_{BD}$-*DHH1R*, *CYH*^{s}*2*, amp^{r}. Made by removing a 0.2 kb *Kpn*I/*Pst*I fragment from pSD1-N.

pSD1-ΔR *TRP1*, *2μARS*, *GAL4* $_{BD}$-*DHH1ΔR*, *CYH*^{s}*2*, amp^{r}. Made by removing a 0.2 kb *Cla*I fragment from pSD1.

pSD1-Q *TRP1*, *2μARS*, *GAL4* $_{BD}$-*DHH1Q*, *CYH*^{s}*2*, amp^{r}. Made by removing 1.3 kb *Kpn*I/*Sma*I fragment from pSD1.

pSE1 *TRP1*, *2μARS*, *GAL4* $_{BD}$-*ELM1*, *CYH*^{s}*2*, amp^{r}. Made by inserting a 2.2 kb *Bsp*EI-*Sal*I fragment containing *ELM1* coding region from p106-7 into the *Xma*I/*Sal*I site of pAS2-1.

Gal4 Activation domain (AD) hybrid

pACT2 *LEU2*, *2μARS*, *GAL4* $_{(768\text{-}881)}$ AD, amp^{r}. (Clontech)

pCD1 *LEU2*, *2μARS*, *GAL4* $_{AD}$-*DHH1*, amp^{r}. Made by inserting a 1.6 kb *Sac*I/*Xma*I fragment containing *DHH1* ORF from pUCDHH1 into the *Sac*I/*Xma*I site of pACT2.

pCS1 *LEU2*, *2μARS*, *GAL4* $_{AD}$-*SSD1*, amp^{r}. Made by inserting a 4.6 kb *Sac*I/*Bam*HI fragment containing *SSD1* coding region from p104-4HS into the *Sac*I/*Bam*HI site of pACT2.

pCC28 *LEU2*, *2μARS*, *GAL4* $_{AD}$ -*CDC28*, amp^{r}. Made by inserting a 0.8 kb

*Bam*HI/*Sal*I fragment containing *CDC28* coding region from pCDC28 into the *Bam*HI/*Xho*I site of pACT2.

Plasmids for *SSD1* gene manipulation (Fig.15)

p104-4 *TRP1*, *CEN6-ARSH4*, *SSD1*, *SPR101*, amp^r. Isolated from a pRS314x-based genomic library on the basis of its ability to complement the FOA$^-$ phenotype of H104.

p104-6 *TRP1*, *CEN6-ARSH4*, *SSD1*, amp^r. Isolated from a pRS314x-based genomic library on the basis of its ability to complement the FOA$^-$ phenotype of H104.

p104-4HS *TRP1*, *CEN6-ARSH4*, *SSD1*, amp^r. Made by removing of a 1.9 kb *Hpa*I/*Sma*I fragment from p104-4.

p104-6E *TRP1*, *CEN6-ARSH4*, amp^r. Made by removing of a 1.4 kb *Eco*RI fragment from p104-6.

p104-6S (YCpSSD1) *TRP1*, *CEN6-ARSH4*, *SSD1*, amp^r. Made by removing a 0.5 kb *Sph*I fragment from p104-6.

YCpΔssd1 ::cgHIS3 *TRP1*, *CEN6-ARSH4*, *ssd1::cgHIS3*, amp^r. Made by inserting a 2.0 kb *Sma*I/*Bcl*I fragment containing *cgHIS3* from pUCcgHIS3 into *Hpa*I/*Bcl*I site of YCpSSD1

Plasmids for *CDC28* gene manipulation (Fig. 19)

p104-12 *TRP1*, *CEN6-ARSH4*, *CDC28*, *YBR161w*, amp^r. Isolated from a pRS314x-based genomic library on the basis of its ability to complement the FOA$^-$ and ts- phenotype of H104.

YCpCDC28 *TRP1*, *CEN6-ARSH4*, *CDC28*, amp^r. Made by inserting a 3.5 Kb *Pst*I fragment of p104-12 containing *CDC28* into the *Pst*I site of pRS314x.

YCpCDC28$_{\Delta CE}$ *TRP1*, *CEN6-ARSH4*, *CDC28*, amp^r. Made by removing a 0.6 kb *Cla*I/*Eco*RI fragment from YCpCDC28.

pCDC28 *CDC28* ORF, amp^r. Made by inserting a 0.8 kb PCR fragment of *CDC28* coding region amplified from genomic DNA of RAY-3AD using primer CDC28-a and CDC28-s, into pT7-blue (Novagen).

Plasmids for *ELM1* gene manipulation (Fig. 20)

p106-6 (YCpELM1) *TRP1*, *CEN6-ARSH4*, *ELM1*, amp^r. Isolated from a pRS314x-based genomic library on the basis of its ability to complement the FOA$^-$ phenotype of H106.

p106-7 *TRP1*, *CEN6-ARSH4*, *ELM1*, amp^r. Isolated from a pRS314x-based genomic library on the basis of its ability to complement the FOA$^-$ phenotype of H106.

YCpΔelm1::cgHIS3 *TRP1*, *CEN6-ARSH4*, *elm1::cgHIS3*, amp^r. This plasmid was made by multiple step. A 0.9 kb *Cla*I/*Sma*I fragment was removed from p106-7 to yield YCpELM1ΔCS. YCpELM1ΔCS were digested *Sal*I and *Sph*I and after fill-in the end, re-circularized to yield YCpELM1ΔΔ. A 2.0 kb *Pst*I/*Bcl*I fragment of *cgHIS3* from pUCcgHIS3 were inserted into the *Pst*I/*Bgl*II site of YCpELM1ΔΔ.

Plasmids for *ELM1* over-expression

pT7-ELM1 *ELM1* ORF, amp^r. Made by inserting a 2.0 kb PCR fragment of *ELM1* ORF amplified from YCpELM1ΔΔ using primer ELM1-s and B, into pT7-blue.

YEpGAL-ELM1 *URA3*, *2μARS*, P_{GAL1}:GFP-*ELM1*, amp^r. A 0.7 kb *Xho*I/*Bam*HI fragment containing 3' half of *ELM1* from pT7-*ELM1* was inserted into the *Xho*I/*Bam*HI site of YEpGAL1 to yield YEpGAL-ELM1C. A 1.5 kb *Xho*I fragment containing 5' half of *ELM1* from pT7-ELM1 was inserted into the *Xho*I site of YEpGAL-ELM1C.

Plasmids for GFP fusion protein expression

pCR-GFP GFP (S65T), amp^r. pCR-script (STRATAGENE) containing 0.8 kb PCR fragment of GFPS65T (Clontech), from S. Tanaka

pT7-PDHH1 *DHH1* promoter, amp^r. Made by inserting a 0.6 kb PCR fragment containing *DHH1* promoter from YCpDHH1 using primer DHH1-5' and PDHH1-AscI, into pT7-blue (Novagen).

pP_{DHH1}-GFP P_{DHH1}:GFP, amp^r. Made by inserting a 0.8 kb *Asc*I/*Sma*I fragment containing GFP from pCR-GFP into the *Asc*I/*Sma*I site of pT7-PDHH1.

YEpDGFP-DHH1 *TRP1*, *2μARS*, P_{DHH1}:GFP-*DHH1*, amp^r. Made by inserting a 1.0 kb *Sma*I/*Sph*I fragment containing P_{DHH1}:GFP from pPDHH1-GFP, intot he *Sma*I/*Sph*I site of YEpGGDH3.

YCpDGFP-DHH1 *TRP1*, *CEN6-ARSH4*, P_{DHH1}:GFP-*DHH1*, amp^r. Made by inserting a 1.2 kb *Sac*I fragment containing *DHH1* from YEPGGDH, into the *Sac*I site of YCpDGFP.

YCpDGFP-DHH13' *TRP1*, *CEN6-ARSH4*, P_{DHH1}:GFP-*DHH1*-T_{DHH1}, amp^r. Made by iserting a 1.5 kb *Bcl*I/*Sph*I fragment containing P_{DHH1}:GFP into the *Bcl*I/*Sph*I site of YCpDHH1.

YCpDGFP *TRP1*, *CEN6-ARSH4*, P_{DHH1}:GFP,amp^r. Made by inserting a 1.0 kb *Sal*i fragment containing P_{DHH1}:GFP from pT7-PDHH1 into the *Sal*I site of pRS314x.

YEpDGFP-ELM1 *TRP1*, *2μARS*, P_{DHH1}:GFP-*ELM1*, amp^r. A 1.5 kb *Xho*I/*Pst*I fragment of P_{DHH1}:GFP from pPDHH1-GFP was inserted into the *Xho*I/*Pst*I site of YEpGAL1-ELM1C to yield YEpDGFP-ELM1C. And a 1.5 kb *Xho*I fragment containing 5' half of *ELM1* from pT7-ELM1 into the *Xho*I site of YEpDGFP-ELM1C.

YCpDGFP-ELM1 *TRP1*, *CEN6-ARSH4*, P_{DHH1}:GFP-*ELM1*, amp^r. Made by inserting a *Sph*I-*Bam*HI fragment of P_{DHH1}:GFP-*ELM1* from YEpDGFP-*ELM1* into the *Sph*I/*Bam*HI site of pRS314x.

pT7-PELM1 *ELM1* promoter, amp^r. Made by inserting a 0.6 kb PCR fragment of *ELM1* promoter amplified from YCpELM1ΔCS using primer A and PELM1-AscI, into pT7-blue (Novagen).

YCpGFP-ELM1 *TRP1*, *CEN6-ARSH4*, P_{ELM1}:GFP-*ELM1*, amp^r. Made by inserting a 0.6 kb *Sph*I/*Asc*I fragment of *ELM1* promoter from pT7-PELM1 into the *Sph*I/*Asc*I site of YCpDGFP-ELM1

3. Growth media and culture conditions

Growth media, culture conditions and genetic manipulations are mostly as described by Guthrie and Fink (1991). All cultures were incubated at 28 °C unless otherwise stated.

4. Manipulation of DNA

The procedures for gene cloning described by Sambrook et al. (1989) were mostly followed. *E. coli* strains used for the manipuraion of plasmid are described in Moriya (1995). Plasmids used in constructions and DNA sequencings were prepared using Flexi prep plasmid purification kit (Pharmacia). DNA sequencing were performed using ABI PRISM 310 Genetic Analyzer following to supplier's protocol.

5. Introduction of DNA into yeast cells

For the introduction of DNA into yeast, lithium-acetate method was used. All procedures were followed as described in Tanaka, 1990.

6. Polymerase chain reaction (PCR)

PCR was performed using the Expand High Fidelity PCR system (Boehringer) by following the manufacture's protocol. To make flushed PCR fragments, the UlTma PCR system (Perkin-Elmer) was used. Synthetic primers used in this study are:

A :	5'-gcttccggctcgtatgttgtgtg-3'
B :	5'-aaaggggatgtgctgcaaggcg-3'
DHH1-a :	5'-gggagctcttaatactggggttgtgac-3'
DHH1-s :	5'-ggacccgggttccatcaataataacttc-3'
fDHH-5' :	5'-gtccctggtgagtctctgtc-3'
fDHH1-3' :	5'-tcccggaaacatgaagatgc-3'
PDHH1a :	5'-ctcgagccattactactattttctttcttg-3'
PDHH1-AscI :	5'-ggggcgcgcctactattttctttcttgtcg-3'
GALU-a :	5'-ggctcgagtcattatagtttttttctcc-3'
GALU-s :	5'-gcggtaccactttgtaactgagctg-3'
GST-s :	5'-ggctcgagcccctatactaggttattgg-3'
GAP3-a :	5'-ggggatccctatgagtaccgcttcgtgg-3'

GAP3-s : 5'-ggggatccagggccagactaatgggagg-3'

ELM1-s : 5'-ctcgagtgtcacctcgacagcttatacc-3'

PELM1-AscI : 5'-ggcgcgcctttcatgctaagtaattattg-3'

CDC28-s : 5'-gagcggtgaattagcaaattac-3'

CDC28-a : 5'-ttatgattcttggaagtagggg-3'

7. Preparation of DNA from *S. cerevisiae*

Chromosomal and plasmid DNA from *S. cerevisiae* were prepared as described in Tanaka (1993) with minor modification. For PCR and Southern blot analysis, Flexi prep kit (Pharmacia) was used for purification of DNA

1. Cells of a 1.5 ml overnight culture were collected and resuspended in 400 μl of breaking buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA).
2. Equal volume of glass beads (0.5 mm diameter) and 400 μl of TE-saturated phenol/chloroform (1:1) were added and vortex vigorously for 5 min.
3. Centrifuged for 5 min. and the supernatant was transferred another tube.
4. 1 ml of ethanol was added and mixed. Centrifuged for 5 min.
5. Precipitate was resuspended in 50 μl of water.
   (To recover plasmid, 2.5 μl of the DNA suspension was used for *E. coli* electro-transformation.)
6. 200 μl of Flexi prep glass matrix (Pharmacia) was added and vertex gently for 1 min.
7. Centrifuged, removed the supernatant and wash precipitate. with 300 μl of wash buffer (Pharmacia).
8. Wash precipitate with 70 % ethanol.
9. Elute DNA with 50 μl of water. (This DNA solution was used for PCR or Southern blot analysis.)

8. Disruption of *DHH1*, *SSD1* and *ELM1*

Gene disruption was performed by following the one-step gene replacement method described by Rothstein (1991).

1. Plasmid containing disruption construct of *DHH1* (p*Δdhh1::URA3* and p*Δdhh1::LEU2*), *SSD1* (YCp*Δssd1::HIS3*) or *ELM1* (YCp*Δelm1::HIS3*) was amplified by PCR using the primers derived from the vector sequences (A and B primers; Dynal-Japan, Tokyo).
2. Amplified fragments were introduced into each strain listed in above by Li-acetate method (Guthrie and Fink, 1991) and spread to synthetic complete (SC) medium lacking the amino acids to be selected.
3. Colonies were picked up and the organization of the target gene and its neighboring regions was examined by PCR using appropriate ORF specific primers followed by Southern blot analysis (Moriya 1995).

9. RcDNA sequencing

9-1. GST fusion protein purification

The procedures of GST-fusion protein purification were mostly followed as described in Ausubel et al. (Vol. 2 13: *Saccharomyces cerevisiae*.).

1. 10 ml of YPD culture of YEpDGDH3 carrying RAYΔDLα was transferred to 400 ml of YPD and cultured 40 hrs.
2. Cells were collected by centrifugation 5 min. at 4 °C.
3. Cells were washed with 3 cell volume of water (1 g cell precipitate is about 1 ml).
4. Cells were resuspended in one cell volume of Zymolyase buffer (50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 1 M sorbitol) containing 30 mM DTT and incubated for 15 min. at room temperature.
5. Cells were collected by centrifugation and resuspended in 3 cell volume of Zymolyase buffer containing 1 mM DTT and add 50 mg of Zymolyase 20T and incubated for 1 hr at 30 °C.
6. Cells were washed with Zymolyase buffer 3 times.
7. Cells were resuspended in 15 ml of PBS containing 1 mM PMSF.
8. Cells were disrupted by dounce homogenizer (about 15 strokes).
9. The Cell lysate was centrifuged 15, 000 rpm for 1 hr at 4 °C.
10. 300 μl of Glutathione sepharose 4B (Pharmacia) pre-saturated by PBS was added into the supernatant and rotate 30 min.

11. 0.5 ml of 20 % Triton X-100 was added and rotate another 15 min.
12. Beads was pelleted by centrifugation and washed with 10 ml of PBS twice.
13. The fusion protein was eluted with 200 μl of elution buffer 3 times for 10 min. at room temperature.
14. 5 μl of each elution fractions were analyzed by 10 % SDS-PAGE and the second elution fraction was used for the RcDNA sequencing.

9-2. RNA recovery from GST-fusion protein

1. 200 μl of GST-DHH1 fusion protein solution and 20 μl of 3 M Na-acetate (pH 5.2) and 1 μl glycogen were mixed.
2. 200 μl of phenol /chloroform (1:1) was added and vortex, then centrifuged.
3. The aqueous layer was extracted by chloroform.
4. 500 μl of ethanol was added and centrifuged.
5. The precipitate was washed with 70 % ethanol and dry under vacuum.
6. The precipitate was resuspended in 10 μl of water and the OD260 was measured the RNA solution to estimate the concentration of RNA.

9-3. cDNA synthesis

cDNA synthesis was performed using TimeSaver^{TM} cDNA synthesis kit (Pharmacia) followed as described in manufacture's protocol with minor modification.

1. 1 μg of RNA in 20 μl of water was treated at 65 °C for 10 min. then chilled on ice.
2. The RNA solution was transfered into First strand mix tube and 1 μl of DTT soln. and 1 μl of random hexamer were added.
3. The tubed was incubated for 1 hr at 37 °C.
4. The reaction was transfered into Second strand mix tube and incubated for 30 min. at 12 °C, then for 1hr at 22 °C.
5. The reaction was heat inactivated for 10 min. at 65 °C, then cooled down to room temperature.
6. Add 100 μl of phenol/chloroform (1:1) to the reaction and vortex.
7. The reaction was centrifuged for 1 min. and aquelious fraction was transfered to Nick^{TM} spin column (Pharmacia).

8. The small aliquot of the elution was ligated into the HincII site of BAP treated pUC118 (TAKARA).
9. Nucleotide sequences of the plasmids containing insert fragment were determined.

10. Preparation of total RNA from *S. cerevisiae*

Total RNA preparation from *S. cerevisiae.* was prepared using acid-phenol method (Iyer and Struhl, 1996) with minor modification.

1. Cells of 5 ml yeast culture were collected and washed with water and resuspended in 400 μl of lysis buffer (10 mM Tris-Cl (pH 7.5), 10 mM EDTA, 0.5 % SDS).
2. 400 μl phenol (solved in water, pH 4.0) and equal volume of acid-washed glass beads (0.5 mm diameter) were added.
3. The tube was incubated 1 hr for 60 min. with vortex occasionally.
4. The tube was chilled on ice 10 min. and centrifuged 10 min at 4 °C.
5. The supernatant was transferred into another tube and extracted by phenol (pH 4.0), then chloroform.
6. 40 μl of 3 M Na-acetate (pH 5.2) and 1 ml of ethanol were added and vortexed, then centrifuged 5 min.
7. The precipitate was washed with 1 ml of 70 % ethanol and dried under vacuum.
8. The precipitate was suspended in 20 μl of H_20.
9. The RNA prepared as this was used for Northern blot analysis. Procedures were followed as described in Amano. 1992.

11. β-galactosidase assay

11-1. β-galactosidase assay of liquid cultures

This assay was followed as described in Ausubel et al. (Vol. 2 **13:** *Saccharomyces cerevisiae.*) with minor modification.

1. Cells from 1 ml yeast culture were collected and resusupended in 550 μl of Z-buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$-$7H_2O$, 50 mM 2-morcaptoethanol / 1 litter H_20.)
2. 50 μl of the aliquot was diluted with 950 μl of water and OD600 was measured.

3. Add 25 μl of 0.1 % SDS and 40 μl of chloroform to the remainder and vortex 20 sec.
4. After pre-incubate 5 min. at 30 °C, 100 μl of ONPG soln. (4 mg/ml in Z-buffer) and incubate at 30 °C until the yellow collor developed.
5. The reaction was stopped by adding 250 μl of 1 M Na_2CO_3. Centrifuged 5 min.
6. 100 μl of the supernatant was diluted in 900 μl of water and OD420 was measured.

11-2. β-galactosidase assay of solid cultures (filter assay)

This assay was followed as described in Ausubel et al. (1997. Vol. 3 **20:** Analysis of protein interactions) with minor modification.

1. Colonies of each strains were picked up and put onto filter paper (Whatman 3MM).
2. Chloroform were added onto the filter to permease the cells.
3. After the filter became dry, Z-buffer containing X-gal (1 mg/ml) was added and incubated at 30 °C until the blue color was developed.

12. Screening for synthetic lethal mutants

The procedure described by Bender et al. (1991) was followed for the isolation of mutants that were lethal when combined with the *dhh1* deletion.

1. Strain HTYΔD (Δ*dhh1::LEU2*) carrying YEpA3D1 were constructed as described above.
2. As the *ade2* red color development of HTYΔD/YEpA3D1 was not fine for unknown reason, first of all, HTYΔD/YEpA3D1 was mated with RAY3-A**a** and spores of the yielded strain were separated on YPD plate.
3. Colonies with fine red color and clear sectors were selected.
4. Cells of these strains were mutagenized with 3% EMS (which resulted in about 40 % survivors) and spread on YPD plates.
5. After about 5 days' incubation at 28°C, the plates were placed at 4 °C for several days to allow red color caused by the *ade2* mutation to develop.
6. About 20,000 colonies were examined under microscope and colonies producing no sector ($SECT^-$) were selected.
7. These colonies were re-streaked twice on YPD plate to make their $SECT^-$ phenotype stable.

8. They were then streaked on SC medium containing 5-fluoro-orotic acid (5-FOA) at a final concentration of 1 mg/ml to examine whether or not they need the plasmid for growth.
9. A single-copy or a multicopy plasmid with or without *DHH1* was then introduced into each SECT$^-$ isolate to confirm whether or not their *DHH1*-dependent growth (*ddg*) phenotype.

13. Identification of genes responsible for the *ddg* phenotype

1. A plasmid library which was constructed with the single-copy vector pRS314x and harbored 4 to 10 kb *Sau*3AI partial digests of the genomic DNA of RAY3-AD was used to transform each of the SECT$^-$ isolated and the resultant transformants were allowed to grow on SC plates lacking tryptophan (SC-W) at 28°C for several days.
2. Colonies were replica-plated onto SC-W containing 5-FOA (1 mg/ml) and the plates incubated at 28°C until colonies became visible.
3. They were then picked up and subjected to plasmid isolation.
4. The nucleotide sequence of the insert in each plasmid was then determined to identify their chromosomal location as well as the corresponding genes/ORFs using the *S. cerevisiae* genomic databases (MIPS, YPD, SGD).

14. Construction of a *ELM1* over-expressing strain

1. Colonies of RAY-3A carrying YEpGAL-ELM1 were inoculated and cultured in SC-W medium for overnight at 28 °C.
2. Cells were collected and resuspended in SC medium lacking uracil and containing 2% galactose and 2% raffinose instead of glucose.
3. Cells after overnight cultivation were analyzed by microscopy or FACS.

15. Microscopic observation

Morphology of the cells of each isolate was monitored under various conditions using a fluorescent microscopy BX60 (Olympus Optical, Tokyo) according to the procedure described by Guthrie and Fink (1991). Images were taken by the SenSys CCD camera system (Photometrics, München) and the data were rendered using the IPLab Spectrum Software

(Image and measurement, Tokyo) and the Photoshop version 3.0 (Adobe) on a Macintosh computer.

For the observation of GFP-fusion protein and *ade2* endogenous fluorophore, cell cultures were directly observed under the fluorescent microscopy using a NIBA filter for the observation of GFP-fusion protein or a WBV filter for the observation of *ade2* endogenous fluorophore .

For the observation of actin,

1. Cells were fixed by adding formaldehyde (final conc. 3.7%) by direct addition of 1/10 volume of 37% formaldehyde to the cell culture.
2. After 5 min. at 28 °C, cells were collected by centrifugation and resuspended in 100 μl of water.
3. 5 μl of Rhodamine-conjugated pharoidin soln. (3.3 μM: Molecular probe) was added and leave it for 5 min. at RT.
4. Cells were observed under fluorescent microscopy using a WBV filter.

For the observation of nuclear and bud scar,

1. Cells were collected and fixed by resuspending in 95% ethanol.
2. After 5 min. at RT, cells were collected and resuspended in, 100 μl of Fluorescent Brighter soln. (0.5 mg/ml: Sigma) for bud scar staining. Or 100 μl of DAPI soln. (2.5 μg/ml) for nuclear staining. Double staining can be also performed.
3. Cells were observed under fluorescent microscopy using a WU filter.

16. FACS analysis

FACS analysis was performed as described by Tanaka et al. (1996).

1. 1.5 ml yeast cultures were spun down, resuspended in 0.2 M Tris-HCl (pH7.5), and fixed overnight in 70 % ethanol at -20 °C.
2. Cells were washed with Tris-HCl, resuspended in the same buffer containing 1 mg/ml RNaseA and incubated for 30 min at 37 °C.
3. The cells were pelleted, washed, resuspended in Tris-HCl containing 0.05 mg/ml of

propidium iodide (PI), chilled on ice for 15 min, pelleted again, and resuspended in Tris-HCl containing 0.01 mg/ml PI.

4. They were then sonicated briefly, and subjected to DNA content and cell size analysis using a FACScan (Beckton-Dickinson).

17. World Wide Web Yeast Genome Databases

MIPS : Munich Information Centre for Protein Sequence
(http://speedy.mips.biochem.mpg.de/mips/yeast/)

SGD : Saccharomyces Genome Database
(http://genome-www.stanford.edu/Saccharomyces/)

YPD : Yeast Protein Database
(http://quest7.proteome.com/YPDhome.html)

RESULTS

1. The phenotypes of a *DHH1* disruptant

The coding region of the *DHH1* gene was amplified by PCR, cloned into plasmid pT7-blue and was disrupted by inserting the maker gene *LEU2* or *URA3* (Fig. 2) as described in MATERIALS AND METHODS. In addition to the previously reported growth retardation phenotype (Strahl-Bolshinger et al., 1993), I found some other phenotypes caused by the disruption of *DHH1*: the *DHH1* disruptant obtained showed reduced viability as judged from the color development assay on Erythrosine-containing YPD plates. In addition, it exhibited a sporulation defect when made homozygous in a diploid (Fig. 3). The *DHH1* deletion in strain W303 showed some additional phenotypes: more pronounced growth retardation on SC or SDS-containing YPD plates in comparison with the growth on YPD plates, similar growth retardation at a high temperature (37°C) in comparison with that at 28°C, and defective *ade2* red color pigment formation (see below). From these results, it was concluded that *DHH1* was not essential for yeast growth, but its disruption affected the growth under certain conditions.

2. Dhh1p is non-specifically associated with RNA *in vivo*

To analyze the function of Dhh1p, I performed an experiment to characterize the RNA molecules associated with Dhh1p, because many RNA helicases are known to be associated with various RNA molecules. The strategy was as follows. 1) A glutathione S-transferase (GST)-Dhh1p fusion protein was expressed and purified directly from yeast cells. 2) RNA fragments co-purified with GST-Dhh1p were recovered by phenol extraction and converted to cDNA using random-primers. 3) These cDNAs were cloned into a plasmid and DNA sequences were determined. Using an analogous system, I found that the *Escherichia coli* RNA helicase HrpA bound ribosomal RNA segments known to be located at the 'platform' site. I also found that it was associated with an mRNA fragment corresponding to the *ppk-ppx* inter-genic region (Moriya and Isono, 1995). Based on that experience, I tried first to express GST-Dhh1p under the control of a *GAL1* promoter, but it did not complement the sporulation defect of the *DHH1* disruptant. This might have been caused by the fact that the

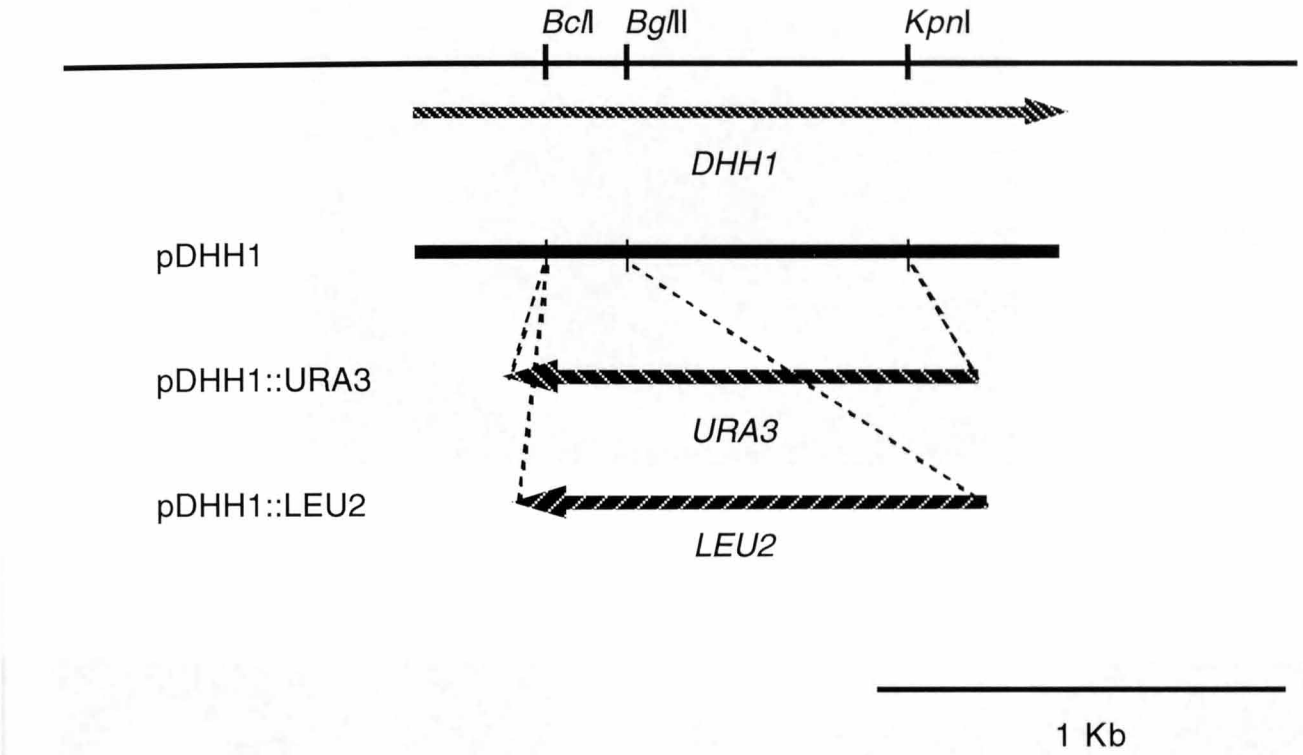


Fig. 2. Disruption of the *DHH1* gene. Part of *DHH1* was replaced by *URA3* or *LEU2* genes at the restriction enzyme cleavage site as indicated. Procedures for the construction of plasmids and *DHH1* disruptants are described in MATERIALS AND METHODS.

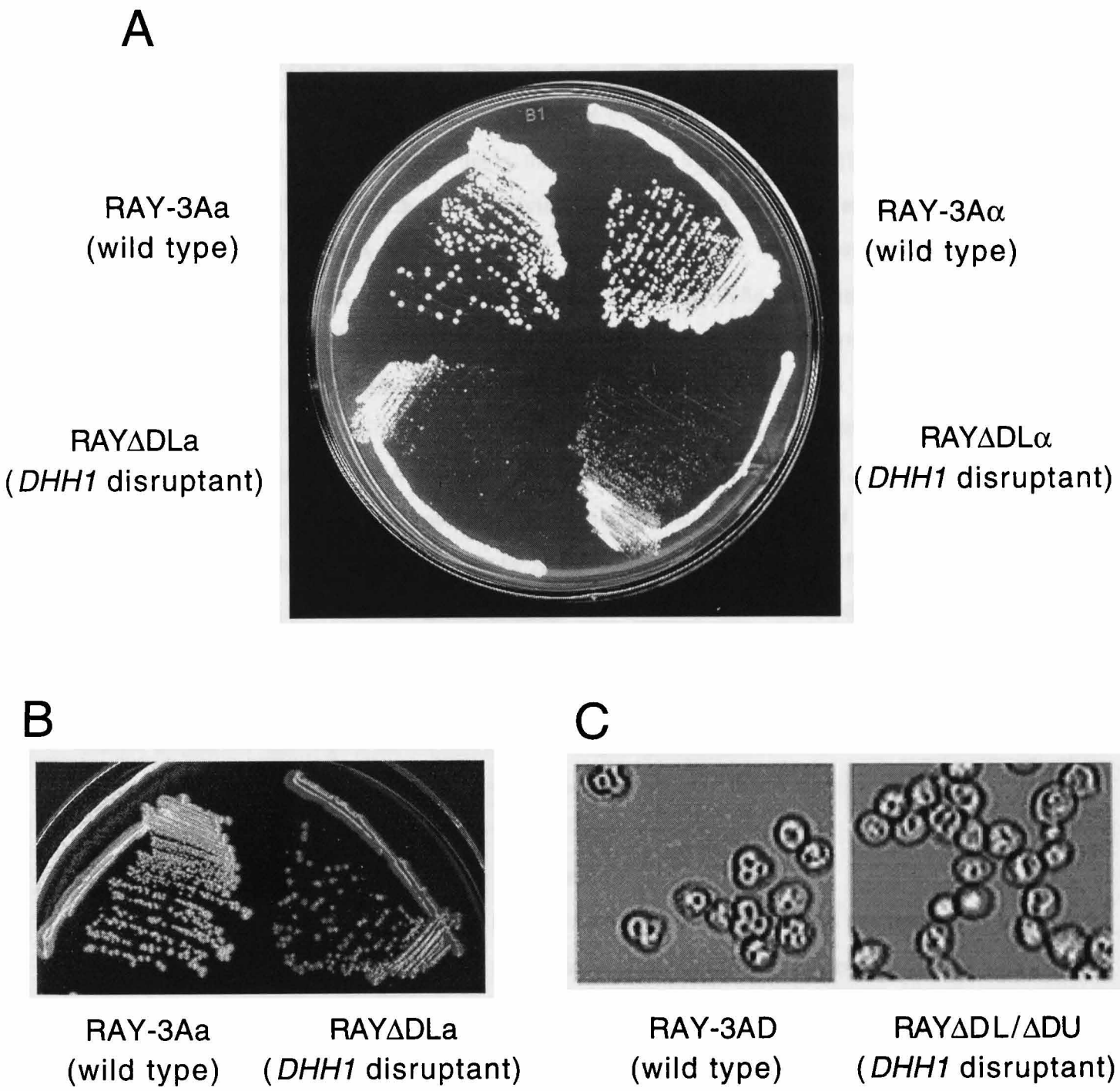


Fig. 3. The phenotypes of *DHH1* disruptants. A, Growth retardation of *DHH1* disruptant. Each strain was streaked on a YPD plate and incubated for two days at 28 ℃. B, Reduced viability. Each strain was streaked on a YPD plate containing erythrosineB and incubated for three days at 28℃. C, Sporulation defect in homolyzous diplid. Each strains were cultured in sporulation medium for one week. Photographs were taken under the microscopy (magnification 400X).

GAL1 promoter does not work under the sporulation condition. Consequently, I changed the *GAL1* promoter to a *DHH1* promoter. This construct (YEpDG-DHH1) was able to complement the growth retardation and the sporulation defect of the *DHH1* disruptant (data not shown). I performed subsequent experiments using this construct. I purified the GST-Dhh1p fusion protein, recovered RNA fragments and converted them into cDNA as described in MATERIALS AND METHODS. The results of sequencing of thirty cDNA clones thus obtained are listed in Table 3. Most of the RNA species were identified as stable RNAs such as ribosomal RNA (18SrRNA, 25SrRNA and 5SrRNA) and spliceosomal RNA (U1snRNA and U4snRNA). In addition, four mRNA fragments encoding glycolytic enzymes glucokinase, glyceraldehyde-triphosphate-dehydrogenase and so on were isolated. From these sequence data, it was obvious that no specific RNA fragments were associated with Dhh1p. Electrophoretic analysis of the recovered RNA species of low molecular weight confirmed this observation and small RNAs except tRNA were found to be co-purified with GST-Dhh1 in their intact length (Fig. 4). As tRNA forms a characteristic structure and binds specific proteins, the result indicated that no tRNA was associated with Dhh1p. Therefore, I concluded that Dhh1p binds RNA, but not in a species- or sequence-specific manner. Since the recovered cDNA clones contained both cytoplasmic RNA (20S RNA) and nuclear RNA (snRNA species), the subcellular localization of Dhh1p could not be determined from this experiment alone.

3. Deletion of *DHH1* affected gene expression

As described above, I found that the association of Dhh1p and RNA seemed not to be specific. However, I also found that the occurrence of some mRNA species associated with Dhh1p was different between the wild type and the *DHH1* disruptant. As shown in Fig. 5, the mRNA abundance of *GLK1* and *YHR110w* was different between the wild type and the *DHH1* disruptant cell grown in acetate media (YEPA). Similarly, the abundance of *TDH1* in the stationary phase cells varied between the wild type and the *DHH1* disruptant. Although I can not say that there is a general relationship between the mRNA abundance and its association with Dhh1p, *DHH1* disruption seems to affect the level of some transcripts. From a subsequent Northern blot analysis, it was suggested that a *DHH1* deletion affected the *TDH1* gene expression. Therefore, I analyzed more quantitatively the effect of *DHH1* deletion on the *TDH1* expression using *lacZ* as a reporter gene. First, I constructed a plasmid which

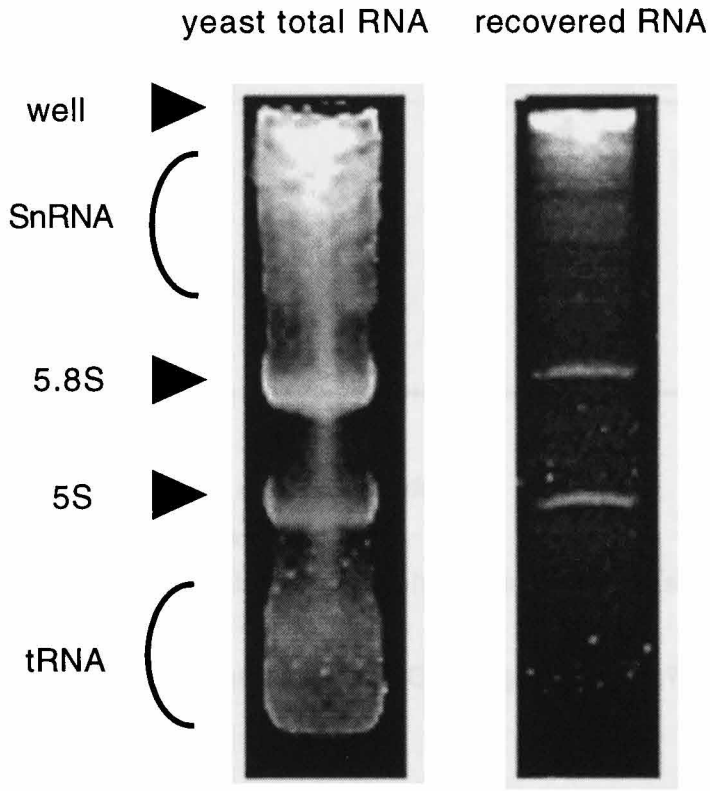


Fig. 4. Electrophoresis of small RNA molecules associtaed with GST-Dhh1p on a 12% denaturing polyacrylamide gel. Each RNA species was judged from its molecular weight as indicated. Yeast total RNA was prepared as described in MATERIALS AND METODS. 'recovered RNA' indicates RNA assocoated with Dhh1p prepared as described in MATERIALS AND METODS.

Table 3. Summary of RNA species associated with GST-Dhh1p

RNA species	Number of clones* 3
mRNA* 1	4
25S rRNA	13
18S rRNA	8
5S rRNA	1
U1 snRNA	1
U4 snRNA	1
20S RNA* 2	2
total 30	

* 1 mRNA containing clones included:

GLK1 (glucokinase),

TDH1 (glyceraldehydephosphate-dehydrogenase),

CDC16,

YHR110w (uncharacterized ORF: glycoprotein?)

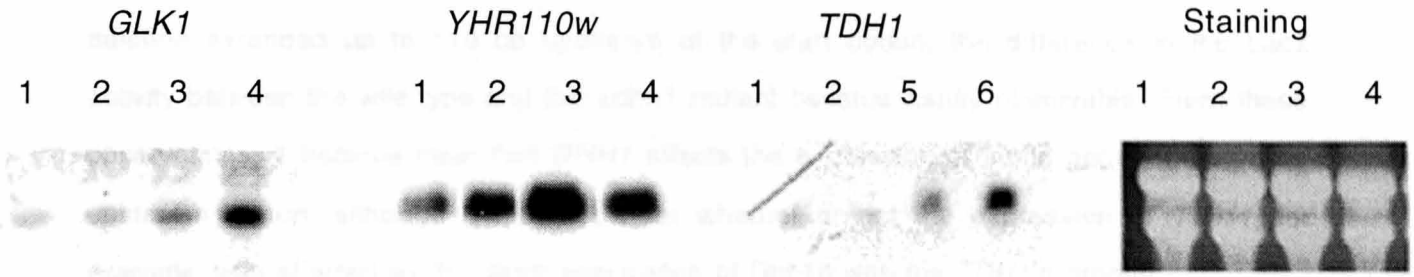
* 2 cytoplasmic virus like RNA

* 3 Additional mRNA containing clones were identified which included:

YHR033w (putative gamma-glutamyl kinase homologue),

DNM1 (dynamin-related protein), and

TEF1 (translational initiation factor 1-alpha)



1: RAY-3Aa	-glucose	4: RAYΔDLa	-acetate
2: RAYΔDLa	-glucose	5: RAY-3Aa	-stationary
3: RAY-3Aa	-acetate	6: RAYΔDLa	-stationary

Fig. 5. Northern blot analysis of mRNA associated with Dhh1p. RNA was prepared from the mid-log phase cells of wild type (RAY-3Aa) and Δ*dhh1* (RAYΔDLa) cultured in YPD (indicated as -glucose) or YEPA (indicated as -acetate), or from cells in the stationary phase cultured in YPD medium (indicated as -stationary) as described in MATERIALS AND METHODS. Radio-labeled DNA probes were prepared from plasmids containing fragments of indicated genes as described in MATERIALS AND METHODS.

contained *lacZ* so as to be expressed under the control of the *TDH1* promoter. In addition, I constructed another plasmid in which *lacZ* would be expressed under the control of an *ACT1* promoter as a control. Results are shown in Fig. 6. The LacZ activity expressed from the *TDH1* promoter was significantly low in the *DHH1* disruptant than in the wild type, especially in the acetate medium. However, the LacZ activity expressed from the *ACT1* promoter was not so much affected at least in log phase in YPD.

To investigate further the effect of a *DHH1* disruption on the *TDH1* expression, I constructed several deletions of the *TDH1* promoter region and found that when the promoter deletion extended up to 110 bp upstream of the start codon, the difference in the LacZ activity between the wild type and the *Δdhh1* mutant became hardly observable. From these observations, it became clear that *DHH1* affects the expression of some genes through the upstream region, although it was not clear whether or not the expression of *TDH1*, for example, was affected by the direct association of Dhh1p with the *TDH1* promoter.

4. Dhh1p has a transcription-stimulating activity at its C-terminal region

To know the function of a gene product, it is usually informative to identify its molecular partner protein. There are several experimental procedures that have been developed to investigate protein interactions. The yeast two-hybrid system is one of the most powerful procedures for such a purpose (Phizicky and Fields, 1995). In the course of studies of Dhh1p by the yeast two hybrid system, I found two characteristic phenomena associated with Dhh1p. One is a transcription-stimulating activity, and another is a self-interacting (dimer forming) activity of Dhh1p. When fused to the Gal4p DNA-binding domain, Dhh1p showed a transcription stimulating activity from a *GAL1* promoter. As shown in Figs. 7-a and -b, the tester strain Y190 carrying plasmid pSD1 showed a little but significantly higher LacZ activity in comparison to the control, i.e. Y190 carrying plasmid pAS2-1. This activity was enhanced several-fold when cells were cultured in a medium containing acetate as a carbon source. By constructing a truncated pSD1 derivatives, I was able to determine the domain which possessed this activity. As shown in Figs. 7-c and -d, a hybrid containing only the C-terminal proline-glutamine rich region of Dhh1p had this transcription-stimulating activity. In contrast, the R-construct (Fig. 7-c) showed a significant but only weak activity. An explanation for this observation is that perhaps it was

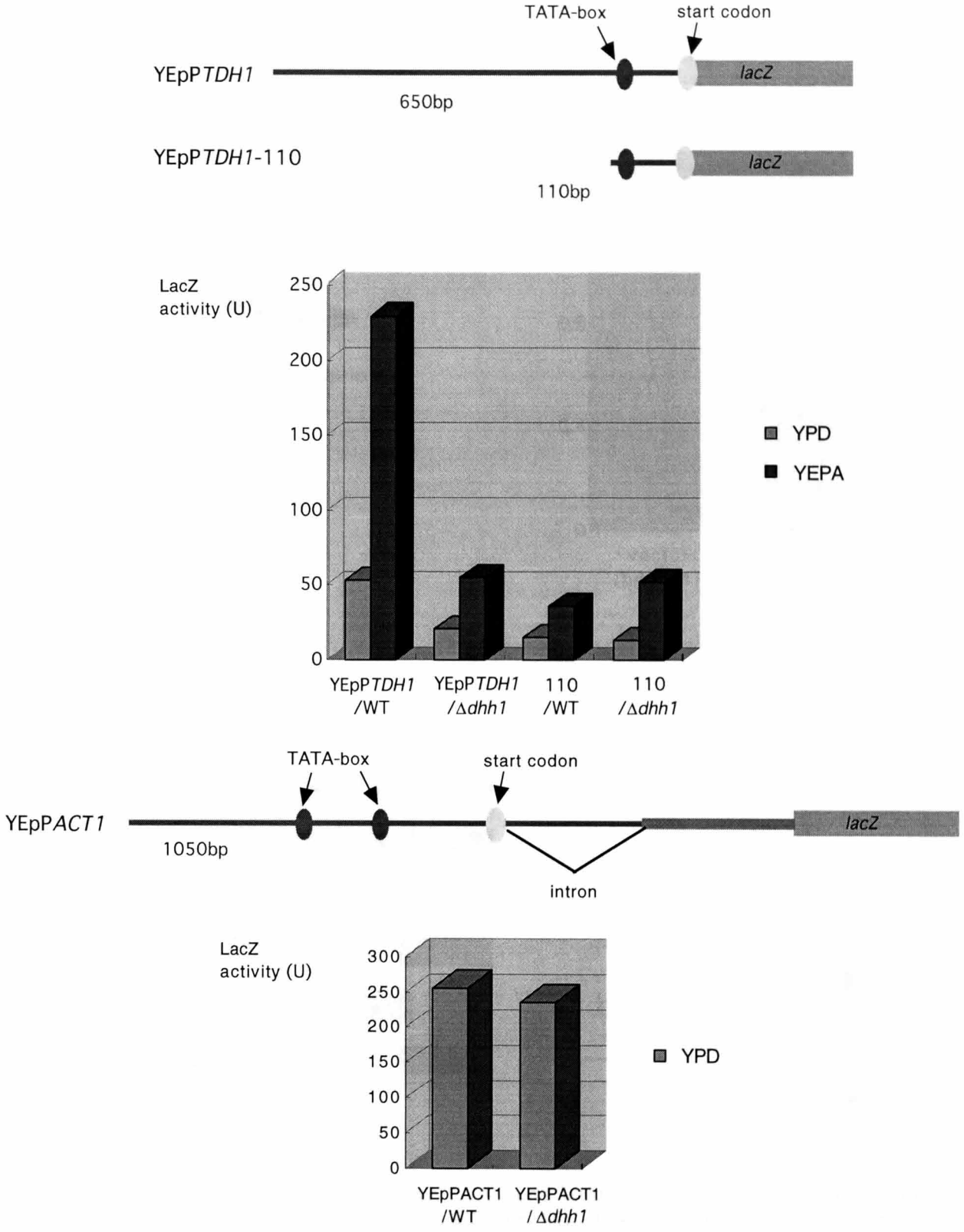


Fig. 6. Effects of *DHH1* disruption on the expression of LacZ from the *TDH1* and *ACT1* promoters. Plasmids used in this experimet are shown at the top. The yeast cells of wild type (WT) and *DHH1* disruptant (*Δdhh1*) carrying indicated plasmids are precultured in SC-U for two days and transfered to YPD (light box) or YEPA (dark box) with 1/2 dilution. After five hours' cultivation, the LacZ activities were determied as described in MATERIALS AND METHODS and presented in Miller units. Each experiment was duplicated and avarage values are shown. The standard deviations in each experiment was less than 20%.

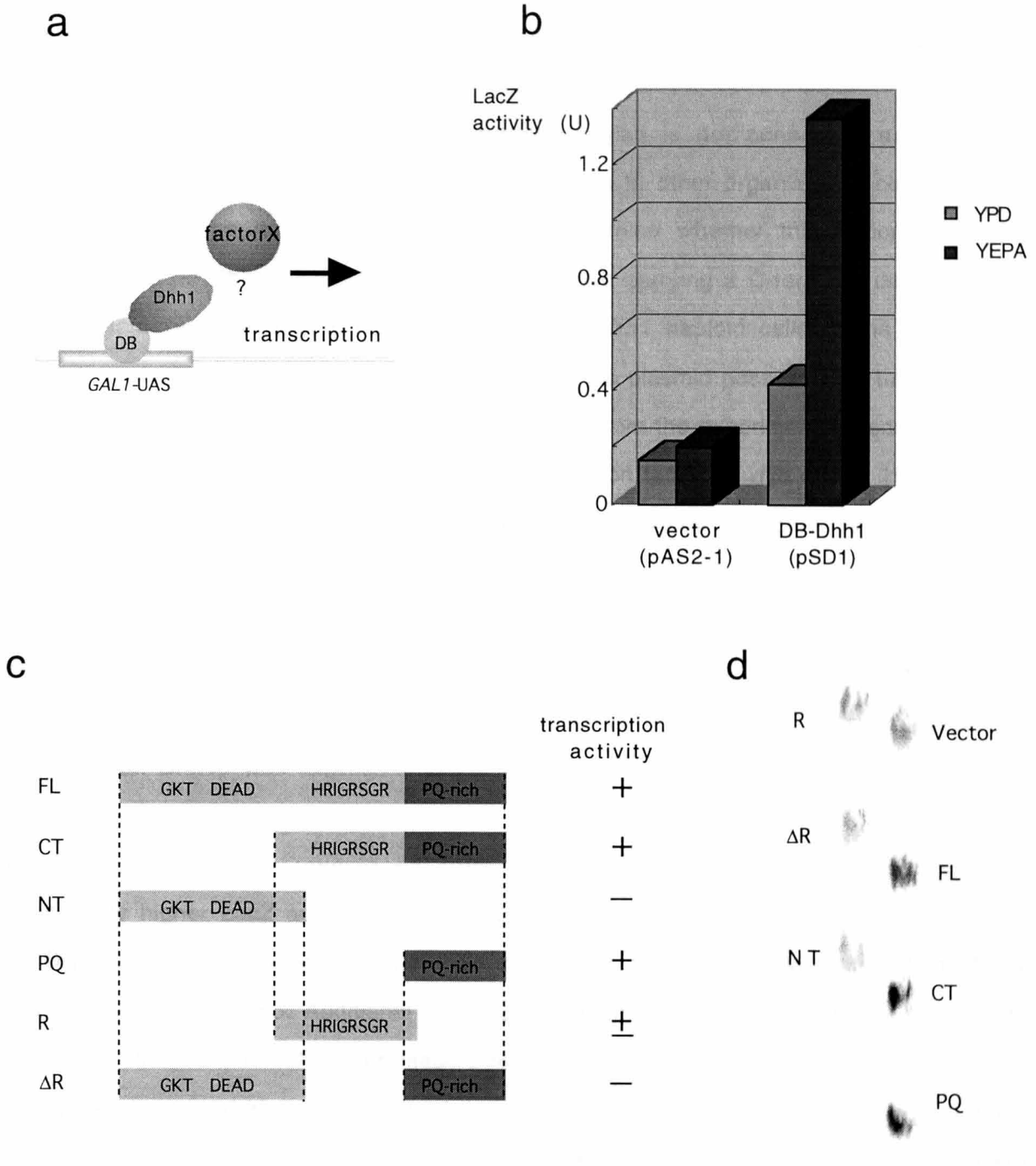


Fig. 7. Functional dissection of the Dhh1p protein. a, A model for the mechanism of one hybrid system. b, The transcription-stimulating activity of Dhh1p. The LacZ activities of tester strain Y190 carrying plasmid pSD1 expressing a hybrid protein consisting of the Gal4p DNA-binding domain (DB) and Dhh1p and plasmid pAS2-1 expressing the Gal4p DB alone. Each strain was cultured for two days in SC-W and transfered to YPD (blue box) or YEPA (red box) by making a 1/2 dilution. After 4.5 hours' cultivation, the LacZ activity of each strain was measured as described in MATHERIALS AND METHODS and expressed in Miller units. c, Various truncated Gal4p DB-Dhh1p hybrid constructs and their transcription stimulating-activities. Truncated Gal4p DB-Dhh1p hybrids were constructed as described in MATERILAS AND METHODS and transformed into tester strain Y190. Colonies on SC-W plates were picked up and their transcription stimulationg activities were determied by the LacZ filter assays as shown in d.

due to the self-interacting activity of Dhh1p (see below). I do not know why the ΔR construct apparently lacked the activity. Maybe, its protein folding or physiological stability was affected.

As the C-terminal proline-glutamine rich region is not conserved among other DEAD-box proteins as well as the *DHH1* homologues in other organisms, I constructed a plasmid harboring a deletion of this region to examine whether this region would be necessary for the Dhh1p function. A resultant plasmid carrying a C-terminal deletion could not complement either the growth retardation of Δ*dhh1* haploid cells or the sporulation defect in homozygous Δ*dhh1* diploid cells, although the plasmid possessed in full-length the conserved region of the DEAD-box proteins (Fig. 8). From the experiments described above, it was not known whether Dhh1p acts as a transcription factor *in vivo* or not. However, the above results indicate that at least Dhh1p acts in the process of nuclear RNA metabolism through the interaction with a protein or proteins which is (are) involved in transcription, and that the C-terminal region which is not conserved among other DEAD-box proteins is essential for the function of Dhh1p. Dhh1p thus appears to have achieved a specific role using its newly acquired region in its C-terminus.

When Dhh1p was expressed in the tester strain Y190 as a protein containing the Gal4 DNA-binding domain (expressed from pSD1) along with a protein harboring a Gal4-transcription activation (expressed from pCD1) domain, the tester strain showed significantly higher LacZ activity in comparison with the tester strain which contained only the DNA-binding domain hybrid pSD1. This means that Dhh1p has a self-interacting (dimer forming) activity *in vivo*. Additional analysis with various truncated hybrid proteins led to the finding that the interaction was made between the N-terminal and the C-terminal halves Dhh1p. Further analysis of the self-interacting domains is currently in progress. I have observed that purified Dhh1p tended to aggregate easily, a phenomenon which additionally indicated the multimer forming activity of Dhh1p.

5. Subcellular localization of Dhh1p

One of the ways to analyze the function of a protein is to obtain information concerning its subcellular localization. This is very useful, especially in the case of a protein associated with RNA, because RNA metabolisms occurring in the nucleus and

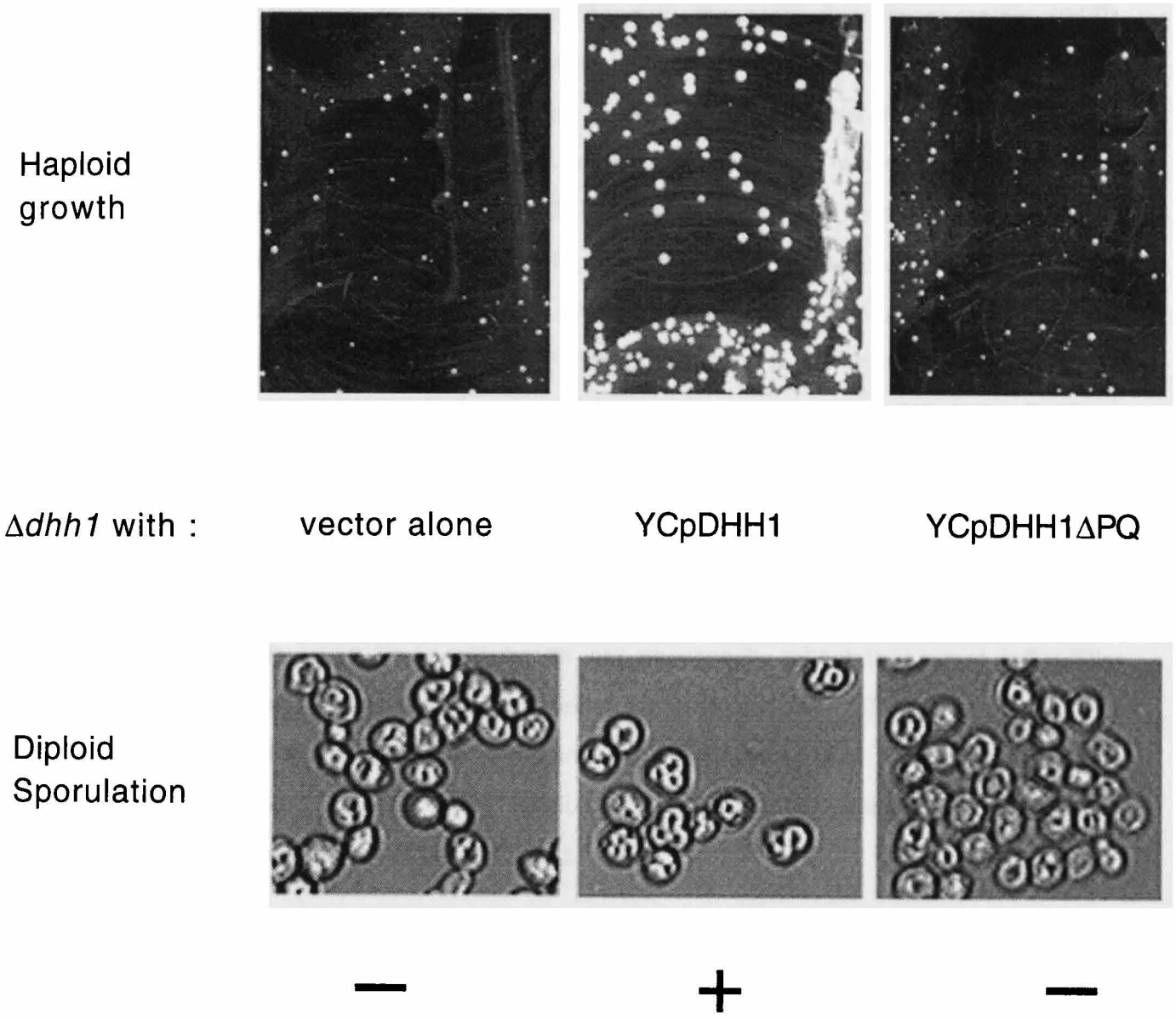


Fig. 8. The C-terminal PQ rich region is essential for the function of *DHH1*. Depletion of Dhh1p from which the PQ-rich region was deleted were unable to complement the growth retardation of a *DHH1* disruptant (upper) and the sporulation defect of a homozygous diploid *DHH1* disruptant (lower). *DHH1* disruptants carrying pRS314x (vector alone), YCpDHH1 or YCpDHH1ΔPQ were cultured on SC-WU plate for 3 days. Similarly, *DHH1* disruptants of homozygous diploid carrying pRS314x (vector alone), YCpDHH1 or YCpDHH1ΔPQ were cultured in sporulation medium for one week and observed under the microscopy (magnification 400X). + and - indicate sporulation-positive and -negative, respectively.

cytoplasm can then be separated. Therefore, I performed an experiment to analyze the intracellular localization of Dhh1p by fusing it to the green-fluorescent-protein (GFP). GFP is an auto-fluorescence-emitting protein isolated from jelly fish, and by expressing a fusion protein containing GFP and a target protein, we can observe the localization of the protein in question in a living cell under fluorescent microscopy without any treatment. A single copy plasmid expressing GFP-Dhh1p (YCpGFP-DHH1, see MATERIALS AND METHODS) was constructed which could complement the growth retardation and *ade2* pigment formation defect of W303ΔD, indicating that this fusion protein contained a functional Dhh1p. As shown in Fig. 9, GFP-Dhh1p was found to be localized in the cytoplasm as many spots in log phase cells grown in YPD. In YEPA medium, and in the stationary phase (data not shown), Dhh1p was localized as single or a very few spots in the cytoplasm. Judging from the staining with DAPI, this spot was not identical with the nucleus. Therefore, these spots remain to be characterized further. These observations, appear to indicate that Dhh1p forms a dimer (multimer ?) *in vivo* and the dimer formation is stronger in YEPA than in YPD. However, these results do not necessarily reflect the actual subcellular localization of Dhh1p, because the localization was analyzed as a GFP-fusion protein. It is possible that GFP fusion to the N-terminal region of Dhh1p had perhaps masked the localization signal. To confirm the localization results further, subcellular fractionation and/or immuno staining must be performed.

6. Screening and characterization of mutants synthetic lethal with a *DHH1* deletion

To analyze the function of *DHH1* further, I performed screening of mutants which showed synthetic lethality with a *DHH1* deletion. A 'synthetic lethal' phenotype can be observed when a mutant yeast can not grow with the combination of mutations in two or more genes, despite that individual mutations are not lethal. Screening of mutants was performed using the *ADE2/ADE3* colony sectoring assay (Fig.10, Bender and Pringle, 1991) described in MATERIALS AND METHODS. I selected about fifty mutants by their non-sectoring and *DHH1* dependent growth phenotype and I named them *ddg* (for *DHH1* dependent growth) mutants (H101 through H150). Nine of these mutants listed in Table 4 were studied in more details. Some of them showed temperature sensitivity (ts) which was rescued by the addition

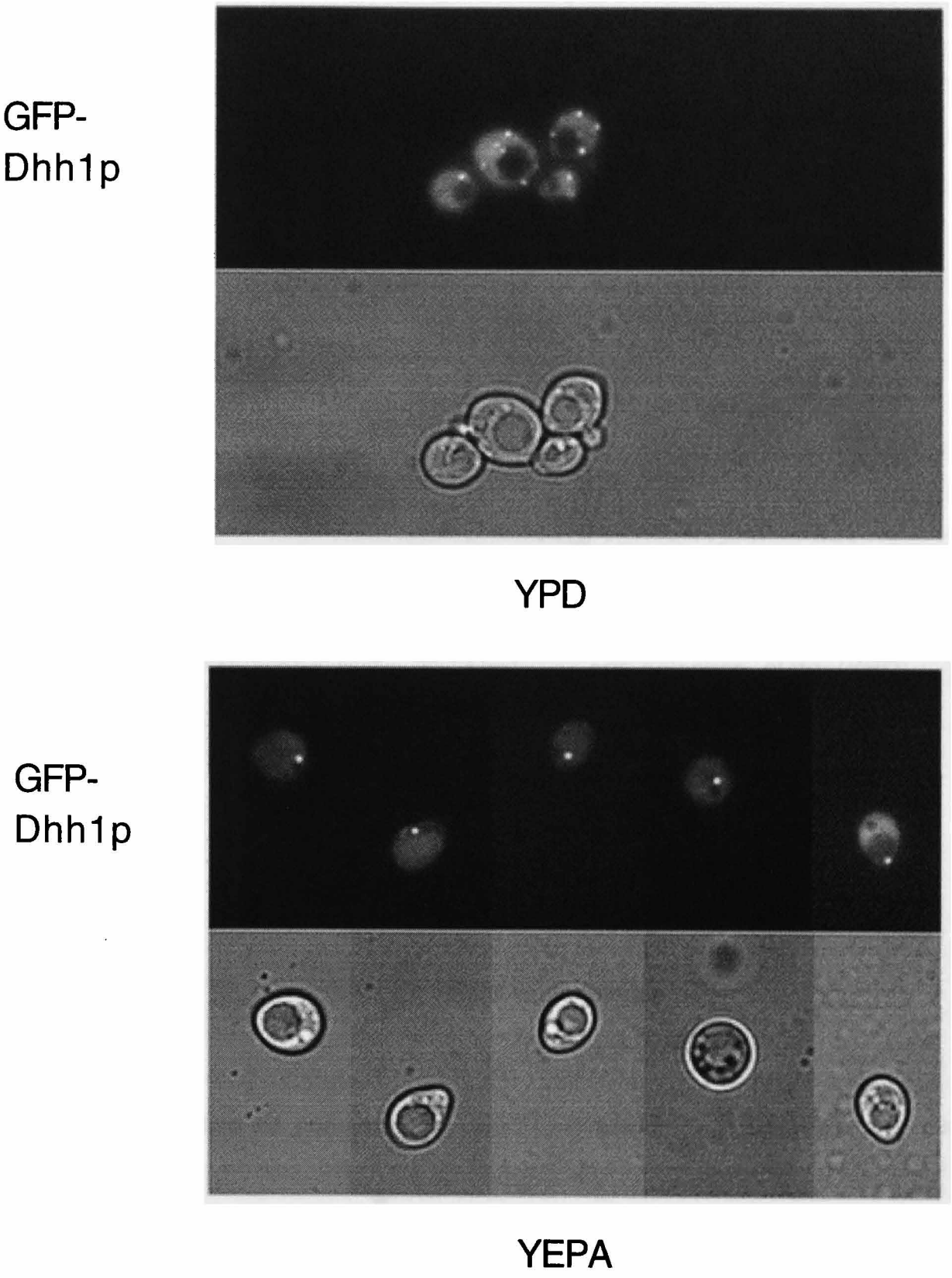
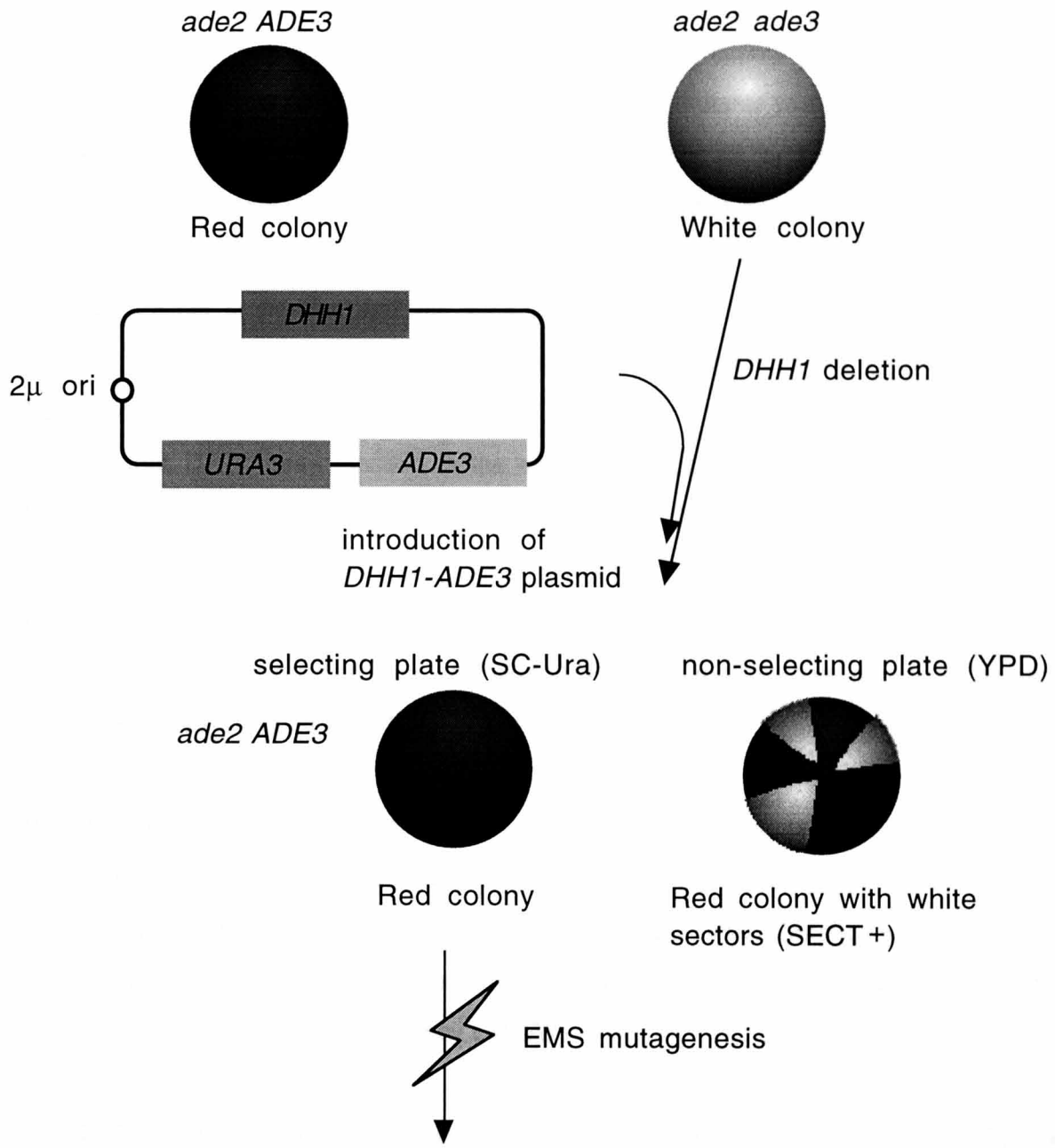


Fig. 9. Subcellular localizations of GFP-Dhh1p. The localization of GFP-Dhh1p in cells of an overnight culture of W303ΔD carrying YCpGFP-DHH1 grown in the indicated medium was visualized under fluorescent microscopy. Magnification was 1000X.

Fig. 10 Isolation of *ddg* (*DHH1* dependent growth) mutants



Microscopic selection of non-sectoring (SECT -) colonies on non-selecting plate

↓

Confirmention of the requirement for the *DHH1* activity and characterization of mutant phenotypes

↓

Identification of the genes defective in *ddg* mutants by screening a yeast genomic DNA library

of 1 M sorbitol as an osmotic stabilizer into the growth media. Some other strains also showed a *ddg* phenotype rescued by sorbitol. These *ddg* mutants may suffer from an osmotic instability of their cell wall. About a half of such *ddg* mutants was rescued by introducing a single copy suppressor gene *SSD1*. *SSD1* was identified as its functional redundancy with *DHH1* in many cellular processes and might be inactivated in strain HTY2-1 that was used for the isolation of the *ddg* mutants (see below). In some mutants, abnormal morphology such as cellular elongation and cell surface projections were observed and these abnormal cellular morphology was more pronounced at higher temperatures (Fig.11 and Fig.12). Some other mutants showed alpha-factor secretion defects (Fig.13). These observations indicate that *DHH1* may function in the cell wall integrity, cellular morphology and alpha-factor secretion. The characteristic features of some of the mutants are summarized below.

H101: All cells were elongated at 37°C, suffering from a cellular separation defect. Alpha-pheromone secretion was defective. This strain may a have defect in the targeted secretion of vesicles to the bud neck growth position.

H102: Most cells showed an abnormal cellular morphology at 28°C resembling a *Paramecium*. Enlarged cells were observed at 37°C. It cannot be rescued by a single copy *DHH1*, but can be rescued by a multicopy *DHH1* (multicopy suppression). Its *ddg* phenotype was not rescued by *SSD1*, while its ts phenotype was rescued. The *ddg* phenotype was also rescued by *CDC28*.

H103: The *ddg* phenotype was rescued by *SSD1*, but its ts phenotype was not. By a single copy genomic library screening, I found the *ERG10* gene which encodes an acetyl-coA acetyltransferase could rescue the *ddg* and ts phenotypes. As *ERG10* is an essential gene, further experiments such as to confirm the synthetic lethality of *ERG10* and *DHH1* has not been performed yet.

H104: Cellular elongation and cell-clamping were observed with this mutant grown at 37°C. Although a single copy *DHH1* rescued its *ddg* phenotype, *DHH1* on a multicopy was more effective. As will be described in greater details later, it possessed a

Table 4. Summary of the phenotype of *ddg* mutants isolated.

Strain	ts [a]	1M sorbitol [b]	Morphology [c] 28℃	Morphology [c] 37℃	α–factor [d]	YCpSSD1 [b]
H101		-		EL	-	-
H102		-	EL		+	-
H103	ts-S $^{+}$	+			+	+
H104	ts-S $^{+}$	+		EL	+	+
H105		-		EL	+	-
H106		+	EL	EL	+	+
H108	ts-S $^{-}$	-			+	-
H109	ts-S $^{+}$	+	EL		±	+
H110		+	EP	EP	-	+

a) S^{+}: ts rescued by the addition of 1 M sorbitol to growth media. S^{-}: ts not rescued by the addition of 1 M sorbitol to growth media. b) +: sectoring, -: non-sectoring. c) EL: elongated, EP: extra cell surface projections. d) +: make a large halo in alpha-factor halo assays, -: make no halos, ±: make a small halo.

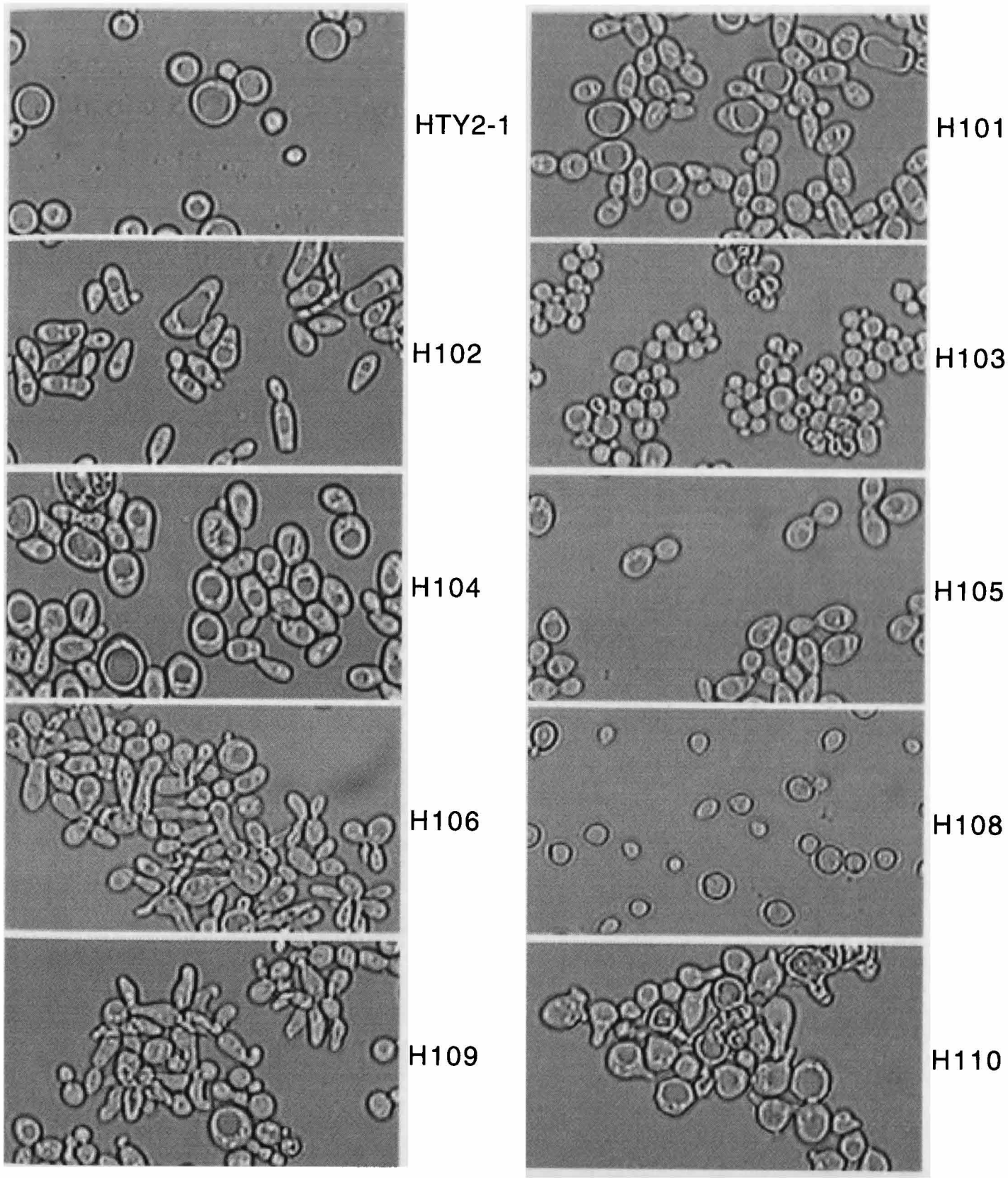


Fig. 11. Morphologies of *ddg* mutants, part 1. Colonies were picked up from YPD plates after incubation for three days at 28℃ and suspended into water. Photographs were

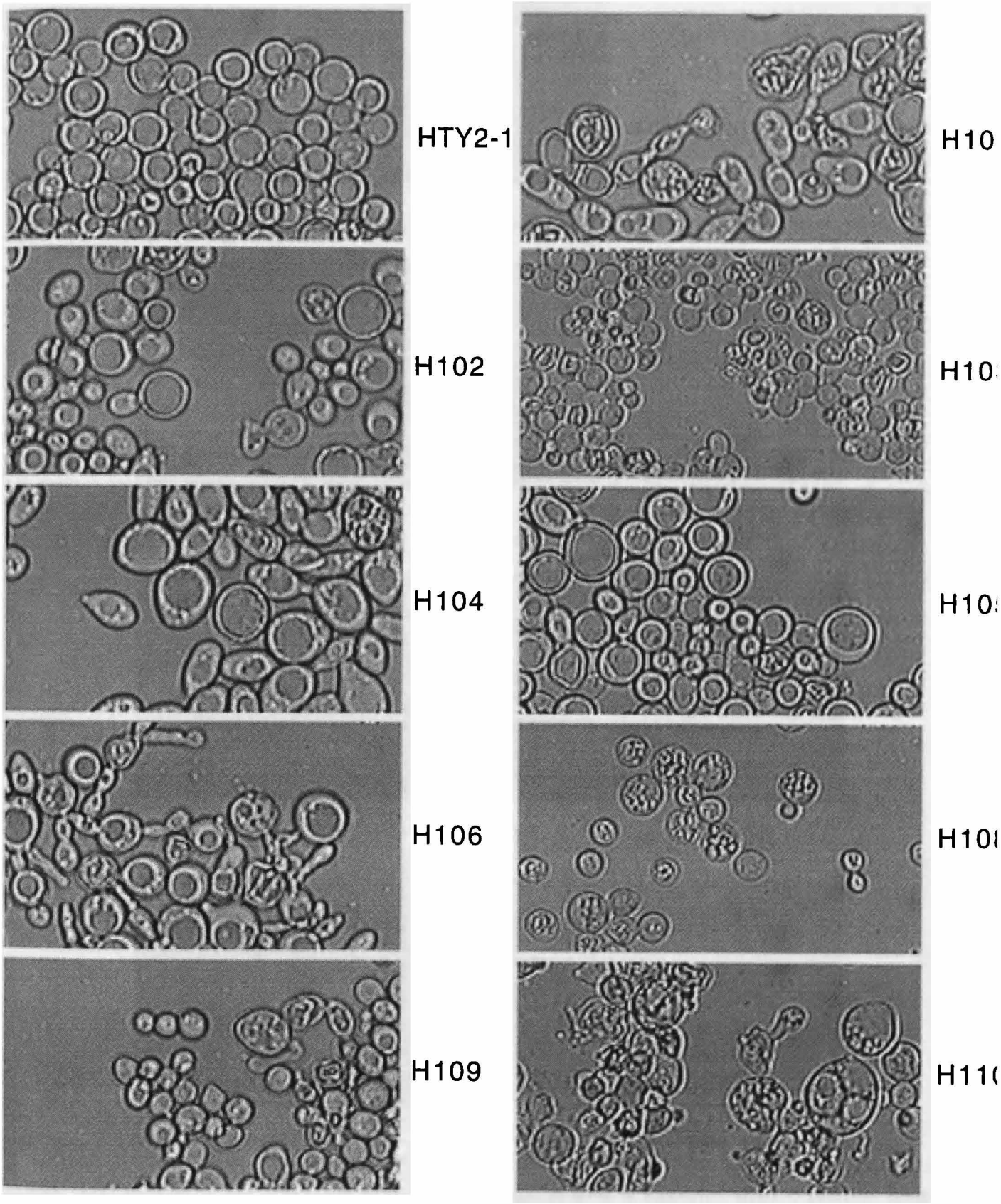


Fig. 12. Morphologies of *ddg* mutants, part 2. The same experiment as in Fig. 11 except for the cultivation temperature which was 37℃.

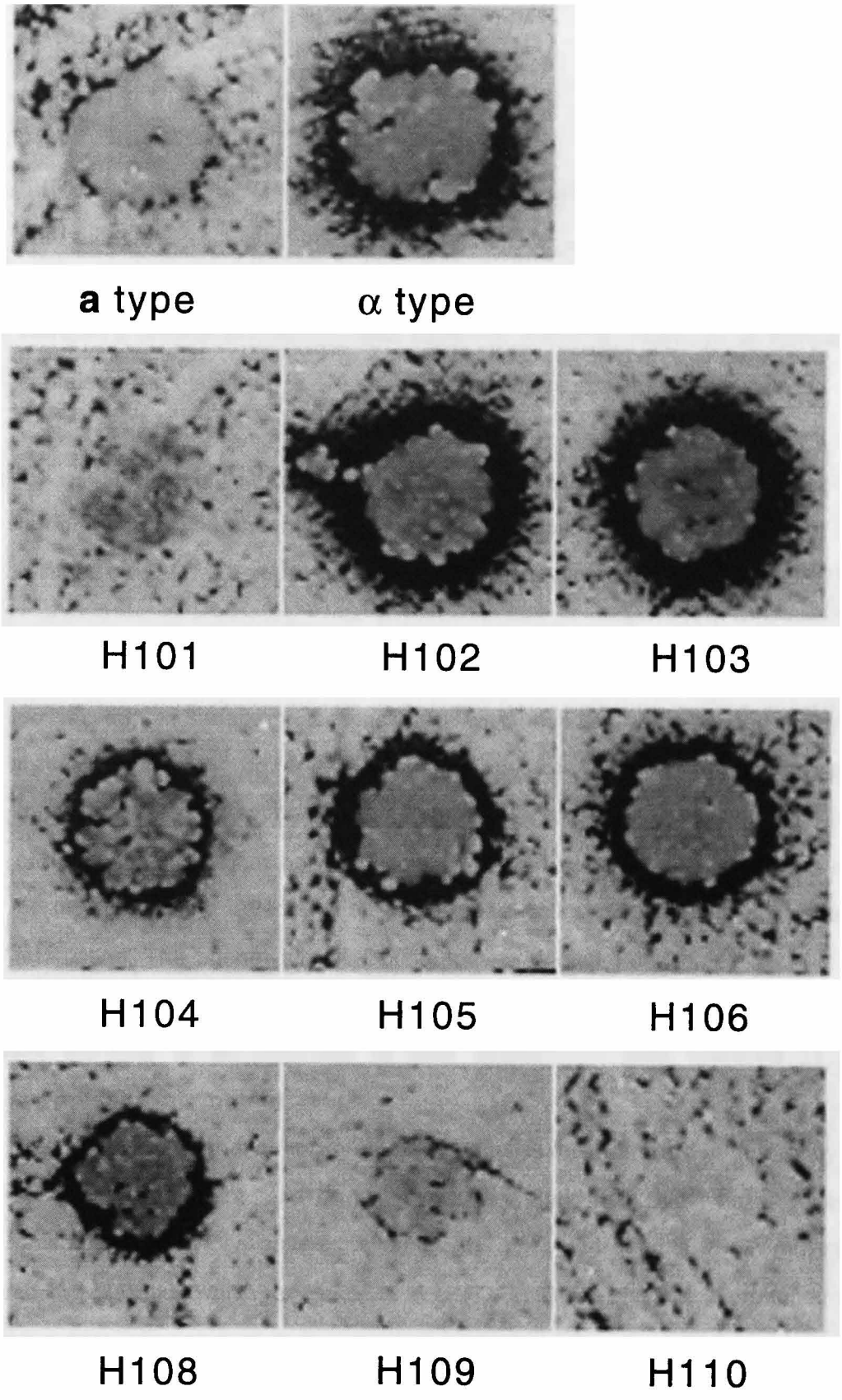


Fig. 13. Alpha-factor halo assays of wild type **a** and α mating type controls and *ddg* mutants. Colonies of each strain were picked up and used to inoculate an YPD plate. After an overnight incubation, the plate was replica-plated to YPD plate on which alpha-factor hyper-sensitive tester strain K1169 was pre-spreaded. After two-days' incubation

mutation in *CDC28* as well.

H105: In the absence of *DHH1*, cells grew very slowly but not lethal. Cells showed very elongated cellular morphology and extensive clamping.

H106: Elongated cellular morphology was observed with this mutant at all temperatures. It contained a mutation in *ELM1* as will be described later in greater details.

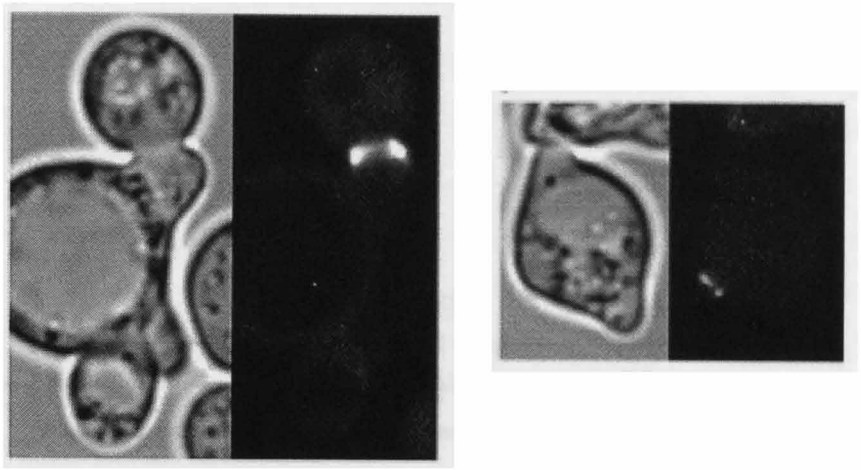
H109: As in the case of mutant H106, elongated cellular morphology was observed at all temperatures. The phenotype appeared to be similar to that of H106, but it was not rescued by *ELM1*. The *ddg* phenotype and ts phenotype were rescued by *SSD1*.

H110: The mutant showed an alpha-factor secretion defect and invasive growth into agar. It possessed cell surface projections which were found to be composed of cortical actin patches normally observed at the stage of budding or mating projection formation.*DHH1* is apparently required in this process. Although the wild type allele of the mutated gene has not been cloned yet, the function of this gene which appears to be involved in the determination of polarized cell surface growth seems to be very interesting. The phenotypic characteristics are depicted in Fig.14.

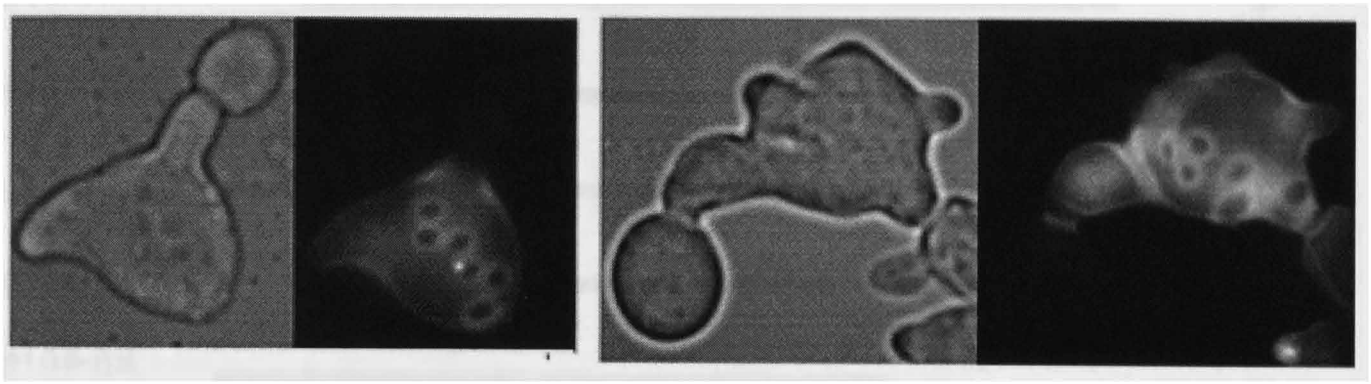
7. *SSD1* thought to be involved in RNA metabolism was isolated as a single copy suppressor of mutant H104

Each of the genomic library clones constructed with the single-copy plasmid pRS314x was introduced into the *ddg* mutants to identify their wild type allele as well as others which would phenotypically complement the observed defects. From mutant H104, I recovered two plasmids which rendered H104 to FOA$^+$. These plasmids were found to contain overlapping DNA fragments of chromosome IV harboring two genes. One of them was *SSD1* and the other was *SRP101*. By truncating the DNA fragments and re-introducing them into mutant H104, I was able to identify that *SSD1* was responsible for the phenotypic rescue of H104 (Fig.15). *SSD1* was previously identified to be polymorphic in various strains and to act as a single-copy suppressor of many genes (Sutton et al., 1991, Costigan et al., 1992,

GFP-Elm1p



Bud scar staining



Actin staining

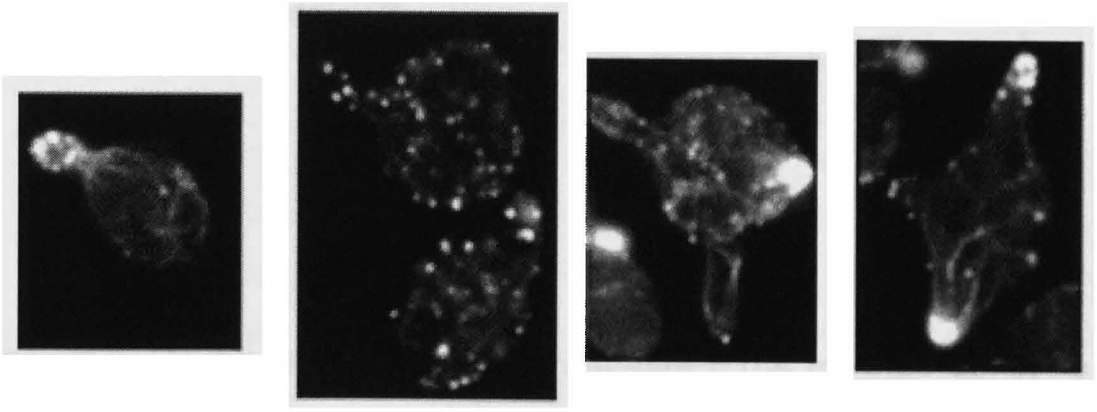


Fig. 14. Fluorescence microscopic observation of mutant H101. GFP-Elm1p (top) was observed after the introduction of plasmid YCpDGFP-ELM into H101. Bud scar (middle) was stained with Fluorescent Brighter 28 and actin (bottom) was stained with Rhodamine-conjugated pharoidin. Photograph were taken under visible light (left) and fluorescent light (right) Magnification is 1000X.

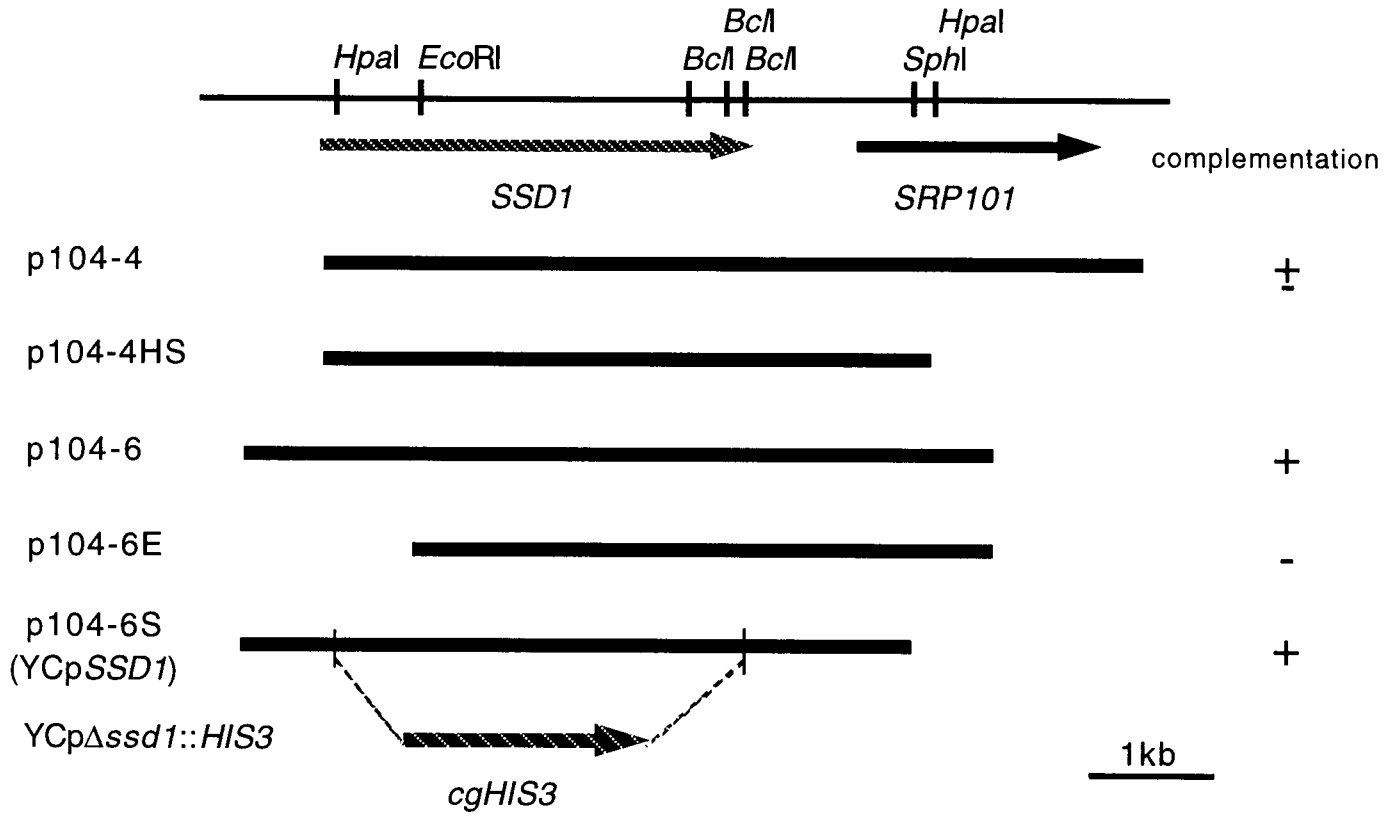


Fig. 15. Plasmids identified by screening on mutant H104 for their ability to render it FOA$^+$. Relevant restriction enzyme cleavage sites are indicated at the top. Horizontal arrows indicate genes in this region and thick bars are the insert found in the plasmids. "Complementation" indicates the abitity to make the mutant cells grow on SC+FOA plates (+: grow well, -: do not grow, ± : grow poorly). The insertion points of cgHIS3 to disrupt SSD1 are shown at the bottom (see also MATERIALS AND METHODS).

Evans and Stark, 1997). Among various laboratory strains, at least two alleles of *SSD1* are known to exist. One is an active allele termed *SSD1-V* (*V* means viable in combination with a *SIT4* deletion) and the other is an inactive allele termed *ssd1-d* (d means dead in combination with the *SIT4* deletion) (Sutton et al., 1991). Thus, there was a possibility that *SSD1* was inactivated already in the parental strain HTY2-1 used for the *ddg* mutant isolation, and *SSD1* can act as a single-copy suppressor because the genomic library used in this work was made from the genomic DNA of RAY-3A strain, which was known to have *SSD1-V* (Uesono et al., 1994). To ascertain this possibility, I disrupted *SSD1* in HTY2-1 strain as described in MATERIALS AND METHODS. In YEpA3D1/HTYΔD strain, *SSD1* disruption did not make the strain *ddg* (data not shown). This indicates that the disruption of both *DHH1* and *SSD1* simultaneously did not cause a lethal phenotype and that the parental strain HTY2-1 carried *ssd1-d*. The result further indicated that H104 had a mutation other than the *ssd1-d* that affected its viability. Indeed, I could isolate another gene which could rescue the *ddg* and ts phenotypes of H104.

To examine whether other *ddg* mutants could also be rescued by *SSD1*, I introduced YCpSSD1 into each of the *ddg* mutants isolated. About a half of the *ddg* mutants became $SECT^+/5\text{-}FOA^+$ and the suppressibility was correlated with whether the *ddg* phenotype could be rescued by the osmotic stabilizer or not (Table 4). This indicates that there are many genes the mutations in which become lethal in combination of *Δdhh1* and *ssd1-d*. Such mutations appear to cause loss of cell wall integrity.

8. The *SSD1* gene has parallel roles with *DHH1* in many processes

W303 strain is known to have an inactivated allele of *SSD1* (*ssd1-d*, Sutton et al., 1991). To test the genetical interaction of *DHH1* and *SSD1*, I disrupted the *DHH1* of W303. One of the resultant derivatives (W303ΔD) was not lethal as predicted, but red color pigment formation due to the *ade2* mutation disappeared, and the colonies of this mutant were white on YPD plates. In addition to a single-copy of *DHH1*, a single-copy of *SSD1* was able to recover the *ade2* pigment formation of W303ΔD, although only partially. In other words, while YCpDHH1/W303ΔD gave rise to red colonies, YCpSSD1/W303ΔD yielded pink colonies. It is known that an *ade2* mutant accumulates endogenous fluorophore (5-aminoimidazole ribonucleotide) in its vacuoles and this fluorescence was observable under

fluorescent microscopy (Weismann et al., 1987). Consequently, the fluorescence of each strain was observed. The fluorescence in vacuoles due to the *ade2* mutation was not observed in the Δ*dhh1* mutant but it became observable by the introduction of a single-copy of either *DHH1* or *SSD1* (Fig. 16). This indicates that *DHH1* and *SSD1* have a parallel role in the development of the *ade2* pigment, most likely by affecting the expression of a gene(s) involved in nucleic acid metabolism. As many *ddg* strains were rescued by osmotic stabilizer, I tested the integrity of the cell wall of W303ΔD by the addition of SDS to growth medium. Results are shown in Fig. 17. The *DHH1* deletion strain showed slower growth on plates containing SDS from 0.005% to 0.01%, which was rescued by a single-copy *SSD1* in addition to *DHH1* itself. I also found that the growth retardation caused by the *DHH1* deletion was severer on a synthetic dextran plate than on a YPD plate, and at a high temperature (37°C) than at the normal growth temperature (28°C). These phenotypic differences were also rescued by a single copy *SSD1* (Fig. 18). These results suggest that redundant roles are played by *DHH1* and *SSD1* in diverse processes such as nucleic acid metabolism, cell wall integrity, growth at high temperature and growth under nutrient limiting conditions, in addition to affecting the growth of various *ddg* mutants. I noted that the direct interaction of *DHH1* and *SSD1* was not detected by the yeast two-hybrid system. This indicates that the functions of *DHH1* and *SSD1* are related but are performed without direct interaction between the two gene products.

9. H104 mutant was rescued by *CDC28*, a cyclin-dependent kinase gene

As summarized above, mutant H104 shows temperature sensitive growth, has a little elongated cellular morphology, and its *ddg* phenotype is rescued by an osmotic stabilizer. Although the *ddg* phenotype of H104 was rescued by a single-copy *SSD1* gene as well, it seemed to harbor another mutation, because the white sector colonies of H104 mutant carrying YCpSSD1 showed very low viability. Therefore, to identify the putative mutated gene, I repeated the library screening once more by selecting for FOA^+ and Ts^+ growth at 37°C. Thus, I actually found that a plasmid which contained a 5.4 kb insert from chromosome II carrying two genes was able to rescue the viability defect. One of the genes was *CDC28*, a cyclin-dependent protein kinase gene which is known to play an essential role in cell cycle progression, while the other was *YBR161w*, a putative gene of unknown

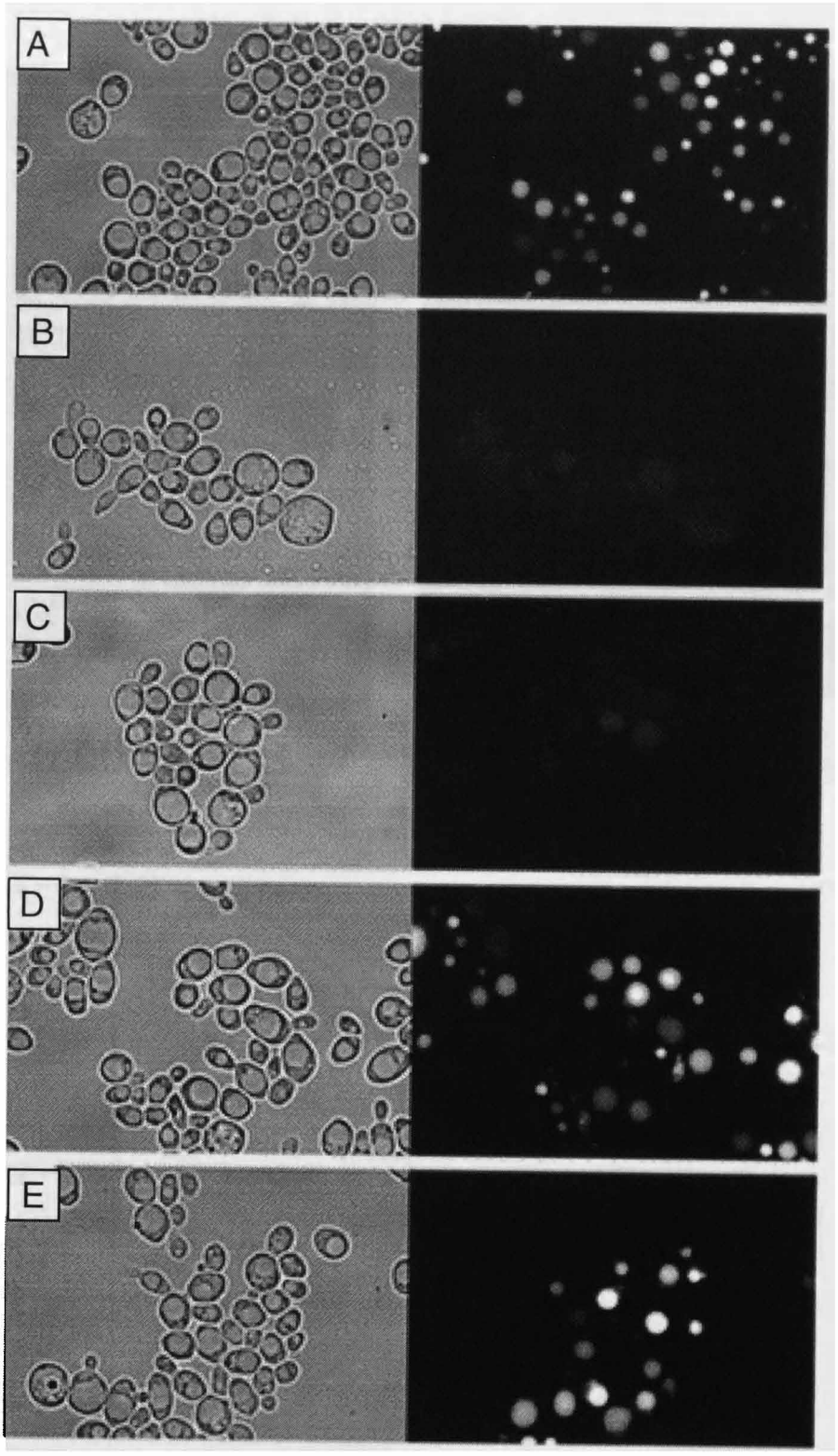
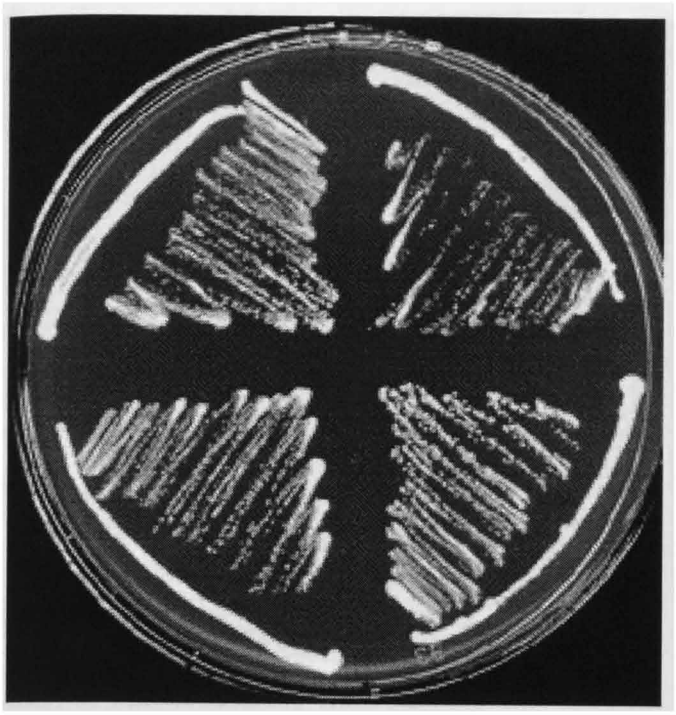
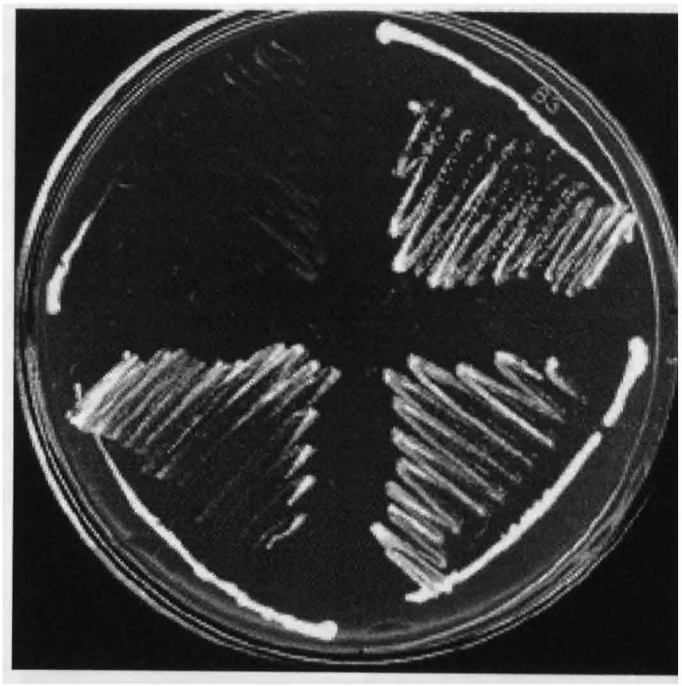


Fig. 16. Images of the ade2 endogenous fluorophore of: A, W303 wild type: B, *DHH1* disruptant (W303ΔD) : C, *DHH1* disruptant carrying the vector pRS314x alone: D, *DHH1*disruptant carrying a single-copy plasmid containing *DHH1* (YCpDHH1): E, *DHH1* disruptant carrying a single-copy plasmid containing *SSD1* (YCpSSD1). Each strain was cultured overnight in YPD medium.



YPD + 0.001%SDS



YPD + 0.005%SDS

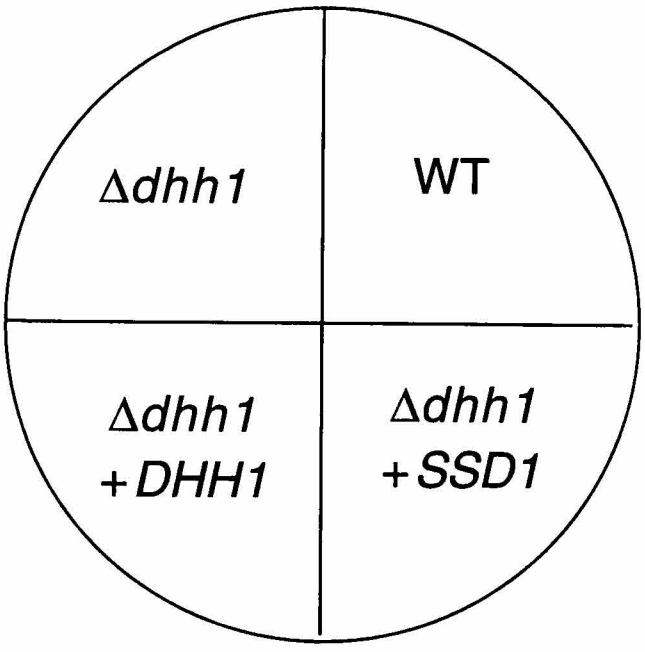
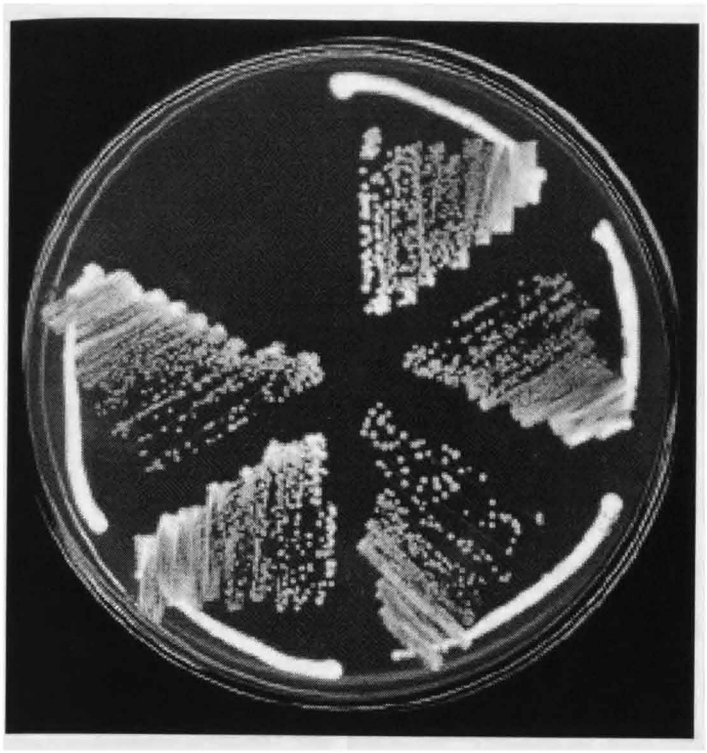
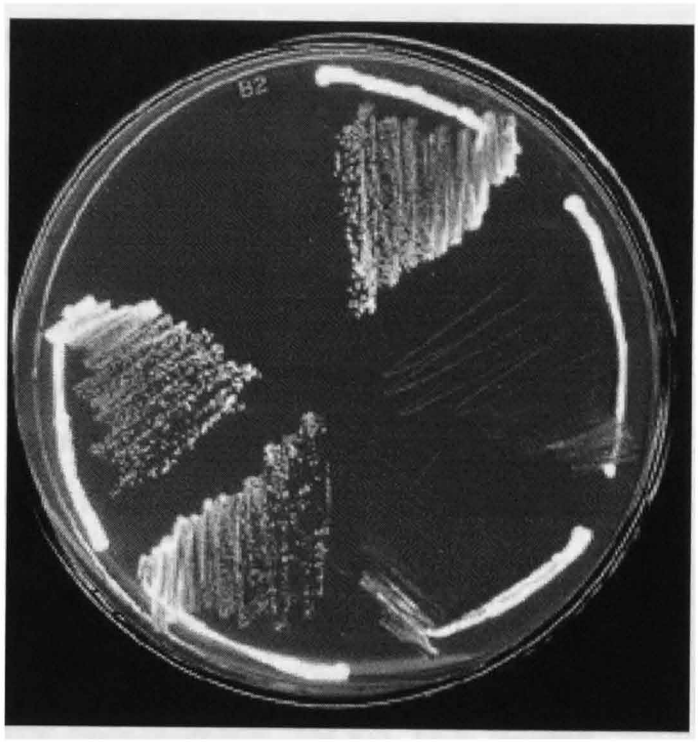


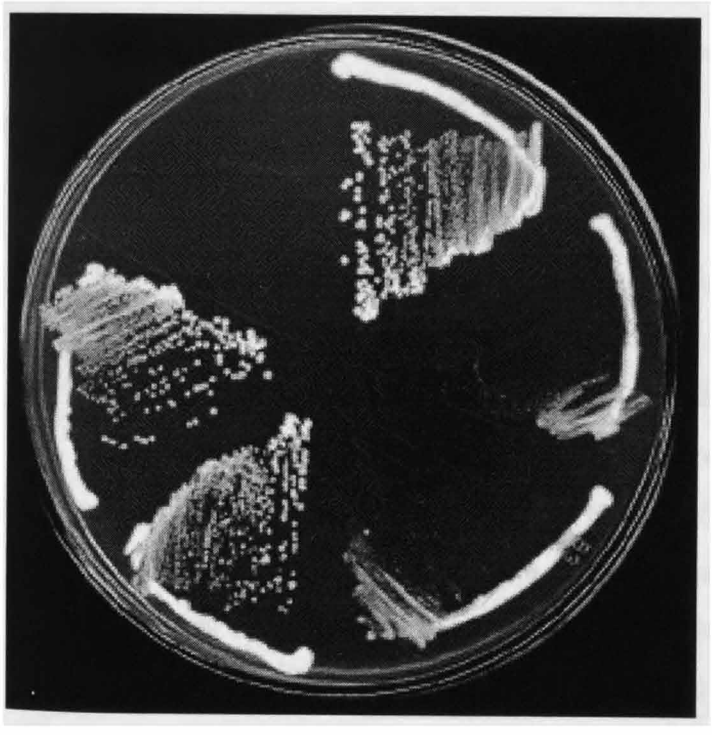
Fig. 17. Rescue of the SDS hyper sensitivity of a *DHH1* disruptant derived from W303. A single-copy plasmid containing *SSD1* (YCpSSD1) or *DHH1* (YCpDHH1) was introduced into a Δ*dhh1* strain, W303ΔD. The resultant transformants and the parental strain were streaked and cultured for 3 days at 28℃ as indicated.



YPD 30℃



SD 30℃



YPD 37℃

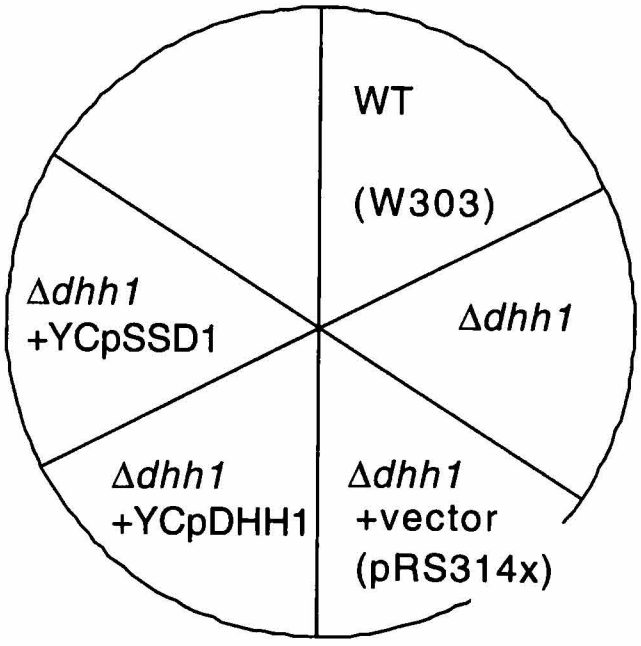


Fig. 18. *DHH1* and *SSD1* have overlapping roles. A single-copy plasmid containing *SSD1* (YCpSSD1) or *DHH1* (YCpDHH1) was introduced into a Δ*dhh1* strain, W303ΔD.The resultant transformants and the parental strain were streaked and incubated on either YPD or SD plates for 4 days at either 28℃ or 37℃ as indicated.

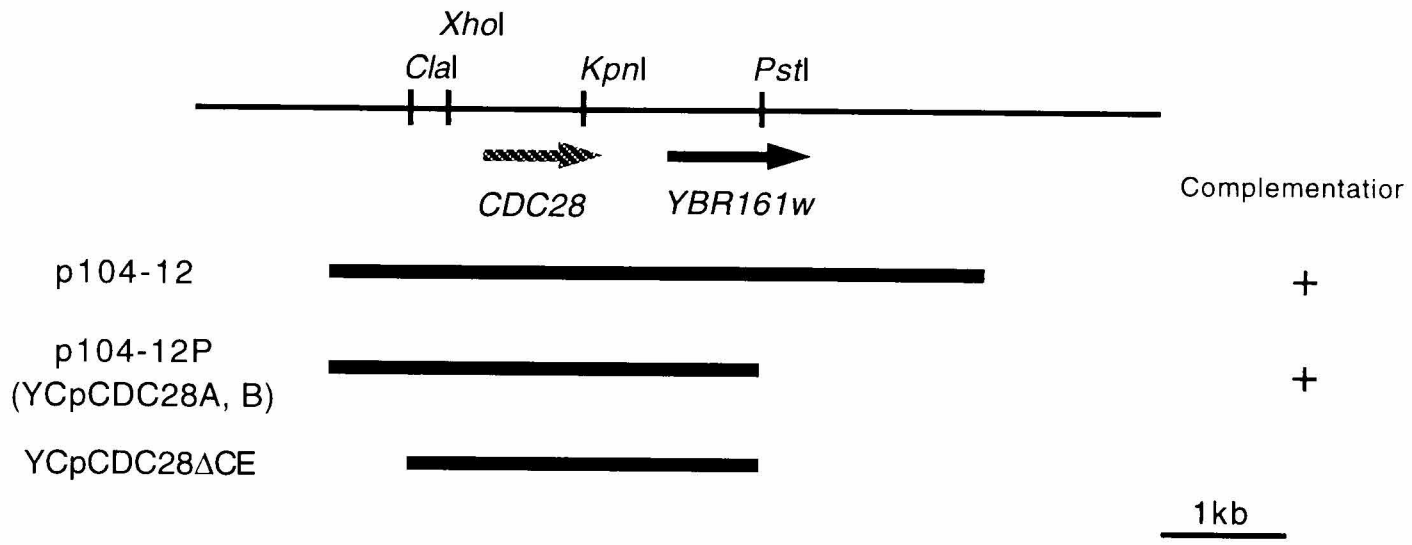
function. By making a subclone, I was able to identify that the *CDC28* gene complemented the *ddg* and temperature sensitive phenotypes of H104 (Fig. 19-a). In addition, I introduced this plasmid into other *ddg* mutants and found that H102 was also complemented by it. As *CDC28* is an essential gene, it was necessary to confirm that the *CDC28* gene of H102 and H104 was indeed mutated which is responsible for the observed *ddg* phenotypes. For this reason, I performed sequencing of the *CDC28* gene of H102 and H104 by PCR amplifying the *CDC28* coding region. In the *CDC28* gene of H104, I found a single mutation changing A to G at the nucleotide position 383. This mutation causes a change of histidine to arginine at the amino acid position 128. As the mixture of two separate PCR runs amplified from H104 showed the same nucleotide sequence, this nucleotide alteration could not be due to an error in PCR.

The validity of the sequence alteration described above was further confirmed by an allele rescue procedure (Rothstein, 1991). Depletion of an *Xho*I-*Kpn*I fragment, which should contain the mutated nucleotide in H104, from YCpCDC28ΔCE (see Fig. 19-a) resulted in the loss of its ability to rescue the *ddg* phenotype of H104. This indicates that the mutation in *CDC28* at position 383 described above was responsible for the *ddg* phenotype of H104, although it can not be ruled out that another mutation or mutations affect its phenotype as well. The *CDC28* gene is known to be involved in the G1/S and G2/M transition. The elongated morphology of mutant H104 may indicate that this mutant is defective in G2/M because of the *CDC28* mutation which is known to result in elongated morphology (Surana et al., 1991). The mutant could be rescued by a multicopy of *DHH1* and cells of this mutant carrying a single copy *DHH1* were very fragile (Fig. 19-b). This indicate that H104 suffers from cell wall fragility and this defect is stabilized by a high dosage of *DHH1*.

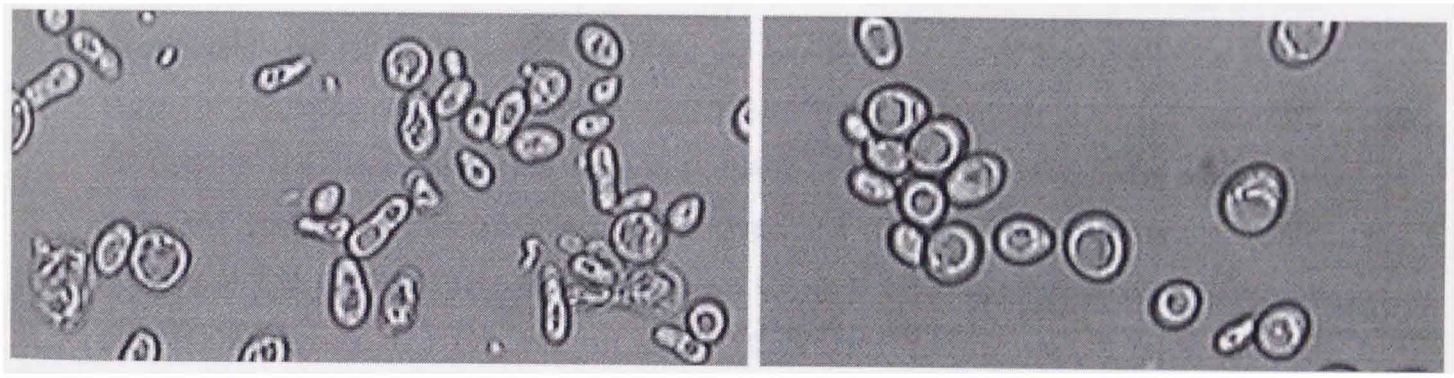
From the analysis of H104, *DHH1* and *SSD1* have somewhat related roles in cell cycle progression. To characterize the *cdc28* mutant allele in H104 further and to establish its functional relationships with *DHH1*, more investigations of detailed cell cycle analysis are necessary after the separation of the mutation.

Unlike the case of H104, the *CDC28* coding region amplified from H102 contained no mutation and the YCpCDC28ΔCE from which the *Xho*I-*Kpn*I fragment was deleted could rescue the *ddg* phenotype. As the *Xho*I-*Kpn*I fragment contained most of the *CDC28* coding region, there is a possibility that H102 has a mutation in the regions flanking *CDC28*. If this is true, then I think this is very interesting, because it indicates the involvement of *DHH1* in

a



b



YCpDHH1/H104

YEpA3D1/H104

Fig. 19. a, Plasmids capable of conferring FOA$^+$ and Ts$^+$ on mutant H104. Relevant restric enzyme cleavage sites are indicated at the top. Horizontal arrows indicate genes/ORFs thick bars chromosomal fragments cloned. "Complementation" indicates the ability to rer the mutant to grow on SC+FOA plate (see also MATERIALS AND METHODS). b, Multic effect of *DHH1* on mutant H104. Left: H104 carrying a single copy *DHH1* plasmid (YCpDHI Right: H104 carrying a multicopy *DHH1* plasmid (YEpA3D1). Cells of each strain were pic up and resuspended in water, then photographs were taken under the microscopy. Magnif tion is 400X.

the regulation of *CDC28* expression.

10. H106 harbors a mutation in *ELM1* that is needed for determining cellular morphology

The cells of mutant H106 were elongated and when growing with an osmotic stabilizer, the colonies became SECT$^+$ (Table 4). The growth of this mutant was rescued by a single-copy *SSD1*, but its morphological defect was not. This indicates the presence of another mutation in addition to the *Δdhh1* and *ssd1-d* that is responsible for its *ddg* phenotype to cause defects in morphology and cell wall integrity. By screening a single-copy genomic library which can complement the FOA$^-$ phenotype of H106, I isolated two plasmids exhibiting the desired phenotype. They contained two mutually overlapping genomic fragments, with the complete coding region of the previously identified *ELM1* gene (Fig. 20-a). This gene encodes a serine-threonine protein kinase. Disruption of *ELM1* was reported to cause elongated cells in haploid yeast strains and pseudophyphal growth in homozygous diploid strains (Blacketer et al., 1993). To confirm that an *ELM1* mutation in the HTYΔD background was lethal, I inactivated *ELM1* by inserting the *cgHIS3* gene into the coding region of chromosomal *ELM1* of strain YEpA3D1/HTYΔD as described in MATERIALS AND METHODS. The strain obtained in this way (YEpA3D1/HTYΔDΔE) showed a *ddg* phenotype, elongated morphology, slow growth and ts phenotype on a YPD plate which was rescued by the addition of 1 M sorbitol. These phenotypes were similar to H106 but somewhat more severe than H106: namely, 1) H106 was not ts but YEpA3D1/HTYΔDΔE was; 2) H106 was not completely *ddg*, and tiny colonies grew on YPD plates. In contrast, no colonies were observed with YEpA3D1/HTYΔDΔE; and 3) the growth of white colonies (i.e. segregants which had lost the *DHH1* plasmid) on plates containing 1 M Sorbitol were the same in size as red colonies (i.e. cells maintaining the *DHH1* plasmid), whereas white colonies were smaller than red ones with YEpA3D1/HTYΔDΔE. Therefore, I concluded that H106 had a mutation in *ELM1* but it was not a null mutation and was slightly leaky.

Previously, Blacketer et al. (1993) reported that the disruption of *ELM1* showed different phenotypes depending on strains used. I found that the *ELM1* disruptant (RAYΔE) of RAY-3A which is known to have *SSD1-V* caused elongation of cells, but not slow growth (see below), while the disruption of *ELM1* in W303 which is known to have *ssd1-d* showed slow

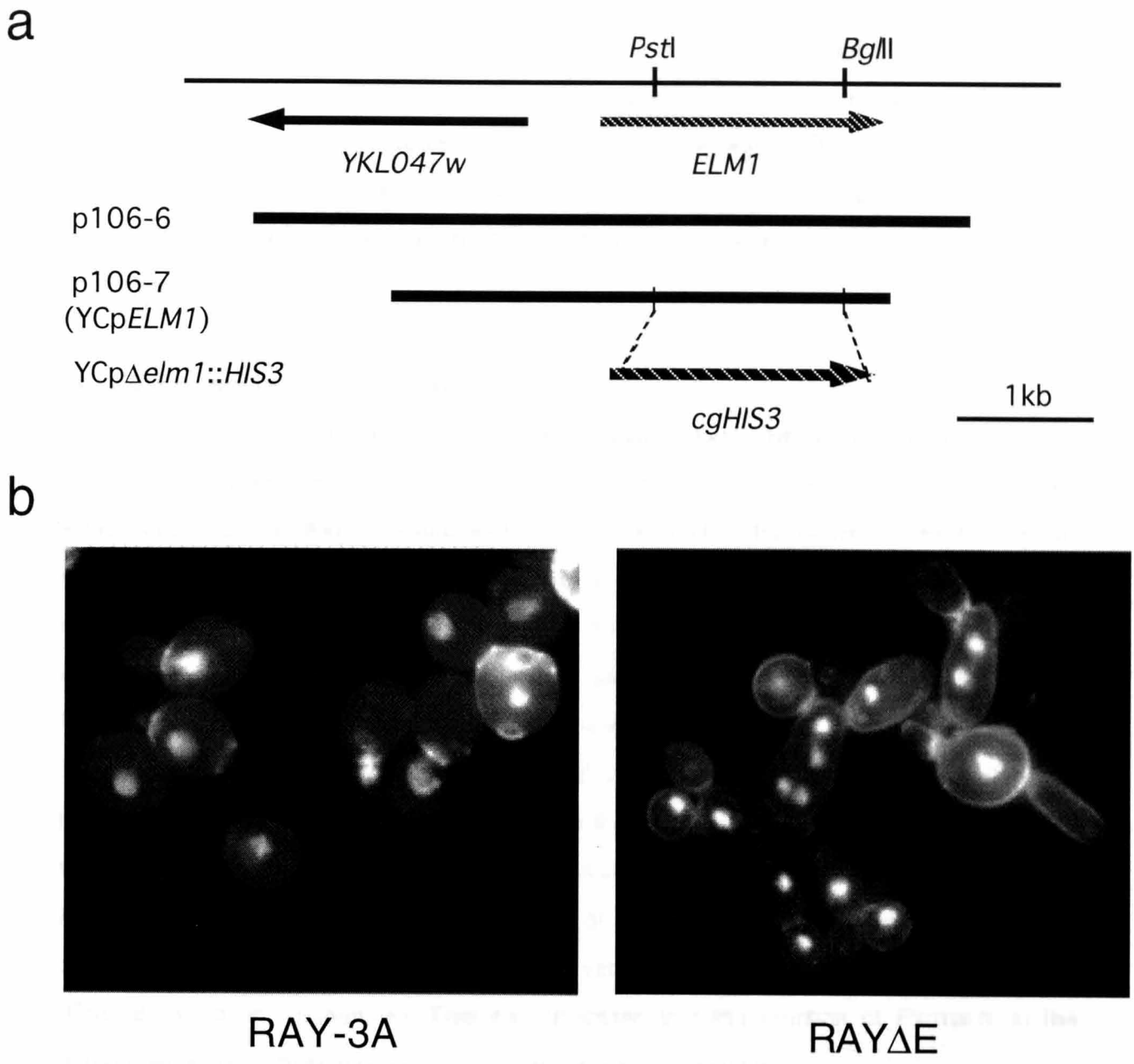


Fig. 20. a, Plasmids capable of conferring FOA$^+$ on mutant H106. Relevant restriction enzyme cleavage sites used are indicated at the top. Horizontal arrows indicate genes/ORFs and thick bars are chromosomal fragments cloned. The insertion points of *cgHIS3* to disrupt *ELM1* are shown at the bottom (see also MATERIALS AND METHODS). b, The morphology of an *ELM1* disruptant in the background of RAY-3A. Nuclei and bud scars of wild type (RAY-3A) and the *ELM1* disruptant (RAYΔE) were stained by DAPI and-Fluorescent Brighter 28. Each strain was cultured overnight in YPD. Magnification is 1000X.

growth and defective in cytokinesis and nuclear partitioning (data not shown). Moreover, these severer defects were not rescued by a single copy of *SSD1* (data not shown). A double mutant of *Δdhh1* and *Δelm1* derived from W303 was not lethal, although its viability was reduced more than a single mutant of each gene (data not shown). Therefore, there may still exist another difference(s) among HTY2-1, W303 and RAY3-A that affects the *ELM1* mutant phenotype.

11. *ELM1* functions in mitosis

It was thus confirmed that an *ELM1* deletion was synthetic lethal with a *DHH1* deletion in the presence of *ssd1-d*, but the molecular function of *ELM1* itself has not been established yet. Therefore, to establish the functional relationship between *DHH1* and *ELM1* more clearly, I performed additional experiments. First of all, I studied the effect of *ELM1* disruption further. An *ELM1* disruptant in the RAY-3A background caused cellular elongation and cell clamping, but the growth rate was indistinguishable from the original RAY-3A. By microscopic observation of the nucleus of RAYΔE by DAPI staining, I found that occasionally multinucleated cells were observed at a low but significant frequency (Fig. 20-b). This may indicate that, in addition to the morphological anomaly, *elm1* disruptants suffered from a nuclear partitioning defect as well. I also constructed cells overexpressing Elm1p by placing the gene under the control of the *GAL1* promoter, and found that the overexpression of Elm1p resulted in cells with very elongated buds with or without nucleus (Figs. 21-a, -b, -c, -d and -e). This also indicates that the function of Elm1p is in the determination of cellular morphology and in nuclear partitioning.

To know the subcellular localization of Elm1p, I constructed a single copy plasmid which expresses a GFP fusion of Elm1p placed under the control of the *ELM1* promoter. This plasmid could completely complement the abnormal cell shape caused by an *ELM1* disruption. As shown in Fig. 22-a, the GFP-Elm1p was found to be localized at a bud neck. As far as I observed, the GFP-ELM1p was localized at bud necks only after buds became visible, and none of the fusion protein localization could be found before bud formation. However, I found also that the GFP-Elm1p expressed from a fusion construct placed under the control of a *DHH1* promoter was localized at bud necks even before buds were visible (Fig. 22-b). These observations suggest that Elm1p interacts with a component(s) of the bud neck such as

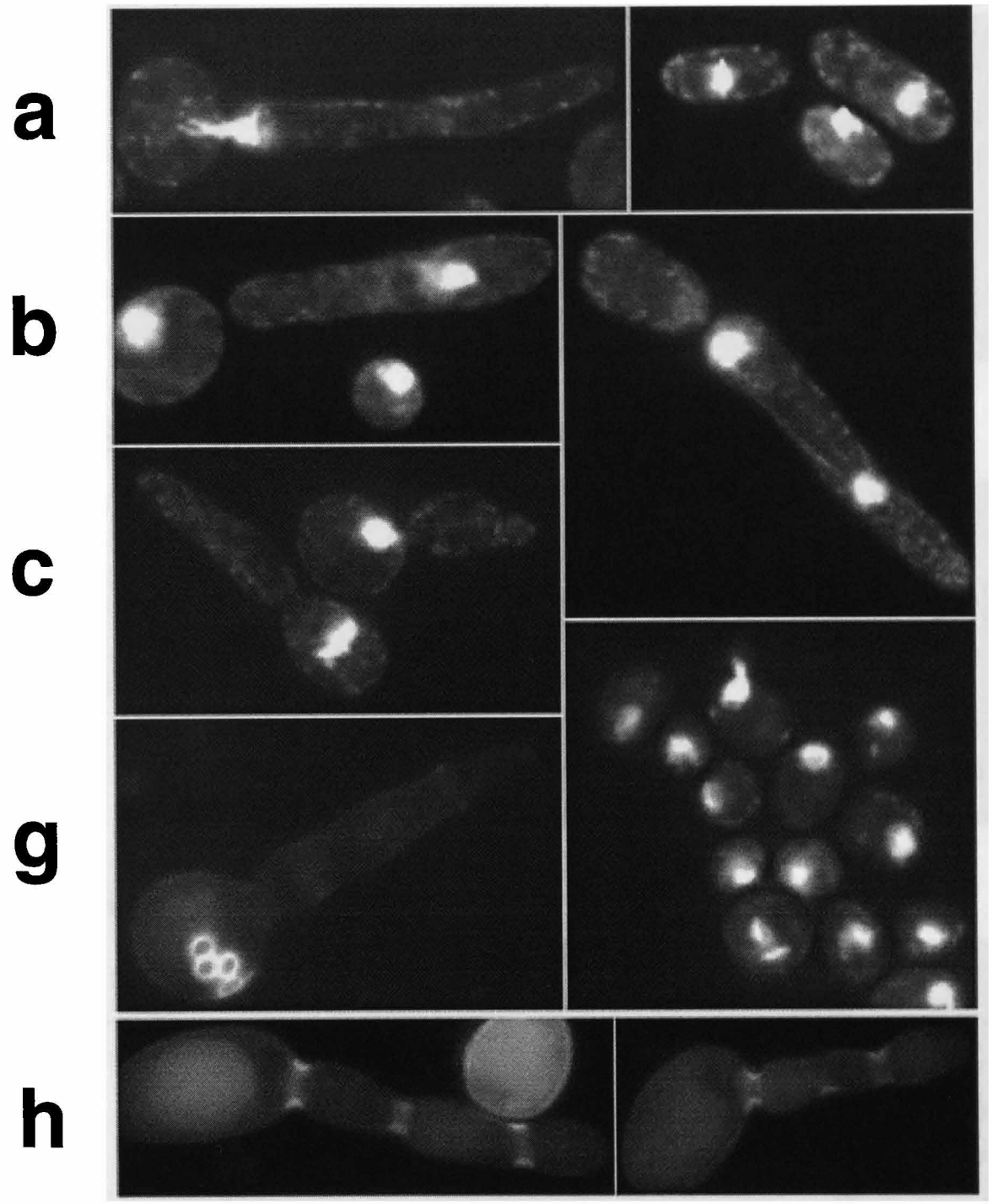
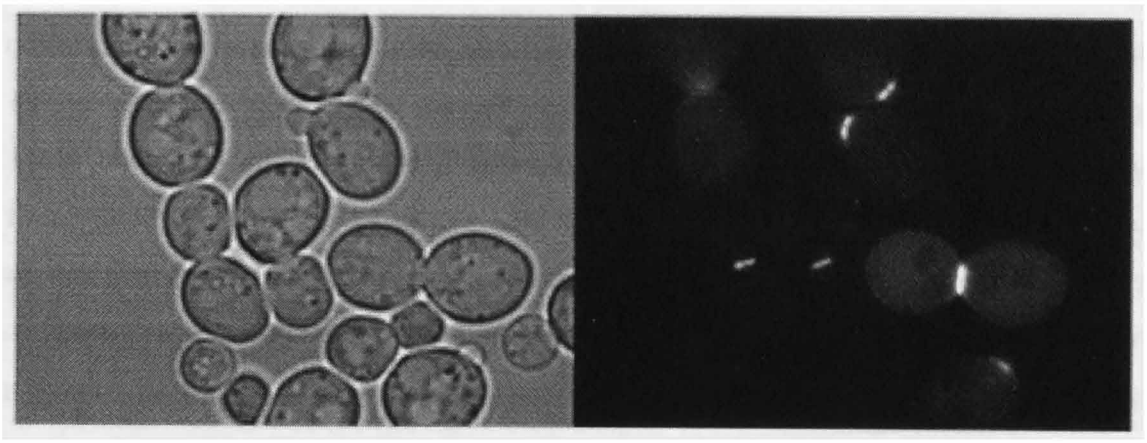
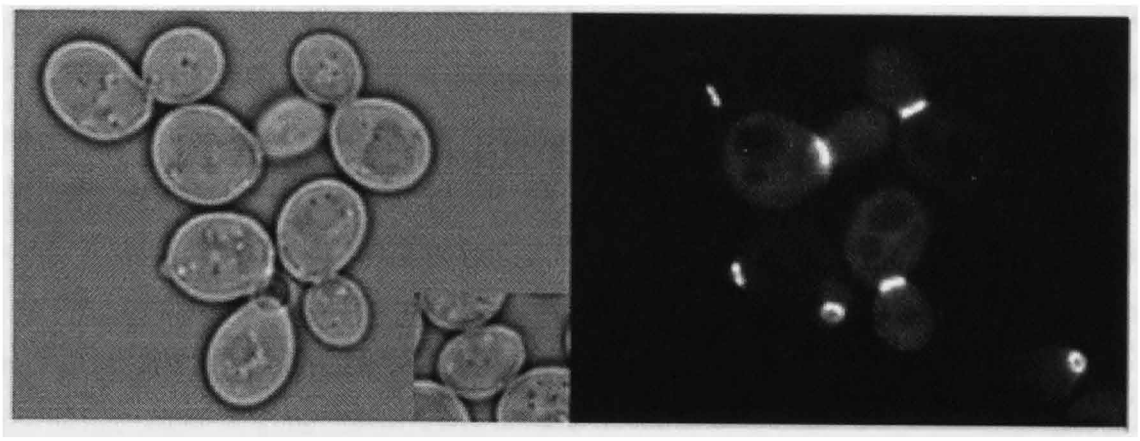


Fig. 21. Fluorescent microsopic images of nuclear staining of the *GAL1* promoter-driven *ELM1* overexpressing strains (a to e) and wild type strain (f), bud scar staining of an *ELM1* overexpressing strain (g), and fluorescent microsopic images of GFP-ELM1 expressed under the control of the *DHH1* promoter on multicopy plasmid (h and i). The *ELM1* overexpressiong strain used was obtained as described in MATERIALS AND METHODS. RAY-3A cells carrying YEpDGFP-ELM1 were cultured 3 days. Magnification is1000X.

a: GFP-Elm1p(*ELM1*promoter)



b: GFP-Elm1p (*DHH1*promoter)



c: GFP (*DHH1*promoter)

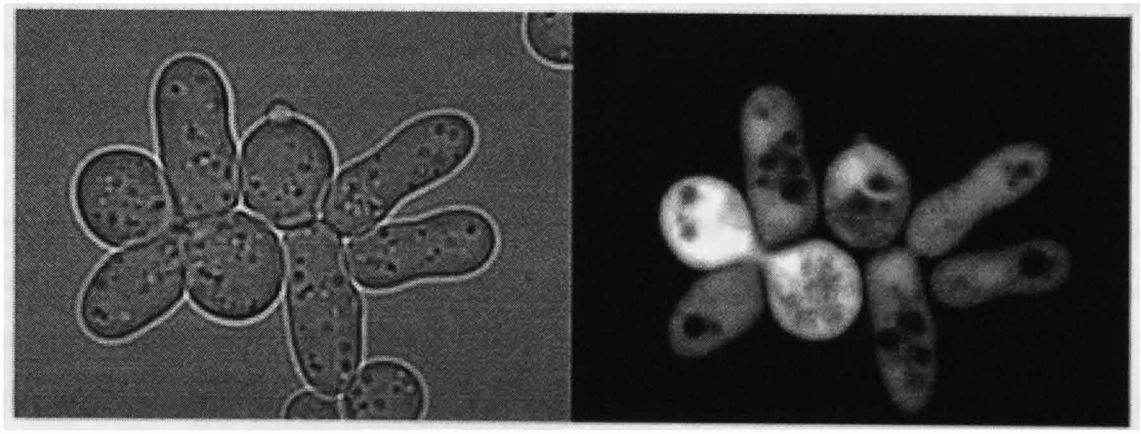


Fig. 22. Fluorescent (right) and optical (left) microscopic images of an *ELM1* disruptant (RAYΔE) carrying: a), a single copy plasmid expressing GFP-ELM1 under the control of the *ELM1* promoter (YCpEGFP-ELM1): b), GFP-ELM1 under the control of the *DHH1* promoter (YCpDGFP-ELM1): c), GFP alone under the control of the *DHH1* promoter (YCpDGFP). Each strain was cultured overnight in SC-W. Magnification is1000X.

sepin protein and its localization to the bud neck is not regulated during the cell cycle but rather its expression is regulated by the cell cycle. Prolonged cultivation of cells expressing GFP-ELM1p under the control of the *DHH1* promoter on a multicopy plasmid occasionally formed elongated bud cells as observed with the Elm1p over-expressing strain, probably because the expression from the *DHH1* promoter was stronger than that from the *ELM1* promoter. Such cells showed the presence of condense bands in elongated buds in addition the band at the bud neck and the shape of these elongated buds was somewhat constricted at the position of these bands (Figs. 21-h and -i). This may indicate that Elm1p has a role in distorting the bud neck.

A FACS analysis of Δ*elm1* and *Elm1p* over-expressing strains showed an increased number of cells containing 2C DNA in comparison with a wild type control (Fig. 23). This indicates that both Δ*elm1* and Elm1p over-expressing strains have a G2/M delay, which in turn strongly suggests the participation of Elm1p during cytokinesis and nuclear partitioning. It thus seems that the function of the *ELM1* gene is not directly related with that of *DHH1*. In this connection it should be noted that no two hybrid interaction between *DHH1* and *ELM1* or *SSD1* and *ELM1* was observed.

12. Genetic interactions of *DHH1*, *SSD1* and *ELM1*

The introduction of a single-copy *SSD1* to HTYΔDΔE rescued its slow growth, ts phenotype and morphological defect, though not completely. To confirm the presence of genetical interactions among the three genes, I introduced a single copy *DHH1*, *SSD1* and *ELM1* into HTYΔDΔE. Each strain was incubated on a plate containing 5-FOA to obtain colonies which have lost YEpA3D1. They were then studied further. A derivative which had lost both *ELM1* and *SSD1* (i.e YCp*DHH1* containing HTYΔDΔE) showed ts and abnormal cellular morphology with elongated nuclei or cells with multiple nuclei (Fig. 24). Similarly, another derivative which had lost both *ELM1* and *DHH1* (i.e YCp*SSD1* containing HTYΔDΔE) showed abnormal cellular morphology with elongated nuclei or cells with multiple nuclei, but its growth was not ts (Fig. 24). These observations indicated that the double mutants suffered from abnormal cellular morphology, and cytokinesis and mitosis defects. In comparison with them, a strain in which both *DHH1* and *SSD1* were inactivated (i.e YCp*ELM1* containing HTYΔDΔE) did not show such a morphological anomaly and nuclear

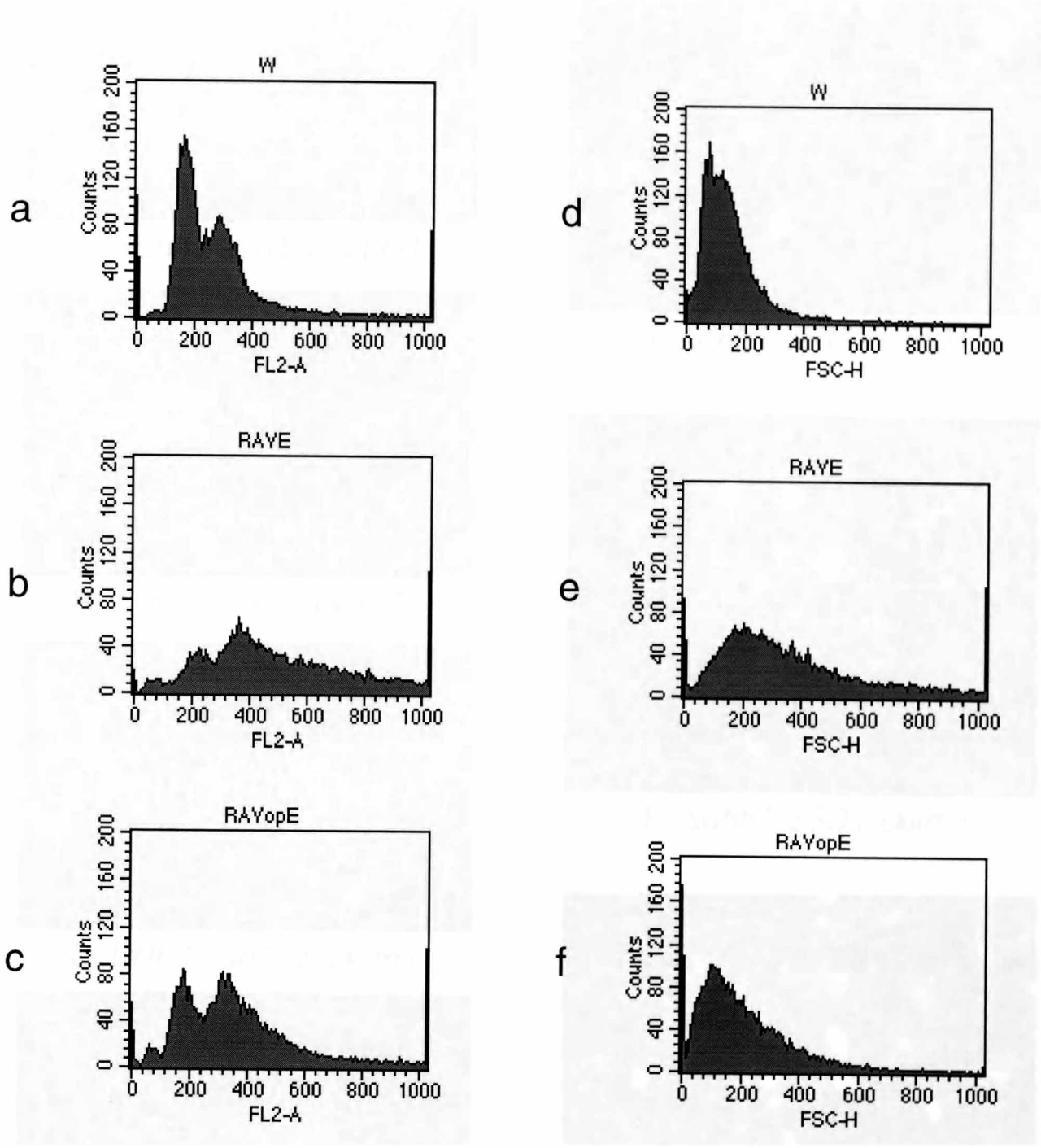
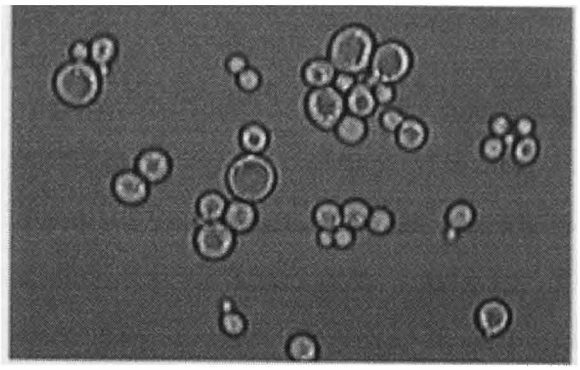
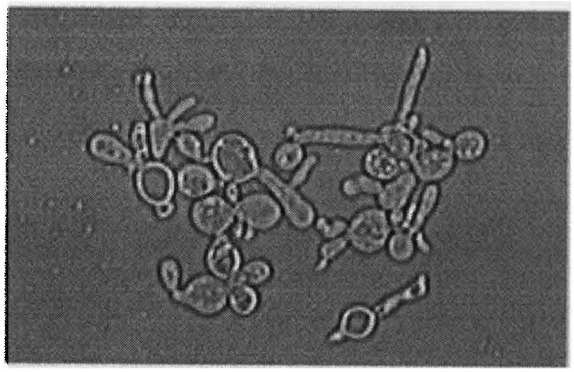


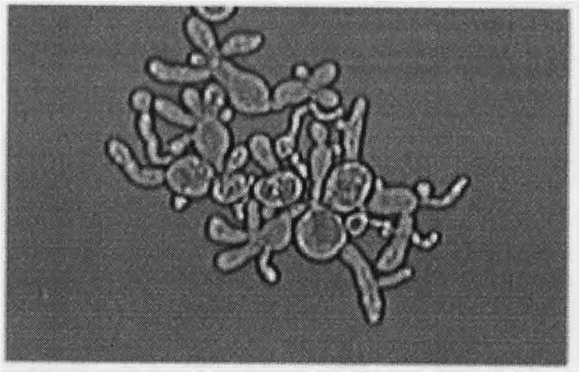
Fig. 23. FACScan analysis of an *ELM1* disruptant and an *ELM1* over-expressing strain. The DNA contents of the wild type RAY-3A (a), an *ELM1* disruptant (b, RAYΔE) and an *ELM1* over-expressing strain (c) were measured as described in MATERIALS AND METHODS. Furthermore, the cell size distribution of RAY-3A (d), RAYΔE (e), and the *ELM1* overexpressing strain (f) was measured. RAY-3A and RAYΔE cells were prepared from log phase culture in YPD. The *ELM1* over-expressing cells were prepared as described in MATERIALS AND METHODS.



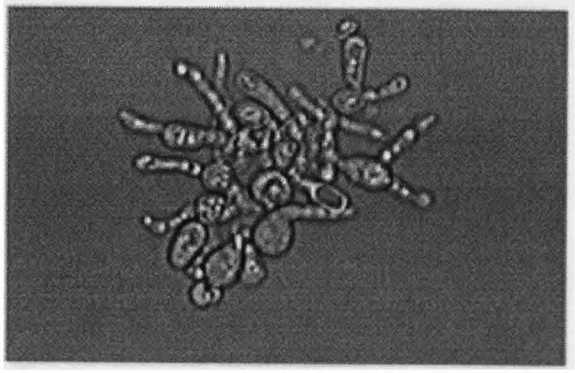
A: Δ*dhh1 ssd1-d ELM1*



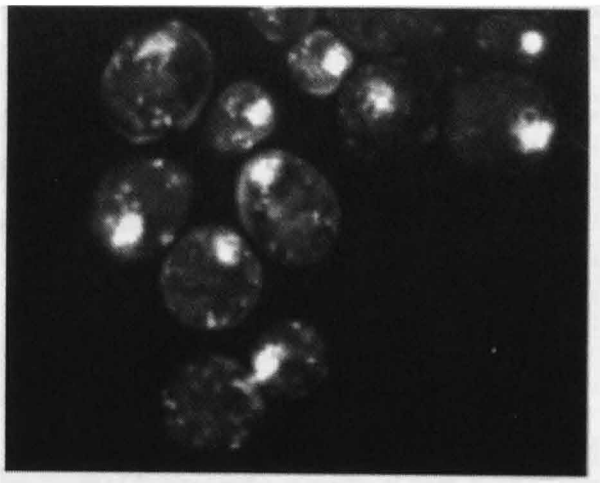
B: Δ*dhh1 SSD1 Delm1*



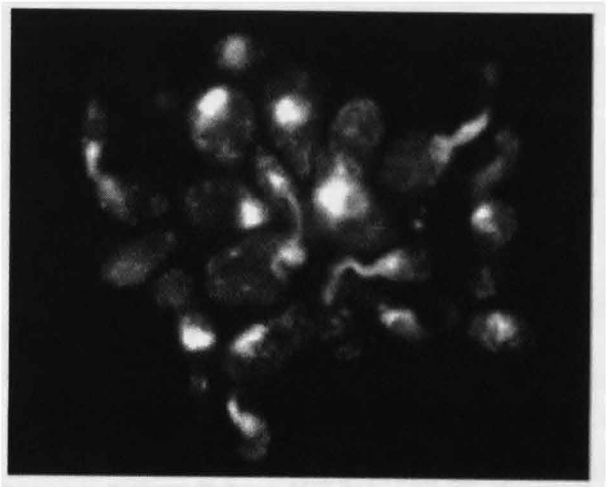
C: *DHH1 ssd1-d Delm1*



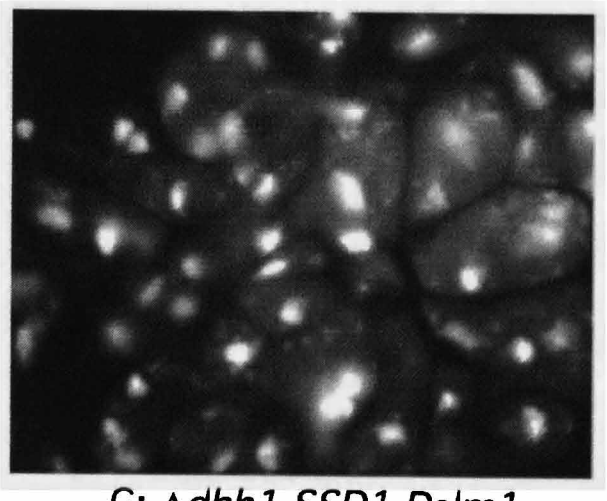
D: Δ*dhh1 ssd1-d Delm1*



E:Δ*dhh1 ssd1-d ELM1*



F: Δ*dhh1 SSD1 Delm1*



G: Δ*dhh1 SSD1 Delm1*

Fig. 24. Morphologies of double and triple mutant strains of *DHH1*, *SSD1* and *ELM1*. Respective mutant was constructed as described in the text and their growth was compared on YPD plates. A-D: Cellular mophologies of indicated strains (magnification 400X), E-G: DAPI staining of indicated strains (magnification 1000X). Photographs were taken under the microscopy.

partitioning defect. A triple disruptant could be obtained by growing YEpA3D1/HTYΔDΔE on YPD containing 1 M sorbitol. The cells of a resultant derivative were very fragile and easily disrupted by an osmotic shock (i.e. transfer to water) or mechanical sharing and cells were observed to be attached to each other (Fig. 24). From these observations, the three genes were found to have somewhat related roles with each other in mitosis and cell wall integrity. Judging from the phenotype of double mutant strains, *DHH1* and *SSD1* are closely related with each other for the growth of *elm1* mutant, and the function of *ELM1* is obviously different from the function of the former two.

DISCUSSION

1. The function of Dhh1p

Until now, many DEAD-box proteins have been identified genetically and biochemically in *S. cerevisiae* most of which are involved in general pathways of RNA processing, such as splicing, initiation of translation and ribosome assembly as described in INTRODUCTION. DEAD-box proteins in these processes are mostly essential for cell viability and the inactivation of their genes leads to an arrest of the respective essential process. In contrast, since yeast cells carrying a null mutation in *DHH1* showed little differences in the expression of the *lacZ* reporter gene which contained an intron and was placed under the control of an *ACT1* promoter (Fig. 6), *DHH1* is not likely to be involved in any one of the essential processes mentioned above. Rather, since the activity of LacZ placed under the control of a *TDH1* promoter was significantly lower in a strain carrying a *DHH1* deletion than in a wild type (Fig. 6), *DHH1* functions in a more gene specific manner. I found that Dhh1p fused to the DNA-binding domain of Gal4p stimulated the transcription from the *GAL1* promoter (Fig. 7), and that the proline-glutamine rich C-terminal domain was responsible for this activity and a derivative of Dhh1p depleted of this domain was not functional. If this reflects the real function of Dhh1p *in vivo*, Dhh1p should interact with a protein or proteins which is (are) involved in the process of transcription. As *DHH1* encodes an RNA helicase, its function is likely to involve RNA. The function of Dhh1p in transcription appears to be a little surprise. However, recent studies implicated that the carboxyl-terminal domain (CTD) of RNA polymerase II directly interacted with factors functioning with RNA processing (for a review, see Eric, 1997). Therefore, it may be that the transcription stimulating activity of Dhh1p is a result of its association with factors interacting with the RNA polymerase II CTD. In fact, Okanan et al. (1997) reported that an *Arabidopsis* DEAD-box protein which was shown to have an RNA helicase activity *in vitro*, interacted directly with a domain conserved among various transcription factors which was known to bind directly to DNA. They explained the function of this protein (AtDRH1) in terms of its interaction with the RNA polymerase CTD. A possible model for the function of RNA helicase associated with the transcription machinery is shown in Fig. 25.

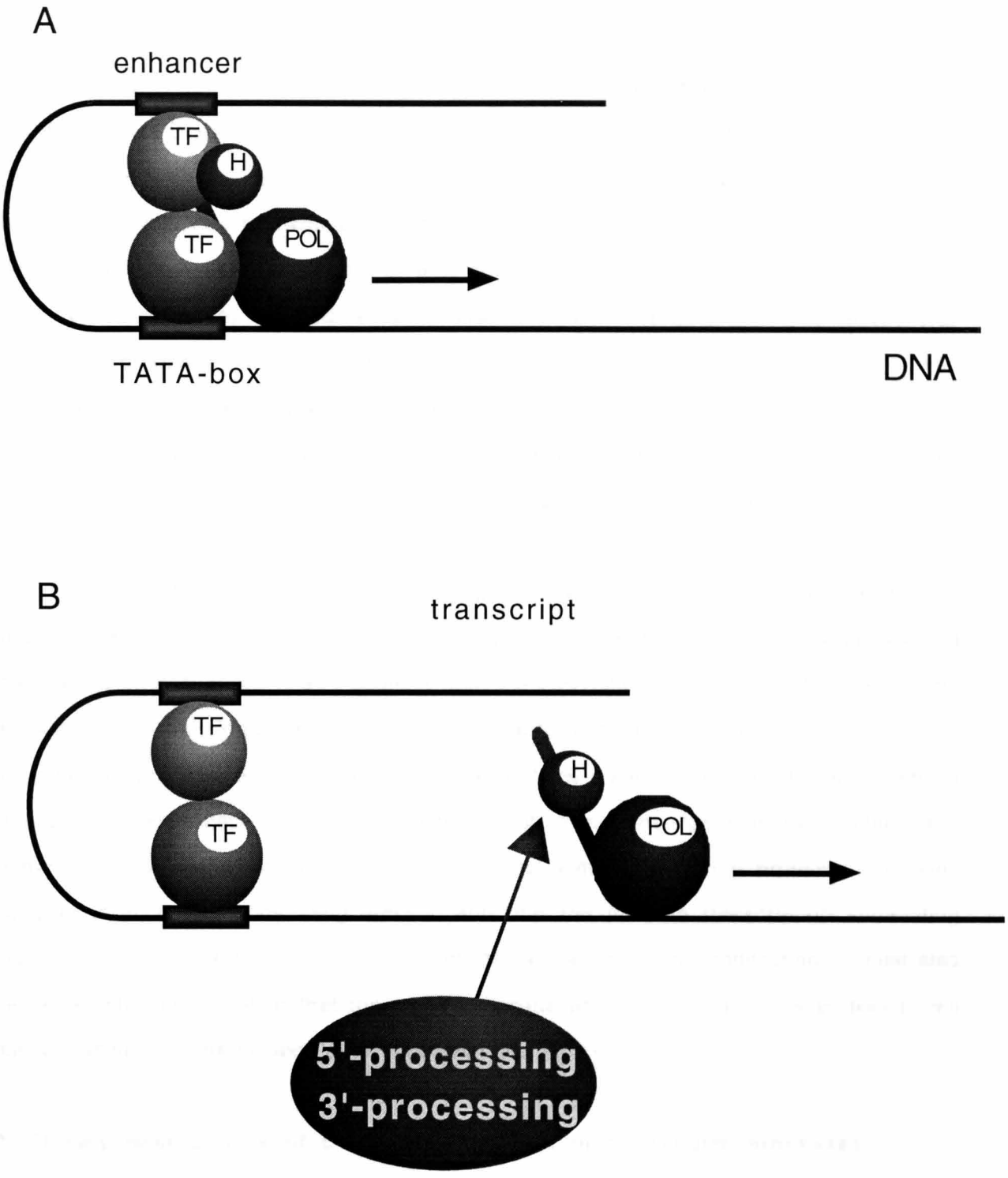


FIG. 25. A possible function of RNA helicase which interacts with the transcription machinery. A, An initiation complex consist of transcription factors (TF), RNA polymerase (POL) and RNA helicase (H) is formed. B, In the transcription reaction, RNA helicase is interacting with RNA polymerase at its C-terminal tail domain and involved 5'- and 3'-processing of the newly transcribed RNA.

If Dhh1p is involved in transcription, it has to be localized in the nucleus. However, a GFP fusion of Dhh1p was largely localized in the cytoplasm in the form of multiple spots in cells grown in YPD (Fig. 9) and of a single or a few spots in cells grown in YEPA (Fig. 9). As this fusion protein was expressed under the control of the *DHH1* promoter on a single copy plasmid (i.e. expressed under more or less natural conditions) and the fusion protein was fully functional, I am inclined to think that this result reflects the true subcellular localization of Dhh1p. However, if that is the case, then its relationship with the observed transcriptional stimulation must be explained.

Very recently, Sakai et al. (1997) reported that a protein termed Pop2p, which was involved in the transcription of glucose repressive genes, formed a large complex *in vivo* which included Dhh1p. Pop2p has a transcription stimulating activity like Dhh1p and is associated directly with Dhh1p *in vivo*. This protein is associated with membrane fraction and interacts with many other proteins localized in cell membrane, cell wall, vacuoles and the nucleus (Katoh et al., 1997). Judging from the GFP-Dhh1p localization, Dhh1p may be associated with the Pop2 complex as multimers and functioning mainly in the cytoplasm in an unknown process. Furthermore, it may be that the transcription stimulating activity of Dhh1p does not reflect the direct function of Dhh1p in transcription in the nucleus, but rather it indicates the interaction of Dhh1p in the cytoplasm with a protein or proteins carrying transcription stimulating activity, and that the putative transcription stimulating protein is shuttling between the nucleus and the cytoplasm. In this connection, it must also be taken into consideration that the place of localization of the majority of protein is not always identical to the location of its functional site.

2. Closely related roles of *DHH1* and *SSD1* in many cellular processes

I have isolated many mutants synthetic lethal with a *DHH1* deletion which were termed *ddg* mutants. By characterizing them, I identified the *SSD1* gene as a single copy suppressor, and found that *SSD1* works cooperatively with *DHH1* in many cellular processes. Previously, the *SSD1* gene has been identified as a single-copy suppressor in many processes such as protein phosphorylation (Sutton et al., 1991, Costigan et al., 1992, Evans et al., 1997), growth at high temperature (Kikuchi et al., 1994), cell cycle

(Curcková and Nasmyth, 1993), cell surface growth (Doseff and Arndt 1995) and transcription (Stetter et al., 1993). Recently, Uesono et al. (1997-a) found that the gene product of *SSD1* showed a low but significant degree of homology with bacterial exoribonuclease II and had an RNA binding activity *in vitro*. They also reported an the an interaction of Ssd1p with a protein which is thought to be involved in mRNA 3' processing (Uesono et al., 1997-b). They suggested that *SSD1* had a role in mRNA degradation, although RNase activity was not detected in the purified Ssd1p. The synthetic effects of the *ssd1-d* allele to many genes may indicate that imbalances of the gene expression at the level in RNA cause defects in many processes.

In this study, I presented various lines of evidence suggesting that Ssd1p and Dhh1p play cooperative roles in the same or closely related processes. In addition, both proteins have been associated function with RNA. Therefore, I think that the function of Dhh1p and Ssd1p are much more related with each other in RNA metabolism than previously suggested based on the analysis of mutants which showed synthetic effects with *SSD1*. This idea was supported in the case of the *elm1* mutation as will be discussed later. Ssd1p is known to be localized in the cytoplasm (Uesono et al., 1997-a), and I confirmed the localization of Dhh1p in the cytoplasm as well (fig. 9). As the direct interaction of Dhh1p and Ssd1p was not detected in the yeast two-hybrid analysis (data not shown), they seem to function in a very related process (es) in the mRNA processing but they are not included within the same complex. The deletion of *DHH1* will cause a very similar defect as does the *SSD1* deletion. Moreover, when both *DHH1* and *SSD1* are deleted, the defects are additive as illustrated in Fig. 26. Processing the transcript of a gene which is involved in growth at high temperatures, nucleotide biosynthesis or cell wall integrity is likely to be regulated by both Ssd1p and Dhh1p at distinct but related steps. When one of the two steps controlled by either Dhh1p or Ssd1p becomes defective, the regulation of the gene in question is affected but can manage to work somehow. However, when both of the two steps become defective, then the regulation of the gene becomes completely abnormal and defects will become discernible.

As all *ddg* mutants rescued by a single copy *SSD1* could also be rescued by an osmotic stabilizer, Δ*dhh1 ssd1-d* mutants seem to suffer from a defect in cell wall integrity. In fact, the Δ*dhh1 ssd1-d* double mutant analyzed showed hyper sensitivity to SDS. Furthermore, I think this cell wall fragility is responsible for the synthetic lethality observed with many

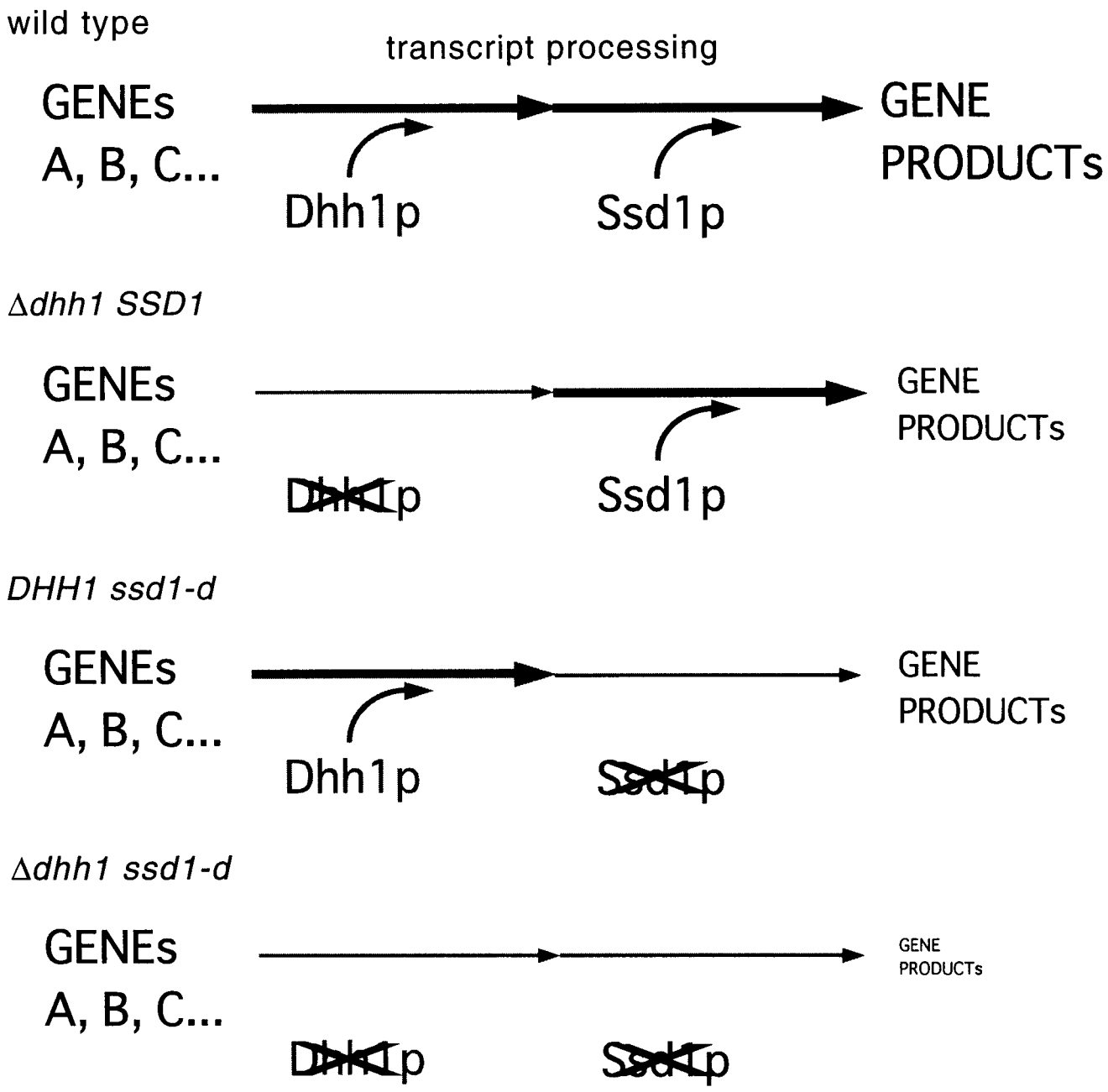


Fig. 26. A model for the overlapping roles played by Dhh1p and Ssd1p in the transcript processing during gene expression. Processing of the transcripts of a *GenesA, B, C...* is regulated by both Ssd1p and Dhh1p at distinct but related steps (wild type). When one of the two steps becomes defective because of the inactivation of either *DHH1* or *SSD1*, the level of the gene product is affected but can still manage to work (*Δdhh1 SSD1* or *DHH1 ssd1-d*). However, when both of the two steps become defective, then the level of the gene product becomes completely abnormal and defects will become discernible (*Δdhh1*

ddg mutants. Mutants with abnormal morphology are likely to suffer from physical stresses in their cell wall such as abnormal tension, and if the wall is fragile, mutant cells can no longer sustain their integrity and results in burst. That is why many *ddg* mutants are caused by morphology mutations.

3. The function of *ELM1* in cytokinesis

In the course of the genetic screening of synthetic lethal mutations with the *DHH1* deletion, I came across with the previously characterized gene *ELM1*. It has been reported that the deletion of *ELM1* causes elongated cell morphology in a haploid and pseudohyphal growth in a diploid yeast, and that the *ELM1* gene product, Elm1p, has a serine-threonine protein kinase activity (Blacketer et al., 1993, Koehler et al., 1997). However, as the function of Elm1p at the molecular level was not very clear, I studied this gene further and found several interesting features suggesting the involvement of Elm1p in mitosis: 1) over-production of Elm1p causes hyper growth of daughter cells with abnormal nuclear distribution; 2) a double mutant either Δ*elm1 ssd1-d* or Δ*elm1* Δ*dhh1* and a triple mutant Δ*elm1 ssd1-d* Δ*dhh1* suffer from severe cytokinesis and cell wall structure defects; and 3) GFP-Elm1p is localized at the bud neck after the emergence of buds. These results suggest that Elm1p is regulated during the cell-cycle and functions in cytokinesis and mitosis, perhaps through its protein phosphorylation activity. Depletion of Elm1p may cause a delay in cytokinesis and, as a result, cell elongation occurs. Over-production of Elm1p, on the other hand, may cause its mis-localization and the cytokinesis signal can not work properly. Koehler et al. (1997) reported that Elm1p had a PEST sequence which has been proposed to be a rapid protein turn-over signal and that the native form of Elm1p was very unstable *in vivo*. This may also suggest the existence of a tight regulation of Elm1p expression.

From these observations, I concluded that Elm1p functions without a direct interaction with Dhh1p, and that the synthetic effect of Δ*elm1* with Δ*dhh1* and *ssd1-d* may be explained as the result of an imbalance in the expression of genes harboring redundant functions with *ELM1*. As shown in Figs. 14 and 15 many *ddg* mutants showed elongated cellular morphology which were not rescued by *ELM1* (data not shown). Perhaps, they possess a mutation in a gene or genes whose products function in parallel with Elm1p in

cytokinesis. In fact, I was able to isolate a *CDC28* mutant from one of the *ddg* mutants, termed H104, which showed elongated morphology. More recently, an *elm7/cdc28* mutant was found to show synthetic lethality with an *elm1* mutation (Myers, personal communication). This indicates that Cdc28p plays a parallel role with Elm1p.

The reason why many mutations show synthetic effects with *ssd1-d* may be explained in terms of the relationships of Δ*elm1* versus Δ*dhh1* and/or *ssd1-d*. A single null mutation in the *ELM1* gene causes cellular elongation but the growth rate is indistinguishable from wild type. Double mutants, Δ*elm1* Δ*dhh1* or Δ*elm1 ssd1-d*, on the other hand shows very similar cell division defects. A triple mutant, Δ*elm1* Δ*dhh1 ssd1-d* is lethal because of its cytokinesis defect and cell wall fragility. As the double mutant Δ*dhh1 ssd1-d* does not show any discernible cell division defect, the function of *ELM1* can be separated from the function of *SSD1* and *DHH1*. This idea is supported from the fact that, whereas the phenotype of Δ*dhh1* and *ssd1-d* mutants is very diverse, the phenotype of an Δ*elm1* mutant is specifically related with mitosis. From these data, the synthetic effect among these genes may be explained as depicted in Fig. 27. When gene A (in this case, *ELM1*) is mutated but the transcript level of a functionally redundant gene X (such as *CDC28* as mentioned above) is normal, only little effect will be observed. However, when the transcript level of gene X becomes also abnormal (i.e. too little or too much), the mutation in gene A becomes critical for cell viability, and if the processing of the gene A transcript is regulated by Dhh1p and Ssd1p, the transcript level is abnormal in a double mutant Δ*dhh1 ssd1-d*, and the double mutant shows synthetic lethality with a gene X mutation.

Many mutations which have previously been identified to show synthetic effects with *ssd1-d* may be explained in a similar manner as described above. If the above explanation is true, then many genes that are not essential by themselves because of the existence of a functionally redundant gene, can similarly be isolated by using the Δ*dhh1 ssd1-d* strain. A morphology mutation such as *elm1*, for example, does not by itself cause a growth defect which makes it difficult to identify. I believe that the Δ*dhh1 ssd1-d* synthetic lethal screening system I have developed here is a good system for isolating mutants which harbor mutations causing abnormal morphological phenotypes, even if individual mutations do not manifest any discernible growth defects.

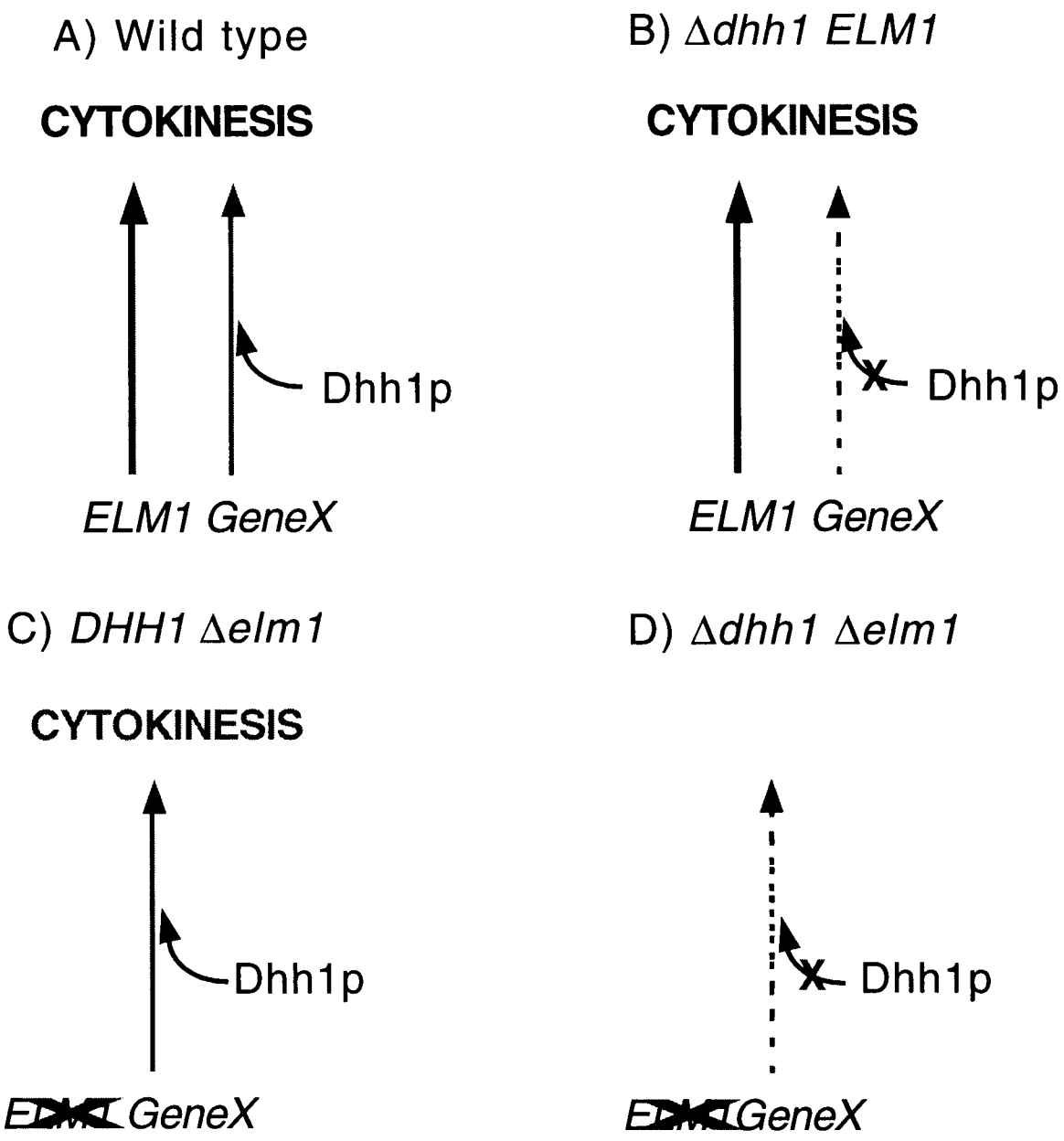


Fig. 27. A possible model for the overlapping function of Dhh1p and Elm1p in the process of cytokinesis. According to the localization of Elm1p and *elm1* mutant morphology, Elm1p may be directly involved in cytokinesis, while Dhh1p may be indirectly involved in cytokinesis, namely via processing of the transcript of a gene (*GeneX*) which is involved directly in cytokinesis. Ssd1p may also be involved in the transcript processing of *GeneX* as shown in Fig. 26. A), In wild type, the level of Elm1p and *GeneX* product are high enough and cytokinesis proceeds normally. B), In a Δ*dhh1* strain, the transcript level of *GeneX* is low but Elm1p isenough to perform cytokinesis. C), In a Δ*elm1* strain, Elm1p does not exist and cytokinesis isdelayed but the product of the level of *GeneX* is high enough to perform cytokinesis. D), In a double mutant, Δ*dhh1* Δ*elm1*, Elm1p is absent and the level of *GeneX* product is too low toperform cytokinesis.

4. Evolutionary conservation among *DHH1* homologues

In multi-cellular eukaryotes, it is known that some DEAD-box proteins are expressed in a tissue-specific manner. Typical examples are *vasa* and ME31b in the germ-line cells of *Drosophila* (Hay et al.,1988, De Valoir et al., 1991). They seem to function towards the RNA needed for specific cellular developments. Homologous genes of *vasa* have been found in *S. cerevisiae*, but their deletion showed growth defects even under vegetative growth conditions. In the case of *DHH1*, homologous genes are known to be involved in the organization and/or maintenance of germ-line cells in some organisms. The deletion of *DHH1* showed a sporulation defect in *S. cerevisiae*, which can be regarded, in a sense, as a germ-line defect, because the process requires meiosis. However, a *DHH1* deletion causes some other defects even in vegetative growth. Moreover, as discussed above, *DHH1* seems to function in diverse processes of gene-expression. *Xenopus* p54, which has a considerable degree of homology with Dhh1p in the conserved region of DEAD-box proteins, is known to be a component of an mRNP complex in the oocyte. It is localized in the cytoplasm and is considered to function as a translational regulator. However, as noted above, I found that the C-terminal PQ rich region of Dhh1p has a transcriptional activity and is essential for the Dhh1 function, although this region is not conserved among the Dhh1p homologues. Mouse and human p54 proteins are very similar to *Xenopus* p54 throughout their structure, expressed in many tissues, and are thought to function as a proto-oncogene. As the conserved region of these DEAD-box proteins are very similar to each other, they are likely to have evolved from a common ancestor and functions towards the RNA of similar structure. Nonetheless, the observations described above indicate that the function of these proteins are not the same.

5. Conclusion of my thesis work and future perspectives

In my doctor course studies described here, I chose *S. cerevisiae* as a most advanced model organism in the study of life, and analyzed *DHH1*, one of the DEAD-box protein genes which is known to from a family with its homologues and other related genes. Its protein product, Dhh1p, plays important roles in RNA metabolism in the cell. As described above I have found several interesting features with this protein. Dhh1p has a transcription

stimulating activity and the deletion of *DHH1* causes a reduction in the expression from the *TDH1* promoter. Since Dhh1p is an RNA helicase and it must have a relation with an RNA, this finding suggests the presence of a hitherto unknown function of a DEAD-box protein in transcription.

In the course of isolation and characterization of synthetic lethal mutants, I came across with the *SSD1* gene. *SSD1* is also a gene of unknown function, except that it has diverse genetic relationships with various genes. Recent work suggests the role of Ssd1p in the 3'-processing of mRNA in the cytoplasm (Uesono et al., 1997-a and b). Nonetheless, the molecular details of the function of Ssd1p is still not well established. From the genetical experiments I have performed, the function of Dhh1p and Ssd1p seem to be closely related with each other. I think that the target transcript(s) of the two proteins are common to both of them. Therefore, an obvious next step is the identification of the target transcript(s) and the determination of a factor(s) which links Dhh1p and Ssd1p.

Through the isolation and subsequent characterization of *ddg* mutants, I obtained several mutant strains exhibiting interesting cellular morphology. One of them showed extremely elongated cells and had a mutation in the *ELM1* gene. Its product, Elm1p, was shown to be localized at the bud neck and it appears to play a role in cytokinesis. Mutant H110 showed a very interesting morphology in which the polarization of cell growth seemed to be abnormal. I could not find any reports in which such a morphological mutant was described. Therefore, I am planning to clone the mutated gene. In addition, there are still many other *ddg* mutants which have not been analyzed yet. The variety of their phenotypes appears to indicate the diverse functions of *DHH1*. Analysis of them will lead to the identification of genes involved in interesting morphology determination.

Throughout my thesis work, I have attempted to clarify the molecular function of Dhh1p and to describe the whole cell scale RNA metabolism in *S. cerevisiae* at the molecular level. However, the results obtained were not satisfactory in this regard. Nonetheless, through the synthetic lethal mutant isolation and other experiments, at least I was able to study and understand many cellular processes involving RNA metabolism, regulation of gene expression such as glucose regulation, cell morphology determination, cell wall organization, secretion, cell polarity determination, mitosis and cell cycle progression in *S. cerevisiae*. I think it is very fascinating that with *S. cerevisiae* we can easily and

systematically study various events occurring in the cell. I was happy to shift my attention towards various phenomena occurring within the cell, although there are still many unknown events and mechanisms in the cell that have to be clarified. Someday, I wish to uncover the whole scale pathway of RNA metabolism and to establish the function of DEAD-box proteins in general.

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