出芽酵母の利己的DNA、*VDE*に関する分子生物学的研究

1999年

野上　識

学位論文

出芽酵母の利己的DNA、*VDE*に関する分子生物学的研究

平成１０年１２月博士（理学）申請

東京大学大学院理学系研究科
生物科学専攻
野上　識

Study of the yeast selfish DNA element, *VDE* encoding a site-specific homing endonuclease

Doctor thesis in University of Tokyo
1999

Satoru Nogami

Table of Contents

Acknowledgment

I wish to express my deepest gratitide to Associate Prof. Yoshikazu Ohya for his constant supervision, advice and patient encouragement throughout this study.

I also wish to express my deepest appreciation to Prof. Yasuhiro Anraku for his supervision and encouragement throughout this study.

I am grateful to Prof. Yoshinori Satow and Prof. Ichio Shimada for their warmful encouragement throughout this study.

I wish to express my deep appreciation to Ms. Sayaka Arai, Masako Sugiura and Shizu Yabe for their helpful technical assistance and valuable discussion during their graduation study, Mr. and Ms. Shunsuke Nakao, Takashi K. Itoh and Shizu Yabe for their technical assistance and discussion during their undergraduate study. I also wish to express my appreciation to Mitsuhiro Abe, Masayo Minemura, Drs. Fumi Kumagai, Kunihiro Ohta, Aiko Hirata and Associate Prof. Seiichiro Hasezawa for collaborations and helpful discussions, and Drs. Ryuta Mizutani, Masato Kawasaki, Akira Matsuura and Ryogo Hirata for valuable comments and discussion.

I am thankful to Drs. Hiroshi Qadota and Hideo Tsubouchi for providing plasmids.

I appreciate warmful encouragement and help to members of Laboratory of Biochemistry and Laboratory of Plant System, Department of Biological Sciences, Graduate School of Science, University of Tokyo.

I thank everybody that supports me throughout the doctor course from the bottom of my heart.

Abbreviations

a.a.:	amino acid(s)
bp:	base pair(s)
χ^2:	chi square
CBB:	coomassie brilliant blue
C. glabrata:	*Candida glabrata*
Cls:	calcium sensitive
DSB:	DNA double-strand break
d.f:	degree of freedom
E. coli	*Escherichia coli*
His:	histidine auxotroph
kb:	kilobase pairs
kDa:	kilodalton
Leu:	leucine auxotroph
NES:	nuclear export signal
NLS:	nuclear localization signal
ORF:	open reading frame
P;	possibility
PCR:	polymerase chain reaction
PAGE:	polyacrylamide gel electrophoresis
PRE:	pheromone response element
S. cerevisiae:	*Saccharomyces cerevisiae*
SDS:	sodium dodecyl sulfate
Trp:	tryptophan auxotroph
Ura:	uracil auxotroph
VDE:	*VMA1*-derived endonuclease
VRS:	VDE recognition sequence
V-ATPase:	vacuolar membrane ATPase
Vma1p:	catalytic subunit of V-ATPase

Summary

VDE, a homing endonuclease with protein splicing activity, is a genomic parasite in the *VMA1* gene of *Saccharomyces cerevisiae*. The *VMA1* gene encodes a catalytic subunit of the vacuolar ATPase and a *Sce VMA1* intein (*in*ternal pro*tein* sequence) that produces VDE. In a heterozygous diploid of a VDE-less *VMA1* allele and a VDE-containing *VMA1* allele, VDE specifically cleaves the VDE-less *VMA1* gene at meiosis. Following homing event converts the VDE-less allele to the VDE-containing allele. In this study, I focused on the gene homing events mediated by VDE and tried to understand physiological functions of the VDE endonuclease. Two novel VDE functions that I found during the doctor course are a gene homing event of another gene and a host-killer effect. I found that VDE was capable of mediating gene homing of another gene at a different locus where the 45-bp recognition sequence of VDE was inserted heterozygously. This indicates that expression of VDE and presence of the recognition sequence are sufficient for the gene homing events at meiosis. I also found that the host-killer effect also occurs at meiosis: presence of the VDE recognition sequence in a homozygous diploid significantly lowered the spore germination ability, but had no deleterious effect on mitotic growth. Using site-directed mutagenesis, I identified amino acid residues that specifically influenced endonuclease activity and gene homing. An arginine to alanine mutant at position 90 exhibited the most severe defect in endonuclease activity, giving this mutant the attributes of intein that only undergoes protein splicing but does not mediate gene homing. Finally, I found that meiosis-specific function of VDE is due to the repression of the endonuclease activity during mitosis.

General Introduction

Mendel's law is one of the most important principle of genetics. Most of eukaryotic genes located on nuclear chromosomes are inherited in a Mendelian manner. Non-Mendelian inheritance is frequently observed in cytoplasmic inheritance and genomic imprinting. A coding region of the homing endonuclease located on chromosome IV of *Saccharomyces cerevisiae* is another striking exception that shows non-Mendelian heredity.

Homing endonucleases are site-specific endonucleases encoded by intron or intein to promote homing of the genetic element into the intronless or inteinless allele (reviewed by Lambowitz and Belfort, 1993). They create a double strand break (DSB) at a defined site of the intronless or inteinless allele (recipient) that is then repaired with the information of intron or intein (donor) (Fig. 1). Many homing endonucleases are identified as site-specific endonucleases and classified into four groups, although few of them have been proved to be involved in DNA rearrangement (Belfort and Roberts, 1997). Among homing endonucleases, a coding sequence of the *Sce VMA1* intein has several unique characteristics (nomenclature for the elements involved protein splicing is defined in Perler *et al.*, 1994). A *VMA1* gene on chromosome IV of *S. cerevisiae* encodes a mature Vma1 protein and a *Sce VMA1* intein, both of which are produced posttranslationally by an autocatalytic protein splicing reaction (Fig. 2; Hirata *et al.*, 1990; Kane *et al.*, 1990). The *Sce VMA1* intein has a site-specific endonuclease activity and is called VDE (for *VMA1*-Derived Endonuclease; also called PI-*Sce*I (Perler *et al.*, 1994)). A sequence recognized by VDE, which is referred to VRS (VDE-Recognition Sequence), is composed of a 31 bp asymmetric sequence (Fig. 3A; Gimble and Thorner, 1992; Gimble

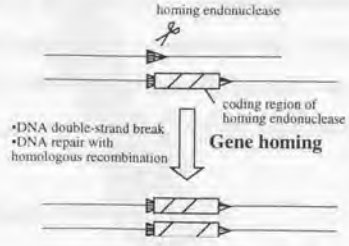


Figure 1 Gene homing. The homing endonuclease cleaves DNA at the recognition site (arrowhead). The recognition site is disrupted by inserting the coding region of the homing endonuclease (hatched box).

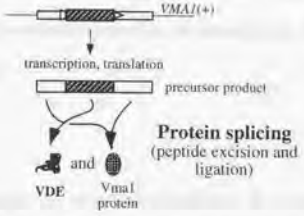


Figure 2 Protein splicing. Open squares and a hatched square indicate regions corresponding the V-ATPase subunit and VDE, respectively.

and Wang, 1996). VRS is located only at the intein insertion site of the intein-less *VMA1* gene (referred to *VMA1*(Δ)) in yeast genome (Bremer *et al.*, 1992). VDE cleaves VRS in the *VMA1*(Δ) allele, but does not cleave the DNA sequence of the intein-containing *VMA1* allele (referred to *VMA1*(+)) (Gimble and Thorner, 1992). VDE mediates gene homing at meiosis. When heterozygous *VMA1*(+)/*VMA1*(Δ) diploid cells undergo a meiotic process, VDE cleaves VRS, initiating gene homing (Fig. 3B). DSB produced by VDE creates a gap in one side of the choromosome, following homologous recombination repaired by *VMA1*(+) allele as a template. Endonuclease activity of VDE together with a recombination system of a host yeast cell results in unidirectional gene conversion of *VMA1*(Δ) to *VMA1*(+) (Gimble and Thorner, 1992). Accordingly, the gene homing process at meiosis spreads the VDE coding sequence in yeast population.

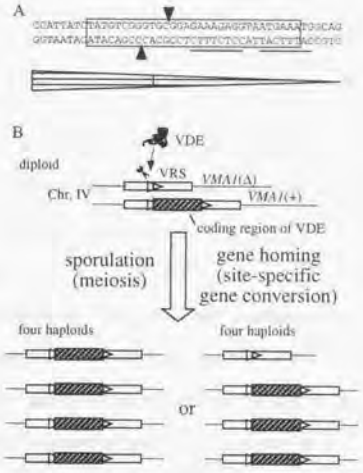


Figure 3 Gene homing and protein splicing mediated by VDE. A. Structure of the VDE-recognition sequence (VRS). Arrowheads, cleavage sites; a box, minimal region for cleavage; a horizontal triangle, region used in this study as VRS; underlines, PRE-like sequence. B. VDE-mediated gene homing at the *VMA1* locus. Same symbols as A are used. Details are described in text.

Proteins whose coding sequence is interrupted by intein with protein splicing activity are still functional, because the reaction is rapid and efficient enough to producing a host protein at a normal rate (Cooper *et al.*, 1993; Nogami *et al.*, 1997). However, it is known that unspliced VDE remains a precursor form and has no endonuclease activity or V-ATPase activity. Mutations that impair the protein splicing reaction result in loss of function of vacuole and therefore must be excluded evolutionary. Endonuclease activity is essential for gene homing, because cleavage of VRS is the first step of the gene homing event. Thus, the protein splicing reaction as well as the endonuclease activity is required for propagation of the VDE sequence in yeast population.

Purpose of this study

Of the two enzymatic activities of VDE, the protein splicing reaction has been well studied in the last decade (for review, see Anraku, 1997; Perler, 1998). Three core regions essential for the splicing reaction have been identified using site-directed and random mutational analysis (Cooper *et al.*, 1993; Kawasaki *et al.*, 1997). A series of *in vitro* biochemical studies of an archaea intein and VDE indicate that a novel type of reaction intermediates, branched inter-

mediate and succinimide ring formation, is proposed to exist transiently (reviewed in Anraku, 1997). The other enzymatic activity of VDE, site-specific endonuclease activity has also been studied from the viewpoint of the substrate recognition using the purified enzyme (Gimble and Stephens, 1995; Gimble and Wang, 1996). Recently a crystal structure of VDE was determined at a 2.1 Å resolution, having two distinct structural domains (Duan *et al.*, 1997). The molecule mimics "a snail", and it consists of Domain I (the first 182 amino acid and the last 44 amino acid) and internal Domain II. While Domain II contains a catalytic center of endonuclease, Domain I seems to have dual functions, including recognition of the DNA substrate, and protein splicing.

Accumulating information of functions of VDE is mainly derived from the *in vitro* studies mentioned above. Detailed *in vivo* functions of VDE still remains to be clear, although at least one of the ultimate aims of VDE is no doubt its propagation in nature. It is noted that there is few amount of information about *in vivo* function of VDE other than gene homing and meiosis specificity of gene homing. I, therefore, focused on to *in vivo* functions of VDE in my thesis study.

Part 1

Novel functions of the endonuclease VDE *in vivo*

Introduction

Gene homing of the *Sce VMA1* intein coding sequence is one of few examples that the homing endonuclease has been shown to be involved in DNA rearrangement. However, detailed function of VDE *in vivo* and the mechanism of meiosis specific gene homing remain to be clear. I am interested in the condition of gene homing driven by VDE. In this part, I focused on the recognition sequence of VDE. I found the two novel activities of VDE other than gene homing. First, I demonstrated that VDE is able to mediate homing of another gene, *URA3*, at a foreign locus, *MSB1*. This indicated that VDE is able to function altruistically like HO endonuclease that mediates yeast mating type switching. Second, I showed that meiosis-specific DNA cleavage by VDE results in reduced germination rate of spores when the VDE-sensitive *VMA1* allele is homozygous. In contrast, expression of VDE was not deleterious when the VDE-sensitive *VMA1* allele and the VDE-insensitive *VMA1* allele are heterozygous. This suggested that the DNA cleavage by VDE is deleterious for the host organism without a template for repair.

Results

1-1) Gene homing of an unrelated gene at another locus

Strain construction and principle of experiments

I investigated whether existence of VRS is a necessary and sufficient condition for gene homing. For this purpose, I planned to examine gene homing of an unrelated gene at a different locus. I chose the *URA3* gene as the unrelated gene (guest), and the *MSB1* locus as the different locus (host). The gene homing will be easily detected as disparity of the segregation of a Ura^+ phenotype. There are three reasons I chose the *MSB1* gene as a host. 1) The *MSB1* gene located on the different chromosome from the *VMA1* gene. 2) Any interaction with the *VMA1* gene and its product has not been reported. 3) Disruption of the *MSB1* gene did not affect yeast cell growth. Fig. 4 is a schematic drawing of the experiments. Briefly, two series of mutant *MSB1* alleles were constructed (Fig. 4A). Two alleles, *msb1-201* and *msb1-203* are used as donor alleles of the genetic information. The *URA3* gene was inserted as a donor marker

into the *Bgl*II gap of the *MSB1* locus to construct alleles, *msb1-201* and *msb1-203*. The *URA3* gene of *msb1-201* is bracketed by VRS to match the sequence to the recipient allele. Meanwhile, alleles *msb1-101* and *msb1-103* are used as recipient alleles of the genetic information. To construct these alleles, the *LEU2* gene was inserted as a recipient marker in the *MSB1* locus 1.0 kb apart from the *URA3* insertion point. In these alleles, corresponding position to the *URA3* insertion point was replaced by VRS (*msb1-101*) or deleted (*msb1-103*). Crossing the donor strains (*msb1-201* or *msb1-203*) made heterozygous *msb1* diploids with the recipient strains (*msb1-101* or *msb1-103*), sporulated and phenotypes of spores were analyzed. Expected segregation of leucine and uracil auxotrophic phenotypes is shown in Fig. 4C. If gene homing of the *URA3* gene occurs, Leu$^+$ Ura$^+$ haploid will appear frequently because the *URA3* gene will be inserted into the recipient allele that is marked with the *LEU2* gene (Fig. 4C, left). On the other hand, if gene homing of the *URA3* gene does not occur, parental ditype will be observed because the *LEU2* gene and the *URA3* gene are tightly linked (Fig. 4C, right).

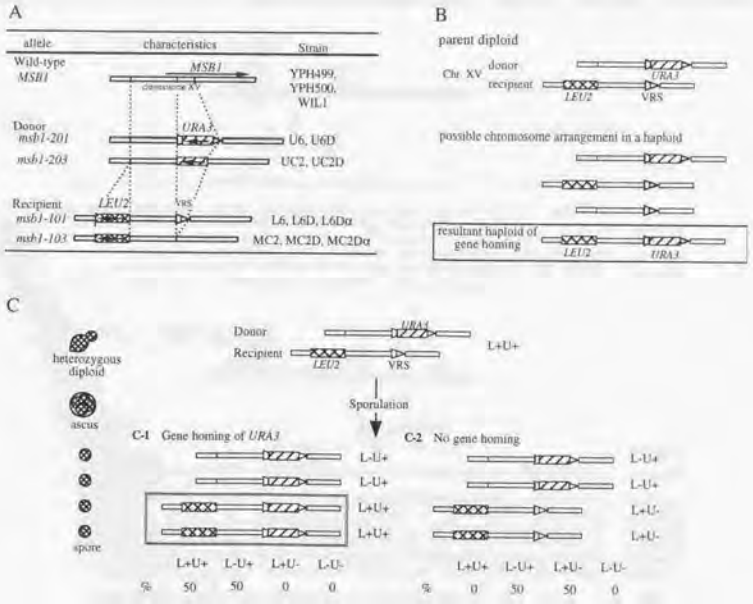


Figure 4 Strategy for investigating gene homing of the *URA3* gene at the *MSB1* locus. A. Mutant *MSB1* alleles constructed in this study. Hatched boxes indicate the *URA3* gene and *LEU2* gene and an arrow indicates the coding region of the *MSB1* gene. Arrowheads indicates (a part of) VRS. B. Possible chromosome arrangement of a parent diploid and spores. C. Expected phenotypes of spores. Typical phenotype of spore that is expected to occur the *URA3* gene homing is boxed.

VDE mediates gene homing of the URA3 gene at the MSB1 locus

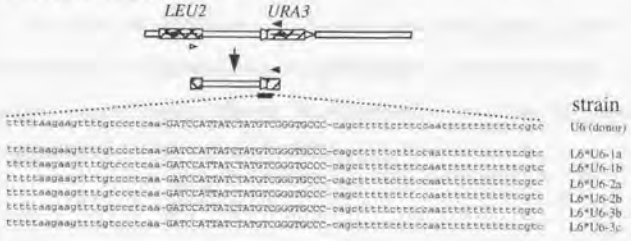
First, I analyzed random spores derived from a heterozygous *msb1-101/msb1-201* diploid strain, made by crossing L6 and U6. This strain contains VRSs on both chromosomes at the *MSB1* locus and is thought to likely exhibit gene homing. Segregation of Leu$^+$ and Ura$^+$ phenotypes that implicates gene homing was in fact observed (Table 1). This result suggests that gene homing of the marker gene occurred even at the different locus. I confirmed this phenomenon with additional tetrad analysis experiments with control strains (Table 2). Strains carrying VRS at the *MSB1* locus showed reduced germination ability of spores, suggesting that the existence of VRS may have deleterious effect for yeast cell germination from a spore (for example, *msb1-101/msb1-201* diploid, Table 3; further discussed below). Although reduced germination ability was observed, segregation of Ura$^+$ and Leu$^+$ phenotypes with tetrad analysis

Table 1 Random spore analysis of *msb1-101/msb1-201* heterozygous diploid

	phenotype (%)*			
	L^+U^+	L^-U^+	L^+U^-	L^-U^-
Expected				
No homing	0	50	50	0
complete homing	50	50	0	0
Observed				
	50.3 (142)	46.5 (131)	1.4 (4)	1.8 (5)

*Percentage of spores with indicated phenotype. Values in parentheses are number of observed spores.

A *MSB1/URA3* junction



B *URA3/MSB1* junction

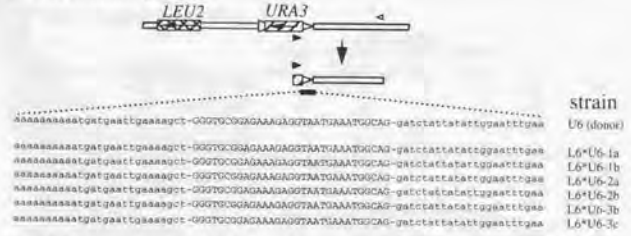


Figure 5 DNA sequence at the *MSB1* locus from strains that was expected to occur the *URA3* gene homing. A. Chromosome arrangement and DNA sequence around the homing site of spores expected to occur gene homing at the *MSB1/URA3* junction. Arrowheads inidicate position of primers for PCR amplification. Filled arrowheads indicate primers used for direct sequencing. Horizontal lines indicate regions whose DNA sequence was determined. Uppercase indicates VRS. B. Chromosome arrangement and DNA sequence around the homing site of spores expected to occur gene homing at the *URA3/MSB1* junction. Same symbols as A are used.

was similar to that with random spore analysis (compare Table 1 with Table 2). Moreover, all tetrads with four viable spores had four Ura$^+$ spores indicating an occurrence of unidirectional gene conversion. Segregation patterns of mating type and His$^+$ phenotypes were normal, suggesting that the unidirectional gene conversion is specific at the *MSB1* locus. Genomic DNA was isolated from the Leu$^+$ Ura$^+$ spores, and DNA sequence surrounding the insertion site of the *URA3* gene was determined. Over 70 bp nucleotide sequences apart from the insertion site on either side were identical to that of the sequence of the donor strain (Fig. 5), indicating that exact gene homing occurred.

Gene homing of the URA3 gene is dependent on both VDE and VRS

Next, I investigated whether this altruistic gene homing at the *MSB1* locus depends on presence of the *cis* element, VRS and the *trans* element, VDE. Diploid strains without VRS on the recipient chromosome (*msb1-103/msb1-201* and *msb1-103/msb1-203*) showed neither typical segregation for gene homing nor reduced spore germination ability (Table 2A). These results suggest that VRS is responsible for gene homing at the *MSB1* locus and reduced spore viability. Dependency on the VDE protein was also tested. I constructed new series of strains based on NNY103 or NNY203 in which the VDE coding sequence is absent. The new strains L6D, MC2D, U6D and UC2D carry alleles of *msb1-101, msb1-103, msb1-201 and msb1-203*, respectively. These donor and recipient strains were crossed similarly as above and phenotypes of spores were analyzed by tetrad analysis. All strains tested exhibit normal segregation of marker genes with high spore viability (Table 2B). This indicates that existence of the VDE protein was required both for homing of the *URA3* gene and reduced spore viability.

Table 2 phenotype of segregants of *msb1* mutant strains

A. *VMA1*(+) background (VDE exist)

		phenotype					
strain	genotype	L^+U^+	L^-U+	L^+U^-	L^-U^-	total	n
L6*U6	*msb1-101/msb1-201*	28	19	2	0	49	39
L6*UC2	*msb1-101/msb1-203*	11	46	2	0	59	20
MC2*U6	*msb1-103/msb1-201*	2	24	39	11	76	20
MC2*UC2	*msb1-103/msb1-203*	0	39	38	0	77	20

B. *VMA1*(Δ) background (VDE absent)

		phenotype					
strain	genotype	L^+U^+	L^-U+	L^+U^-	L^-U^-	total	n
L6D*U6D	*msb1-101/msb1-201*	0	38	38	0	76	20
L6D*UC2D	*msb1-101/msb1-203*	0	36	38	0	74	20
MC2D*U6D	*msb1-103/msb1-201*	0	40	40	0	80	20
MC2D*UC2D	*msb1-103/msb1-203*	0	36	33	0	69	19

n; number of asci tested.
The other numerals indicate number of spores with indicated phenotypes.

1-2) Deleterious effect of VDE to the host cell

DSB by VDE at the MSB1 locus is toxic in yeast

Reduced germination ability of spores was observed in some diploid strains used in the experiment mentioned above. For example, only 3 tetrads of a total of 39 produced four viable spores from *msb1-101/msb1-201* diploid (Table 3). This depended on existence of both VRS and the VDE protein (Table 3). Moreover, backcross of L6 with YPH500 (*msb1-101/MSB1* diploid) revealed that the number of viable Leu^- spores is larger than that of Leu^+ spores (Leu^+: Leu^- = 75: 135 in total 228 spores tested). Because the *LEU2* gene is linked to VRS, the existence of VRS may result in the biased segregation of Leu phenotype. These results suggest that the existence of VRS (and presumably resultant DSB) may cause loss of chromosome that contains VRS and has deleterious effect to yeast cell. However, there is an alternative explanation may be possible that the heterogeneity of the *MSB1* locus besides VRS may result in these phenomena. To distinguish these possibilities, I constructed three new diploid strains. A difference among these strains was only the presence of VRS at the *MSB1* locus: *msb1-101/msb1-101*, *msb1-101/msb1-103* and *msb1-103/msb1-103* had two, one and no VRSs, respectively, at the *MSB1* locus. Spores derived from diploids homozygous or heterozygous for VRS (*msb1-101/msb1-101* and *msb1-101/msb1-103*) showed reduced germination ability, whereas spores derived from diploids without VRS (*msb1-103/msb1-103*) had normal germination ability (Table 3). This suggests that the existence of VRS is ascribable to the reduced germination ability, referred here to a host-killer effect.

Table 3 germination ability of spores of *msb1* diploids

genotype of the diploids	*VMA1*(+) background							*VMA1*(Δ) background						
	number of spores							number of spores						
	4	3	2	1	0	total	%[a]	4	3	2	1	0	total	%[a]
msb1-101/msb1-201	3	2	9	13	12	39	8	17	2	1	0	0	20	85
msb1-101/msb1-203	7	6	6	1	0	20	35	16	3	1	0	0	20	80
msb1-103/msb1-201	17	2	1	0	0	20	85	20	0	0	0	0	20	100
msb1-103/msb1-203	18	1	1	0	0	20	90	16	3	0	0	0	19	84
msb1-101/MSB1	34	15	21	5	3	78	44	17	2	0	0	1	20	85
msb1-103/MSB1	34	3	3	0	0	40	85							
MSB1/MSB1	15	5	0	0	0	20	75							
msb1-101/msb1-101	14	6	10	6	4	40	35							
msb1-101/msb1-103	18	6	10	2	4	40	45							
msb1-103/msb1-103	33	2	4	1	0	40	83							

Numerals are number of tetrads with indicated number of viable spores.
a, ratio of tetrads with four viable spores to total tetrads tested.

DSB by VDE at the VMA1 locus is toxic in yeast

To analyze the host-killer effect of VDE at the *VMA1* locus, I constructed four additional *VMA1* alleles (Table 4). *VMA1-101*, *VMA1-103* and *VMA1-105* possessed mutations of VRS and are all *VMA1*(Δ), encoding functional VDE-less Vma1 protein. Sensitivity of VRS mutants to the endonuclease was different from each other *in vitro* due to

Table 4 characteristics of *VMA1* mutant allele

allele	presence of VDE	endonuclease activity	VRS sequence[b]	VRS sensitivity (%)[c]
VMA1 (wild-type)	+	active	-TAT GTC GGG TGC/GGA GAA-	0
VMA1-101	-		-TAT GTC GGG TGC GGA GAA-	100
VMA1-103	-		-TAT TCT GGC TGC GGA GAA-	0
VMA1-104	+	inactive[a]	-TAT GTC GGG TGC/GGA GAA-	0
VMA1-105	-		-TAT GTC GGC TGC GGA GAA-	50

a, endonuclease activity of VDE in the *VMA1-104* is lost because of a point mutation in the VDE-coding sequence.
b, changed bases are underlined. A slash indicates insertion site of the VDE-coding region.
c, relative sensitivity to wild-type VDE protein *in vitro* (*VMA1-101* = 100%).

extent of base substitution(s) (Table 4, Gimble and Wang, 1996 and data not shown). *VMA1-104* is an intein-containing *VMA1* allele (*VMA1*(+)), but carries a point mutation (A1826 to T from start codon of the *VMA1* gene) in the VDE coding sequence (Nogami *et al.*, 1997). This mutation (corresponding to asparagine to valine mutation at position 326 of the mature VDE protein) causes loss of endonuclease activity with no effects for protein splicing activity (Gimble and Stephens, 1995; Kawasaki *et al.*, 1997). The *VMA1-104* allele therefore produces both proteins by protein splicing, but endonuclease activity of VDE is lost. Alleles *VMA1* and *VMA1-104* are insensitive to VDE *in vitro* because their VRS is disrupted by inserting the intein coding sequence (Table 4). Haploid strains each carrying a mutant *VMA1* allele were crossed to make heterozygous diploid strains and spore germination ability was analyzed by tetrad analysis. Since functional VDE was produced in neither strain, active VDE was expressed from the introduced plasmid (Fig. 6A). A *VMA1-101/VMA1-101* diploid is the striking example that showed reduction of spore viability. When VDE was expressed, only ten tetrads of a total of 40 produced four viable spores, whereas 39 tetrads of a total of 40 produced

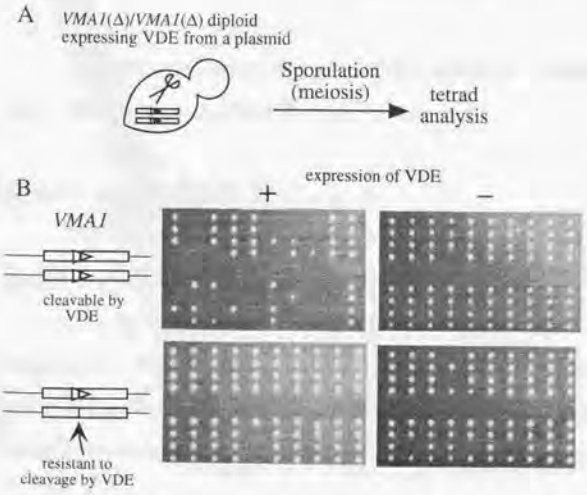


Figure 6 Strategy for investigating deleterious effect of VDE at meiosis. A. Experimental procedure. Scissors indicate VDE. B. Tetrad analysis of a homozygous *VMA1-101/VMA1-101* diploid (upper panels) and a heterozygous *VMA1-101/VMA1-103* diploid (lower panels). A plasmid expressing wild-type VDE (+) or a control plasmid (-) was introduced into the diploid strain.

four viable spores when VDE was absent (Fig. 6B and Table 5). The rate of tetrads with four viable spores to total tetrads tested depended on VDE ($P < 10^{-9}$; $\chi^2 = 40$ with 1 d.f.). Significant difference is also observed in *VMA1-101/VMA1-105* diploid and *VMA1-105/VMA1-105* diploid (Table 5, $P < 0.01$ for each diploid; $\chi^2 > 7$ with 1 d.f.). The *VMA1* alleles of these diploids contained either *VMA1-101* or *VMA1-105*, which are both sensitive to VDE *in vitro* (Table 5). Other combinations of the *VMA1* alleles exhibited no significant reduction of the spore germination (Table 5). These results indicated that the host-killer effect occurred when the VDE sensitive alleles were homozygous. Reduced germination ability was also dependent on the existence of VDE (Fig. 6B and Table 5).

Table 5 spore germination ability of various *VMA1* mutants

strain	*VMA1*(+) background							*VMA1*(Δ) background						
	number of spores							number of spores						
	4	3	2	1	0	n[a]	%[b]	4	3	2	1	0	n[a]	%[b]
NNY311	10	6	6	10	7	39	26	39	1	0	0	0	40	98
NNY351	26	10	4	0	0	40	65	37	2	1	0	0	40	93
NNY355	23	12	3	1	1	40	58	37	2	1	0	0	40	93
NNY313	33	5	1	1	0	40	83	35	3	1	1	0	40	88
NNY314	16	3	1	0	0	20	80	17	0	2	0	0	19	89
NNY333	32	5	1	1	0	39	82	15	1	2	0	0	18	83
NNY353	18	0	0	0	1	19	95	18	2	0	0	0	20	90
NNY343	20	0	0	0	0	20	100	15	3	2	0	0	20	75
NNY344	18	2	0	0	0	20	90	12	5	2	0	0	19	63
NNY354	20	0	0	0	0	20	100	17	2	1	0	0	20	85

numerals are number of asci with indicated number of viable spores.
a, number of asci tested.
b, ratio of asci with four viable spores to total asci tested.

Discussion

In this part, I reported two novel activities of VDE, gene homing of another gene and the host-killer. Based on these results, I discuss its function as a selfish gene product.

Altruistic function of VDE

First, I showed that the ectopically inserted VRS could lead to gene homing of an unrelated gene. The *URA3* gene that was artificially embedded in the *MSB1* locus was able to 'home' into the VRS that was embedded in the corresponding site of the sister chromosome. This ectopic homing was dependent on existence of VRS and VDE, suggesting that combination of the *cis* element, VRS and the *trans* element, VDE is a sufficient condition for gene homing. The ability of VDE to mediate genetic rearrangement of an unrelated gene gives us an altruistic insight into homing endonucleases. In fact, altruistic endonuclease has been reported in *S. cerevisiae*. An endonuclease called HO

is known to initiate mating-type switching by cleaving at a site in the *MAT* locus on chromosome III (reviewed by Herskowitz *et al.*, 1992). DSB produced by the HO endonuclease is followed by unidirectional gene conversion that replaces the information at the *MAT* locus with information copied from either of two homologous loci that reside near the telomeres of chromosome III. Interestingly, HO endonuclease and VDE share 34% identity and are evolutionary close to each other, belonging to a LAGLIDADG homing endonuclease family from phylogenic analysis (Dalgaard *et al.*, 1997; Hirata *et al.*, 1990; Perler *et al.*, 1997). In spite of the enzymatic and structural relationship between them, their function to the host cell is opposite. Functional difference of these two enzymes may result from the different location of the recognition sequence. If an endonuclease recognizes DNA at the position that the coding region of itself should be inserted, the enzyme would function selfishly. In contrast, If it cleaves at the position that the non-self gene should be inserted, the function would be altruistic.

VDE is not silent to its host

The second fact that I found is the deleterious effect of VDE to the host cell. This is not so surprising because a failure to repair a single DSB may be lethal in yeast (Bennett *et al.*, 1993; Malone and Esposito, 1980). In the previous experiments by another group, however, no obvious difference between intein-less strains and intein-containing strains had been observed (Gimble and Thorner, 1992). The host-killer effect is the first obvious difference between them. I suppose that the sequence specificity and life-stage specificity of VDE would explain the phenotypic silence of VDE. First, in the natural condition, cleavage of DNA occurs only when the template for repair is present in the heterozygous *VMA1*(Δ)/*VMA1*(+) diploid. In the *VMA1*(Δ) homozygous diploid, DNA cleavage will not occur because VDE is absent. In the *VMA1*(+) homozygous diploid, DNA cleavage will not also occur since VRS is absent. Second, cleavage by VDE observes only at meiosis with unknown mechanisms (Gimble and Thorner, 1992). The rate of DSBs is elevated at meiosis and DSBs are efficiently repaired with homologous recombination systems (reviewed by Shinohara and Ogawa, 1995). Controlled DNA cleavages by VDE may facilitate the DNA repair and maintain high spore germination ability in the natural condition.

Difference between the MSB1 locus and the VMA1 locus in the host-killer effect

Spore viability was reduced when VRS was inserted either into the *MSB1* locus or the *VMA1* locus. At the *VMA1* locus, host-killer effect was observed when the VDE sensitive site was homozygous. At the *MSB1* locus, however, it was observed even when VDE sensitive site was heterozygous (Table 3, *msb1-101/msb1-103* diploid). Moreover, a VDE sensitive *MSB1* allele tended to be lost. I suppose that sensitivity of VRS is different *in vivo* between

these two loci. The difference may be due to accessibility of VDE to VRS. The *VMA1* locus may be protected from VDE by a certain DNA-binding protein. Alternatively, homology search of cleaved ends may be unsuccessful at the *MSB1* locus.

The fact that heterozygous host-killer effect depends on locus where VRS is present is interesting in the context of propagation of the selfish gene, because it could be explained why the VDE sensitive site is unique in the yeast genome. It also suggests that several genes would be involved in this process. In other organisms, plural inteins are found and some of them are thought to have multiplied from common ancestors in the host genome (Perler *et al.*, 1997). Meanwhile, VDE cleaves yeast genomic DNA at a single site (Bremer *et al.*, 1992). Difference of host-killer effect between the *MSB1* locus and the original *VMA1* locus might prevent a mobile element from spreading horizontally in the host genome.

Toxic DSB in biological contexts

Host-killer effect after DSB and distortion of segregation that I observed is similar to the previous reports of cell death programmed by selfish genes. Host-killer with DSB has been reported in type II restriction-modification systems as genomic parasites (Kusano *et al.*, 1995; Naito *et al.*, 1995). Distortion of chromosome in the viable spores indicates that the VDE coding sequence is a potent meiotic drive gene. *Segregation Distorter* of *Drosophila melanogaster* is a well-described example for meiotic drive gene that spreads in the population by killing progeny with one chromosome that do not contain the gene (reviewed by Hurst *et al.*, 1996). Thus, endonucleases cause cell death and works on their maintenance in host genome.

In summary, probing of genes involved in gene homing or host-killer effect will provide understandings of the molecular mechanism(s) of meiosis-specific gene homing. A system of the *URA3* gene homing I constructed will be useful for this purpose. In addition, altruistic property of gene homing may provide a novel gene manipulation method of yeast: for example, site directed integration of the interest DNA.

PART 2

Mutational analysis of the bifunctional protein VDE

Introduction

VDE has two different enzymatic activities required for selfishness of its coding region. Of the two enzymatic activities of VDE, the protein splicing reaction has been well studied (for review, see Anraku, 1997; Perler, 1998). Several amino acid residues at the junction sites essential for the protein splicing reaction were identified from the site-directed mutagenesis study (Hirata and Anraku, 1992; Cooper *et al.*, 1993). From the random mutagenesis study, a histidine residue highly conserved among inteins was found to be essential for the splicing reaction (Kawasaki *et al.*, 1997).

Another enzymatic activity of VDE, site-specific endonuclease activity has also been studied from the viewpoint of the substrate recognition and DNA cleavage mainly using the purified enzyme (Gimble and Stephens, 1995; Gimble and Wang, 1996). Recently a crystal structure of VDE was determined at a medium resolution, having two structural domains (Duan *et al.*, 1997; Mizutani *et al*, unpublished). Domain II possesses the endonuclease catalytic site and Domain I (the first 182 amino acid and the last 44 amino acid) is a bifunctional domain composed of the putative endonuclease DNA recognition region and the catalytic site of protein splicing (Fig. 7).

I noticed that basic residues in Domain I align on the surface of the VDE molecule (Fig. 7). These positive-charged residues might be involved in the endonuclease function by interacting negative charges of the phosphate backbone of DNA. I therefore made mutants of which the basic residue was substituted with alanine. I characterized these mutants together with the mutants previously constructed in this laboratory.

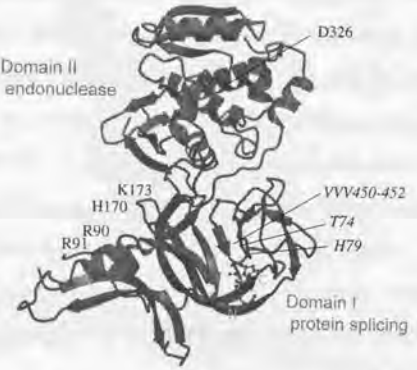


Figure 7 Crystal structure of VDE. Residues involved in the endoncuclease activitiy (D326) and the protein splicing activity (italic) are shown. Charged amino acid residues in Domain I are also shown. Original ribbon diagram are kindly provided by Dr. R. Mizutani.

Results

VDE mutants constructed

Several basic residues in Domain I are on the surface of the VDE molecule. Among of these residues, I selected R90, R91, H170 and K173 (numerals indicate a number of amino acid residue from N-terminal of the VDE protein) because they locate close to each other and project from molecular surface (Fig. 7). Alanine substituted mutant VDEs were constructed, each of which carries a mutation R90A, R91A, H170A or K173A. Beside them, VDE mutants that have been constructed in this laboratory were also characterized, some of which have a protein splicing defect and the other of which have endonuclease defect (T74S H79L, VVV450-452VKS and D326V). All mutations but D326V are located at bifunctional Domain I and D326 is a reaction center of endonuclease in Domain II (Duan *et al.*, 1997). Phenotypes analyzed in this study were: 1) gene homing activity at the *VMA1* locus, 2) gene homing activity of the *URA3* gene at the *MSB1* locus, 3) host-killer activity, 4) endonuclease activity of the purified enzyme and 5) protein splicing activity. Details of each experiment are described in Materials and Methods and Figures, and all results were summarized in Table 9. In experiments 1), 2) and 3), the VDE-coding region was expressed directly under the *VMA1* promoter without *VMA1* gene in order to exclude the effect of protein splicing activity. In experiments 4), mutant VDEs bracketed external amino acids were expressed in *E. coli* under the T7 promoter. Protein splicing activity was examined in experiment 5) by introducing plasmids expressing the Vma1 product with mutant VDE into the *VMA1* disrupted strain.

2-1) Gene homing at the *VMA1* locus mediated by VDE mutants

Gene homing ability of mutant VDEs at the *VMA1* locus was examined with a diploid strain NNY341 containing a heterozygous *VMA1-101/VMA1-104*. A diploid strain NNY341 was transformed with the plasmids that express mutant VDEs under the *VMA1* promoter. The gene homing ability was examined by testing segregation of *VMA1* alleles by colony PCR after tetrad dissection (Fig. 8). Colony PCR of spores can detect alleles *VMA1-101* and *VMA1-104* as a 0.45 kb and 1.8 kb DNA, respectively. If gene homing does not happen, spores from a heterozygous *VMA1-101/VMA1-104* cell will show 2: 2 segregation of *VMA1-101: VMA1-104* (Fig. 8C, left panel). On the other hand, if gene homing occurs, the *VMA1-101* allele will convert to the *VMA1-104* allele, resulting in 1:3 or 0:4 segregation of *VMA1-101: VMA1-104* (Fig. 8C, middle and right panel).

Results of tetrad analysis are shown in Table 6. Of the alanine-substituted VDE mutants, VDE carrying a R90A mutation completely lost the gene homing ability. The catalytic center mutant, D326V exhibit the same pheno-

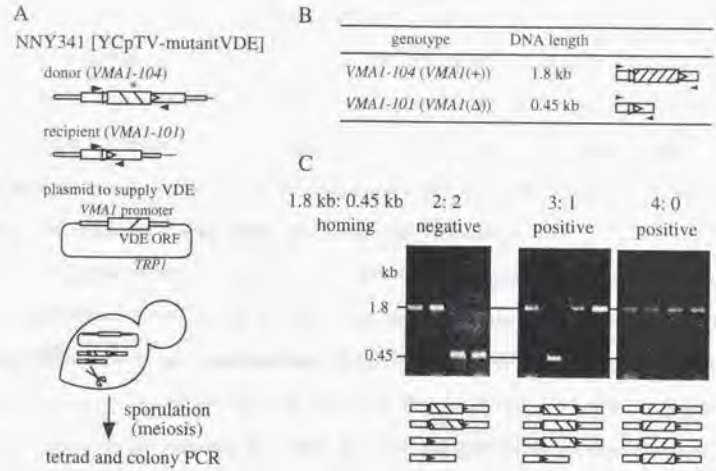


Figure 8 Schematic drawing of the experiments to detect gene homing at the *VMA1* locus mediated by mutant VDEs. A. Construction of a heterozygous *VMA1*(+)/*VMA1*(Δ) diploid strain. Open squares, coding regions corresponding to the V-ATPase subunit; a hatched square, the VDE-coding region; a triangle, VRS; arrowheads, position and direction of primers for colony PCR. A mutation in the chromosomal *VMA1-104* gene (asterisk) abolishes the endonuclease activity of VDE produced from this gene. B. DNA length expected to be amplified with primers shown in A. C. Typical segregation of the *VMA1* alleles detected with colony PCR of spores. Each panel contains the result of colony PCR of four segregants from one tetrad. A homing negative tetrad shows 2:2 segregation (left panel) and a homing positive tetrad shows 3: 1 (middle) or 4: 0 (right) segregation.

type. Results of H170A and K173A mutant VDE suggested occurrence of gene homing, although its efficiency was significantly lower than that of wild type. These results suggest that the VDE function on gene homing was impaired in H170A and K173A. On the contrary, R91A mutant showed mainly 4: 0 segregation for *VMA1*(+): *VMA1*(Δ), suggesting gene homing activity remains normal in this mutant. Of the mutants defective in the protein splicing reaction, T74S H79L showed a normal gene homing ability. On the other hand, VVV450-452VKS showed a relatively low gene homing ability. These results suggest that T74 and/or H79 are required specific for protein splicing, but VVV450-452 is required for both protein splicing and gene homing. Alternatively, mutations VVV450-452VKS might cause conformational change of the VDE protein that lowers both activities.

Table 6 Gene homing mediated by mutant VDEs at the *VMA1* locus

mutant	tetrad tested	tetrad with 4 viable spores carrying a plasmid	segregation of the *VMA1* allele (1.8 kb: 0.45 kb fragment)		
			4:0	3:1	2:2
wild type	70	19	19	0	0
vector	18	8	0	0	8
D326V	19	11	0	0	11
R90	49	15	0	0	15
R91	59	13	9	2	2
H170	65	11	1	2	8
K173	40	14	2	0	12
T74S H79L	20	10	9	0	1
VVV to VKS	19	9	3	1	5

2-2) Gene homing of the *URA3* gene at the *MSB1* locus

I next examined the gene homing ability of mutant VDEs at the *MSB1* locus, as shown in Fig. 4. Experiments were done in diploid strain L6DU6D whose *VMA1* locus carries *VMA1-103*. This allele lacks the coding region of VDE and is resistant to cleavage by VDE. To express the mutant VDEs, plasmids harboring the mutant VDEs under the *VMA1* promoter were introduced into L6DU6D. Altruistic gene homing was then examined by checking segregation of the nutrient auxotrophs after tetrad dissection. Results were summarized in Table 7.

As shown in the previous part, wild type VDE showed gene homing of the *URA3* gene at high efficiency. T74S H79L and VVV450-452VKS, protein splicing deficient mutants, also showed gene homing of the *URA3* gene although the efficiency was different. Strains carrying R91A mutant VDE also showed gene homing of the *URA3* gene at high efficiency. Strains carrying H170A and K173A mutant VDE also showed gene homing of the *URA3* gene, although its efficiency was low, suggesting that VDE function on altruistic gene homing was partially impaired in these mutants. Strains carrying D326V and R90A mutant showed normal segregation of Leu$^+$ and Ura$^+$, suggesting that these mutations have severe defects in gene homing function even at the *MSB1* locus.

Table 7 Gene homing mediated by mutant VDEs at the *MSB1* locus

mutant	tetrad tested	tetrad with 4 viable spores	tetrad with 4 viable spores carrying a plasmid	phenotypes of segregants				'random spore' presentation*			
				U4L2	U3L2	U2L2	other	U+L+	U+L-	U-L+	U-L-
wild type	18	8	5	3	0	0	2	16	21	0	0
T74S H79L	19	2	1	1	0	0	0	10	13	2	1
VVV to VKS	20	11	8	5	0	2	1	18	29	5	1
D326V	17	15	13	0	0	13	0	0	29	28	0
R90	19	17	9	0	0	9	0	0	20	19	0
R91	18	4	4	3	0	0	1	23	23	1	0
H170	16	12	9	2	4	3	0	10	23	11	0
K173	19	15	7	2	2	3	0	9	20	9	0

* Ura and Leu phenotypes are counted for all spores carrying a plasmid.

2-3) Host-killer effect at the *VMA1* locus

I constructed homozygous *VMA1-101/VMA1-101* diploid cells named NNY311. This strain lacks the coding region of VDE and has homozygous VRSs. As shown in the previous part, a host-killer effect was observed when wild type VDE was expressed (Fig. 6 and Table 5). Similarly, the host-killer effect by the VDE mutants was examined by transforming NNY311 with a plasmid that expresses the wild type or mutant VDE protein under the *VMA1* promoter, following with tetrad analysis (Table 8).

Germination ability of spores was reduced when mutant VDEs carrying T74S H79L or R91A mutation(s) was expressed, indicating that the host-killer activity remains in these mutants. H170A, K173A and VVV450-452VKS mutations resulted in moderate reduced spore

Table 8 Host killing effect mediated by mutant VDEs

mutant	tetrad tested	number of viable spores				
		4	3	2	1	0
wild type	20	4	0	2	2	12
vector	20	15	4	0	0	1
D326V	20	15	2	3	0	0
R90	18	12	4	2	0	0
R91	20	4	3	3	2	8
H170	19	11	4	3	1	0
K173	19	5	7	4	2	1
T74S H79L	19	5	2	3	3	6
VVV to VKS	20	7	9	1	2	1

germination, suggesting that the host-killer effect was partially impaired in these mutants. In strains carrying D326V or R90A mutant, spore germination ability is as high as the vector-control, indicating that these mutations have severe defects in the host-killer effect.

2-4) Endonuclease activity of purified VDE protein

Mutational analysis of the three activities mentioned above indicates that the VDE mutants were classified into three groups. The first group mutants, R90A and D326V exhibited a severe defect in gene homing at the *VMA1* and *MSB1* locus and in the host-killer activity. The second group mutants, H170A, K173A and VVV450-452VKS resulted in a moderate defect in gene homing and in the host-killer activity. The third group mutants, T74S H79L and

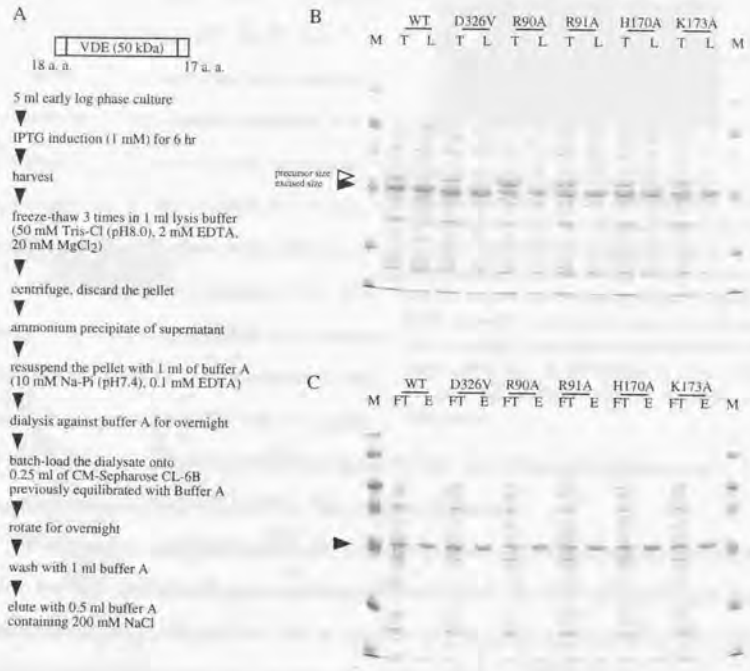


Figure 9 Expression and purification of mutant VDEs. A. Construction of recombinant VDE and purification procedure. VDEs bracketed by 18 proximal and 17 distal a.a. were expressed under the control of the T7 promoter. B. CBB staining of the lysate and centrifuge-clarified lysate prepared from VDE-expressing *E. coli*. T, total cell lysate; S, supernatant of the lysate after centrifuge. Ten µl of each fraction was analyzed on 10% of SDS-PAGE gel. The precursor size (open arrowhead) and the excised size (filled arrowhead) are indicated. C. CBB staining of teh purified recombinant VDEs. FT, flow-through fraction of the resin-loaded dialysate; E, fraction with 0.5 ml buffer A containing 200 mM NaCl. Ten µl of each fraction was analyzed on 10% of SDS-PAGE gel. A filled arrowhead indicates the size of VDE.

R91A exhibited no defect in three activities. Since these three *in vivo* activities are all related to the endonuclease activity, I examined the endonuclease activity of VDE mutants with purified protein.

In order to examine endonuclease activity of alanine-substituted mutant VDEs, I purified the mutant VDE protein expressed in *Escherichia coli* according to Kawasaki *et al.* (1997). VDE bracketed by 18 proximal and 17 distal amino acids were expressed under the control of the T7 promoter (Fig. 9A). When expressed at 25°C, two protein bands were observed around 50 kDa (Fig. 9B, lanes T). It is plausible that the minor upper band represents the precursor and that the major lower band represents the excised VDE by protein splicing since the upper band disappeared after purification (Fig. 9B, lanes S and Fig. 9C). This suggests that the introduced mutations do not affect the protein splicing reaction in *E. coli* (see below). Mutant VDE proteins were then purified by ammonium sulfate precipitation and CM-Sepharose CL-6B chromatography (Fig. 9C). Endonuclease activity of the purified VDEs was qualitatively analyzed (Fig. 10). A 8.0 kb linearized plasmid with the VRS was incubated with purified enzymes. If VDE cleaves this plasmid, a 4.7 kb and a 3.3 kb DNA fragment will be generated.

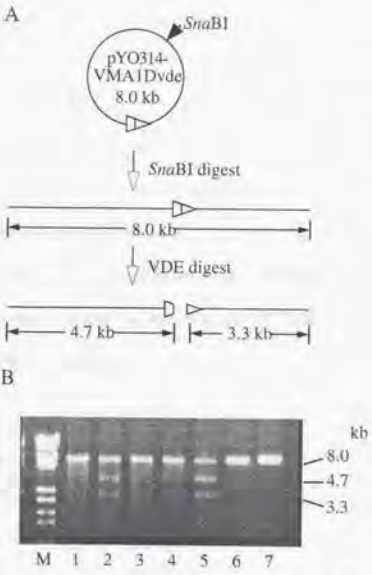


Figure 10 Endonuclease activity of mutant VDEs. A. Experimental procedure. A 8.0 kb *Sna*BI-linearized pYO314-VMA1Dvde that contains a VRS was used as a substrate. Digestion with VDE results in appearance of a 4.7 kb and a 3.3 kb fragment. See Materials and Methods for detail. B. Endonuclease activity of mutant VDEs purified from *E. coli*. M; λ-StyI marker. A Linearized substrate (lane 7) was incubated with VDE carrying following substitutions; R90A (lane 1), R91A (lane 2), H170A (lane 3), K173A (lane 4), wild-type (lane 5) and D326V (lane 6). M indicates λ-*Sty*I DNA marker.

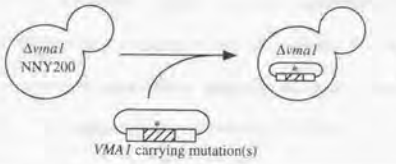
Wild-type and a mutant VDE carrying a R91A mutation generated a 4.7 kb and a 3.3 kb DNA fragment well. However, the D326V and R90A mutants showed no digested DNA fragment. The H170A and K173 mutants generated a faint amount of digested DNA fragments. Endonuclease activity of the protein splicing mutants (T74S H79L and VVV450-452VKS) was previously shown to remain normal (Kawasaki *et al.*, 1997; Nogami *et al.*, 1997).

2-5) Protein splicing activity of mutant VDE in yeast

It has been already shown that T74S H79L and VVV450-452VKS mutants impair the protein splicing reaction but that D326V mutant remains the full protein splicing activity (Kawasaki *et al.*, 1997; Nogami *et al.*, 1997). It

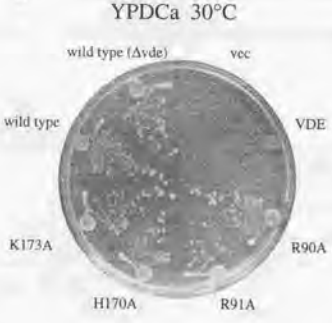
was suggested that the substituted alanine mutations do not affect the protein splicing reaction in *E. coli* (Fig. 9). Protein splicing ability of alanine-substituted VDEs was examined also in yeast cell. Plasmids expressing the *VMA1* gene with mutant VDE were introduced into the *VMA1*-disrupted strain, NNY200. NNY200 shows calcium sensitive phenotype typical to V-ATPase defective mutant. Transformants of NNY200 carrying the mutant *VMA1* gene was able to grow on calcium-rich medium, indicating that a functional V-ATPase was produced (Fig. 11B). This suggests that the protein splicing reaction occurred in these strains. Next, Western blotting analysis of whole cell extracts in these strains was performed. If the splicing reaction occurs, 70 kDa and 50 kDa products can be detected with an anti-Vma1p antibody and an anti-VDE antibody, respectively. All alanine substituted mutants produced the 70 kDa and 50 kDa products, indicating that the mutations do not impair the protein splicing activity (Fig. 11C).

A



protein splicing	pruduct	calcium sensitivity (Cls)	Western analysis
negative		Cls^-	120 kDa
positive		Cls^+	70 kDa + 50 kDa

B



C

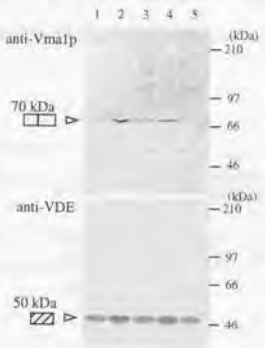


Figure 11 Protein splicing of mutant VDEs in yeast. A. Schematic drawing of the experiments. A lack of the V-ATPase activity confers inability to grow on calcium-rich medium (Cls^- phenotype). Therefore protein splicing defects of mutant *VMA1* can be detected as inability to complement of calcium sensitive phenotype of the *VMA1*-disrupted strain. B. Growth phenotypes of alanine-substituted mutant in the *VMA1* gene on a YPDCa plate. Yeast cells harboring a mutant *VMA1* gene carrying indicating mutation were streaked on YPDCa medium and incubated for 3 days at 30°C. C. Western analysis of yeast cell extracts. Same strain as B were used. Mutation in the VDE ORF was R90A (lane 1), R91A (lane 2), H170A (lane 3), K173A (lane 4), and wild type (lane 5).

Discussion

I identified the basic amino acid residues R90, H170 and K173 that are involved in the endonuclease activity of VDE. Alanine-substituted mutations of these residues impaired the endonuclease activity of purified VDE, the gene homing ability at the *VMA1* and *MSB1* locus and the host-killer ability. In the crystal structure of VDE, these residues locate in Domain I apart from the putative catalytic center (D218 and D326) in Domain II, suggesting that they are not directly involved in the catalytic reaction (Fig. 7; Duan *et al.*, 1997). It is known that charged amino acid residues on the surface of a protein molecule are often involved in interaction with other molecules. To interact with DNA, basic residues are likely important, because their positive charges form salt bridge with negative charges of the phosphate backbone of DNA. Basic residues that I identified may be important for binding or recognition of DNA.

Table 9 summarizes the phenotypes of the VDE mutants. Protein splicing mutants T74S H79L remained activities involved in the endonuclease activity, indicating that these residues are specifically involved in the protein splicing reaction. Contrary, mutants in Domain I (R90, H170 and K173) whose endonuclease activity was low still remained the protein splicing activity, suggesting that these residues are specifically involved in the endonuclease activity. These results suggest that different residues are involved in the two activities of VDE, protein splicing and gene homing, although both are located in Domain I.

No mutants were found in this study that impair gene homing but retain endonuclease activity *in vitro*. Mutations showing these characteristics are thought to be involved specifically in the gene homing event. Further functional dissection of bifunctional VDE might identify residues required for gene homing at meiosis, but not for endonuclease activity.

Table 9 Chraracteristic of mutant VDEs

	endonuclease activity				protein splicing	
	in vivo			in vitro		
	gene homing					
mutant	*VMA1* locus	*MSB1* locus	killer effect	endonuclease activity	Calcium sensitivity	Western blotting
wild type	++	++	++	++	++	++
R91A	+	++	++	+	++	++
H170A	±	+	+	±	++	++
K173A	±	+	+	±	++	++
R90A	–	–	–	–	++	++
D326V	–	–	–	–	++	++
T74S H79L	++	++	++	++	–	–
VVV450-452VKS	+	+	+	++	+	+

Part 3

Meiosis-specific function of the endonuclease VDE

Introduction

A number of homing endonuclease with protein splicing activity has been identified in the last decade. Some have been found in the process of genome sequence project and homology search (reviewed in Perler *et al.*, 1997). Some of them were examined for their endonuclease activity with the purified protein (Gimble and Thorner, 1992; Hodges *et al.*, 1992). Among them, VDE is an only example of the intein whose homing ability was experimentally examined. Gene homing of VDE occurs specifically at meiosis (Gimble and Thorner, 1992).

When the coding region of VDE is regarded as a selfish DNA element, it is purposive for VDE to function specifically at meiosis. Since sister chromosomes are separated from each other in mitosis, DSB caused by VDE in diploid cells would remain unrepaired, resulting in the cell death. On the contrary, in meiosis, DSB caused by VDE would be repaired efficiently by homologous recombination host system that is activated in meiosis. In addition, meiotic gene homing is preferable, since alignment of homologous chromosomes guarantees accessibility to the template for repair.

Since the discovery of meiosis specific gene homing in 1992, there was no report about the VDE function *in vivo*. In this part, I focused on the meiosis specific function of VDE. I first examined whether two novel functions of VDE that I found in part 1 are also specific at meiosis. Then I investigated the molecular basis of meiosis specificity of the VDE function.

Results

3-1) *URA3* gene homing at the *MSB1* locus at mitosis

I found that VDE is able to mediate homing of another gene at a different locus (part 1). In this experiment, however, it was not clear whether gene homing at the *MSB1* locus would occur specifically at meiosis. I therefore analyzed frequency of the altruistic gene homing event at mitosis. For this purpose, I developed an experimental system that detects gene homing even at a low frequency. Since the donor and the recipient were marked with *LEU2* and *URA3*, respectively, the homing product harbored *LEU2* and *URA3* gene on the same chromosome. This DNA fragment was detected by PCR with primers, which anneal the different strand of *LEU2* and *URA3*.

Since relative amount of the homing product is expected to be very low, high sensitivity and specificity of the detection system are therefore required. For this purpose, I tried to detect this DNA construct with a semi-nested PCR method that the second PCR is done with primers that anneals inside the first PCR fragment.

First I designed PCR primers. Primers, LEU2atg and LEU2atg-2, were annealed to the top strand of the *LEU2* gene and URA3taa was annealed to the bottom strand of the *URA3* gene (Fig. 12A). A 1.4 kb (with primers LEU2atg and URA3taa) or a 1.3 kb (with primers LEU2atg-2 and URA3taa) DNA fragment will be amplified only when the *LEU2* gene and the *URA3* gene locate on the same chromosome. In fact, no obvious PCR product was observed when the template DNA was isolated from a mitotic haploid strain L6 or U6 (Fig. 12B, lanes 1 and 2) with primers LEU2atg and URA3taa. On the other hand, a 1.4 kb PCR product was obtained when the template DNA was isolated from a haploid strain LU66-1a, a sergeant of LU66 showing a Leu$^+$ Ura$^+$ phenotype and expected to occur gene homing (Fig. 12B, lane 3).

Then I tested the nested PCR method with a mixture of the parent DNA and the homing product as a template. The protocol is described in Materials and Methods and Fig. 12C. The first PCR reaction was done under a weak

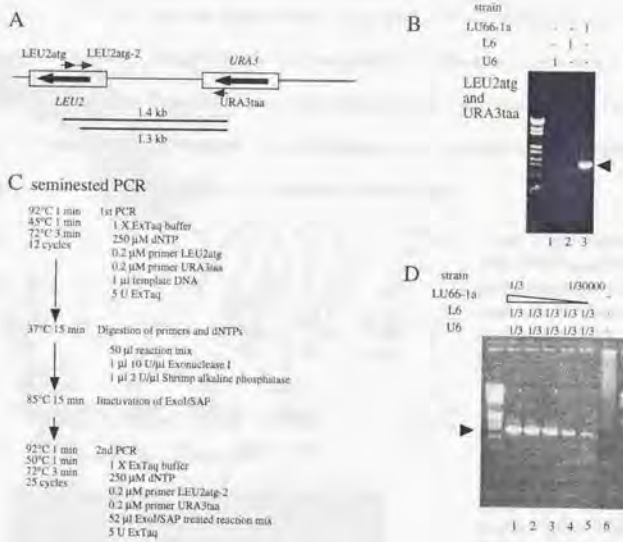


Figure 12 Stratgy to detect gene homing of the *URA3* gene at the *MSB1* locus at mitosis. A. Location of primers and expected length of a PCR product Small arrows, primers; large arrows, direction and location of ORF; horizontal lines, regions expected to amplify. B. Yeast genomic PCR with primers URA3taa and LEU2atg. A 1.4 kb fragment (arrowhead) was amplified from genomic DNA of LU66-1a. C. Condition of semi-nested PCR. D. Semi-nested PCR of mixture of DNA. Relative concentration to the undiluted DNA solution are indicated. A 1.3 kb fragment (arrowhead) was detected with semi-nested PCR.

condition with decreased cycle number because the purpose of this step is to amplify a template for the second reaction. After digestion of excess primers for the first PCR reaction and dNTPs, the second PCR reaction was done with primers URA3taa and LEU2atg-2 that anneals inside the target fragment of the first PCR. A 1.3 kb fragment was amplified from a mixture of DNA from LU66-1a, L6 and U6 without nonspecific amplification (Fig. 12D, lanes 1-5). The minimum detectable ratio of the LU66-1a genome to the L6 and the U6 genomes was 1: 20000. Although a blank control sample shows nonspecific amplification, a 1.3 kb fragment was not amplified (Fig. 12D, lane 6). These results indicate that this system is specific and sensitive enough to detect gene homing at mitosis.

I applied this protocol to examine the frequency of gene homing of the *URA3* gene in mitotic and meiotic diploid cells. Three diploid strains were used in this study. LU66 and L6U6 were independently obtained by crossing L6 and U6 and shown to exhibit the altruistic homing. L6DU6D lacking the VDE-coding region did not exhibit gene homing. This was used as a negative control strain of LU66 and L6U6. Yeast cells were continuously grown in YPD medium and sampled for genome isolation every day. The third day's culture was shifted to the sporulation step (Fig. 13). The spore formation rate was approximately 20-30% (as complete ascus with four ascospores) or 40-50% (including incomplete ascus). The 1.3 kb DNA fragment that was the indication of gene homing was detected, when DNA of sporulated LU66 and L6U6 was used as a template of the semi-nested PCR (Fig. 13B, lanes 20 and 21). Other stage of the cell exhibited no DNA band. A faint band of 1.3 kb was, when DNA obtained from sporulated L6DU6D was used as a template (Fig. 13B, lane 22). I suppose that this band reflected a meiotic recombination at this locus. These results showed that occurrence of altruistic homing at mitosis was undetectable by the most sensitive method, suggesting that gene homing event even at the *MSB1* locus seldom occurs at mitosis, if any.

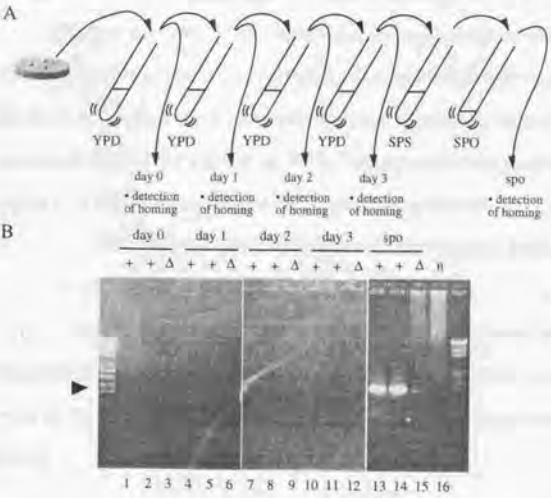


Figure 13 Gene homing of the *URA3* gene at the *MSB1* locus at mitosis. A. Experimental procedure. The culture were diluted with the 100-fold YPD medium at each day. Presporulation was done in SPS medium and sporulation was done in 1.5 ml of 1% potassium acetate with appropriate nutrients. Yeast DNA was isolated from 1 ml of the culture (containing 2-3 x 10^7 cells). B. Nested PCR to detect gene homing. +, a strain with VDE expected gene homing at meiosis (LU66 and L6U6); Δ, a strain without VDE expected no gene homing at meiosis (L6DU6D); n, negative control without template.

3-2) Host-killer effect at mitosis

Diploid NNY311 cells with the homozygous *VMA1* alleles that can be cleaved by VDE were transformed with plasmids YCpTV-VDE(s) expressing wild type VDE. This strain shows low spore germination ability, indicating the host-killer effect at meiosis (see part 1). Here, I examined the viability of this strain at mitosis. Single cells without a bud were collected from log-phase culture incubated in YNBD-Trp, placed on YPD plate with micromanipulator, and incubated at 30°C for 3 days. Almost all cells formed as large colonies on YPD as a vector control strain, suggesting that VDE did not affect viability of growing cell at mitosis (Fig. 14).

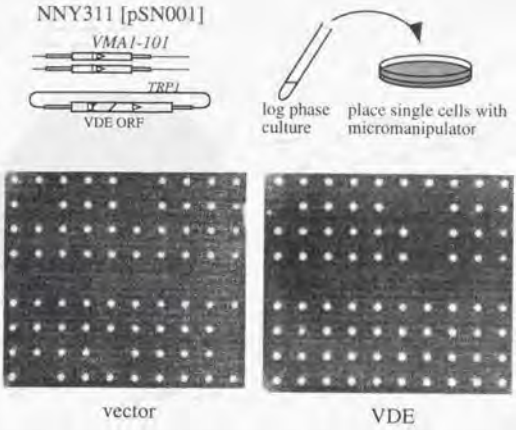


Figure 14 Cell growth of strains carrying VRS is not inhibited when VDE is expressed at mitosis. Diploid strain carrying VRSs at the VMA1 locus was transformed with pYO314 (vector) or pSN101 (VDE), and a transformant was cultured in YPD medium. Single cells without a bud were placed on YPD medium with micromanipulator and cultured at 30°C for 3 days.

3-3) What defines meiosis specific gene homing?

Previous and current studies revealed that endonuclease-driven VDE functions *in vivo* are meiosis specific. However, the VDE protein exists in the yeast cell even at mitosis and is detected with Western blotting analysis. Moreover, endonuclease activity and its characteristics of purified VDE from mitotic yeast cell extract are similar to that from *E. coli* (Gimble and Thorner, 1993). Two explanations are possible why the functions of VDE functions related to its endonuclease activity are specifically observed at meiosis.

I) DSB(s) caused by VDE is rapidly repaired by repair system at mitosis

II) DSB caused by VDE does not occur at mitosis.

To genetically distinguish these two possibilities, I used mutants defective in the DSB repair. If the first possibility is correct, a mutation in the repair gene will result in harmful effect because DSB by VDE remains to be repaired. On the contrary, if DSB does not occur, mutations in the repair system will not show any defects for cell growth.

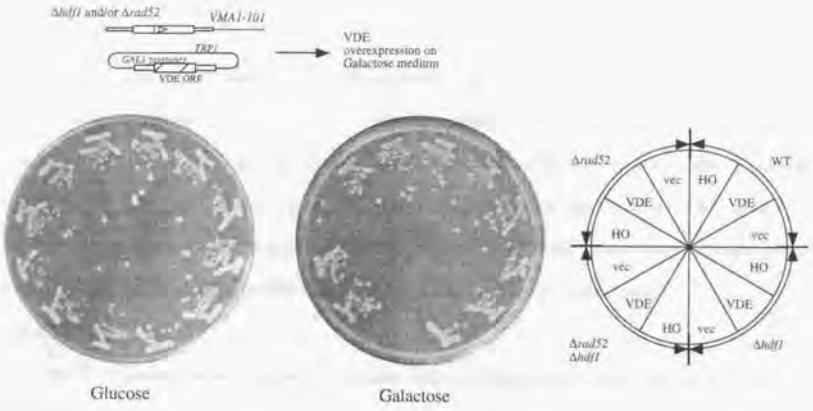


Figure 15 Overexpression of the VDE protein does not affect cell growth at mitosis in repair mutants. Plasmids YCpTG578T (vec), pSAY21 (VDE) and pJH283 (HO) were introduced into strains NNY201 (wild type), NNY201Dr (*Δrad52*), NNY101Dh (*Δhdf1*) and NNY101DhDr (*Δhdf1 Δrad52*). Cells were incubated on plates containing glucose (Glucose) or galactose (Galactose) as a carbon source for 4 days at 30°C.

To examine whether repair of DSB is involved in meiosis specific function of VDE, I disrupted the *HDF1* gene and the *RAD52* gene using the *HIS3* gene and *LEU2* gene of *Candida glabrata*, respectively. The *RAD52* gene has been reported to be involved in homologous recombination, a major pathway for DSB repair in yeast (reviewed by Shinohara and Ogawa, 1995). Sensitivity to DSB is known to be high in *rad52* mutant. For example, the creation of an unrepairable DSB induced by HO endonuclease at the *MAT* locus, caused *rad52* haploid cells to die (Malone and Esposito, 1980). The *HDF1* gene has been reported to be involved in end-joining type DNA repair, another DSB repair pathway in yeast (reviewed by Tsukamoto and Ikeda, 1998). Sensitivity to DSB is higher in *rad52 hdf1* double mutant than in *rad52*. If a DSB by VDE occurs at mitosis, growth defect will be observed in these mutants. If DSB by VDE does not happen at mitosis instead, nothing will happen. Mutants were transformed with a plasmid harboring a Galactose-inducible VDE construct. Transformants were streaked on YNBD-Trp or YNBGS-Trp and incubated at 25°C for 3 days. Mutations in the gene involved in DSB repair did not affect cell viability when VDE was overexpressed, strongly suggesting that DSB by VDE does not occur at mitosis (Fig. 15). On the contrary, overexpression of the *HO* gene resulted in the growth defect in the *RAD52*-disrupted strain, as reported previously (Fig. 15; Malone and Esposito, 1980). These results are consistent with the idea that DSB by VDE does not occur at mitosis. It also suggests existence of the other mechanism to explain meiosis-specific gene homing by VDE.

Discussion

Mechanism(s) of Meiosis specificity of VDE-mediated homing

As discussed in Introduction of this part, it seems purposive that gene homing of the VDE ORF was regulated to occur specifically at meiosis. In this part, I found that gene homing at the *MSB1* locus and host-killer effect are also specific at meiosis, as in the case of gene homing at the *VMA1* locus. I also showed that a VDE-mediated DSB does not occur suggesting the existence of a system(s) to repress the endonuclease activity of VDE at mitosis. Several possible mechanisms for meiosis specificity of VDE-mediated gene homing can be postulated as follow.

1) Localization

The VDE protein may be excluded from nucleus at mitosis and imported into the nucleus only at meiosis. VDE has neither obvious nuclear localization signals (NLS) nor nuclear export signals (NES). Molecular mass of VDE is 50 kDa that is about upper limit to pass the protein through the nuclear pore with diffusion, suggesting a selective transportation mechanism. Alternatively, VDE may interact with any proteins with NLS that is imported into nuclear at meiosis.

2) DNA structure

Accessibility of VDE to DNA may be restricted at mitosis because of the DNA structure around VRS. Chromatin structure is one of the probable barriers against VDE attack. Chromatin structure is known to dramatically change at recombination initiation sites during meiosis (Ohta *et al.*, 1994; Wu and Lichten, 1994). Transient DSBs at recombination hot spots by meiosis-specific endonuclease, Spo11p, occur during meiosis and are thought to initiate homologous recombination between chromosomes (Keeney *et al.*, 1997). Changes in chromatin structure around VRS in premeiotic and meiotic cells may results in different accessibility of VDE to VRS. Alternatively, a protein that binds the VRS specifically at mitosis may interfere VDE to access VRS. Actually, VRS contains two copies of pheromone response element (PRE; Gimble and Thorner, 1992). PRE is a famous upstream activating sequence in yeast and binds the transcriptional activator Ste12 (Dolan *et al.*, 1989). Binding of the Ste12 protein to PRE of VRS at mitosis and dissociation at meiosis may explain meiosis specific function of VDE.

3) Interacting molecule(s)

Endonuclease activity of VDE may be regulated by interacting molecule(s) that binds to VDE specifically at mitosis or meiosis. Several explanations are possible. One is that a protein that binds to VDE specifically at mitosis represses the endonuclease activity of VDE at mitosis. Another explanation is that a protein that binds to VDE specifically at meiosis activates the endonuclease activity of VDE at meiosis. Alternatively, there may be a factor that

changes site-preference or site-specificity. An endonuclease of LAGLIDADG family in which VDE is contained is known to form complex to another protein. Endo-*Sce*I, an endonuclease localized in mitochondria and involved in recombination of mitochondria DNA, consists of two-polypeptide subunits. One is a catalytic subunit and has homology to VDE with LAGLIDADG motifs (Nakagawa *et al.*, 1991). The other is a member of HSP70 family (Morishima *et al.*, 1990). HSP70 is thought to enhance the site-specificity of catalytic subunit.

4) Modification

Modification of VDE itself or its interacting protein may regulate meiosis specific endonuclease activity. An idea that direct modification of VDE regulates its endonuclease activity is unlikely, because purified VDE from cells in mitosis when the endonuclease activity is repressed still remains endonuclease activity *in vitro* (Gimble and Thorner, 1993). Modification of a protein that interacts with VDE regulates endonuclease activity of VDE may be possible.

Following experiments will distinguish the possibilities discussed above: determination of localization of the VDE protein, observation of chromatin configuration in premeiotic and meiotic yeast cells, screening of interacting molecules with VDE using two-hybrid system or immunoprecipitation with VDE. Since any host factors are likely involved in meiosis specificity, genetic screening to identify mutants in which VDE cleaves VRS even in mitosis may be informative.

Conclusions

1. I found two novel activities of VDE in yeast cells. First, VDE mediates gene homing of the *URA3* gene at the *MSB1* locus. This indicates that the 45 bp-long recognition site of VDE is necessary and sufficient for gene homing by VDE. Second, existence of VDE reduces spore germination ability when VRS is homozygous in diploid. This indicates that the DSB produced by VDE at meiosis is potentially deleterious for yeast.

2. I identified amino acid residues required for the endonuclease activity of VDE. Residues identified locate Domain I of three-dimension structure of VDE.

3. I found that all of the endonuclease activities of VDE *in vivo* are meiosis specific. This meiosis specificity is achieved by repressing the endonuclease activity at mitosis by an uncertain mechanism.

Materials and Methods

Strains

Yeast strains used in this study are listed in Table 10. All strains were derivatives of YPH499, YPH500 (Sikorski and Hieter, 1989) and constructed by transformation using the lithium acetate procedure (Ito *et al.*, 1983) or by standard genetic crosses (Kaiser *et al.*, 1994). Characteristics of mutant alleles of the *VMA1* (Table 4) and the *MSB1* (Fig. 4A) locus are shown.

Construction of VMA1 mutant

VMA1 mutant strains were constructed as follows. Strains NNY100 (*MAT*α *vma1::URA3*) and NNY200 (*MAT***a** *vma1::URA3*) are Ura$^+$ segregants of diploid that was made by crossing NY101 (Nogami *et al.*, 1997) with YPH500. They showed calcium sensitive phenotype typical to *vma1* disruption mutant (Hirata *et al.*, 1990; Nogami *et al.*, 1997; Ohya *et al.*, 1991). A DNA fragment containing the mutant *VMA1* allele was integrated into the *VMA1* locus of NNY100 or NNY200 and plated on a YPDCa medium. Because every mutant allele produces a functional vacuolar ATPase subunit, integrants whose *VMA1* allele was substituted by introducing DNA were able to grow on YPDCa medium, complementing the calcium sensitive phenotype of the *vma1* mutant. These Cls$^+$ integrants also showed Ura$^-$ phenotype.

Disruption of the CAN1 gene

One-step gene disruption of the *CAN1* gene was carried out to make the strain WIL1. WIL1 strain was constructed in four steps. The *Eco*RI site and the *Spe*I site of pBluescript KS+ were removed by a fill-in reaction to make a plasmid pBluescript DEDS. A 1.3 kb *Hin*dIII fragment of pRS319 (containing a *CAN1* gene, Sikorski and Boeke, 1991) was cloned into the *Hin*dIII site of pBluescript DEDS to make pAOCAN1. A 1.8 kb *Eco*RI-*Xba*I fragment of pJJ217 (containing a *HIS3* gene, Jones and Prakash, 1990) was replaced with a 230 bp *Eco*RI-*Spe*I fragment of pAOCAN1 to make pAOCAN2. pAOCAN2 was linearized with *Xba*I and *Sal*I and introduced into YPH499. His$^+$ transformant was able to grow on YNBD plate containing 60 mg/l canavanine and designated WIL1.

Construction of the msb1-101 and the msb1-103 allele and introduction into yeast

The mutant *MSB1* alleles *msb1-101* and *msb1-103* were constructed in two steps as follows. A plasmid YEpT-MSB1 was yeast multi-copy plasmid containing a 5.5 kb *Sal*I-*Bam*HI fragment including a *MSB1* gene and marked with *TRP1* (H. Qadota, unpublished). To construct plasmids pMA and pMV, a 496 bp *Bgl*II fragment of YEpT-MSB1 was deleted (pMA) or substituted with an oligonucleotide cassette (pMV1). Oligonucleotide cassette were made by annealing VDE-F and VDE-R (Oligonucleotides used in this study are listed in Table 11). Resultant plasmids carry a deletion mutation at the nucleotide positions 280-776 from the start codon of the *MSB1* gene. *In vitro*, pMA was insensitive to VDE, whereas pMV1 was sensitive because pMV1 contains VRS (data not shown). Next, a 1.5 kb *Ssp*I-*Ssp*I fragment of pJJ282 (containing a *LEU2* gene, Jones and Prakash, 1990) was inserted into a blunted *Nhe*I site of pMA and pMV1 to make plasmids pML2ND and pMV12N, respectively. The *Nhe*I site is located at 862 bp upstream of the ATG of the *MSB1* gene.

Strains carrying the *msb1-101* allele and the *msb1-103* allele were constructed as follows. A *Pvu*II fragment of pML2ND (containing the *msb1-101* allele) and pMV12N (containing the *msb1-103* allele) was introduced into WIL1, and Leu$^+$ transformant was named as MC2 and L6, respectively. Strains MC2D, MC2Da, L6D and L6Da were

isogenic to MC2 and L6, except that the *VMA1* locus was substituted with intein-less allele, *VMA1-103*. They were derived from backcrossing with strain NNY103 and selecting haploid derivatives that showed same phenotypes as MC2 and L6. The *VMA1-103* allele of haploid derivatives was detected as a 0.45 kb DNA fragment using colony PCR of yeast with primers H and MK-23.

Construction of the msb1-201 and the msb1-203 allele and introduction into yeast

Mutant alleles, *msb1-201* and *msb1-203* were constructed in two steps as follows. A plasmid pMS1 was derived from YEpT-MSB1 by replacing a 496 bp *Bgl*II fragment with an oligonucleotide cassette that was made by annealing oligonucleotides, SMA-F and SMA-R. This oligonucleotide cassette contains a VRS splitted by a *Sma*I recognition sequence. Next, plasmids pMU2 and pMSU12 were constructed by inserting a blunted 1.1 kb *Hin*dIII-*Hin*dIII fragment of pJJ242 (containing a *URA3* gene, Jones and Prakash, 1990) into a *Bgl*II gap of YEpT-MSB1 (pMU2) or a *Sma*I gap of pMS1 (pMSU12).

Strains carrying the *msb1-201* allele and the *msb1-203* allele were constructed as follows. A *Pvu*II fragment of pMU2 (containing the *msb1-203* allele) and pMSU12 (containing the *msb1-201* allele) was introduced into YPH500, and Ura$^+$ transformant was named as UC2 and U6, respectively. UC2D and U6D were isogenic to UC2 and U6, respectively, except that the *VMA1* locus was substituted with intein-less allele, *VMA1-103*. They were derived from backcrossing with strain NNY203 and selecting haploid derivatives that showed same phenotypes as UC2 and U6. The *VMA1-103* allele of UC2D and U6D was confirmed by detecting a 0.45 kb DNA fragment using colony PCR of yeast with primers H and MK-23.

Disruption of the HDF1 gene and the RAD52 gene

The *HDF1* gene and the *RAD52* gene were successively disrupted in diploid strains NNY311, NNY333 and NNY355. The *HDF1* gene was disrupted with the *Candida glabrata HIS3* gene (Cg*HIS3*). The Cg*HIS3* gene was amplified with primers hdf1-HIS3 F and HIS3-hdf1 R. Each primer has extra oligonucleotide sequence at the 5' tail that anneals the *HDF1* gene of *S. cerevisiae*. Amplified DNA was purified with ethanol precipitation and used for transformation. His$^+$ transformants were selected and disruption of the *HDF1* gene by the Cg*HIS3* gene was confirmed with PCR using primers HDF1p F and HDF1t R, annealing with the upstream and downstream of the *HDF1* gene, respectively. Next, the *RAD52* gene was disrupted with the *C. glabrata LEU2* gene (Cg*LEU2*). Primers used were: rad52-LEU2 F and LEU2-rad52 R for amplifying the Cg*LEU2* gene, RAD52p F and RAD52t R for confirming disruption, annealing with the upstream and downstream of the RAD52 gene, respectively. The resulted *HDF1/hdf1::HIS3 RAD52/rad52::LEU2* diploids were sporulated and subjected to tetrad analysis.

Plasmids

Plasmid used in this study and their characteristics are listed in Table 12. pYO314, pSN001, pSN101 are described previously (Nogami *et al.*, 1997). pYO314-VMA1Dvde, YCpTV-PT, YCpTV-VDE(s), pSAY21 and pJH283 were routinely used in this study and other plasmids were derived from these plasmids.

pYO314-VMA1Dvde was identical to pSN101 except that the VDE-coding region was entirely deleted. This plasmid produces a functional catalytic subunit of V-ATPase without VDE in yeast cell. This plasmid was also used as a substrate for examining the endonuclease activity of mutant VDEs.

To generate pSAY21, a 1.3 kb *Eco*RI fragment of pUC-VDE (M. Kawasaki, unpublished) was inserted into the *Eco*RI site of YCpTG578T that locates downstream of the *GAL1* promoter. pSAY21 contains a whole VDE ORF

with a methionine preceding the first cysteine and expresses VDE in yeast cells when cultured in the galactose media.

YCpTV-PT was constructed as follows. A 4.2 kb *Spl*I-*Aff*II fragment of pSN001 was inserted into the multiple cloning site of pYO314 to generate pYO314-VMA1(S-A). A 5.5 kb fragment was amplified from pYO314-VMA1 (S-A) by PCR with VMA1 promoter R and VMA1 terminator F as primers. The amplified fragment was digested with *Kpn*I and self-ligated to generate YCpTV-PT. YCpTV-PT contains the *VMA1* promoter and terminator regions that were connected with multiple restriction sites of *Nco*I, *Kpn*I, *Xba*I and *Bgl*II.

To generate YCpTV-VDE(s), a 1.3 kb *Kpn*I-*Bam*HI fragment of pSAY21 that contains VDE-coding region was inserted into the *Kpn*I-*Bgl*II gap of YCpTV-PT. YCpTV-VDE(s) expresses VDE under the constitutive *VMA1* promoter.

pJH283 contains galactose inducible *HO* gene marked with *TRP1* (Rudin *et al.*, 1989).

Culture conditions

Cells were grown in rich medium (YPD) and selective medium (YNBD) as described (Kaiser *et al.*, 1994). To examine the calcium sensitive phenotype, YPD supplemented with 100 mM $CaCl_2$ (YPDCa) was used. Presporulation medium was YPA or SPS medium. YPA contained 1% yeast extract, 2% polypeptone and 1% potassium acetate. SPS contained 0.5% yeast extract, 1% polypeptone, 0.17% yeast nitrogen base, 1% potassium acetate, 0.5% ammonium sulfate and 0.05 M potassium phthalate buffer (pH 5.0). Sporulation medium (SPO) contained 1% potassium acetate and was supplemented with appropriate nutritional ingredients. Standard methods were used for cross, transformation and other genetic procedures (Kaiser *et al.*, 1994). For *GAL1* promoter induction, medium containing 2% galactose and 0.1% sucrose was used as carbon sources (YNBGS).

Continuous culture

Yeast diploid strains were pregrown in 2 ml YPD at 30°C for overnight (2-3 x 10^7 cells/ml). Every day, 50 µl of the culture were diluted with 5 ml YPD and cultivated at 30°C for 24 hours. Genomic DNA from mitotic cell was isolated from 1 ml of the culture (2-3 x 10^7 cells) before dilution. Sporulation procedure was as follows. The third day's culture was harvested, (once washed with SPS), and cultivated in 2 ml of SPS for 1-2 days. One ml of the late-log culture (2-3 x 10^7 cells) was harvested, washed once with sterile water and cultivated in 1.5 ml of SPO for 2-3 days with good aeration. Genomic DNA of spores was isolated from spheroplast prepared from sporulation culture as described below. Number of spores and unsporulated cells were counted with a hemocytometer.

In vivo assay system for detecting gene homing at the VMA1 locus

Gene homing at the *VMA1* locus was detected using a strain, NNY341. NNY341 contains a heterozygous *VMA1-101/VMA1-104* (Fig. 8). The *VMA1-101* allele lacks the coding region of VDE and has a sequence that is able to cleave by VDE. This allele therefore works as a recipient. The *VMA1-104* allele contains the coding region of VDE, but the endonuclease activity is lost because of a mutation at the putative reaction center of VDE (D326V). This allele therefore works as a sole donor of genetic information. NNY341 was transformed with a plasmid that expresses the wild type or mutant VDE protein under the *VMA1* promoter, and gene homing ability of mutants was analyzed with tetrad following a colony PCR of segregants with primers MK-23 and H. The final reaction mixture (50 µl) contained 10 p moles of each primer and a tooth-pickful of yeast cells. The reaction was taken through 30 cycles: each cycle

consisted of 94°C for one minute, 50°C for one minute, and 72°C for two minutes. In this PCR condition, the *VMA1-101* allele and the *VMA1-104* allele are detected as a 0.45 kb and a 1.8 kb of amplified fragment, respectively. If gene homing is negative, spores from a heterozygous *VMA1-101(VMA1(Δ))/VMA1-104(VMA1(+))* cell will show 2: 2 segregation of *VMA1-101*: *VMA1-104*. On the other hand, if gene homing is positive, the *VMA1-101* allele will convert to the *VMA1-104* allele, resulting in 1: 3 or 0: 4 segregation of *VMA1-101*: *VMA1-104*.

In vivo assay system for detecting altruistic gene homing (gene homing of the URA3 gene at the MSB1 locus)

Diploid strain heterozygous of *msb1::LEU2/msb1::URA3* was sporulated and phenotypes of segregants were analyzed with random spore analysis or tetrad analysis. Direct sequencing was done for determining a DNA sequence of the homing site. Primers used for amplifying the yeast genomic DNA were: LEU2atg and URA3taa for the *MSB1-URA3* junction, and URA3atg and MSB1sph for the *URA3-MSB1* junction (shown in Fig. 5, arrowheads). DNA sequencing of the amplified fragment was carried out using URA3atg or URA3taa (Fig. 5, filled arrowheads) as a sequencing primer with an automated DNA sequencer (Applied Biosystems, 373A) according to the manufacturer's protocol.

In vivo assay system for detecting host-killer effect

Diploid strain NNY311 was transformed with plasmids expressing a mutant VDE under the *VMA1* promoter. Trp^+ transformants were sporulated and analyzed with tetrad analysis. Number of viable spores is counted.

PCR to detect homing products at the MSB1 locus

Preparation of template DNA

Genomic DNA isolation from mitotic growing cells was done by 'smash and grab' method (Strathern and Higgins, 1991) from 1-5 x 10^7 cells. To isolate DNA from spores, they were pretreated with 100 μg/ml Zymolyase (Seikagaku-Kogyo) in 1M sorbitol at 37°C for one hour before suspending STES buffer. Ethanol-precipitated DNA was dissolved in 200 μl of TE (pH 8.0).

Primers

Primers used for amplifying the homing products were LEU2atg, LEU2atg-2 and URA3taa. LEU2atg and LEU2atg-2 were hybridized with nucleotide positions 166-147 and 8-(-12) of the *LEU2* gene, respectively (numbers indicate positions from start methionine of the *MSB1* gene). URA3taa was hybridized with nucleotide positions 13-32 of the *URA3* gene (numbers indicate positions from the termination codon). Positions of hybridization are depicted in Fig 12.

Semi-nested PCR

First amplification was done with ExTaq between LEU2atg and URA3taa. The final reaction mixture (50 μl) contained 10 p moles of each primer and 1 μl of yeast DNA solution. The reaction was taken through 10-12 cycles: each cycle consisted of 94°C for one minute, 45°C for one minute and 72°C for three minutes. The PCR products were treated with 10 Units of Exonuclease I (ExoI: Amersham) and 2 Units of shrimp alkaline phosphatase (SAP: Amersham) at 37°C for 15 minutes to digest excess primers and dNTPs, and the enzymes were heat inactivated (85°C, 15 minutes).

Second amplification was done between primers LEU2atg-2 and URA3taa. The final reaction mixture (60 µl) contained 10 p moles of each primer and 52 µl of the ExoI/SAP treated PCR products as a template. The reaction was taken through 25 cycles: each cycle consisted of 94°C for one minute, 50°C for one minute and 72°C for three minutes. Five µl of the second PCR products were subjected to electrophoresis with 0.7% agarose gel in TAE system, and stained with ethidium bromide for visualizing the amplified DNA.

Host-killer effect at mitosis

Diploid strain NNY311 were transformed with YCpTV-VDE(s) and Trp^+ transformant was cultured in 2 ml of YNBD + casamino acid -Trp at 30°C for one day. Ten µl of culture are spotted onto YPD and single cells without a bud were placed onto grids with micromanipulator, and cultured at 30°C for 3 days.

Effect of repair mutant on host-killer by VDE

Haploid cells with a single and double mutation of the *HDF1* gene and the *RAD52* gene were transformed with pSAY21 (carrying the ORF of wild type VDE under the *GAL1* promoter), pJH283 (carrying the *HO* gene under the *GAL1* promoter) or YCpTG578T (vector). Trp^+ transformants were streaked on YNBD + casamino acid -Trp (VDE or HO is not expressed), YNBGS + casamino acid -Trp (VDE or HO is expressed) and incubated at 25°C for 3 days. In Fig. 15, results of strains carrying *VMA1-101* allele are shown.

Table 10 Strain list

strain	genotype	reference*
YPH499	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1*	a
YPH500	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1*	a
NY101	*MATa ade2 his3 leu2 lys2 trp1 ura3 vma1::URA3*	b
NNY100	*MATα ade2 his3 leu2 lys2 trp1 ura3 vma1::URA3*	c
NNY200	*MATa ade2 his3 leu2 lys2 trp1 ura3 vma1::URA3*	c
NNY101	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-101*	c
NNY201	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1-101*	c
NNY103	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-103*	c
NNY203	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1-103*	c
NNY104	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-104*	d
NNY204	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1-104*	d
NNY105	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-105*	c
NNY205	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1-105*	c
WIL1	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1 can1::HIS3*	e
L6	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1 can1::HIS3 msh1-101*	e
MC2	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1 can1::HIS3 msh1-103*	e
U6	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1 msh1-201*	e
UC2	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1 msh1-203*	e
L6D	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1-103 can1::HIS3 msh1-101*	e
MC2D	*MATa ade2 his3 leu2 lys2 trp1 ura3 VMA1-103 can1::HIS3 msh1-103*	e
U6D	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-103 msh1-201*	e
UC2D	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-103 msh1-203*	e
L6Dα	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-103 can1::HIS3 msh1-101*	e
MC2Dα	*MATα ade2 his3 leu2 lys2 trp1 ura3 VMA1-103 can1::HIS3 msh1-103*	e
LU66	L6 X U6	e
L6U6	L6 X U6	e
L6UC2	L6 X UC2	e
MC2U6	MC2 X U6	e
MC2UC2	MC2 X UC2	e
L6DU6D	L6D X U6D	e
L6DUC2D	L6D X UC2D	e
MC2DU6D	MC2D X U6D	e
MC2DUC2D	MC2D X UC2D	e
L6L6Dα	L6 X L6Dα	e
L6MC2Dα	L6 X MC2Dα	e
LU66-1a	a segregant of LU66 showing Leu^+ and Ura^+ phenotype	e
LU66-1b	a segregant of LU66 showing Leu^+ and Ura^+ phenotype	e
LU66-2a	a segregant of LU66 showing Leu^+ and Ura^+ phenotype	e
LU66-2b	a segregant of LU66 showing Leu^+ and Ura^+ phenotype	e
LU66-3b	a segregant of LU66 showing Leu^+ and Ura^+ phenotype	e
LU66-3c	a segregant of LU66 showing Leu^+ and Ura^+ phenotype	e
NNY311	NNY101 X NNY201	c
NNY313	NNY101 X NNY203	c
NNY333	NNY103 X NNY203	c
NNY341	NNY104 X NNY201	c
NNY343	NNY104 X NNY203	c
NNY344	NNY104 X NNY204	c
NNY351	NNY105 X NNY201	c
NNY353	NNY105 X NNY203	c
NNY354	NNY105 X NNY204	c
NNY355	NNY105 X NNY205	c
NNY367	NNY106 X NNY207	c
311Dh	NNY311 with heterozygous *HDF1/hdf1::CgHIS3*	c
333Dh	NNY333 with heterozygous *HDF1/hdf1::CgHIS3*	c
355Dh	NNY355 with heterozygous *HDF1/hdf1::CgHIS3*	c
311DhDr	NNY311 with heterozygous *HDF1/hdf1::CgHIS3 RAD52/rad52::CgLEU2*	c
333DhDr	NNY333 with heterozygous *HDF1/hdf1::CgHIS3 RAD52/rad52::CgLEU2*	c
355DhDr	NNY355 with heterozygous *HDF1/hdf1::CgHIS3 RAD52/rad52::CgLEU2*	c
NNY101Dh	NNY101 with *hdf1::CgHIS3* (segregant of 311DhDr)	c
NNY201Dh	NNY201 with *hdf1::CgHIS3* (segregant of 311DhDr)	c
NNY101Dr	NNY101 with *rad52::CgLEU2* (segregant of 311DhDr)	c
NNY201Dr	NNY201 with *rad52::CgLEU2* (segregant of 311DhDr)	c
NNY101DhDr	NNY101 with *hdf1::CgHIS3 rad52::CgLEU2* (segregant of 311DhDr)	c
NNY201DhDr	NNY201 with *hdf1::CgHIS3 rad52::CgLEU2* (segregant of 311DhDr)	c

*a. Sikorski and Hieter (1989); b. Nogami et al. (1997); c. This study; d. S. Nakao; e. M. Sugiura; f. S. Yabe

Table 11 Oligonucleotide list

oligonucleotide	sequence
VDE-F	GAT CCA TTA TCT ATG TCG GGT GCG GAG AAA GAG GTA ATG AAA TGG CAG
VDE-R	GAT CCT GCC ATT TCA TTA CCT CTT TCT CCG CAC CCG ACA TAG ATA ATG
SMA-F	GAT CCA TTA TCT ATG TCG GGT GCC CCG GGT GCG GAG AAA GAG GTA ATG AAA TGG CAG
SMA-R	GAT CCT GCC ATT TCA TTA CCT CTT TCT CCG CAC CCG GGG CAC CCG ACA TAG ATA ATG
LEU2atg	CAC CTG TAG CAT CGA TAG CA
URA3atg	CAG GAC TAG GAT GAG TAG CA
URA3taa	TAA GTA AAT GCA TGT ATA CT
MSBlsph	GCT TCT AAT GTT ATC ATA TAG CTT C
LEU2atg-2	GCA GAC ATT AGA ATG GTA TA
H	TAC TTG GCC TGT TCG TGT TCC
MK-23	TCT CAA AGC TTC AGC CCA TCT
VMA1promoter R	GGG GTA CCA GCC ATG GTT TCT TTT CTT CTC TAT TTG C
VMA1terminator F	GGG GTA CCT CTA GAT CTA AGT GAT TAA GAT ATA TGT AGC ATT
R90A-F	TTA GAA CAC CTC GTA GTG TCG CGC GCT TGT CTC GTA CCA TTA A
R90A-R	TTA ATG GTA CGA GAC AAG CGC GCG ACA CTA CGA GGT GTT CTA A
R91A-F	GAA CAC CTC GTA GTG TCC GCG CGT TGT CTC GTA CCA TTA AGG G
R91A-R	CCC TTA ATG GTA CGA GAC AAC GCG CGG ACA CTA CGA GGT GTT C
H170A-F	GCC AGA GAT CTT TCT CTG TTG GGT TCC GCG GTT CGT AAA GCT AGC TAC
K173A-F	GCC AGA GAT CTT TCT CTG TTG GGT TCC CAT GTT CGT GCA GCT AGC TAC CAG ACT TAC
VDE end R BclI	TTC ATT ACC TCT TTC TGA TCA ATT ATG
HDF1p F	GGA AGC TGA AGT GCA AGG
HDF1t R	CTG AGA AAG TAC TGT CGA
hdf1-HIS3 F	ATG CGC TCA GTC ACT AAT GCA TTT GGC AAT AGT GGA GAA CTT AAC CGA TCA ACG TAC AGT GG
HIS3-hdf1 R	ATT GTA TGT AAC GTT ATA GAT ATG AAG GAT TTC AAT CGT CTT TAT GAC AAT CTG GCA GCT CG
RAD52p F	CCT ATG GAC ATA TGT GCG
RAD52t R	AAG TAA TCG AGA TCA AGC
rad52-LEU2 F	GCG GCG GGC GGG TTA CGC GAC CGG TAT CGA ATG GCG TTT TTA AGC GAA ATG TGT GCT CTG TAA TCA
LEU2-rad52 R	CGG CCA GGA AGC GTT TCA AGT AGG CTT GCG TGC ATG CAG GGG ATT TTA CGT AAA GTT CGT TTG CCG

Table 12 Plasmid list

plasmid	origin[1)]	note	reference[2)]
pBluescript KS+		*E. coli* high copy vector	a
pET-17b		*E. coli* expression vector	b
pRS314		yeast centromere vector marked with *TRP1*	c
pRS316		yeast centromere vector marked with *URA3*	c
pRS319		containing the *CAN1* gene	d
pIJ217		containing the *HIS3* gene	e
pIJ242		containing the *URA3* gene	e
pIJ282		containing the *LEU2* gene	e
pYO314	A, B	yeast centromere vector marked with *TRP1*	f
pSN001	C		f
pSN101	C		f
pYO314-VMA1Dvde	C	3.4 kb fragement containing the *VMA1* gene without VDE ORF	g
pYO314-VMA1(S-A)	C	4.2 kb fragement containing the *VMA1* gene with VDE ORF	g
YCpTV-PT	D	containing the *VMA1* promoter and the *VMA1* terminator	g
YCpTV-VDE(s)	E	VDE ORF is connected directly under the *VMA1* promoter	g
pET-17b VDE: WT	G	for expressing wild type VDE in *E.coli*	h
pBluescript DEDS		pBluescript KS+	g
pAOCAN1		containing the *CAN1* gene in the MCS of pBluescript DEDS	i
pAOCAN2		containing the *can1::HIS3* construct in pBluescript DEDS	i
YEpT-MSB1		containing the 5.8 kb fragment including the *MSB1* gene	j
pMA	J	containing the *msb1* allele whose 0.5 kb *Bgl*II fragment is deleted	i
pMV1	I	containing the *msb1* allele whose 0.5 kb *Bgl*II fragment is deleted	i
pML2ND	I	containing the *msb1-101* allele	i
pMV12N	I	containing the *msb1-103* allele	i
pMS1	I	containing the *msb1* allele whose 0.5 kb *Bgl*II fragment is deleted	i
pMU2	I	containing the *msb1-203* allele	i
pMSU12	I	containing the *msb1-201* allele	i
YCpTV-VDE D326V	F	carrying a mutation D326V	g
YCpTV-VDE T74S H79L	F	carrying mutations T74S and H79L	g
YCpTV-VDE VVV-VKS	F	carrying mutations VVV450-452VKS	g
pSZ-R90	F	carrying a mutation R90A	k
pSZ-R91	F	carrying a mutation R91A	k
pSZ-H170	F	carrying a mutation H170A	k
pSZ-K173	F	carrying a mutation K173A	k
pYB-R90	D	carrying a mutation R90A	k
pYB-R91	D	carrying a mutation R91A	k
pYB-H170	D	carrying a mutation H170A	k
pYB-K173	D	carrying a mutation K173A	k
pET17b-VDE 64-2	H	for expressing mutant VDE (D326V) in *E.coli*	h
pET17b-VDE R90A	H	for expressing mutant VDE (R90A) in *E.coli*	g
pET17b-VDE R91A	H	for expressing mutant VDE (R91A) in *E.coli*	g
pET17b-VDE H170A	H	for expressing mutant VDE (H170A) in *E.coli*	g
pET17b-VDE K173A	H	for expressing mutant VDE (K173A) in *E.coli*	g
YCpTG578T	B	containing the *GAL1* promoter and marked with *TRP1*	l
pSAY21		VDE ORF is cloned under the *GAL1* promoter of YCpTG578T	m
pJH283		containing the galactose-inducible HO gene, marked with *TRP1*	n

1) A, pUC19; B, pRS314; C, pYO314; D, pYO314-VMA1(S-A); E, YCpTV-PT; F, YCpTV-VDE(s); G, pET-17b; H, pET-17b VDE: WT; i, YEpT-MSB1

2) a, STRATAGENE; b, NOVAGENE Inc.; c, Sikorski and Hieter (1989); d, Sikorski and Boeke (1991); e, Jones and Prakash (1990); f, Nogami *et al.* (1997); g, this study; h, Kawasaki *et al.* (1997); i, M. Sugiura; j, H. Qadota; k, S. Yabe; l, Y. Ohya; m, S. Arai; n, Rudin *et al.* (1989).

References

Anraku, Y. (1997) Protein splicing: its chemistry and biology. *Genes to Cells*, **2**, 359-367.

Belfort, M. and Roberts, R. J. (1997) Homing endonucleases: keeping the house in order. *Nucleic Acids Res.*, **25**, 3379-3388.

Bennett, C. B., Lewis, A. L., Baldwin, K. K. and Resnick, M. A. (1993) Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid. *Proc. Natl. Acad. Sci. USA*, **90**, 5613-5617.

Bremer, M. C. D., Gimble, F. S., Thorner, J. and Smith, C. L. (1992) VDE endonuclease cleaves *Saccharomyces cerevisiae* genomic DNA at a single site: physical mapping of the *VMA1* gene. *Nucleic Acids Res.*, **20**, 5484.

Cooper, A. A., Chen, Y.-J., Lindorfer, M. A. and Stevens, T. H. (1993) Protein splicing of the yeast *TFP1* intervening protein sequence: a model for self-excision. *EMBO J.*, **12**, 2575-2583.

Dalgaard, J. Z., Klar, A. J., Moser, M. J., Holley, W. R., Chatterjee, A. and Mian, I. S. (1997) Statistical modeling and analysis of the LAGLIDADG family of site-specific endonucleases and identification of an intein that encodes a site-specific endonuclease of the HNH family. *Nucleic Acids Res.*, **25**, 4626-4638.

Dolan, J. W., Kirkman, C. and Fields, S. (1989) The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. *Proc. Natl. Acad. Sci. USA*, **86**, 5703-5707.

Duan, X., Gimble, F. S. and Quiocho, F. A. (1997) Crystal structure of PI-SceI, a homing endonuclease with protein splicing activity. *Cell*, **89**, 555-564.

Gimble, F. S. and Stephens, B. W. (1995) Substitutions in conserved dodecapeptide motifs that uncouple the DNA binding and DNA cleavage activities of PI-*Sce*I endonuclease. *J. Biol. Chem.*, **270**, 5849-5856.

Gimble, F. S. and Thorner, J. (1992) Homing of a DNA endonuclease gene by meiotic gene conversion in *Saccharomyces cerevisiae*. *Nature*, **357**, 301-306.

Gimble, F. S. and Thorner, J. (1993) Purification and characterization of VDE, a site-specific endonuclease from the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **268**, 21844-53.

Gimble, F. S. and Wang, J. (1996) Substrate recognition and induced DNA distortion by the PI-*Sce*I endonuclease, an enzyme generated by protein splicing. *J. Mol. Biol.*, **263**, 163-180.

Herskowitz, I., Rine, J. and Strathern, J. (1992) Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*, pp. 583-656. In E. W. Jones, J. R. Pringle and J. R. Broach (eds.) *The molecular and cellular biology of the yeast Saccharomyces: Gene expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hirata, R., Ohsumi, Y., Nakano, A., Kawasaki, H., Suzuki, K. and Anraku, Y. (1990) Molecular structure of a gene, *VMA1*, encoding the catalytic subunit of H^+-translocating adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 6726-6733.

Mutations at the putative junction sites of the yeast Vma1 protein, the catalytic subunit of the vacuolar membrane H^+-ATPase, inhibit its processing by protein splicing. *Biochem. Biophys. Res. Commun.*, **188**, 40-47.

Hodges, R. A., Perler, F. B., Noren, C. J. and Jack, W. E. (1992) Protein splicing removes intervening sequences in an archaea DNA polymerase. *Nucleic Acids Res.*, **20**, 6153-6157.

Hurst, L. D., Atlan, A. and Bengtsson, B. O. (1996) Genetic conflicts. *Q. Rev. Biol.*, **71**, 317-364.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163-168.

Jones, J. S. and Prakash, L. (1990) Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast*, **6**, 363-366.

Kaiser, C., Michaelis, S. and Mitchell, A. (1994) *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kane, P. M., Yamashiro, C. T., Wolczyk, D. F., Neff, N., Goebl, M. and Stevens, T. H. (1990) Protein splicing converts the yeast *TFP1* gene product to the 69-kD subunit of the vacuolar H^+-adenosine triphosphatase. *Science*, **250**, 651-657.

Kawasaki, M., Nogami, S., Satow, Y., Ohya, Y. and Anraku, Y. (1997) Identification of three core regions essential for protein splicing of the Yeast Vma1 protozyme. A random mutagenesis study of the entire *VMA1*-derived endonuclease sequence. *J. Biol. Chem.*, **272**, 15668-15674.

Keeney, S., Giroux, C. N. and Kleckner, N. (1997) Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of widely conserved protein family. *Cell*, **88**, 375-384.

Kusano, K., Naito, T., Handa, N. and Kobayashi, I. (1995) Restriction-modification systems as genomic parasites in competition for specific sequences. *Proc. Natl. Acad. Sci. USA*, **92**, 11095-11099.

Lambowitz, A. M. and Belfort, M. (1993) Introns as mobile genetic elements. *Annu. Rev. Biochem.*, **62**, 587-622.

Malone, R. E. and Esposito, R. E. (1980) The *RAD52* gene is required for homothallic interconversion of mating types and spontaneous mitotic recombination in yeast. *Proc. Natl. Acad. Sci. USA*, **77**, 503-507.

Morishima, N., Nakagawa, K., Yamamoto, E. and Shibata, T. (1990) A subunit of yeast site-specific endonuclease *SceI* is a mitochondrial version of the 70-kDa heat shock protein. *J. Biol. Chem.*, **265**, 15189-15197.

Naito, T., Kusano, K. and Kobayashi, I. (1995) Selfish behavior of restriction-modification systems. *Science*, **267**, 897-899.

Nakagawa, K., Morishima, N. and Shibata, T. (1991) A maturase-like subunit of the sequence-specific endonuclease endo.*Sce*I from yeast mitochondria. *J. Biol. Chem.*, **266**, 1977-1984.

Nogami, S., Satow, Y., Ohya, Y. and Anraku, Y. (1997) Probing novel elements for protein splicing in the Yeast Vma1 protozyme: a study of replacement mutagenesis and intragenic suppression. *Genetics*, **147**, 73-85.

Ohta, K., Shibata, T. and Nicolas, A. (1994) Changes in chromatin structure at recombination initiation sites during yeast meiosis. *EMBO J.*, **13**, 5754-5763.

Ohya, Y., Umemoto, N., Tanida, I., Ohta, A., Iida, H. and Anraku, Y. (1991) Calcium-sensitive *cls* mutants of *Saccharomyces cerevisiae* showing a Pet$^-$ phenotype are ascribable to defects of vacuolar membrane H^+-ATPase activity. *J. Biol. Chem.*, **266**, 13971-13977.

Perler, F. B. (1998) Protein splicing of inteins and Hedgehog autoproteiolysis: structure, function, and evolution. *Cell*, **92**, 1-4.

Perler, F. B., Davis, E. O., Dean, G. E., Gimble, F. S., Jack, W. E., Neff, N., Noren, C. J., Thorner, J. and Belfort, M. (1994) Protein splicing elements: inteins and exteins—a definition of terms and recommended nomenclature. *Nucleic Acids Res.*, **22**, 1125-1127.

Perler, F. B., Olsen, G. J. and Adam, E. (1997) Compilation and analysis of intein sequences. *Nucleic Acids Res.*, **25**, 1087-1093.

Rudin, N., Sugarman, E. and Haber, J. (1989) Genetic and physical analysis of double-strand break repair and recombination in *Saccharomyces cerevisiae*. *Genetics*, **122**, 519-534.

Shinohara, A. and Ogawa, T. (1995) Homologous recombination and the roles of double strand breaks. *Trends Biochem. Sci.*, **20**, 387-391.

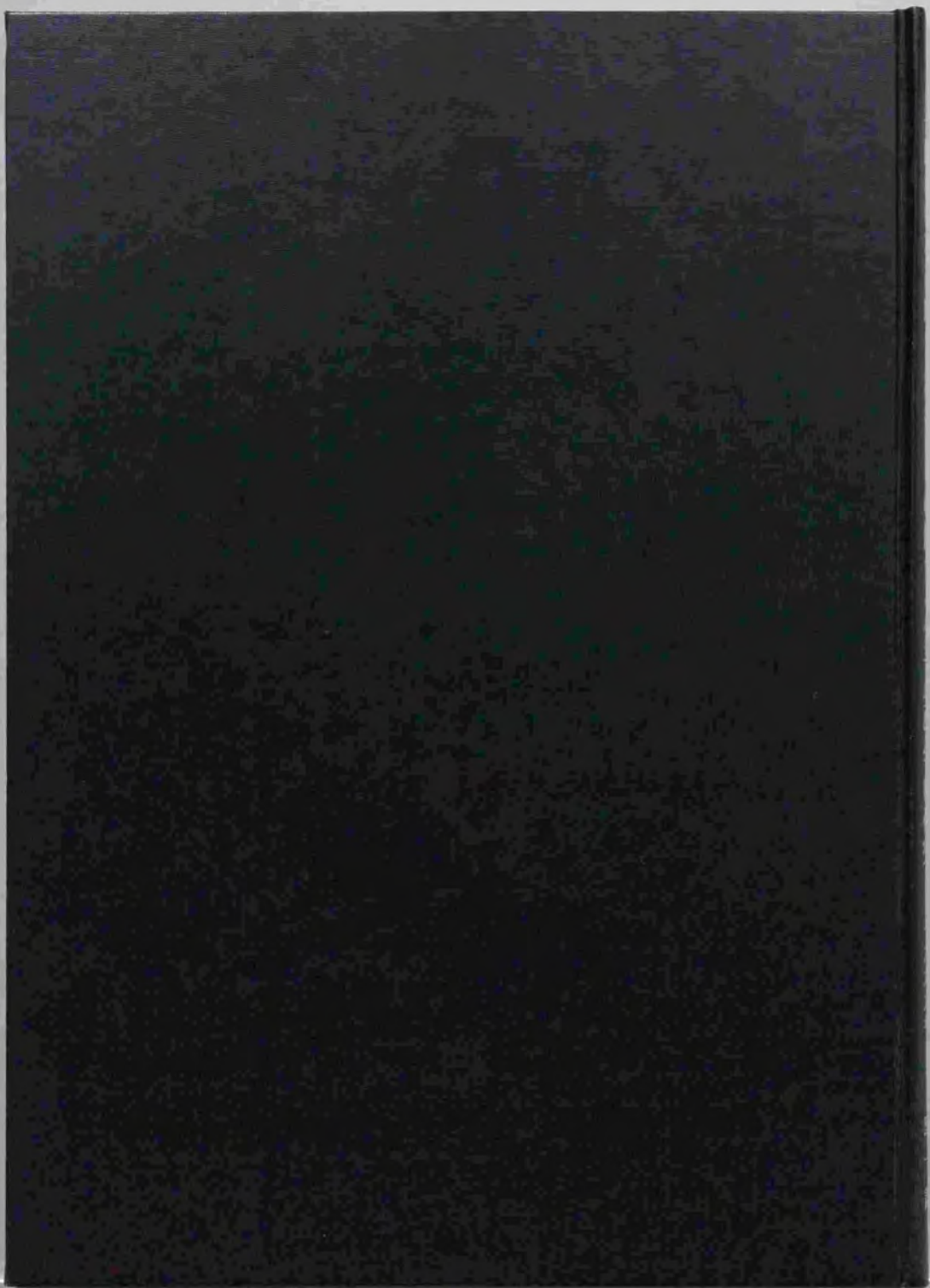
Sikorski, R. S. and Boeke, J. D. (1991) In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Methods Enzymol.*, **194**, 302-318.

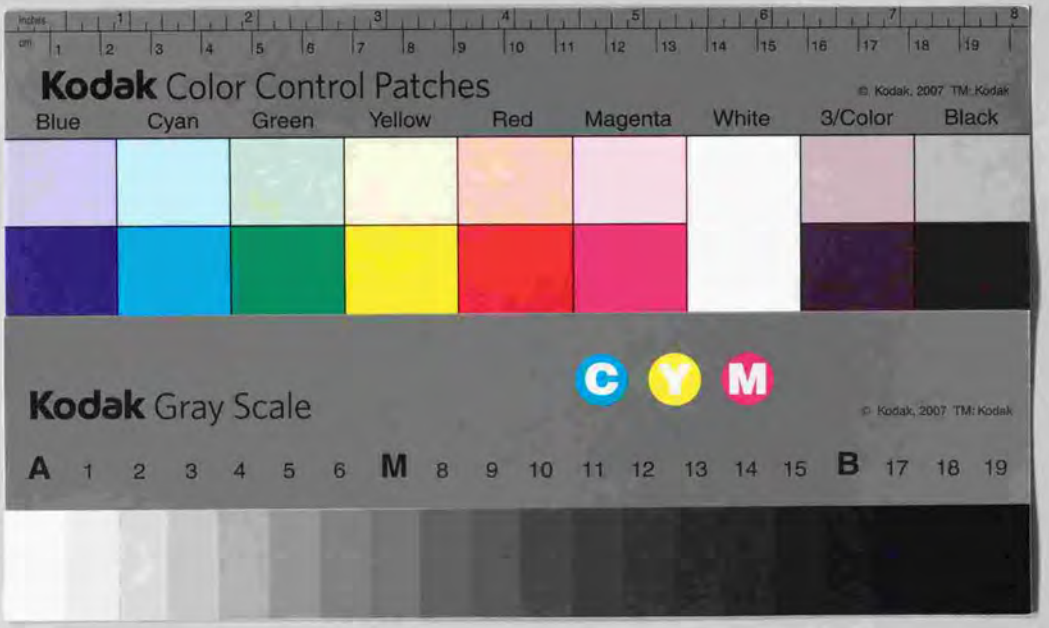
Sikorski, R. S. and Hieter, P. (1989) A system of shuttle vector and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19-27.

Strathern, J. N. and Higgins, D. R. (1991) Recovery of plasmids from yeast into *Escherichia coli*: shuttle vectors. *Methods Enzymol.*, **194**, 319-329.

Tsukamoto, Y. and Ikeda, H. (1998) Double-strand break repair mediated by DNA end-joining. *Genes to Cells*, **3**, 135-144.

Wu, T.-C. and Lichten, M. (1994) Meiosis-induced double-strand break sites determined by yeast chromatin structure. *Science*, **263**, 515-517.




Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19