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**An essential gene *ESR1*
which is
required for both
DNA repair and meiotic recombination
in
*Saccharomyces cerevisiae***

by

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May, 1992

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Abstract

A new mutant was isolated in *Saccharomyces cerevisiae* which is sensitive to both methyl-methane sulfonate (MMS) and ultra-violet light (UV), and defective in meiotic recombination. The gene was cloned by complementation of MMS sensitivity of the mutant. This gene, *ESR1*, is found to be essential for cell growth since the deleted haploid strain is lethal. The *ESR1* gene is adjacent to the *CKS1* gene on chromosome II, and encodes a putative 2368 amino acids protein with molecular weight 273K. Transcripts of the *ESR1* gene are induced by DNA damage and in meiosis. Among viable mutants isolated in the *ESR1* gene, *esr1-2* mutant does not undergo cytokinesis at restrictive temperature, and shows reduced level of meiotic recombination than that at permissive temperature. These results suggest that the *ESR1* gene has a common function in mitosis and meiosis.

Introduction

During meiosis, a single round of DNA replication is followed by two rounds of chromosome segregation (meiosis I and II), and four haploid progenies are formed. Meiosis II is similar to a mitotic division. However, meiosis I is unique and it involves homologous chromosome pairing, synaptonemal complex formation and chromosome segregation. These events are related to meiotic recombination occurs at a very high level in meiosis I (von Wettstein et al. 1984; Roeder 1990; Kleckner et al. 1991).

To analyze meiotic recombination, many genes which are required for meiotic recombination have been isolated in *Saccharomyces cerevisiae*, and they are classified into two groups (recently reviewed in Petes et al. 1991). One group consists of the genes affecting meiotic recombination alone (*HOP1*:Hollingsworth and Byers 1989; *MER1*:Engbrecht and Roeder 1989; *MEI4*:Menees and Roeder 1989; *MRE4(MEK1)*:Leem and Ogawa 1992; Rockmill and Roeder 1991; *REC102,104,107,114*:Malone et al. 1991; *RED1*:Rockmill and Roeder 1988; *SPO11*:Esposito and Esposito 1969), and the other group consists of the genes that have additional mutant phenotypes in mitosis as well deficiency in meiotic recombination (*CDC40*:Kassir and Simchen 1978; *MRE11*:Ajimura et al. 1992; *RAD6*:Game et al. 1980; *RAD50-57*:Baker et al. 1976; Game et al. 1980; Haynes and Kunz 1981). Mutants in the genes of the latter group show phenotypes in mitosis as follows; deficiency in cell growth, deficiency in DNA repair, or hypo- or hyper- mitotic recombination. Existence of the mutants that show deficiency in both DNA repair and recombination are thought to reflect the presence of recombinational repair system. And the *RAD50-57* genes work on both meiotic recombination and recombinational repair (Friedberg 1988; Petes et al. 1991). Recently, Ajimura et.al. (1992) obtained mutants which showed deficiency in meiotic recombination by directly screened using

disomic haploid. Among them, one mutant (*mre11*) showed deficiency in DNA repair (MMS sensitivity) as additional mitotic phenotype. This result suggests that the genes affecting both meiotic recombination and mitotic recombinational repair may not be saturated.

Thus, I attempted to screen new mutants that are defective in both meiotic recombination and DNA repair using a disomic haploid strain for chromosome III. The original disomic haploid strain was constructed by Roth and Fogel (1971). Although this strain is haploid, both information of *MATa* and *MATα* on the disome allowed it to initiate meiosis and to carry out meiotic recombination. The recombinants are detected by returning the cells to mitotic growth (Esposito and Esposito 1974). The mutagenized cells were screened primarily by deficiency of DNA repair (Prakash and Prakash 1977), and then selected for defect in meiotic recombination ability.

In this manuscript, I will describe one of the mutants obtained that is deficient in meiotic recombination and sensitive to both MMS and UV. The gene was cloned by complementation for MMS sensitivity of the mutant. The deletion mutant of the gene was not viable. Therefore, the gene is essential for cell growth, and I named the gene *ESR1* (an essential gene required for both DNA repair and meiotic recombination). Molecular features of the *ESR1* gene and genetic characters in the *esr1* non-lethal mutants will be shown.

Materials and Methods

Strains and genetic methods:

The genotypes of *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. A strain, 116-7B, which is disomic for only chromosome III, was used for mutants isolation because the strain could enter meiosis and monitor recombination (Roth and Fogel 1971). NK110-3DU is a *ura3* derivative of NK110-3D selected by 5-fluoro-orotic-acid (5-FOA) resistance (Boeke et al. 1984). *rad50-57* and *mre11* mutant strains (not shown in Table 1) used for complementation test are the same as described previously (Ajimura et al. 1992). Yeast standard genetic methods were described by Guthrie and Fink (1991).

E.coli strains used are as follows; DH5 (Sambrook et al. 1989) and MC1066 (Casadaban et al. 1983), for plasmid transformation and plasmid DNA preparation, DM2525 (*mutD5* as JM103) (Maruyama et al. 1983; Kenneth et al. 1984) for mutagenesis of cloned DNA. Mutator ability of DM2525 was tested by generation of rifampicin resistant cells before use.

Media:

All solid media contain 1.5% agar: MYPD (0.3% malt extract, 0.3% yeast extract, 0.5% polypeptone, 1% glucose), MYPL (2% lactate (pH 6.5) instead of glucose in MYPD), ACG (0.5% yeast extract, 1% bacto-peptone, 2% potassium acetate (pH 7.0), 0.67% yeast nitrogen base, with amino acid supplement), SD (2% glucose, 0.67% yeast nitrogen base, with amino acid supplement), SPM-plate (0.25% yeast extract, 0.1% glucose, 2% potassium acetate (pH 7.0), with amino acid supplement). Other media containing a drug were already described (canavanine, cycloheximide and 5-FOA were in Rose et.al (1990), MMS was in Prakash and Prakash (1977); Ajimura et al.

(1992)). Liquid media used here were; YPD (1% yeast extract, 2% bacto-peptone, 2% glucose), YPA (2% potassium acetate (pH 7.0) instead of glucose in YPD), SD (same as SD in solid media), SPM (0.3% potassium acetate, 0.03% raffinose). In this manuscript, a synthetic medium lacking a particular amino acid was noticed SD-(amino acid), for example; SD-ura shows a medium lacking uracil.

Mutant isolation:

Logarithmically growing cells of a wild type strain, 116-7B, were harvested, then washed and re-suspended in 50mM Tris-Malate (TM) buffer (pH7.0) at 5×10^7 cells/ml. The cells were exposed to 40 μ g/ml of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for 60 min at 30°C. After washing in TM buffer twice and then in YPD once, the cells were re-suspended in YPD at 2×10^7 cells/ml, and grown to stationary phase by shaking for 48 hours. The cells were plated onto MYPD, and colonies grown on MYPD were replicated to MYPD and MYPD containing 0.04% MMS. After incubation of the plates for four days, I compared to the plates each other, and selected MMS sensitive candidates. The candidates were tested again for their MMS sensitivity by streaking on MYPD containing 0.01% MMS and were streaked to ACG to exclude respiration deficient cells. The MMS sensitive mutants were incubated on SPM-plate for four days, and then streaked to SD-leu and SD-his. After incubation, mutants that is defective in meiotic recombination yielded less than 100 prototroph per streak on these plates, while a wild type strain, 116-7B, yielded about 1,000 prototroph per streak. The candidates were tested again for their reduce in meiotic recombination frequency as follows. Fresh single colonies of the candidates were cultured in YPD to saturation, and the cells were diluted 1:100 in YPA and then grown to approximately 2×10^7 cells/ml. The cells were harvested, washed and re-suspended in SPM at 5×10^7 cells/ml. After 2 days incubation, the cells were plated onto SD-leu and SD-his plates.

The original mutant strains were reconstructed into complete haploid by

chromosome loss using methyl benzimidazole-2-yl-carbamate (MBC) (Wood 1982). The segregants were mated with *rad50-57* and *mre11* mutant strains, and the zygotes were streaked onto MYPD containing 0.01% MMS for complementation test. The mutants which could complement MMS sensitivity of the known mutant strains were checked for their UV sensitivity with exposure to UV at a dose of 40 J/m²

The *MATa* segregants of the mutant candidates were crossed with a wild type strain P7BA-11. The zygotes were sporulated and haploid random spore clones carrying *ura3*, *can1*, *cyh2* and *ade6* were selected. These haploid tester strains were checked for mutant loci by complementation of their MMS sensitivity to original segregants. The *MATα* segregants of each mutant were crossed with a wild type strain N22D-1, and resultant diploids were sporulated and the tetrads were analyzed. The spore clones were tested for their MMS and UV sensitivity, streaking to 0.01% MMS plates and irradiation of UV (20J/m²), respectively. On the other hand, the tetrad clones were mated with either *MATa* or *MATα* tester strains, and resultant zygotes were incubated for four days on SPM-plates, then meiotic recombination was tested streaking on SD-leu, SD-his, SD+canavanine and MYPD+cycloheximide plates.

Measurement of UV or MMS sensitivity:

Fresh single colonies were cultured and grown to stationary phase in YPD. The cells were washed, diluted, plated onto MYPD or SD plates, and irradiated with various dosage of UV for measurement of UV sensitivity. To measure MMS sensitivity, cells were plated onto MYPD or SD plates containing various concentration of MMS.

Observation of meiotic recombination (return-to-growth method):

Cells were grown on MYPD (MYPL in *sk1* or *sk1*-derivative strain). The single colonies were picked and grown to saturation in YPD. The culture was diluted into 1:100 in YPA and then grown for 12 hours to approximately 2×10^7 cells/ml. The cells

were harvested, washed with sterile water, and re-suspended at the same density in SPM. At each time point, cells were plated onto synthetic selective plates to score recombinant prototroph.

Cloning and sub-clonig of the *ESR1* gene:

The *ESR1* gene was cloned by complementation of MMS sensitivity of the *esr1-1* mutant strain. The library used here contains *Sau3A*I partial digested yeast genomic DNA fragments into *Bam*HI site of YCp50 vector (Rose et al. 1987). The mutant strain PK110A-15 was transformed with this library by Lithium methods (Ito et al. 1983), and the transformants were selected on SD-ura plates containing 0.01% MMS. Plasmid DNAs rescued from the URA⁺ and MMS resistant transformants were re-introduced to diploid *esr1-1* strain NR110ABU. Two plasmids (pMK5 and pMK9) could complement both of the MMS sensitivity and deficiency of meiotic recombination. Using restriction sites, I constructed three sub-cloned plasmids which contained over-lapping region of pMK5 and pMK9. These are as follows; pRK900: a *Spe*I-*Spe*I 8.1kb fragment from pMK9 were inserted into *Spe*I site of Ycp50, pRK910: a *Bgl*III-*Bgl*III 7.4kb fragment from pMK9 were inserted into *Bgl*III site of Ycp50, pRK920: pMK9 was digested with *Pvu*II and *Sal*I to remove the left region of insert fragment in pMK9, end filled, and self ligated (see Fig.2A).

DNA sequencing:

A 8.7kb *Sac*II-*Spe*I DNA fragment were separated into five segments and they were sub-cloned into pUC118 and pUC119 phagemids (Sambrook et al. 1989) with both direction; i) *Sac*II-*Spe*I 0.6kb, ii) *Spe*I-*Xba*I 1.5kb, iii) *Xba*I-*Xba*I 3.2kb, iv) *Xba*I-*Kpn*I 1.8kb and v) *Kpn*I-*Spe*I 1.6kb. Serial deletions of those fragments were constructed by *Exo*III-S1 method (Henikoff 1984; Sambrook et al. 1989). Single strand DNAs were prepared from these deletion phagemids, using *E.coli* AK101 (Horii et al. 1992) and

helper phage M13KO7 (Sambrook et al. 1989). Some DNA fragments were sub-cloned onto pBluescript II KS+, and ssDNAs were prepared using *E.coli* dg98 and helper phage VCSM13 (Stratagene). The DNA sequences of both strands were determined by the dideoxy chain termination method (Sanger et al. 1977) using *Taq* DNA polymerase and dye primers (Applied Biosystems) and then analyzed with an ABI DNA sequencer 373A. In some cases, sequence reactions were carried out as a template of double strand DNA by the dideoxy method using Sequenase version 2.0 (U.S.B. Co.) and α -³²P-dATP. Junctions between i) and ii), and iv) and v) of each DNA fragments were determined used another over-lapping sub-clones. Junctions between ii) and iii), and iii) and iv) were sequenced using synthetic oligo-nucleotide primers.

RNA isolation and Northern blot analysis:

RNA was isolated from wild type sk1 strain, NKY1059/1068, using glass-bead method (Carlson and Botstein 1982). Isolation of poly-A⁺ RNA was carried out as described by Sambrook et al.(1989). To isolate RNA from mitotic cells treated with MMS, yeast cells were grown to 5x10⁶ cells/ml and then cultured in the presence of 0.01% MMS. For isolation of RNA from meiotic cells, grown cells in YPA were sporulated as described in a return-to-growth experiment (see above). In both case, the culture was taken at each time point for RNA preparation. Denatured RNA samples were electrophoresed by formaldehyde gel (0.6 % agarose), and then transferred to nylon membrane (NYTRAN, Schleicher & Schuell). After UV fixation, the membrane was probed with DNA fragments labeled with ³²P by the random primer method (Feinberg and Vogelstein 1984). The amount of transcripts was measured as the intensity of bands quantified by FUJIX Bio-image analyzer BAS2000 (Fuji Photo Film Co.,ltd).

Gene disruption:

esr1-d1 was constructed as follows; In a 9.0 kb *BglII-SpeI* fragment of pMK9, a *SpeI-SpeI* region containing all of the *ESR1* gene were replaced with the 2.2kb *Sall-XhoI* *LEU2* fragment from YEp13 (Broach et al. 1979). *esr1-d2* as follows; in a 6.5 kb *SpeI-KpnI* fragment of pRK900, a *XbaI-XbaI* region which was internal of the *ESR1* gene was replaced with a 3.8 kb *BamHI-BglII* fragment containing *hisG::URA3::hisG* isolated from pNKY51 (Alani et al. 1987). And NKY1059/1068 diploid cells were transformed with each DNA fragment (see Fig.3C). The disruption of the *ESR1* gene was confirmed by Southern blot analysis (Southern 1975).

Mutagenesis of cloned DNA fragment using *E.coli mutD*:

A 8.1 kb *SpeI-SpeI* fragment from pRK900 was re-cloned to *SpeI* site of pRS316 (Sikorski and Hieter 1989), and resultant plasmid was named pRK901. It was confirmed that the plasmid, pRK901, had an ability to complement MMS and UV sensitivity and deficiency of meiotic recombination in the *esr1-1* mutant strain NR110ABU. *E.coli* DM2525 was transformed with the pRK901 plasmid and all of the transformants on a plate were collected. The bacterial cells were added to terrific-broth (Sambrook et al. 1989) containing ampicillin, grown over-night to saturation, and then plasmid DNAs were purified. The *ESR1/esr1-d1* diploid, KR1001, were transformed with the mutated plasmids, and the transformants were directly collected from plates and then add to YPA at a cell density of 1×10^6 cells/ml. As described above, cells were sporulated and haploid random spore clones carrying *LEU2* and *URA3* by selecting on SD-leu,ura plates were germinated at 23°C or 34°C. The plates were replicated onto two piece of SD-leu,ura and each plate were incubated at 23°C and 34°C, respectively. The candidates of growth conditional mutants by temperature were streaked onto SD-leu,ura and SD-leu,ura containing 0.01% MMS, and then incubated at both temperature. The plasmid DNAs were recovered from the mutant candidates, and re-introduced to *ESR1/esr1-d1* diploid, KR1001. The transformants were checked for

temperature or MMS sensitivity, to know whether the mutant phenotype was not caused by the mutation in the *ESR1* gene but other region on the plasmid (e.g. *CEN*, *ARS*). The resultant transformants were sporulated and each of random spore clone carrying *LEU2*, *URA3*, *MAT α* and *his4X*, or *LEU2*, *URA3*, *MAT α* and *his4X-ADE2-his4B* was selected. These haploids were mated to examine mitotic and meiotic properties, and the diploids were named KR1003.

Mutant integration:

A 3.8 kb of *URA3* fragment from pNKY51 was inserted into *SpII* site of 8.1kb *SpeI-SpeI* fragment from pRK901 which have *esr1-2* allele, and resultant fragment was inserted to *SpeI* site of pBluescriptKS+. KJ1-1D and KJ2-2A haploid cells were transformed with this plasmid DNA cutted with *SpeI*. The replacement of chromosomal *ESR1* gene with *esr1-2* allele marked with *URA3* was confirmed by Southern blot analysis.

Nucleotide sequence accession number:

The nucleotide sequence data reported in this paper wil appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession number D11088.

Results

Isolation of the mutants defective in both DNA repair and meiotic recombination:

To know the mechanisms of meiotic recombination and DNA repair, and the relationship of them, I tried to isolate new mutants which show deficiencies in meiotic recombination and DNA repair.

A disomic haploid strain, 116-7B, was used for the mutant isolation. As described in Materials and Methods, growing cells were mutagenized with 40 $\mu\text{g/ml}$ of NTG for 60 minutes and plated onto MYPD. The colonies grown on MYPD were replicated to MYPD containing 0.04% of MMS. Prints of wild type strain could grow at this MMS concentration. Among 13,000 mutagenized colonies, about 500 were primary screened as MMS sensitive candidates. Next, these candidates were streaked on plates containing 0.01% MMS, and ACG plates for rho^+ selection. 95 strains that were MMS sensitive and rho^+ were saved.

To screen meiotic recombination-defective mutants among them, these MMS sensitive mutants were examined for their meiotic recombination ability by streak test (Materials and Methods). The candidates which defect in meiotic recombination were inoculated in sporulation medium for 2 days, and then plated on to SD-leu and SD-his plates to measure their meiotic recombination frequencies. In wild type strain, recombination frequency of the *leu2* or *his4* hetero-alleles increases approximately 10^3 -fold compared in meiosis with that in mitotic cell. By this screening, I collected 28 mutant strains that showed no or a reduced meiotic recombination (less than 1/10 of the wild type).

I constructed mutant haploid strains from the original disomic haploid mutant strains (Materials and Methods) to do a complementation test with known MMS

sensitive and meiotic recombination defective mutants. Each mutant haploid was mated with the *rad50-57* and the *mre11* mutant strains (Game et al. 1980; Ajimura et al. 1992), and the obtained zygotes were streaked on a plate containing 0.01% MMS. In 28 mutant strains, the 12 mutants were not complemented with one of the known mutants; they were three *rad50*, two *rad51*, two *rad52*, two *rad55*, one *rad57* and two *mre11* mutants.

The remaining sixteen mutants were checked for their UV sensitivity with exposure to UV at a dose of 40 J/m². Wild type strain, 116-7B, showed 0.14 of survival fraction at this UV dose. The fourteen mutant strains were slightly sensitive to UV (about 5X10⁻² to 2X10⁻¹ of survival at a dose of 40 J/m²), and two mutant strains showed distinct sensitivity of less than 5X10⁻³ survivals at the same dose of UV. Although *rad50-57* mutants show deficiency in both meiotic recombination and DNA repair, they are extremely sensitive to X/γ-ray and MMS, but slightly to UV. Thus, the mutants defective in meiotic recombination and sensitive to both MMS and UV were unique, and I analyzed them in detail.

To examine whether the three phenotypes (MMS sensitivity, UV sensitivity and meiotic recombination deficiency) were caused by a single gene mutation or not, tetrad analysis was carried out for these two mutants. In one mutant, all three phenotypes were segregated separately. Therefore, this mutant has triple mutations in different genes. In the other mutant, three phenotypes were co-segregated with Mendelian type of 2:2 segregation pattern for 17 asci tested. When I assume that three phenotypes are based on mutations in different genes, our results indicate that their genes will be closely located within 3 centi-Morgan on the map distance. Or this result indicates that three phenotypes are based on mutation of a single gene. Further analysis described below shows that these multi-phenotypes were caused by a mutation on a single gene. Therefore, I named the identified gene as the *ESR1* and the mutant as the *esr1-1*.

Mitotic and meiotic phenotypes of the *esr1-1* mutant:

I examined MMS and UV sensitivity of the *esr1-1* mutant strain. While wild type cells were resistant to MMS up to concentration of 0.01%, *esr1-1* cells showed marked sensitivity to MMS (10^{-4} survival at 0.005% MMS). In addition, the *esr1-1* cells were also 2 to 3 times sensitive to UV irradiation compared with the wild type cells (Fig.1). *esr1-1* cells grew slower about 1.7 times than wild type cells at 30°C, however, they could grow at high (34°C) and low (23°C) temperatures. After transferring the *esr1-1* mutant into sporulation medium, pre-meiotic DNA synthesis occurred but very low level of spore formation (2%) was observed (data not shown). In the *esr1-1* mutant, mitotic inter-gene recombination (*can1-ura3* or *ade6-cyh2*) was 30 to 60 times more frequent than in wild type, but intra-gene recombination (*leu2-1/leu2-27*) was the same as in wild type. The frequencies of meiotic inter-gene and intra-gene recombination in the *esr1-1* mutant were reduced to about 1/5 to 1/10 of those in wild type. The increments of the meiotic inter-gene recombination frequencies from those in mitosis were lower than those in intra-gene frequency (Table 2).

Cloning and structure of the *ESR1* gene:

The *ESR1* gene was cloned from yeast genomic DNA library by complementation of the MMS sensitivity of the *esr1-1* mutant. Two plasmids, pMK5 and pMK9, were isolated as candidates which carried 13 Kb (pMK5) and 11.6 Kb (pMK9) insertion in the YCp50 vector, respectively, and they had about 9 Kb over-lapping region on their restriction maps. To localize the *ESR1* gene on the cloned DNA fragment, several plasmids containing a part of the over-lapping region were constructed. As shown in Fig.2, the plasmid pRK900 had the smallest fragment (*SacII-SpeI*, 8.7 Kb) containing an ability to complement both deficiency in DNA repair and meiotic recombination.

As described in Materials and Methods, the nucleotide sequence of a *SacII-SpeI*

8.7 Kb DNA fragment was determined on both strands (Fig.3). Three ORFs are found in the fragment (Fig.2B and 3). A truncated ORF was found at the *SacII* end of the fragment. It consists of 119 amino acid residues and lacks N-terminal protein. Compared the nucleotide sequence of the truncated ORF with other sequences in the data bases, Gen-Bank and EMBL, this ORF is the same as the C-terminal protein of the Cks1 protein (Hadwigwer et al. 1989). The second ORF is long which encode a putative 273,397 Da protein consisting of 2368 amino acid residues. The third ORF is composed of 179 amino acids with a putative 20,453 Da protein. A *BglII-KpnI* 7.4 kb fragment (pRK910) which lacks C-terminal of the second ORF could not complement MMS sensitivity of the *esr1-1* mutant. A pRK920, removed from *PvuII* site to left end of insert fragment in pMK9, which contains entire of the third ORF and the N-terminal-truncated second ORF could not also complement (Fig.2A). The results of the complementation tests of the sub-cloned fragments indicated that the *ESR1* gene encodes the second large ORF.

The Esr1 protein contains 41.3% nonpolar, 32.8% polar, 11.5% acidic and 14.4% basic residues. Predicted isoelectric point is 8.28. There are two hydrophobic regions which contain nonpolar amino acids more than 75%; "VSALALLLEYNPFLVM" at a position 74-90 amino acids, and "IKFLIWVINDILVPAFW" at a position 1153-1169 amino acids. A hydrophilic region was found at 847-879 as "NFEKDKRHGSKYKNINNWTDDQEAFQKKLQDN". However, I could not find any significant homologies by comparing the *ESR1* amino acid sequence with the sequences in the NBRF-PIR and SWISS-PROT databases.

As described above, nucleotide sequence analysis revealed that the *ESR1* gene is adjacent to the *CKS1* gene mapped between *CDC28* and *LYS2* on right arm of chromosome II (Hadwigwer et al. 1989). To confirm this result, yeast chromosomes were separated with CHEF-DRII (Bio-Rad Inc.) and analyzed by Southern blot hybridization. The result showed that the *ESR1* gene was located on chromosome II

(data not shown). The *esr1-1* strain, NK110A-3D, was mated with a *lys2* strain, IFO10092, and the resultant diploid were sporulated and the spores were dissected. Tetrad analysis show that the *ESR1* gene was located at about 12 cM distance from *LYS2* (13 parental ditypes[PD], 0 nonparental ditypes[NPD], and 4 tetratypes[T]).

Increase of *ESR1* transcripts in meiosis and after MMS treatment in mitosis:

Several genes inducible in meiosis carry a URS sequence in their 5' flanking region (Buckingham et al. 1990). In this gene *ESR1*, a URS-like sequence is found at a position 623 to 632, 90 base pair upstream from the start codon of the second ORF. To examine the expression of the *ESR1* gene in meiosis, total RNA was isolated from wild type cells at various time points after transfer of the cells into meiosis, and analyzed by Northern blotting analysis as described in Materials and Methods. Fig.4 shows that the *ESR1* gene is transcribed at a low level in mitosis, but in meiosis, the transcripts increased and the amount reached a maximum 20-fold of the mitotic level at 6 hours after transfer to sporulation medium, and then reduced gradually.

Expression of some DNA repair genes are also induced when the DNA is damaged by radiations, alkylating agents and blocking DNA synthesis. To test whether the expression of the *ESR1* gene is induced by DNA damage, wild type logarithmic growing cells were exposed to a final concentration of 0.01% MMS, and total RNA was isolated at various time points. The *ESR1* transcripts were identified by Northern blotting analysis. Measurement of the transcripts showed that the *ESR1* transcript was induced 2.5-fold at 4 hours incubation after the addition of MMS (Fig.4B). No mitotic regulatory sequence was found as; *MluI* site required for cell cycle regulated expression (Pizzagalli et al. 1988), and DRS sequence required for expression by DNA damage (Cole and Mortimer 1989). The length of the *ESR1* transcript was estimated as about 8.0 Kb compared with single strand RNA size marker (Fig.4A). This length is

sufficient to encode a 273 kDa protein.

The *ESR1* gene is essential for cell growth:

To examine the phenotypes of the *esr1* null mutants, I constructed two different deletion alleles; *esr1-d1*, in which the entire coding region was replaced by *LEU2*, and *esr1-d2*, in which a middle part of 1/3 of the ORF was deleted and replaced by *URA3* (Fig.2C). Dissection of 142 tetrads following sporulation of the *ESR1/esr1-d1* diploid, showed that two spores were viable but the other two were inviable in 138 asci (Fig.5B). The remaining 4 asci produced no or one viable spores. Among the 138 asci, 27 asci were picked out randomly and their genetic markers of viable spore clones were examined. They were all *Leu*⁻ and did not show MMS sensitivity. In the another strain *ESR1/esr1-d2*, the similar results were obtained; two were viable but remained two were inviable in a tetrad. All viable spores were *Ura*⁻ and MMS resistant.

These results suggest that the *ESR1* gene is essential for cell growth or spore germination or both. To distinguish these possibilities, *ESR1/esr1-d1* diploid was transformed with pRK901 plasmid containing *ARS*, *CEN*, *URA3* and the *ESR1* gene, and tetrad analysis were carried out. In every cases, three or four spores were viable (Fig.5C), and every *Leu*⁺ spore clone carrying the deletion *esr1-d1* mutation, contained the *URA3* marker (that is a pRK901 plasmid). Even after the *Leu*⁺ *Ura*⁺ spore clones were cultured for several generations in a non-selective medium, YPD, no *Leu*⁺ *Ura*⁻ segregant could be selected on 5-FOA medium. Thus, the *ESR1* gene is likely to be essential for cell growth. To examine spore germination ability of the *esr1* deletion mutant, the hetero-zygous diploid strain, *ESR1/esr1-d1*, was sporulated and after dissection of the tetrad, and the spore generation was observed under microscopy. Majority (66%) of the inviable spores which can not form colonies on growth medium, had undergone two cell divisions, 18% one division, and 13% three or four divisions (Table 3). This indicates that almost all (96%) *esr1* deletion spores can germinate but

have a defect after germination. Staining of the cells germinated from the spores with DNA specific fluorescent dye 4',6'-diamidino 2-phenylindole (DAPI) revealed that some cells lost nuclear DNA (Fig.6).

Conditional *esr1* mutants:

As described above, a homologous diploid strain for the *esr1-1* mutation is viable but defective in DNA repair and meiotic recombination, while the *esr1-d1* deletion haploid strain can not grow mitotically. These results suggest that the *ESR1* gene may have multiple functions involving in cell growth, DNA repair and meiotic recombination. To analyze these gene functions, *esr1* point mutants conferring temperature sensitive growth and/or MMS sensitive phenotypes were isolated. The method used for mutant screening was shown in Fig.7. The *ESR1* gene cloned on a shuttle vector was mutagenized through in *E.coli mutD* strain. The plasmids recovered from the *mutD* cells were introduced into *ESR1/esr1-d1* diploid strain. After sporulation of the transformants, random spore clones that had the plasmid and the *esr1-d1* mutation, were selected and the mutants were screened for temperature sensitivity in growth and for MMS sensitivity. Among total 27,000 colonies examined, eight mutants were isolated as candidates. I named those mutants *esr1-2* to *esr1-9*.

Mitotic and meiotic phenotypes of these mutants were examined using homozygous *esr1-d1* deletion diploid strain, KR1003, harboring pRK901 plasmid which carried wild type or mutant *ESR1* gene. The strains which have the mutant *esr1* gene were examined for their temperature sensitivity in mitotic growth and survival at 0.01% concentration of MMS (Table 4). From these mitotic phenotypes, the mutants were classified into three groups; i) temperature sensitive growth and slightly sensitive to MMS (*esr1-2,3*), ii) distinctly sensitive to MMS (*esr1-4,5,6*) but not show temperature sensitivity, iii) temperature sensitive growth and distinctly sensitive to MMS (*esr1-7,8,9*).

To measure meiotic recombination, I used a recombination system carrying

directly repeated *his4X* and *his4B* heteroalleles separated by an *ADE2* gene on one chromosome and a single *his4X* allele on the other chromosome (Bishop et al. 1992). Yeast cells grown in permissive condition were transferred into sporulation medium, divided and inoculated at 23°C or 34°C, and then cells were returned to growth in mitosis at 23°C to measure meiotic recombination. All of the mutants except *esr1-6* showed approximately the same meiotic recombination frequency as the wild type and were able to sporulate at both low and high temperature (Table 5). In the mutant, *esr1-6*, however, no spore formation and decreased level of meiotic recombination (about 1/40 fold lower than wild type) was observed at both temperature. Among His⁺ recombinants obtained from this construct, those which retain the central marker (His⁺Ade⁺) are thought to arise by gene conversion, whereas those which have lost a central marker (His⁺Ade⁻) are thought to arise by different (pop-out) mechanism. As shown in Table 5, all *esr1* mutants included the *esr1-6* mutant were showed approximately the same rates in each type of recombination.

***esr1-2* point mutant is temperature sensitive in both cell growth and meiotic recombination:**

To examine properties of the temperature sensitive growth mutant, the *esr1-2* mutation was introduced at chromosomal location in a wild type strain, KJ1-1D and KJ2-2A, by targeted integration. A homozygous diploid strain, KR1102, was constructed by mating the haploid strains which carried chromosomal *esr1-2* mutation. Using this strain, KR1102, the cell morphological change was observed at high temperature. The temperature of the cells growing in SD medium was shifted from 23°C to 37°C, and after incubation of the cells for 12 hours at 37°C, cell morphology was observed by microscopy. As shown in Fig.8, the *esr1-2* mutant developed multiple elongated buds that attached to the parent cell and became multi-nucleate at high temperature (37°C). To know whether the arrested cells are viable or not when return to permissive

temperature, after incubation the mutant cells at 37°C for 48 hours the temperature was shifted to 23°C. After incubation of the cells for 3 days at 23°C, no formation of colonies were observed. The result indicates that the growth temperature sensitivity of the *esr1-2* mutant is irreversible.

I measured mitotic and meiotic recombination in *esr1-2* mutant strain, KR1102, at permissive (23°C) and restrictive temperature (34°C). Since wild type yeast cells can not sporulate at 37°C, I examined the sporulation of the cells at semi-restrictive temperature (34°C). In mitosis, inter-gene recombination (*ade6-cyh2*) was more frequent in the mutant than in wild type, but intra-gene recombination (*his4-4/his4-290*) was the same as in wild type. Although previously showed above, no reduced frequencies of inter-chromosomal "pop-out" recombination was observed in meiosis (Table 5), both of intra- and inter- gene recombination was induced about 1/3 fold of wild type at permissive temperature but the frequency was reduced 1/30 fold of wild type at restrictive temperature in the *esr1-2* mutant (Table 6). The frequency of spore formation in the *esr1-2* mutant strain, was reduced to 1/10 fold at high temperature (Table 7). Spores formed at permissive temperature could form colonies at a low frequency (11.8%) and 63% of asci had no viable spore.

Discussion

I isolated new genes which mutants were defective in both DNA repair and meiotic recombination to know their mechanisms and the relationships. One of the genes termed *ESR1* was cloned and sequenced. From the facts that the *ESR1* gene is mapped adjacent to *CKS1* gene on chromosome II, and the nucleotide sequence, the *ESR1* gene is a new gene which is not isolated previously. This gene is essential in mitotic cell growth since disruption of the gene conferred lethality upon a haploid cell. Viable conditional mutants of the *ESR1* gene were isolated, and they showed various levels of deficiency in mitosis and meiosis. These results suggest the *ESR1* gene is multi-functional through mitosis and meiosis.

Expression of the *ESR1* gene:

About 8.0 kb *ESR1* transcript was detected both in mitosis and meiosis. The length of these transcripts is large enough to encode a 273 kDa protein. There are no intron splicing consensus sequences (Teem et al. 1984), suggesting that the transcripts are not spliced.

Amount of the transcript increases about 20-fold at 6 hours after entry to meiosis. An URS (Upstream Repressor Sequence) consensus sequence, TAGCCGCC^G/CA (Buckingham et al. 1990; Leem and Ogawa 1992), which is conserved among genes inducible in meiosis, is found at 90 bp upstream to the first ATG. The *ESR1* gene is probably regulated by this sequence in meiosis.

In mitosis, when cells are exposed with 0.01% of MMS, amount of the *ESR1* transcript increases about 2.5-fold. Among the genes involved in DNA repair, three genes, *RAD2*, *51* and *54*, have been shown to be induced by DNA-damaging agent (Madura and Prakash 1986; Robinson et al. 1986; Cole et al. 1987; Cole and Mortimer

1989; Shinohara et al. 1992). Comparing the 5' flanking region of the *ESR1* gene with upstream nucleotide sequences of the *RAD2,51* and *54* genes, I can not find any specific homology to the genes. Thus, DNA damage inducible transcription of the *ESR1* gene is likely to be regulated by different system from the regulation of the *RAD2,51* and *54* genes.

The role of the *ESR1* gene in mitosis and in meiosis:

The *ESR1* gene plays an essential role in mitotic growth because disruption of the gene makes cells lethal. Many mitotic essential genes have already been isolated, including the genes of DNA- and RNA- polymerases. The size of the main subunits of DNA- and RNA- polymerase are very large similar to that of the Esr1 protein. But no consensus sequence for DNA- and RNA- polymerases (Allison et al. 1985; Ito and Braithwaite 1991), is found in the amino acid sequence of the Esr1 protein. Furthermore, terminal morphology of the mutant cells deficient in DNA replication at a restrictive condition is dumbbell-shaped with nucleus at the isthmus (Pringle and Hartwell 1981), while the cells germinated from the spores carrying the *esr1* null mutation stalled in their growth mainly at four cells stage with irregular distribution of nuclear DNA. Therefore, it is unlikely that the *ESR1* gene encode a DNA- nor RNA- polymerase. In other essential *CDC* genes, many protein kinases were found. But no consensus sequence for protein kinases (Hanks et al. 1988) is found in the Esr1 protein.

Terminal morphology of the *esr1-2* mutant is similar to those of the *cdc3,10,11* and *12* mutants, which shown to be blocked in cytokinesis step in the cell cycle in a restrictive condition (Hartwell 1971). However, the *CDC3,10* and *11* genes (*CDC12* was not examined) are not essential for meiosis and spore formation (Simchen 1974). On the other hand, the *ESR1* gene is necessary in meiosis as well as in mitosis. Thus, the function of the *ESR1* gene is different from that of those *CDC* genes. But the *RAD9*

gene, whose mutant is defective in DNA repair, is required for G₂ arrest in cell cycle when DNA is damaged (Schiestl et al. 1989). It suggests that the mitotic cell cycle and DNA repair systems are coupled. The *ESR1* gene might work to connect the two systems but not like the *RAD9* gene, because the *ESR1* gene is essential but the *RAD9* gene is not.

Some of the mutants in DNA repair genes and *CDC* genes, show defect in sporulation and/or meiotic recombination (Pringle and Hartwell 1981; Haynes and Kunz 1981). This fact indicate that mitosis and meiosis share common mechanism to drive a cell cycle. And the *ESR1* gene might be a member of them, because the *esr1* mutant showed multiple phenotypes; defect in cell growth and DNA repair in mitosis; and defect in recombination in meiosis. The linked temperature dependency of mitotic growth and meiotic recombination in *esr1-2* mutant, might reflect that the *ESR1* gene has a common function in mitosis and meiosis.

***esr1* mutants show hyper crossing-over but normal gene conversion in mitosis:**

In the *esr1-1* and *esr1-2* mutants, although the mitotic spontaneous level of intra-gene recombination (gene conversion) is the same as that in wild type, the frequency of inter-gene recombination (crossing-over) is 10 to 50 fold higher than wild type. Increase of spontaneous recombination in mitosis is observed in various radiation sensitive mutants; *rad3,6,18,50*, *mms9,13,21*, and *mre11*, in cell cycle mutants; *cdc2,5,6,8,9,13,14* and *17*, and in many other mutants (Montelone et al. 1988; Petes et al. 1991; Ajimura et al. 1992). However, they show hyper-rec phenotype in both gene conversion and crossing-over. Only *rad1* and *rad4* mutants show a normal level of gene conversion but elevated level of crossing-over (Montelone et al. 1988) as the *esr1-1* and *esr1-2* mutants. These results suggest that gene conversion and crossing-over may be separable processes in mitosis. In *esr1* mutant, meiotic recombination is

reduced in both gene conversion and crossing over. And *rad1* and *rad4* mutants show normal level of meiotic recombination. The simplest interpretation of these facts is that gene conversion and crossing-over might be mechanistically connected in meiosis.

***esr1-2* mutant shows different frequency of inter- and intra-chromosomal recombination in meiosis:**

In meiosis, the *esr1-2* mutant is defective in inter-chromosomal recombination, while it shows the same frequency of intra-chromosomal "pop-out" recombination between adjacent directly repeated sequences as in wild type. In *dmc1* mutant, reduced level of meiotic recombination was observed only in inter-chromosomal recombination but not in intra-chromosomal recombination (Bishop et al. 1992). This phenotype of the *dmc1* mutant is similar to that of *esr1-2*. These results could be interpreted as follows. Each inter- and intra- chromosomal recombination system might have independent recombination mechanisms. The *ESR1* gene might play a role in both meiotic mechanisms, as the other mutant, *esr1-6*, shows defect in intra-chromosomal recombination. Alternatively, inter- and intra- gene recombination system might share a common mechanism. In *esr1* and *dmc1* mutants, meiotic recombination might be arrested at the same points due to the lack of each gene function. When the cells are returned to growth from meiosis to mitosis, the recombination intermediates might be converted into recombinants by intra-chromosomal system but not by inter-chromosomal system. The difference of recombination frequency between inter- and intra- chromosomal system may reflect by physical distance of alleles in mitosis; in intra-chromosomal system, two alleles lie very close to one another on the same molecule, but in inter-chromosomal system, two alleles lie on two different molecules.

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References

Ajimura,M.,S.-H.Leem,and H.Ogawa. 1992. Identifivation of new genes (*MRE*) required for meiotic recombination in *Saccharomyces cerevisiae*. Genetics: submitted

Alani,E.,L.Cao,and N.Kleckner. 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics 116: 541-545

Allison L.A.,M.Mole,M.shales,and J.Ingles. 1985. Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell 42: 599-610

Baker,B.S.,A.T.C.Carpenter,M.S.Esposito,R.E.Esposito,and L.Sandler. 1976. The genetic control of meiosis. Annu.Rev.Genet. 10: 53-134

Bishop,D.K.,D.Park,L.Xu,and N.Kleckner. 1992. *DMC1*: a meiosis-specific yeast homolog of bacterial *recA* required for meiotic recombination, synaptonemal complex and cell cycle progression. Cell: in press

Boeke,J.,F.LaCroute,and G.R.Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. Mol.Gen.Genet. 197: 345-346

Broach,J.R.,J.N.Strathern,and J.B.Hicks. 1979. Transformation in yeast: development of a hybrid cloning vectar and isolation of the *CAN1* gene. Gene 8:121-133

- Buckingham, L.E., H.-T. Wang, Z.T. Elder, R.M. McCarroll, M.R. Slater, and R.E. Esposito. 1990. Nucleotide sequence and promoter analysis of *SPO13*, a meiosis-specific gene of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 87: 9406-9410
- Carlson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell* 28: 145-154
- Casadaban, M.J., A. Martinez-Arias, S.K. Shapira, and J. Chou. 1983. β -Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. In *Methods Enzymol.* 100: pp.293-308
- Cole, G.M., D. Schild, S.T. Lovett, and R.K. Mortimer. 1987. Regulation of *RAD54* and *RAD52-lacZ* gene fusions in *Saccharomyces cerevisiae* in response to DNA damage. *Mol. Cell. Biol.* 7: 1078-1084
- Cole, G.M., and R.K. Mortimer. 1989. Failure to induce a DNA repair gene, *RAD54*, in *Saccharomyces cerevisiae* does not affect DNA repair or recombination phenotypes. *Mol. Cell. Biol.* 9: 3314-3322
- Engerbrecht, J., and G.S. Roeder. 1989. Yeast *mer1* mutants display reduced levels of meiotic recombination. *Genetics* 121: 237-247
- Esposito, R.E., and M.S. Esposito. 1969. The genetic control of sporulation in *Saccharomyces*. I. The isolation of temperature-sensitive sporulation-deficient mutants. *Genetics* 61: 79-89

Esposito,R.E.,and M.S.Esposito. 1974. Genetic recombination and commitment to meiosis in *Saccharomyces*. Proc.Natl.Acad.Sci.USA 71: 3172-3176

Friedberg,E.C. 1988. Deoxyribonucleic acid repair in yeast *Saccharomyces cerevisiae*. Micro.Rev. 52: 70-102

Feinberg,A.P.,and B.Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal.Biochem. 137: 266-267

Game,J.C.,T.J.Zamb,R.J.Braun,M.A.Resnick,and R.M.Roth. 1980. The role of radiation (*rad*) genes in meiotic recombination in yeast. Genetics 94: 51-68

Guthrie,C.,and G.R.Fink. eds. 1991. Guide to yeast genetics and molecular biology. Methods Enzmol. 194

Hadwiger,J.A.,C.Wittenberg,M.D.Mendenhall,and S.I.Reed. 1989. The *Saccharomyces cerevisiae* *CKS1* gene, a homolog of the *Schizosaccharomyces pombe* *suc1⁺* gene, encode a subunit of the Cdc28 protein kinase complex. Mol.Cel.Biol. 9: 2034-2041

Hanks,S.K.,A.M.Quinn,and T.Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241: 42-52

Hartwell,L.H. 1971. Genetic control of the cell division cycle in yeast, IV. Genes controlling bud emergence and cytokinesis. Exp.Cell.Res. 69: 265-276

Haynes,R.,and B.,Kunz. 1981. DNA repair and mutagenesis in yeast. In the Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance, J.Strathern,

E.W.Jones, and J.Broach eds. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, pp.371-414

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene*. 28: 351-359

Hollingworth, N.M., and B.Byers. 1989. *HOP1*: a yeast meiotic pairing gene. *Genetics* 96: 589-611

Horii T., N.Ozawa, T.Ogawa, and H.Ogawa. 1992. Inhibitory effects of N- and C-terminal truncated *Escherichia coli recA* gene products on functions of the wild-type *recA* gene. *J.Mol.Biol.* 223: 105-114

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

Ito J., and D.K.Braithwaite. 1991. Compilation and alignment of DNA polymerase sequences. *Nuc.Aci.Res.* 19: 4045-4057

Kassir, Y., and G.Simchen 1978. Meiotic recombination and DNA synthesis in a new cell cycle mutant of *Saccharomyces cerevisiae*. *Genetics* 90: 49-68

Kennith, F., J.W.Little, and D.W.Mount. 1984. Rapid mutational analysis of regulatory loci in *Escherichia coli* K-12 using bacteriophage M13. *Proc.Natl.Acad.Sci.USA* 81: 3801-3805

Kleckner, N., R.Padmores, and D.K.Bishop. 1991. Meiotic chromosome metabolism: one

coherent view. Cold Spring Harbor Symp.Quant.Biol. 56: in press.

Leem,S.-H.,and H.Ogawa. 1992. The *MRE4* gene encodes a novel protein kinase homologue required for meiotic recombination in *Saccharomyces cerevisiae*. Nuc.Aci.Res. 20: 449-457

Madura,K.,and S.Prakash. Nucleotide sequence, transcript mapping, and regulatio of the *RAD2* gene of *Saccharomyces cerevisiae*. J.Bacteriol. 166: 914-923

Maruyama,M.,T.Horiuchi,H.Maki,and M.Sekiguchi. 1983. A dominant (*mutD5*) and recessive (*dnaQ49*) mutator of *Escherichia coli*. J.Mol.Biol. 167: 757-771

Menees,T.T.,and G.S.Roeder. 1989. *MEI4*: a yeast genes required for meiotic recombination. Genetics 123: 675-682

Malone,R.E.,S.Bullard,M.Hermiston,R.Rieger,M.Cool,and A.Galbraith. 1991. Isolation of mutants defective in early steps of meiotic recombination in the yeast *Saccharomyces cerevisiae*. Genetics 128: 79-88

Montelone,B.A.,M.H.Hoekstra,and R.E.Malone. 1988. Spontaneous mitotic recombination in yeast: Hyper-recombinational *rem1* mutations are alleles of the *RAD3* gene. Genetics 119: 289-301

Petes,T.D.,R.E.Malone,and L.S.Symington. 1991. Recombination in yeast. In the Molecular Biology of the Yeast *Saccharomyces*: Genome Dynamics, Protein Synthesis, and Energetics, J.R.Broach,J.R.Pringle,and E.W.Jones eds. Cold Spring Harbor,New York:Cold Spring Harbor Laboratory Press, pp.407-521

Pizzagalli,A.,P.Valsasnini,P.Plevani,and G.Lucchini. 1988. DNA polymerase I gene of *Saccharomyces cerevisiae*: nucleotide sequence, mapping of a temperature-sensitive mutation, and protein homology with other DNA polymerases. *Proc.Natl.Acad.Sci.USA*

Prakash,L.,and S.Prakash. 1977. Isolation and characterization of MMS-sensitive mutants of *Saccharomyces cerevisiae*. *Genetics* 86: 33-55

Pringle,J.R.,and L.H.Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. In *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, J.Strathern, E.W.Jones, and J.Broach eds. Cold Spring Harbor,New York:Cold Spring Harbor Laboratory, pp.97-142

Robinson,G.W.,C.M.Nicolet,D.Kalainov,and E.C.Friedberg. 1986. A yeast excision-repair gene is inducible by DNA damaging agents. *Proc.Natl.Acad.Sci.USA* 83: 1842-1846

Rockmill,B.,and G.S.Roeder. 1988. *RED1*: a yeast gene required for the segregation of chromosomes during the reductional division of meiosis. *Proc.Natl.Acad.Sci.USA* 85: 6057-6061

Rockmill,B.,and G.S.Roeder. 1991. A meiosis-specific protein kinase homolog required for chromosome synapsis and recombination. *Genes Dev.* 1991: 2392-2404

Roeder,G.S. 1990. Chromosome synapsis and genetic recombination: their roles in meiotic chromosome segregation. *Trends Genet.* 6: 385-389.

Rose,M.D.,P.Novick,J.H.Thomas,D.Botstein,and G.R.Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on centromere-containing shuttle vector. Gene 60: 237-243

Rose,M.D.,F.Winston,and P.Hieter. 1990. Methods in yeast genetics, a laboratory course manual. Cold Spring Harbor,New York:Cold Spring Harbor Laboratory Press.

Roth,R.,and S.Fogel. 1971. A system selective for yeast mutants deficient in meiotic recombination. Mol.Gen.Gent. 112: 295-305

Sambrook,J.,E.F.Fritsch,and T.Manias. 1989. Molecular cloning, a laboratory manual (2nd ed.). Cold Spring Harbor,New York:Cold Spring Harbor Laboratory Press.

Sanger,F.,S.Nicklen,and A.R.Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc.Natl.Acad.Sci.USA. 74: 5463-5467

Schiestl,R.H.,P.Reynolds,S.Prakash,and L.Prakash. 1989. Cloning and sequence analysis of the *Saccharomyces cerevisiae* *RAD9* gene and further evidence that its product is required for cell cycle arrest induced by DNA damage. Mol.Cel.Biol. 9: 1882-1896

Shinohara,A.,H.Ogawa,and T.Ogawa. 1992. Rad51 protein involved in repair and recombination in *Saccharomyces cerevisiae* is a RecA-like protein. Cell: in press

Simchen,G. 1974. Are mitotic functions required in meiosis? Genetics 76: 745-753

Sikorski,R.S.,and P.Hieter. 1989. A system of shuttle vectors and yeast host strains

designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19-27

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J.Mol.Biol. 98: 503-517

Teem, J.L., N.Abovich, N.F.Kaufer, W.F.Schwindinger, J.R.Warner, A.Levy, J.Woolford, R.J.Leer, M.M.C.van Raamsdonk-Duin, W.H.Mager, R.J.Planta, L.Schultz, J.D.Frieson, H.Fried, and M.Rosbash. 1984. A comparison of yeast ribosomal protein gene DNA sequences. Nucl.Aci.Res. 12: 8295-8312

von Wettstein D., S.W.Rasmussen, and P.B.Holm. 1984. The synaptonemal complex in genetic segregation. Ann.Rev.Genet. 18: 331-413

Wood, J.S. 1982. Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae*. Mol.Cel.Biol. 2: 1064-1079

Table Legends

Table 1.

(a): These strains were derived from the strain sk1, and (b): these strains are hybrids, not pure sk1. (c): This strain was obtained from Institute for Fermentation, Osaka.

Table 2.

The recombination frequency was measured by return-to-growth experiments. Mitotic recombination was measured at 0 hour after transfer of the cells into sporulation medium, and meiotic recombination at 48 hours. Relative recombination frequencies were showed as 1.0 at mitotic levels in the parentheses.

Table 3.

Tetrads of KR1001 were prepared as described in Fig.5 legend. The dissected spores were germinated for 48 hours, and they were observed by microscopy. Each of spores were classified by their of cell numbers; observation of one cell shows no cell division, two cells one division, three or four cells two divisions, five to eight cells three divisions, and nine to sixteen cells four divisions. In the parentheses, the percentage of the spore clones in the total were showed.

Table 4.

All experiments were studied in KR1003 background. Survival at 34°C was calculated as colony forming unit (cfu) at 34°C versus cfu at 23°C. *esr1-5* mutant showed slightly cold sensitivity. MMS sensitivity was showed as cell survival fraction at 0.01% MMS.

Table 5.

All experiments were studied in KR1003 background. Yeast cells cultured in permissive condition were transferred to sporulation medium, divided into two and incubated at 23°C or 34°C. After 9 hours, cells were plated onto plates to measure recombination frequency, and the plates were incubated at permissive temperature. Spore formation was observed 48 hours after transfer to sporulation medium.

Table 6.

Yeast cells cultured in permissive condition were transferred to sporulation medium, divided into two and incubated each at 23°C and 34°C. Mitotic recombination frequency was measured at 0 hours after transfer to sporulation medium, and meiotic frequency at 36 hours. Because the *esr1-2* mutant strain can not grow at 34°C, mitotic recombination can not be measured at 34°C. Relative recombination frequencies were showed in the parentheses as 1.0 of mitotic levels.

Table 7.

Yeast cells prepared as described in table 6 legend were sporulated for 48 hours at both temperature, and resultant spores were counted and tetrad analysis was carried out. The dissected tetrads were germinated at a permissive condition in each case.

given below the nucleotide sequence. Numbers to the left and right count nucleotide and amino acid residues, respectively. A promoter consensus sequence (TATA) at nucleotide 529, and the putative regulatory sequence (URS), TACCCGGCGA, at nucleotide 623 are both indicated by underline.

Fig. 4.

Northern blot analysis of *ESR1* RNA.

(A) 10 µg of poly(A)⁺ RNA isolated from mitotic logarithmically growing cells was probed with DNA fragment containing the *ESR1* gene. Numbers on the left indicate the size in kb of single stranded RNA standards (GIBCO BRL) stained with ethidium bromide.

(B) RNA was isolated from wild type yeast cells at various time points after addition of MMS (final concentration was 0.01%). 20µg of total RNA was electrophoresed, analyzed by Northern blot and intensity of bands was measured and plotted.

(C) RNA was isolated from yeast cells after transfer into sporulation medium at each time point. 20µg of total RNA was electrophoresed, and analyzed by Northern blot and intensity of bands was measured and plotted.

Fig. 5.

Tetrad analysis of diploids heterozygous at the *esr1* locus shows the *ESR1* gene is essential. In all cases, fresh colonies grown on MYPL were streaked on SPM-plate and sporulated for four days at 30°C, and then the tetrad were dissected.

(A) Wild type diploid, NKY1059/1068. Almost four viable spores are observed.

(B) *ESR1/esr1-d1* diploid, KR1001. Only two spores grew from each tetrad.

(C) KR1001 which harbors a plasmid carrying *ESR1*. Three or four viable spores could grow.

Fig. 6.

Spores lacking the *ESR1* gene almost undergo one or two cell divisions. The tetrads of the *esr1* hetero-disruptant strain, KR1001, was prepared as described in Fig.5 legend. After germination the dissected spores for 24 hours at 30°C, the spores were stained with DAPI and observed by microscopy. **A** and **C** were viewed by light optics, and **B** and **D** were by fluorescent optics. Upper panells (**A** and **B**) are showed the same sample, and lower panells (**C** and **D**) are also the same.

Fig. 7.

Scheme for generation and screening of point mutants in the *ESR1* gene. Details are described in Materials and Methods.

Fig. 8.

Terminal morphology of the *esr1-2* mutant. **A** and **B** show wild type strain, KJC101, after incubation at 37°C for 12 hours. **C** and **D** show the *esr1-2* mutant strain, KR1102, growing at 23°C. **E** and **F** show the KR1102 cells after 12 hours incubation at 37°C shifted from 23°C. The *esr1-2* cells can not undergo cytokinesis and arrest with elongated buds having multi-nuclei in it at restrictive temperature. The left paneles (**A**, **C** and **E**) show the light microscopy pictures, and the right paneles (**B**, **D** and **F**) show the fluorecence pictures.

Table 1. Genotypes of Yeast Strains

Strain	Genotype	Source
116-7B	<u>MATa leu2-1 his4-4 trp1 met2 ade2</u> <u>MATa leu2-27 his4-290</u>	M. Ajimura
R110	116-7B derivative with <i>esr1-1</i>	This work
P7BA-11	<u>MATa leu2-1 his4-4 can1 ura3 cyh2 ade6 ade2</u>	M. Ajimura
N22D-1	<u>MATa leu2-27 his4-290 trp1 met2 ade2</u>	M. Ajimura
P7BAB	diploid with P7BA-11 and N22D-1	M. Ajimura
PK110A-15	<u>MATa leu2-1 his4 can1 ura3 cyh2 ade6 ade2 esr1-1</u>	This work
NK110A-3D	<u>MATa leu2-27 his4 trp1 met2 ade2 esr1-1</u>	This work
NK110A-3DU	NK110A-3D derivative with <i>ura3</i>	This work
NR110AB	diploid with PK110A-15 and NK110A-3D	This work
NR110ABU	diploid with PK110A-15 and NK110A-3DU	This work
IFO10092	<u>MATa ade1 cdc19 gal1 his7 leu2 lys2 met8 pet9 tyr1</u>	This work
NKY1059/1068 (a)	<u>MATa ho::L YS2 lys2 ura3 leu2::hisG ade2::LK his4X</u> <u>MATa ho::L YS2 lys2 ura3 lue2::hisG ade2::LK his4X-ADE2-his4B</u>	(c) N. Kleckner
KR1001	(a) NKY1059/1068 derivative with <i>ESR1/esr1-d1::LEU2</i> hetero-disruptant	This work
KR1002	(a) NKY1059/1068 derivative with <i>ESR1/esr1-d2::URA3</i> hetero-disruptant	This work
KR1003	(a) NKY1059/1068 derivative with <i>esr1-d1::LEU2/esr1-d1::LEU2</i> harboring pRK901 which carries <i>ESR1</i> gene	This work
KJ1-1D	(b) <u>MATa ho::L YS2 leu2 his4-4 trp1 ura3 ade2 lys2</u>	K. Jozuka
KJ2-2A	(b) <u>MATa ho::L YS2 leu2 his4-290 trp1 ura3 ade2 lys2 ade6 cyh2 can1</u>	K. Jozuka
KJC101	(b) diploid with KJ1-1D and KJ2-2A	K. Jozuka
KR1102	(b) KJC101 derivative with <i>esr1-2::URA3/esr1-2::URA3</i>	This work

Table 3. Numbers of Cell Divisions in Lethal Spores

Numbers of Cell Division	Scored Spores	(%)
0	6	(4.0)
1	27	(18)
2	99	(66)
3	13	(8.7)
4	5	(3.3)
> 4	0	(0.0)
Total	150	(100)

Table 4. Mitotic Phenotypes of Conditional *esr1* Mutants

	Survival at 34°C (%)	Survival at 0.01% of MMS (%)
wild type	100	91
<i>esr1-2</i>	2.1	22
<i>esr1-3</i>	3.7	39
<i>esr1-4</i>	260	0.54
<i>esr1-5</i>	39	0.04
<i>esr1-6</i>	71	0.76
<i>esr1-7</i>	3.7	0.54
<i>esr1-8</i>	7	6.4
<i>esr1-9</i>	12	2.8

Table 5. Meiotic Phenotypes of Conditional *esr1* Mutants

	Spore Formation (%)		Meiotic Recombination			
	23°C	34°C	Frequency (X 10 ⁻³)	Ratio of ade ⁻ /His ⁺	Frequency (X 10 ⁻³)	Ratio of ade ⁻ /His ⁺
wild type	55	37	49	0.44	31	0.47
<i>esr1-2</i>	27	13	50	0.57	26	0.50
<i>esr1-3</i>	13	0.8	55	0.41	34	0.45
<i>esr1-4</i>	22	42	54	0.39	47	0.54
<i>esr1-5</i>	15	5.7	49	0.44	43	0.40
<i>esr1-6</i>	< 0.1	< 0.1	1.2	0.56	1.0	0.65
<i>esr1-7</i>	12	0.6	25	0.47	24	0.53
<i>esr1-8</i>	10	2.9	34	0.49	25	0.44
<i>esr1-9</i>	9.9	2.6	46	0.44	31	0.48

Table 6. Mitotic and Meiotic Recombination of *esr1-2* Mutant

Strain	Relevant Genotype	Mitotic Recombination		Meiotic Recombination	
		23°c	23°c	23°c	34°c
<i>his4-4/his4-290</i>					
KJC101	wild type	1.4 X 10 ⁻⁴	(1.0)	470 X 10 ⁻⁴	210 X 10 ⁻⁴ (150)
KR1102	<i>esr1-2</i>	1.1 X 10 ⁻⁴	(1.0)	160 X 10 ⁻⁴	6.3 X 10 ⁻⁴ (6)
<i>ade6-cyh2</i>					
KJC101	wild type	2.7 X 10 ⁻⁴	(1.0)	24 X 10 ⁻²	35 X 10 ⁻² (1300)
KR1102	<i>esr1-2</i>	19 X 10 ⁻⁴	(1.0)	6.8 X 10 ⁻²	1.8 X 10 ⁻² (9)

Table 7. Spore Formation and Spore Viability of *esr1-2* Mutant

Temp.	Strain	Relevant Genotype	Spore Formation (%)	Viable Spores / Ascus					Spore Viability (%)
				4	3	2	1	0	
23°C	KJC101	wild type	91.1	44	4	3	0	0	95.1
	KR1102	<i>esr1-2</i>	27.6	0	1	14	40	95	11.8
34°C	KJC101	wild type	70.2	26	4	2	1	0	91.7
	KR1102	<i>esr1-2</i>	2.5	-	-	-	-	-	-

Fig.1.

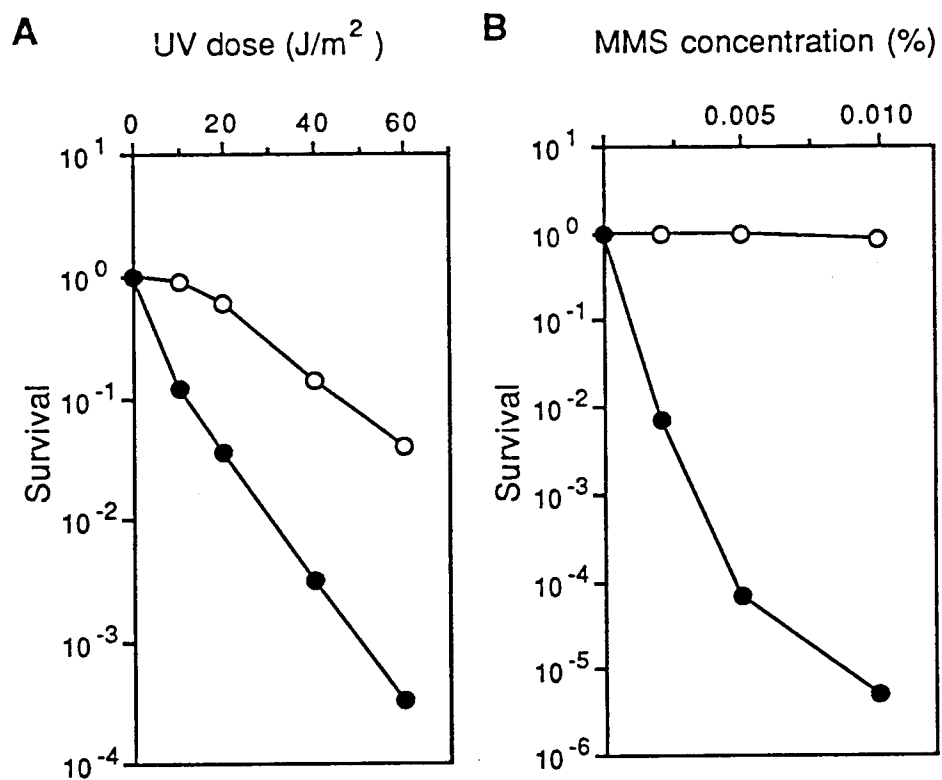
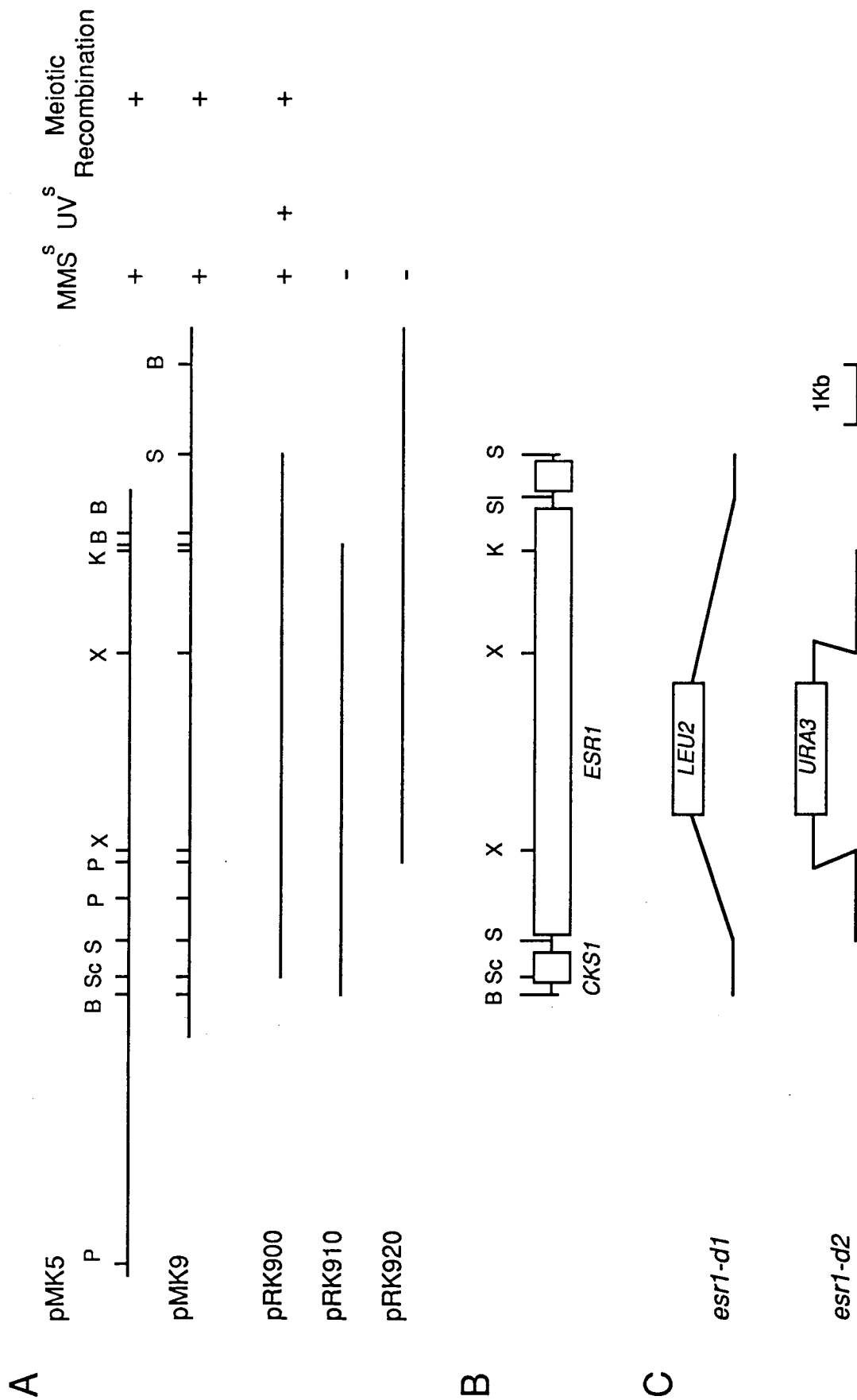


Fig.2



[illegible]

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Fig.4

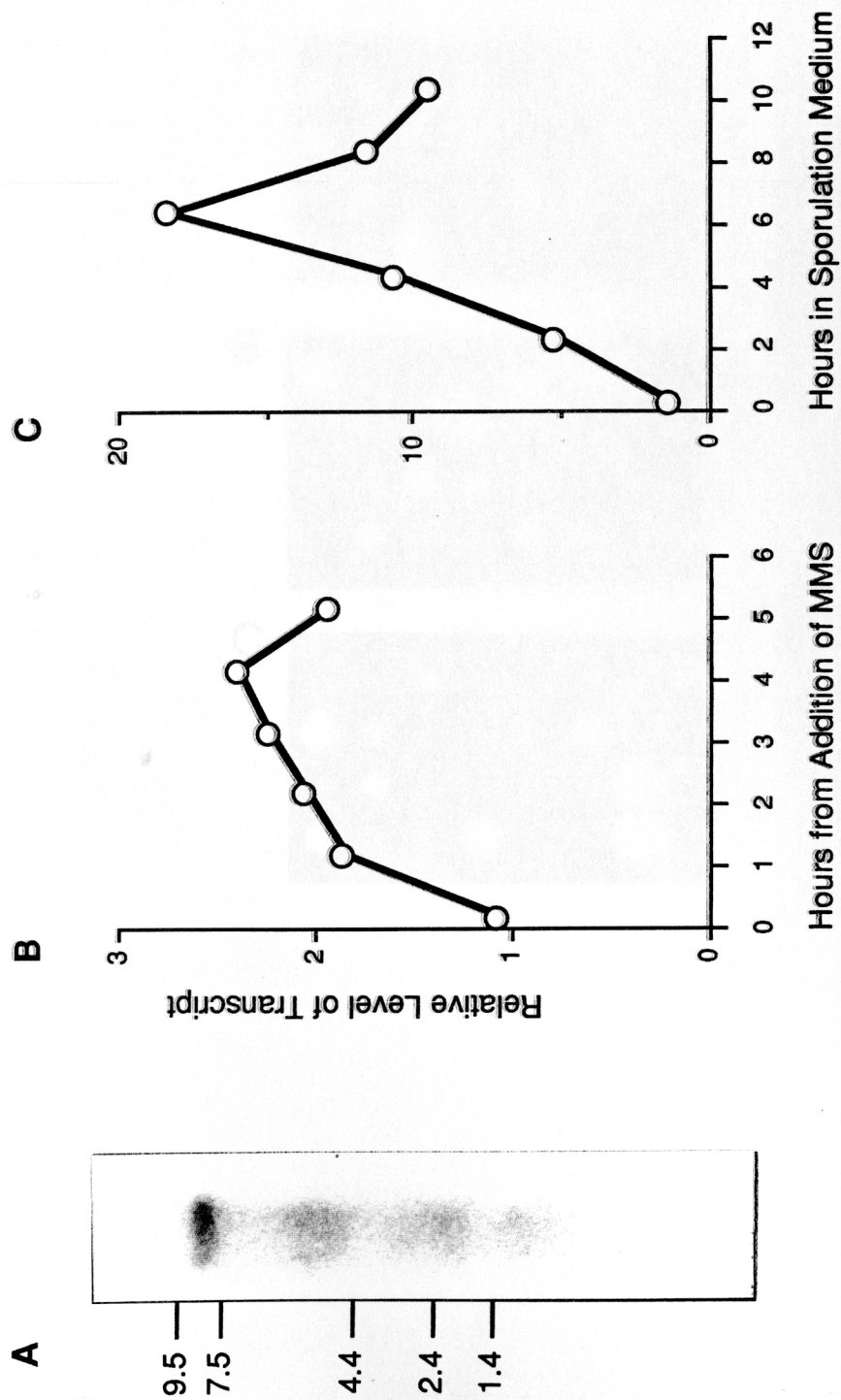


Fig.5

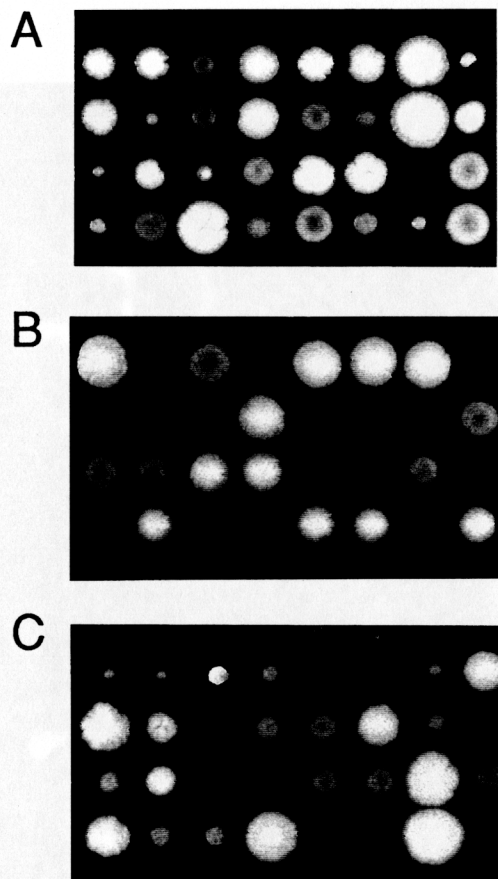


Fig.6

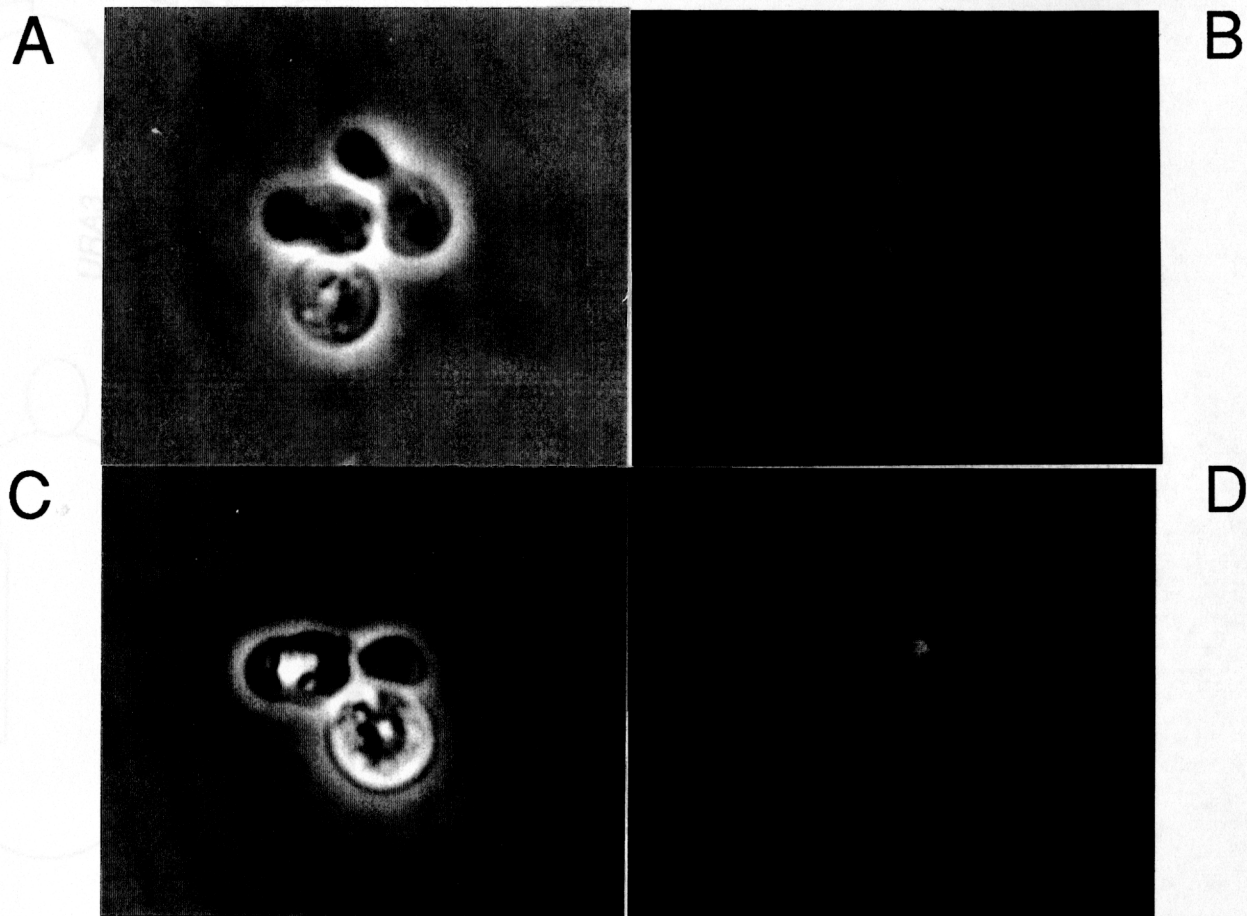


Fig.7

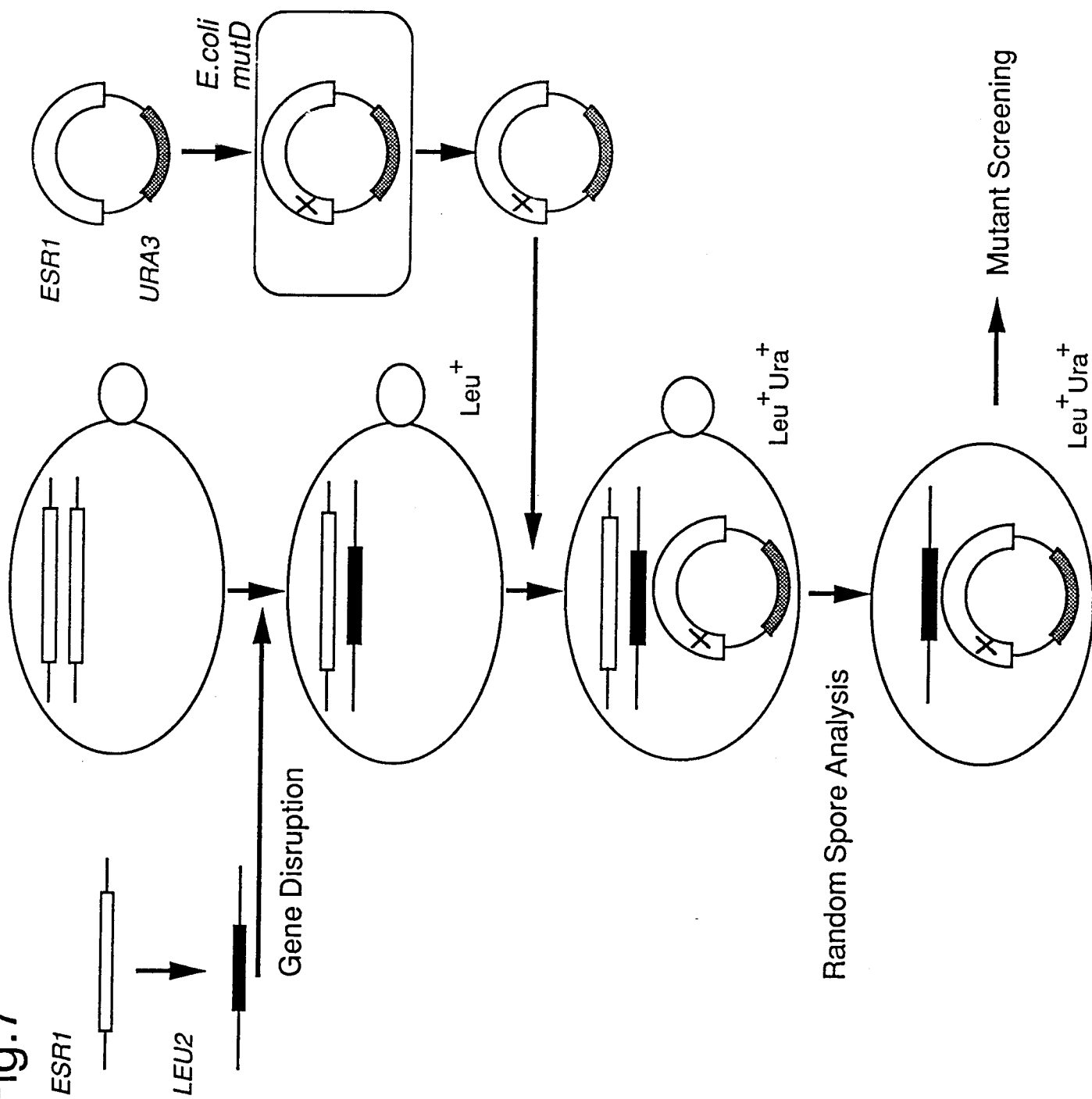


Fig.8

