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Osaka University

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Akira Shinohara

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This thesis is dedicated to my parents and some my friends.

Introduction

Genetic recombination is a process that exchange DNAs, producing new information in DNAs. This process is required for several biological phenomenons. First, recombination can construct new linkage of DNAs on progenitor by reshuffling parental DNAs to creat diversities in genome. This exchnages are observed prominently in meiosis, in which one gamete produces four new progenies with different sets of genes from parentals through two concecutive cell division. In mitosis, some specialized cell carry out recombination to generate diversities in genome. In chickin and rabbitt, B-cells perfoms gene conversion, exchange of materials in one direction, to produce the diversites in V_H regon in antibody genes.

Second, recombination is necessary for a repair of the lesions in DNA. This repair system is specific from other systems; excision repair and error-prone repair, in terms of that it can restore missing information in DNA; gapped DNA., using another homologue in diploids or sister chromatide after replication.

Third, the completion of meiosis requires recombination. In some organisms, crossing-over between homologous chromosomes, which is often observed as chiasma, ensures the chromosome disjunction in meiosis one reductional division. In *Saccharomyces cerevisiae* and *Drosophila melanogaster*, high ratio of non-disjunction of chromosome is observed in recombination dificient mutants.

Our knowledge about genetic recombination has come from studies of recombination in prokayote and funge. Some models have been proposed to account for the phenomeons observed in the above system(Meselson and Radding, 1976; Szostock et al., 1984). Some proteins involved in recombination have also identified in prokayote; RecA protein, RecBCD exonuclese, uvsX protein from T4 phage. However, exact molecular figure of genetic recombination, together with proteins, remains to be solved.

Recombination is known to consist of at least several steps as follows; initiation like single-strand break or double strand break, synapsis which is alignment of homologous chromosome or DNAs with long distance interaction, exchange of materials, repair synthesis of DNAs, branch migration; movement of the junction with extension of the heteroduplex region and maturation of recombinants by disruption of Holliday junction.

In addition to above process, eukaryote bears specific features in recombination. Meiotic recombination accompanies with specific structures. Synaptonemal Complex(SC) is tripartite structure seen in pachytene, which consists of lateral elements and central elements. This structure was considered to be required for recombination, but recent analysis have shown meiotic recombination does not require integral SC structure. At present the role of SC in meiotic recombination remains obscure. Additionally, Recombination Nodule(RM) is another specific structure seen on SC in meiosis. The position of RM often coincides with the position of chiasma, crossing-over point on chromosome, showing that RM plays more direct role in recombination; search for the homology or the determination of the initiation site for recombination or the site for resolution. Furthermore there are two types of RM existed. One emerge at zygotene or early pachytene prior to the formation of full-length SC, while another appears at late pachytene as mentioned above. What relationship between these structure and recombination?

Another issue is about outcome of recombination. Two types of outcome have been known genetically; Crossing-over is a reciprocal exchange of genetic information while gene conversion is a transfer of one donor information to its recipient. These two events is closely interrelated. Some conversion often associate with crossing-over and vice versa. There, however, are mutants which is defective in only one type of recombination in fruit fly and budding yeast; mei218 and mei9 in *Drosophila* is defective in crossing-over but proficient in gene conversion and mer1MER2 double mutant in yeast is deficient in crossing-

over but not in conversion, suggesting that these two events are related but independent processes in some points. If two processes are independent, what is the role of each in recombination?

Saccharomyces cerevisiae, budding yeast, provides us with good opportunity to analyze genetic recombination. This organism is suitable for not only for genetics but for molecular biology, biochemistry and cell cytology compared to other systems like fruit fly. Furthermore there is huge collection of mutants defective in recombination and repair process. Among them *RAD52* epistasis genes; *RAD50*, *51*, *52*, *54*, *55*, *57* and *MRE11* are quite interesting. Mutants defective in genes in this epistasis group are deficient in some feature of recombination as well as repair of double-strand break by ionizing radiation. Recent studies have shown that these genes also work in meiotic recombination. Meiotic recombination is reduced ten to hundred folds in these mutants.

RAD51, *52*, *54* genes are particularly important since these genes are involved in many types of recombination in yeast. Among them *rad52* mutant has been studied extensively, showing it is defective in spontaneous mitotic gene conversion between homologous chromosomes, in mitotic crossing-over between homologs, in radiation induced mitotic recombination between homologs and sister chromatids, in spontaneous mitotic recombination between directly repeated sequences, in meiotic recombination and in mating type switching. For *RAD51* and *RAD54* genes, a few data are available. These analyses suggest that these genes are involved in homology search between DNAs.

In this thesis, I focus on the genes which work in homology search. Irrespective of the extensive studies of prokaryotic systems; RecA and UvsX proteins, molecular mechanism underlying homology search, which is how the homology between DNA are searched and whether proteins called strand-transferase helps

the ability of DNAs to search homology or can look for the sequence, remains to be solved. In eukaryote, even proteins catalyzing this process have not been identified. Some proteins have been purified from yeast, fruit fly, lileis and human, but still are the genetical evidence lacking that these proteins are engaged in recombination or repair.

Analyzing the *RAD51* and *RAD52* gene provides us with oppotunities to acknowledge the mechanism of homologous recombination, especially the search of homology since there are lot of genetical evidences that these genes play important role in recombination. Furthermore, it might promote comprehending the relationship between recombiantion and specific structure like RM, since repair process and the morphology of RM is closely related in *Drosophila*; *mei281* mutant is defective in the repair of thelesion by alkylating agents and bears morphrogically altered RM.

In this thesis, I describe molecular characterization of the *RAD51* and *RAD52* gene and their products as follows. 1), *RAD51* is homologius to RecA gene which is well-known strand transfere in prokayote(Chapte I). This is the first RecA homologue in eukaryote. 2), *RAD51* is ATP-dependent DNA binding protein(Chapter III). 3), *RAD52* protien has DNA pairing activitiy *in vitro*(Chapter II), that is the first strand-transferase in eukayote with genetical proof that this gene is involved in recombination. 4), *RAD52* and *RAD51* can interact directly(Chapter III), spporting the idea that protien complex is crucial in homology search. In Chapter III the relationship between these genes and RM or conversion will be presented.

Chapter I

Structual analysis of the *RAD51* gene

The *RAD51* gene in *S. cerevisiae* encodes a protein homologous to RecA protein.

Summary

The *RAD51* gene in *Saccharomyces cerevisiae* belong to *RAD52* epistasis group, which is involved in many type of recombination in yeast, including repair of double strand break and meiotic recombination. The *RAD51* gene was cloned and sequenced. Sequence analysis revealed *RAD51* gene encoded 43kd protein, which was homologous to RecA protein with 50% homology. This is the first report of the protein homologous to RecA in eukaryote. In meiosis *RAD51* mRNA was induced 20 folds. The time of maximal induction coincided with the time of meiotic recombination. These results suggest that the *RAD51* gene play an important role in homology search between DNA in homologous recombination.

I. Introduction

Genetic recombination is a process required for the repair of the lesion in DNAs and the completion of meiosis and generates the specific diversity of the organisms. The exchange of genetic information in the parental DNAs produces new combinations of genes and the process involves following steps: Initiation by introduction of a single strand nick or a double strand break to DNA, strand invasion with homology search, branch migration and resolution of the recombination intermediates. A few leading models of recombination adopting above steps have been proposed (Meselson and Radding, 1975; Szostak *et al.*, 1981), but the knowledge on the molecular mechanism on the action of recombination enzymes remains to be solved in Eukaryote.

The genes in RAD52 epistasis group of *Saccharomyces cerevisiae*, *RAD50*, *51*, *52*, *54*, *55*, *57* and *MRE11* have been identified and is shown to be involved in genetic recombination and repair (For reviews, Game, 1984 and Resnick, 1988; Ajimura and Ogawa, 1991). The mutants defective in these RAD genes were isolated primarily as X-ray or gamma ray sensitive ones (Game and Mortimer, 1974) and later were shown to be deficient also in genetic recombination (Game *et.al.*, 1980). The *mre11* mutants were isolated as the mutants defective specifically in the meiotic recombination among Methyl Methan Sulfonate (MMS) -sensitive mutants (Ajimura and Ogawa, 1991). This epistasis group can be further divided into three subgroups based on their characteristic properties (see below).

As the first approach to elucidate the molecular mechanism of the process of homology search between DNAs in Eukaryote, we try to analyze the action of the protein of the genes *RAD51*, *52* and *54* belonging in the second subgroup. The structural analysis of *RAD52* gene has been described by Adzuma, et al., (1984). And the biochemical analysis of the protein will be published in accompanying paper (Shinohara, et al., 1991), but little is known about the structures and the action of proteins on *RAD51* and *54* genes. The detail genetic analysis of these two genes

showed that the properties of *rad51* mutant are almost same as those of *rad52* mutant, but some differences were observed in *rad54* mutant (cited in Cole *et.al.*, 1988), suggesting *RAD51* and *RAD52* proteins acts together in the same process of the genetic recombination (Saeki *et al.*, 1980; Morrison and Hastings, 1979; Kuntz *et al.*, 1982).

In this paper, we describe the organization of *RAD51* gene on the analysis of structure by the DNA sequences, the expression of the gene by measuring the amount of the mRNA synthesis and the identification of the gene product. Analysis of its amino acid sequence revealed that the 60% of the region at a side of C-terminus of the protein had homology with the region containing a nucleotide binding domain of *E. coli* *recA* protein (Shinohara and Ogawa, Ogawa and Shinohara and Story and Steize unpublished results). This is the first case that the recombination gene carrying a homologous amino acid sequence to the *recA* gene of *E.coli* was identified in Eukaryote. The mRNA synthesized from the *RAD51* gene was induced to 20 fold in meiosis and also induced to fold by treatment of MMS in mitotic cells.

II. Results

(1) The subgrouping of the genes in *RAD52* epistasis group of *S.cerevisiae* based on their characteristic properties.

The genetic properties of the genes in *RAD52* epistasis group have been studied extensively. We further classified these genes into three subgroups based on their characteristics as shown in Table 1. The genes belonging to the first subgroup are *RAD50* and *MRE11*. The mutants in this subgroup were X-ray or MMS sensitive, and were observed neither formation of synaptonemal complex nor incision of double strand breaks at recombination hot spots in meiotic recombination (Cao *et al.*, 1990; Jozuka *et al.*, unpublished data). Both are thought to be involved in the repair of damaged DNA caused by exposure to X-ray or MMS and in the initiation events of meiotic recombination which is essential for the homology search at chromosome level (Alani *et al.*, 1990; Jozuka *et al.*, unpublished results), and follows to create the initiation signals responsible for reciprocal recombination (Cao, *et al.*, 1990; Jozuka, *et al.*, unpublished results). On the contrary to the deficiencies in meiotic recombination and in the repair of damaged DNA in mitotic state, the mutants are proficient in mitotic recombination.

On the other hand, meiotic recombination is required for formation of viable spores. Therefore, mutants isolated from genes in *RAD52* epistatic groups are unable to form viable spores. However, the inability to form viable spore of *rad50* and *mre11* mutants can be rescued by bypassing Meiosis division I (a reductional division in meiosis) in the presence of *spo13* mutation (Malone and Esposito, 1980), that is, the double mutant with the *spo13* mutation forms viable diad spores (Alani *et al.*, 1990; Ajimura and Ogawa, 1991). One of interpretations of this phenomenon is that the mutants that yield viable spores in the presence of *spo13* mutation are blocked at an early step in the recombination process. In addition to *RAD50* and *MRE11* mutations, the inability to form viable spore of *spo11* mutant can be rescued by the formation of double mutant with *spo13* (Klapholz, *et al.*, 1985). *Spo11* mutants only affect

meiotic recombination (Lovett *et al*, 1987; Kuwahara and Ogawa, in preparation). The inviable spore formation of these mutants is not rescued by the formation of double mutant with *spo13* mutation, but suppressed by the formation of triple mutant with *rad50 spo13* or *spo11 spo13* mutations (Kuwahara, et al., in preparation; Game, personal communication). Thus, the genes in the third group are likely to act in the intermediate steps between or after the first and the second subgroup genes.

(2) Cloning and Localization of the *RAD51* gene.

The *RAD51* gene was cloned from the yeast genomic DNA library carried by a vector YRp7 (Adzuma *et al.*, 1984) as to complement the MMS sensitivity of *rad51-1* mutant. Two independent colonies that endowed the MMS resistance of *rad51-1* mutant among 10^4 *trp*⁺ transformants were selected. The plasmids recovered from these transformants contained 3.7kb BamH1 fragment as a inserted yeast chromosomal DNA. Transformants of *rad51-1*, *trp1* strain with these purified plasmids endowed MMS resistance and had the ability to carry out meiotic recombination. This result strongly suggests that the 3.7 kb BamH1 fragment contains RAD51 gene. A restriction map of the 3.7kb DNA fragment coincided well with the map reported (Calederon,1983). To determine the region that is responsible for MMS resistance of *rad51-1* mutant, the serial deletions of the fragment from both ends were constructed by digestion of 3.7kb fragment with *ExoIII-S1* (Henihoff, 1984) on another shuttle vector, pRS316 (Sikorski and Hieter, 1988). The regions carried by various plasmids and the results on the MMS resistancy on *rad51-1* mutatin carrying each of these plasmids are presented in the Figure 1. The 1.5kb region located at position from 615 to 2152 on the 3.7kb BamH1 fragment confferred complete MMS resistance to the *rad51-1* mutation.

(2) Nucleotide sequence of the 3.7kb BamH1 fragment and structural gene of RAD51.

Using these series of deletions, the DNA sequence of 3.7kb fragment was

Phenotype of *rad* mutants in *S.cerevisiae*

	Mitotic Phenotype			Meiotic Phenotype		
	X-Ray (MMS) Sensitivity	Mitotic Recombination	Mating Type Switching	Meiotic Recombination	Rescue by <i>spo13</i> mutation	
<i>RAD50</i>	Sensitive	Hyper	+	--	+	
<i>MRE11</i>	Sensitive	Hyper	+	--	+	
<i>RAD51</i>	Sensitive	-	-	-	-	
<i>RAD52</i>	Sensitive	-	-	-	-	
<i>RAD54</i>	Sensitive	-	-	-	-	
<i>RAD55</i>	Sensitive	±	+	-	-	
<i>RAD57</i>	Sensitive	±	+	-	-	

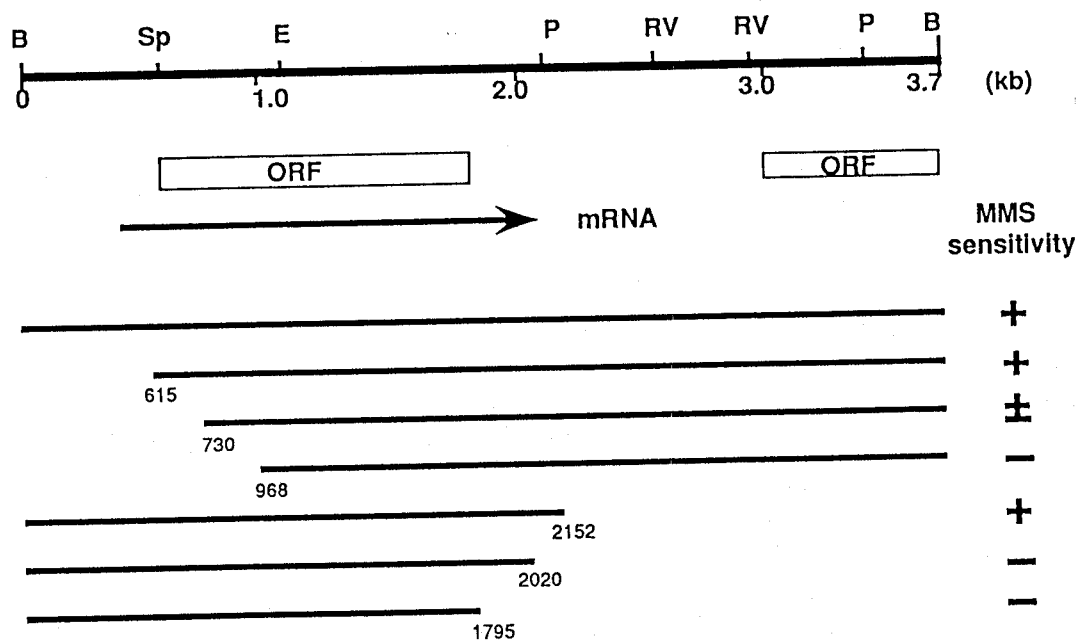


Figure 1. Restriction map of 3.7kb BamHI fragment and complementation test of the deletion fragments.

The upper line shows a restriction map of 3.7kb DNA fragment which complements the defects of *rad51-1* strain. Putative ORFs deduced from sequence analysis (Figure 2) are shown by the white arrow. Solid bars below indicate the deletion fragments on pRS316 vector, used in the completion analysis. The number is the end point of the fragment. On the right hand, the results of the complementation test to check the sensitivity of the XS3672-3C *rad51* strain bearing each deletion plasmid to MMS are shown. "+" and "-" denote resistant to MMS and sensitive to MMS, respectively. "±" indicate the partial sensitive between "+" and "-" phenotype above. The transformants carrying the plasmid were streaked on the MYPD plate containing 0.01% of MMS. After 2 days, the growth of the yeast on the plate was checked.

completely determined on the both DNA strands. A whole DNA sequences is presented in Figure 2. The left end of the upper strand of the 3.7 kb BamH1 fragment is taken as the reference position (position 1).

The analysis of coding frames in the fragment revealed that the only one open reading frame (ORF) that can represent the protein is a leftward frame between two translational termination codons at 644 and 1844. This region coincide well to the region determined by the ability to complement MMS resistance, and contains ORF consisting of 1,200 bases with 400 amino acid residues. The initiation codon located at position 644 and the termination codon at 1848. The predicted molecular weight of the putative RAD51 protein was 42,900. In addition to the ORF, the regions located upstream 30 bps from the initiation codon and downstream 301 bps from the termination codon are essentially required for the ability to endow MMS resistancy of *rad51-1* mutant. The other ORF initiating at position 3029 did not required for the complementation of MMS sensitivity for *rad51-1* mutant, and no termination codon of this frame was found in the 3.7kb fragment.

(3) RAD 51 protein is homologous to *E. coli* RecA protein.

Homology search of the putative ORF in the data base NBRF-PIR and SWISS-Pross. revealed that RAD51 protein is significantly homologous to RecA protein of *E. coli* in the amino acid sequences. Amino acid sequences of RecA proteins were well conserved in ten bacteria species and two cyanobacteria(Ogawa, et al., in preparation). Figure.3 shows a homologous alignment between *E. coli* RecA and RAD51 proteins with permission of small gaps. The homologous region extends over 210 amino acids. Identical amino acids and homologous amino acids in the region were 63 (30%) and 50 (24%), respectively. And it corresponds to the main domain of *E. coli* RecA protein, which is responsible for nucleotide binding, nucleotide hydrolysis and DNA binding (Kawashima, et al., 1984; Ogawa,T. and Shinohara, A. in preparation).

A paper in this issue reports a new gene called *DMC1* in *S. cerevisiae* which is

10 20 30 40 50 60
 5' GGATCCGACA TTTTTTTT ATGCTTTATT CACTGTTCAA TATTTTACC ACAATTCGCA
 70 80 90 100 110 120
 AGAAACGCAC TCTACTTCGA AACTACGGTT CAAACTTACT TAGCAGCTTC CCGATTAAAT
 130 140 150 160 170 180
 TGGCCTTTCT ACTATGCCAT AAACCTTTTC TTCCTCTCTT TTCATCGCCC CTGCATTTCG
 190 200 210 220 230 240
 ACTTTTTTGC CACCGGCAGT GCCATCCGGT CACATGACTA CACCACGTTA ATAGCGATCT
 250 260 270 280 290 300
 GGCTTATCAT TGTCACAGAG TAAATTAATA TGGACGGTAA ATGTGGGAAA TGCACCACTA
 310 320 330 340 350 360
 CCGTTCTTCA ACCAATCTAG TTTAGCTATC CTGCAACAGG TGGCCTTCTT GAGCATTCCC
 370 380 390 400 410 420
 TGAGCATTC AACCGGTTGT ATCAGTGTTC TATCACCGTC TCACCATATC CCACGACTAG
 430 440 450 460 470 480
 GCCACACTTC GTTACCCTAT GCTACGCGTC ATTTCCGCTA TTTCTGTCCT GGTITGTTTA
 490 500 510 520 530 540
 CAGTACGCGT GGTGGGACCA TAAAGGGGAA TAGTGGGGAC TGGAAAAAAA ATTTTCTCAU
 550 560 570 580 590 600
 TTACTTCTTC TATCTTCCGT AGTTCCATA TACTAGTACT TGAGTGTAGC GACAAAGAGC
 610 620 630 640
 AGACGTAGTT ATTTGTAAAA GGCCTACTAA TTTGTTATCG TCAT

653 662 671 680 689 698
 5' ATG TGT CAA GTT CAA GAA CAA CAT ATA TCA GAG TCA CAG CTT CAG TAC GGG AAC
 Met Ser Gln Val Gln Gln His Ile Ser Glu Ser Gln Leu Gln Tyr Gln Asn
 707 716 725 734 743 752
 GGT TCG TTG ATG TCC ACT GTA CCA GCA GAC CTT TCA CAG TCA GTC GTT GAT GGA
 Gly Ser Leu Met Ser Thr Val Pro Ala Asp Leu Ser Gln Ser Val Val Asp Gly
 761 770 779 788 797 806
 AAC GGC AAC GGT AGC AGC GAA GAT ATT GAG GCC ACC AAC GGC TCC GGC GAT GGT
 Asn Gly Asn Gly Ser Ser Glu Asp Ile Glu Ala Thr Asn Gly Ser Gly Asp Gly
 815 824 833 842 851 860
 GCG GGA TTG CAG GAG CAA CCG GAA GCG CAA GGT GAA ATG GAG GAT GAA GCA TAC
 Gly Gly Leu Gln Glu Gln Ala Glu Ala Gln Gly Glu Met Glu Asp Glu Ala Tyr
 869 878 887 896 905 914
 GAT GAA GCT GCC TTA GGT TCG TTT GTG CCA ATA GAA AAA CTG CAA GTG AAC GGG
 Asp Glu Ala Ala Leu Gly Ser Phe Val Pro Ile Glu Lys Leu Gln Val Asn Gly
 923 932 941 950 959 968
 ATT ACT ATG GCG GAT GTG AAA AAA CTA AGG GAG AGT GGG CTT CAC ACT GCT GAA
 Ile Thr Met Ala Asp Val Lys Lys Leu Arg Glu Ser Gly Leu His Thr Ala Glu
 977 986 995 1004 1013 1022
 GCG GTA GCA TAT GCT CCC AGA AAG GAT TTA TTG GAA ATC AAA GGT ATA TCG GAA
 Ala Val Ala Tyr Ala Pro Arg Lys Asp Leu Leu Glu Ile Lys Gly Ile Ser Glu
 1031 1040 1049 1058 1067 1076
 GCT AAG GCA GAT AAG TTG CTA AAC GAA GCG GCA AGG CTA GTG CCT ATG GGA TTT
 Ala Lys Ala Asp Lys Leu Leu Asn Glu Ala Ala Arg Leu Val Pro Met Gly Phe
 1085 1094 1103 1112 1121 1130
 GTC ACG GCT GCT TTT CAT ATG AGA AGA TCG GAG CTG ATT TGT TTG ACA ACG
 Val Thr Ala Ala Asp Phe His Met Arg Arg Ser Glu Leu Ile Cys Leu Thr Thr
 1139 1148 1157 1166 1175 1184
 GGT TCT AAG AAT TTG GAC ACT CTT TTG GGT GGT GTG GAA ACT GGT TCT ATT
 Gly Ser Lys Asn Leu Asp Thr Leu Leu Gly Gly Gly Val Glu Thr Gly Ser Ile
 1193 1202 1211 1220 1229 1238
 ACT GAG CTT TTC GGT GAA TTC AGG ACA GGT AAG TCC CAG CTA TGT CAC ACT TTG
 Thr Glu Leu Phe Gly Glu Phe Arg Thr Gly Lys Ser Gln Leu Cys His Thr Leu
 1247 1256 1265 1274 1283 1292
 GCC GTG ACA TGC CAA ATT CCA TTG GAT ATT GGT GGC GGT GAA GGT AAG TGT TTG
 Ala Val Thr Cys Gln Ile Pro Leu Asp Ile Gly Gly Gly Glu Gly Lys Cys Leu
 1301 1310 1319 1328 1337 1346
 TAT ATC GAT ACC GAA GGT ACT TTC AGG CCG GTA AGA TTG GTA TCC ATA GCT CAG
 Tyr Ile Asp Thr Glu Gly Thr Phe Arg Pro Val Arg Leu Val Ser Ile Ala Gln
 1355 1364 1373 1382 1391 1400
 CCG TTC GGA TTA GAC CCG GAT GAT GCT TTG AAC AAC GTT GCG TAT GCA AGA GCC
 Arg Phe Gly Leu Asp Pro Asp Asp Ala Leu Asn Asn Val Ala Tyr Ala Arg Ala
 1409 1418 1427 1436 1445 1454
 TAT AAC GCC GAT CAT CAG TTA AGA CTT CTG GAT GCT GCT GCC CAA ATG ATG AGC
 Tyr Asn Ala Asp His Gln Leu Arg Leu Leu Asp Ala Ala Ala Gln Met Met Ser
 1463 1472 1481 1490 1499 1508
 GAG TCT CCG TTT TCC TTG ATT GTG GTC GAT TCT GTT ATG GCT CTA TAC CGT ACG
 Glu Ser Arg Phe Ser Leu Ile Val Val Asp Ser Val Met Ala Leu Tyr Arg Thr
 1517 1526 1535 1544 1553 1562
 GAT TTT TCT GGT CBT GGT GAA CTA AGC GCA AGG CAA ATG CAT TTA GCC AAA TTT
 Asp Phe Ser Gly Arg Gly Glu Leu Ser Ala Arg Gln Met His Leu Ala Lys Phe
 1571 1580 1589 1598 1607 1616
 ATG CGT GCT TTG CAA AGG CTG GCC GAC CAA TTT GGT GTT GCA GTC GTC GTT ACT
 Met Arg Ala Leu Gln Arg Leu Ala Asp Gln Phe Gly Val Ala Val Val Val Thr
 1625 1634 1643 1652 1661 1670
 AAC CAA GTG GTC GCC CAA GTT GAT GGT GGT ATG GCT TTT AAT CCA GAT CCA AAG
 Asn Gln Val Val Ala Gln Val Asp Gly Gly Met Ala Phe Asn Pro Asp Pro Lys
 1679 1688 1697 1706 1715 1724
 AAG CCT ATC GGT GGT AAT ATT ATG GCA CAT TCT TCC ACC ACG CGA TTA GGT TTC
 Lys Pro Ile Gly Gly Asn Ile Met Ala His Ser Ser Thr Thr Arg Leu Gly Phe
 1733 1742 1751 1760 1769 1778
 AAA AAG GGT AAG GGA TGT CAA AGA TTA TCC AAA GTT GTT GAC TCA CCT TGC TTA
 Lys Lys Gly Lys Gly Cys Gln Arg Leu Cys Lys Val Val Asp Ser Pro Cys Leu
 1787 1796 1805 1814 1823 1832
 CCA GAG GCT GAA TGT GTG TTC GCG ATC TAT GAA GAT GGT GTT GGT GAC CCC AGA
 Pro Glu Ala Glu Cys Val Phe Ala Ile Tyr Glu Asp Gly Val Gly Asp Pro Arg
 1841
 GAA GAA GAC GAG TAG 3'
 Glu Glu Asp Glu ***

					850	1860
					GTA	TTTGGTCTCT
						1920
1870	1880	1890	1900	1910		GGTTGCGTTC
TGTCTCTATT	TATTTACACA	GGTTTACTTT	CAATTCCTCT	CTTTTCTTA		1980
1930	1940	1950	1960	1970		2040
CGTACATTTT	TATCTTCATT	TCCATCCACT	GCTTAGATT	TTTGCATATA		TAACCTTCGT
1990	2000	2010	2020	2030		2100
TACCTCGCAA	CCCTACTGCG	GTCTTAACCT	TTTTTTCAG	TTCTTTTAAA		2160
2050	2060	2070	2080	2090		2220
ATTGTCTGTC	ACCCATGAAA	TATATGTATT	TTTCTACTCT	TCTTCCCGAT		GACTACTTCC
2110	2120	2130	2140	2150		2280
TCCTCGAGGG	TCCGCGCGCG	TTTATCCTTT	TAGGGGAGTG	AAGAGAAAAA		TTTTCGTATA
2170	2180	2190	2200	2210		2240
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YAGAACAATA	CTTTTGCTAA	CAGCAAGAGA	AGTACGGTTG	ACATTACGAG		TATCCCTTTG
2350	2360	2370	2380	2390		2420
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CCCTGAAATT	GGAGCTTATA	ATGAGGGTGA	TAACAGTTGG	AATCAAGCGG		ATTCTCCCT
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GATTCCTCTC	TGATATTGAA	TAAACCAAAA	GTTTCGTCAAT	ACTGGTCAAT		CGTTTCATCT
2530	2540	2550	2560	2570		2600
CATAITTCAC	GCTCAGGTGA	TGTGTTTACA	AATGATAAAG	AAAAAATTTC		ATCCTCTATT
2590	2600	2610	2620	2630		2660
GATGAGGATG	CGATGGATAT	CGACGCTTGG	CCCTCATTGA	TTGAAAAAAT		AACTCITTT
2650	2660	2670	2680	2690		2720
CCACAAGAAA	GATTCTTCCT	GAGCAAGGAG	AGTTTGAGAA	CGACGTTGAG		GACGATGCTT
2710	2720	2730	2740	2750		2780
CTTCCTCACT	GAAGGAAAAA	TCACAAGGTT	CTTGGAATAT	TGAAATTGCT		TCTGAAATTT
2770	2780	2790	2800	2810		2840
CATCCGAAAT	TTTAAATGGC	ACATCGGCAG	ACGGTAATTC	CGAGTTTCAC		GACTTCGCTG
2830	2840	2850	2860	2870		2900
AGCCCCCTCC	CTCTCAGAAT	GAATCTGTGC	CTCTGTCTTT	TAGTCAATCG		AATGATTTGG
2890	2900	2910	2920	2930		2960
ACTTTTCGAA	TAATCCAAGC	GGATCAGGCT	CTTCAACGAT	ATCAATAGAA		GTACAAGTTC
2950	2960	2970	2980	2990		3020
CATTTCACTT	CCTAGACATG	TGTCACCTAGA	TTTTAACGTT	TACAATAGTC		TTTGCCTCAC
3010	3020	3030	3040	3050		3080
AAATGAGGTG	ACTGCATCAG	AATCACATAA	TGTGGCCAAA	TTTACCTTTG		GCAAGGAAAA
3070	3080	3090	3100	3110		3140
CAAGAAGAGC	TTGCTACCAA	GATGGAAAAA	CATTGAAATG	TACCGGGGAG		GTAGTTAAGA
3130	3140	3150	3160	3170		3200
AAACTCAAGA	CATATATICT	AATTTTCAGT	ATGCACAATA	CATTTTAAAG		GTAGGGTTGG
3190	3200	3210	3220	3230		3260
ATACCGAAAA	ACTACACGAA	CTTGTTAAAG	AACTGGAGGA	TGAAAGCAAT		AGTCTCTCTG
3250	3260	3270	3280	3290		3320
TAGATTCCTT	AAAGGAATAT	TTAGTGAACG	ATGCCAAAGT	TATTTTGAAG		AGACTAAGTG
3310	3320	3330	3340	3350		3380
CCGTTGGATA	CCCTGATGCA	CAGTACCTTC	TAGGTGACGC	CTACTCCTCT		GGAGTTTTCG
3370	3380	3390	3400	3410		3440
GTAATAAAAA	GAATAGAAGA	GCATTTCTTT	TATTCCTCCG	TGCAGCCAAA		AGAATGCACA
3430	3440	3450	3460	3470		3500
TCGAAAGTGT	TFACAGAACT	GCCATTIGCT	ATGAGTGCGG	CTTGGGTGTA		ACCAGAAATG
3490	3500	3510	3520	3530		3560
CACCGAAGGC	GGTTAACTTT	TTGACTTTTG	CTGCAACTAA	GAACCATCCT		GCAGCAATGT
3550	3560	3570	3580	3590		3620
ACAAATTAGG	AGTATATICG	TATCATGGTT	TGATGGGTCT	TCCADATGAT		ATTCTAACCA
3610	3620	3630	3640	3650		3680
AAATGGATGG	TTATAGATGG	TTGCGAAGGG	CTACATCTAT	GGCTAGCAGC		TTTGTITGTG
3670	3680	3690	3700	3710		3720
GTGCTCCTTT	TGAATTAGCC	AATATTATA	TGACGGGATA	TAAAGATCTT		ATCATTTCGG

ATCC 3'

Figure 2. Nucleotide sequence of 3.7kb fragment with putative open reading frames. The nucleotide sequence is shown along with the deduced primary sequence of *RAD51* gene and that of downstream gene. The 5' ends of mRNA determined by primer extension analysis are shown by the asterisks. 1st, 2nd, and 3rd. ATG are underlined. A- and B-type nucleotide binding consensus sequence (Walker et al., 1982) are also underlined. The arrow donates 10 bps direct repeat sequence in 5'-portion of *RAD51* gene. The homologous sequences found in *RAD52* gene are boxed. The deletion junction of the plasmid used in the complementation test are indicated by the arrows.

specifically involved in meiotic reciprocal recombination (Bishop, D. and Kleckner, N., personal communication). RAD51 is more closely related to DMC1 than *E. coli* RecA protein. The homology extends over whose region of RAD51 protein (Figure 5). The extent of homology was 52% and 73% for identical and homologous residues, respectively.

To get an insight about the activity of RAD51 protein, we searched some known conserved sequences in relation to some defined functions; DNA binding, nucleotide binding, protein kinase and so on in the protein. As expected from the homology with *E. coli* RecA proteins, RAD51 protein bore both nucleotide binding A- and B-type consensus sequences. The A-type region located at position from 179th to 199th residues that constitutes β -sheet - turn (GT/SGKT/S) - α -helix (Walker et al. 1982) responsible for the binding to β -phosphate of nucleotide (Dever et al., 1987). The B-type consensus sequence contains hydrophobic stretch ended with aspartic acid at position from 276th to 280th residues responsible for binding to Mg^{2+} ion (Figure 4). RAD51 protein did not contain conserved sequence for guanine base seen in GTP binding protein superfamily (Dever, et al., 1987) suggesting that RAD51 protein bind to ATP. The C-terminal portion of the protein was rich in negatively charged amino acid residues, 7 out of 12 were asparagine and glutamine. This characteristics is often observed in DNA binding proteins, although its functional role is obscure (Friedberg,1988). It is worth to note that zinc finger like sequence Cys 3X Cys 5X Cys 5X Cys in the carboxyl terminal region was found, although the sequence dose not match completely with the consensus sequences for zinc finger (Berg, 1986). Amino acid sequence of the truncated gene found downstrem of RAD51 gene in the cloned 3.7 kb BamH1 showed a significant homology with Actin proteion.

(4). 5' and 3' flanking sequnces of the *RAD51* gene

Among the recombination genes in *S. cerevisiae*, DNA sequences were known for the genes, *RAD50*, *RAD52*, *RAD55* and *MRE11* (Alani et al., 1989; Adzuma et al.,

170	180	190	200	210	220	230	240	250
TTGSKNLDTL	GGVETGSIT	ELFGEFRT	GKSQLCH	TAVTCQI	PLDIGG	GEGK	CLYIDTE	GTFR
:		:			:			:
STGSLSLD	IAGAGGL	PMGRIVE	IYGPES	GKTTTL	LQVIAA	AQR	EGKTC	AFIDA
50	60	70	80	90	100	110	120	
260	270	280	290	300	310	320	330	340
QLRLL	DAAQMM	SESRF	SLIVV	DVSM	ALY	RTDF	SGR	GELS
:	:	:		:		:	:	:
QALEIC	DALA	RS	GA	V	DVIV	VDS	VAA	LTP
130	140	150	160	170	180	190	200	210
350	360	370	380	390	400			
GGN	MAHS	STTRL	GFKK	GKGC	QRLCK	VVDSP	CLPE	AECV
:	:	:		:	:	:	:	:
GGN	AL	KFY	AS	VR	LDI	RRIG	AV	KE
220	230	240	250	260	270			

Figure 3. Amino acid sequence comparison of *E.coli* RecA protein and *RAD51* protein.

The upper sequence represents 162 to C-terminal 400th residue of the *RAD51* protein. The bottom sequence is the region, 40 to 275th in RecA protein (Horii et al., 1981). Vertical bars and colons indicate identical and similar amino acid, respectively. The similar amino acids in this case are, Asp and Glu, Asn and Gln, Thr and Ser, Arg and Lys, and Phe and Tyr. Small hydrophobic residues are Ala, Leu, Met and Val. Bulky amino acids are Ile, Trp, Tyr Phe and Leu.

1984; Kuwahara et al., in press; Jouzuka K and Ogawa H. unpublished result). To know the possibility whether a set of the same functional genes is expressed in a same mode, the upstream sequences of the *RAD51* gene were compared with those of the above genes, of *RAD54* gene in the same subgroup and of *RAD3*, *RAD2* and *PHR* genes which are responsible for repair of DNA damage (Sebastian et al., 1990 and also those of the meiosis specific genes, *SPO11*, *HOP1*, *RED1* and *MER1* (*SPO11*; Atcheson et al., 1987; *HOP1*; Hollingsworth et al., 1990; *RED1*; Thompson and Roeder, 1989; *MER1*; Engrechant and Roeder, 1990). Two similar sequences indicated as BoxA and BoxB in the *RAD52* gene in Figure 6 were found at the 5' flanking region of the *RAD51* gene, but not *RAD50*, *RAD55*, and *MRE11*, which belong to a different subgroup as described in Table 1. The BoxA sequence consists of imperfect inverted repeat. One inverted repeat (BoxA) and the half repeat (A') exist in the upstream region of *RAD52* gene which significantly affected the induction of *RAD52* protein in meiosis (Nabetani and Ogawa, T. unpublished results). Although the function of BoxB sequence has not been identified yet, the homologous sequence was also found. Homologous sequences with *RAD54* gene were found at two regions from 458 to 493 and 443 to 451, and with damage responsible sequences (DRS) that was identified at the 5' flanking region of *HPR* and *RAD2* genes (Sebastian et al., 1990), was found at position from 488 to 477 as shown in Figure 8. No homology with *RAD3* epistasis group genes and meiotic specific genes, *SPO11*, *HOP1*, *RED1* and *MER1* was found. These results suggest that the *RAD51*, *RAD52* and *RAD54* genes in the same subgroup would be regulated co-ordinately in meiosis and expressed to function in the same process of meiotic recombination. And *RAD51* gene would also be co-regulated as damage induced gene in mitosis. These possibilities were supported by the inducible synthesis of the transcripts in meiosis and in mitotic cells treated with MMS (see below).

The sequence required for the termination of mRNA transcription, TAG-X1-14-TAGGT(AT rich) TTT was not found at position between 1848 and 2152 which was observed to be essential for the gene function. However, any particular known

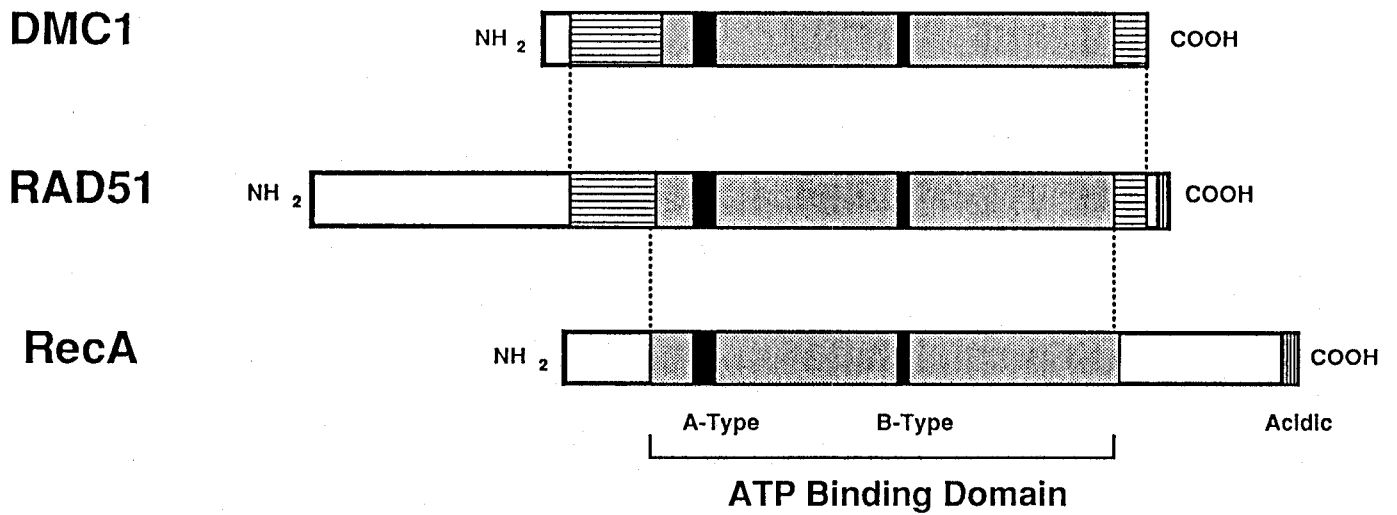


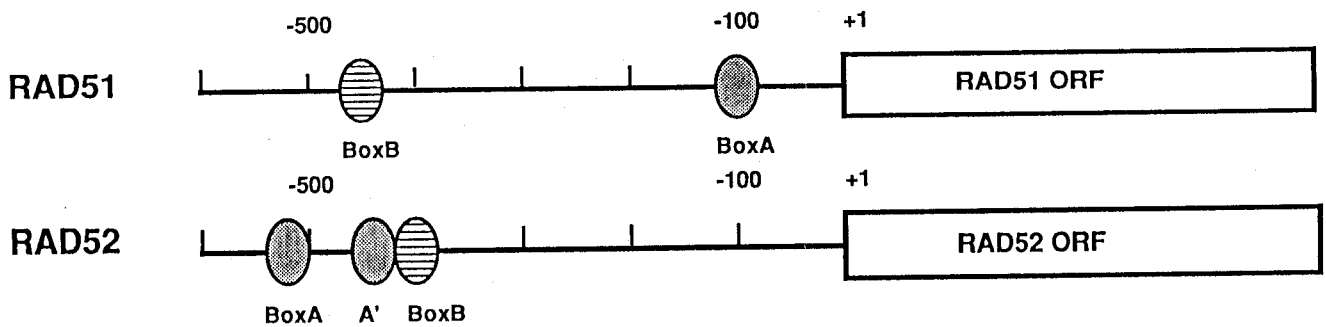
Figure 4 Schematic figure of RecA protein superfamily

The amino acid sequence of DMC1(Bishop Personal communication), RAD51 and RecA protein(Horii et al., 1981) are illustrated by the bars. The hatched and striped region indicate the homologous region among three proteins and between RAD51 and DMC1, respectively. Black boxes show the nucleotide binding consensus sequences.

Homology between RAD51 and DMC1 gene

	110	120	130	140	150	160	170
<u>RAD51</u>	EAVAYAPRKDLLEIKGISEAKADKLLNEAARLVPMGFVTAADFHMRRSELI	CLTTGSKNLDTLLGGGVET					
	:	:		: : : :		::	: :
<u>DMC1</u>	NTVLSTTRRHLCIKGLSEVKVEKLKEAAGKIIQVGFIPATVQLDIRQRVYSLSTGSKQLDSILGGGIMT						
	10	20	30	40	50	60	70
	180	190	200	210	220	230	240
<u>RAD51</u>	GSITELFGEFRTGKSQ LCHTLAVTCQIPLDIGGEGKCLYIDTEGTFRPVRLVSI	AQRFGLDPDDALNNV					
	:		: :		:	::	
<u>DMC1</u>	MSITEVFGFRCGKTQMSHTLCVTTQLPREMGGGEGKVAYIDTEGTFRPERIKQIAEGYELDPESCLANV						
	80	90	100	110	120	130	140
	250	260	270	280	290	300	310
<u>RAD51</u>	AYARAYNADIQLRLLDAAAQMMSES RFSLIVVDSVMALYRTDFSGRGELSARQ	MHLAKFMRALQRLADQF					
	:	: :	: :	:		:	:
<u>DMC1</u>	SYARALNSEHQMELVEQLGEELSSGDYRLIVVDSIMANFRVDYCGRGELSERQQKLNQHLFKLNRLAEEF						
	150	160	170	180	190	200	210
	320	330	340	350	360	370	380
<u>RAD51</u>	GVAVVVTNQVVAQVDGGMAFNPDPKKPIGGNIMAHSS TTRLGFKKGKGCQRLCK	VVDSPCLPEAECVF					
	:		:		:		:
<u>DMC1</u>	NVAVFLTNQVQSDPGASLFASVDGRKPIGGHVLAHASATRILLRKGRGDERVAKLQDSPDMPKENVST						
	220	230	240	250	260	270	

Figure 5. Amino acid sequence homology between RAD51 and DMC1 protein. Homologous alignment are shown between RAD51 and DMC1 protein(Bishop, Personol communication). Bars and stars indicate identical and homologous amino acids, respectively.



BoxA Sequence

RAD51 GGGACTGGAGAAAAAATTTTCTCAGTT
 ||| ||||| ||||| ||||| |||||
 RAD52 GGGCCTGGA AAAAG TTTTCGCAGTT
 ||||| ||||| |||
 A' TTGCCTGGAAAAAG

BoxB Sequence

RAD51 TTGCCACCGGCAGTGCCA
 ||| ||| || ||||| |||
 RAD52 TTGTCAG CGTCAGTGACA

Figure 6. Sequence homology in 5' portion of *RAD51* and *RAD52* gene.

The upper figure illustrates the schematic diagram of the upstream region of *RAD51* and *RAD52* genes. Number represents the position from 1st ATG as 1. The bottom shows homologous sequence seen in the 5' flanking region of *RAD51* and *RAD52* genes. Bars indicate identical base between two sequences. Box A sequence consists of an imperfect inverted repeat and is necessary for meiotic induction of *RAD52* gene.

A.

Gene	Position	Sequence	Matching
<i>RAD54</i>		TACGCGTTA	
<i>RAD51-1</i>	485-493	TACGCGTCA	8 / 9
<i>RAD51-2</i>	443-451	TACGCGTGG	7 / 9

B.

Gene	Position	Sequence	Matching
<i>PHR1-1</i>	-103- -104	CGAGGAAGCA	
<i>PHR1-2</i>	-109- -118	CGAGGAAGAA	9 / 10
<i>RAD2</i>	-168- -157	CGTGGAGGCA	8 / 10
<i>RNR2</i>	-424- -413	CGAGGTCGCA	8 / 10
<i>RAD51</i>	488- 477	CGTGGTGGGA	6 / 10

Figure 8 Homologous sequences seen in genes whose expression increase in response to DNA damages.

A. Table represents the homologous sequence observed between RAD51 and RAD54 genes. The sequence of RAD54 gene is the part of that required for the induction by MMS and UV(Cole et al., 1988). B. The nucleotide sequence seen among damage responsible genes.

sequence was not found. The sequence required for polyA addition in Eukaryote, AAUAAA was found at position 2252.

(5) Induction of *RAD51* mRNA in meiosis and in mitotic cells treated with MMS.

Most of genes (*RAD50*, *RAD52*, *RAD54*, *RAD55*, *MRE1*, *MRE2*, *MRE4* and *MRE11*) required for meiotic recombination are inducible in meiosis (Wendy, 1990 ; Cole et.al., 1988; Nabetani and Ogawa, unpublished results; Kuwahara and Ogawa in preparation; Leem, et al., unpublished results; Jouzuka and Ogawa, unpublished result). To know whether or not the expression of the *RAD51* gene is induced in meiosis, amounts of mRNA synthesized in mitotic and meiotic cells were measured by northern blotting experiments with genomic mRNA (Figure 7). A slight amount of 1.6 kb mRNA was detected in mitotic cells (see time 0 min in Fig. 6), however, the synthesis of the same size of mRNA started to increase immediately after transferring the cells into sporulation medium, and reached at 20 fold after 6 hrs incubation. After reaching the maximum amount of mRNA, the amount declined to 80% of the maximum and was kept at that level for several hours. The kinetics of the mRNA induction in meiosis was coincided well with that of the induction of His⁺ recombinants in *his4* allele in meiosis (data not shown).

The kinetics of the induction of mRNA in meiosis were carried out with other genes essential for meiotic recombination, *RAD50*, *RAD52*, *RAD55* and *MRE11* by using the same RNA preparation (see Kuwahara and Ogawa in press). The results indicated that the time when each mRNA reached at the most abundant level was almost the same except that *RAD55* gene was expressed very earlier stage than others in meiosis.

While, the kinetics of the induction of mRNA of *RAD51* treated with MMS in mitotic stage showed that the synthesis can be induced by MMS treatment. The amount of mRNA starts to increase at 1 hr after addition of MMS, and reached at 4 fold in the absence of MMS showing that *RAD51* gene is efficiently induced by DNA damage.

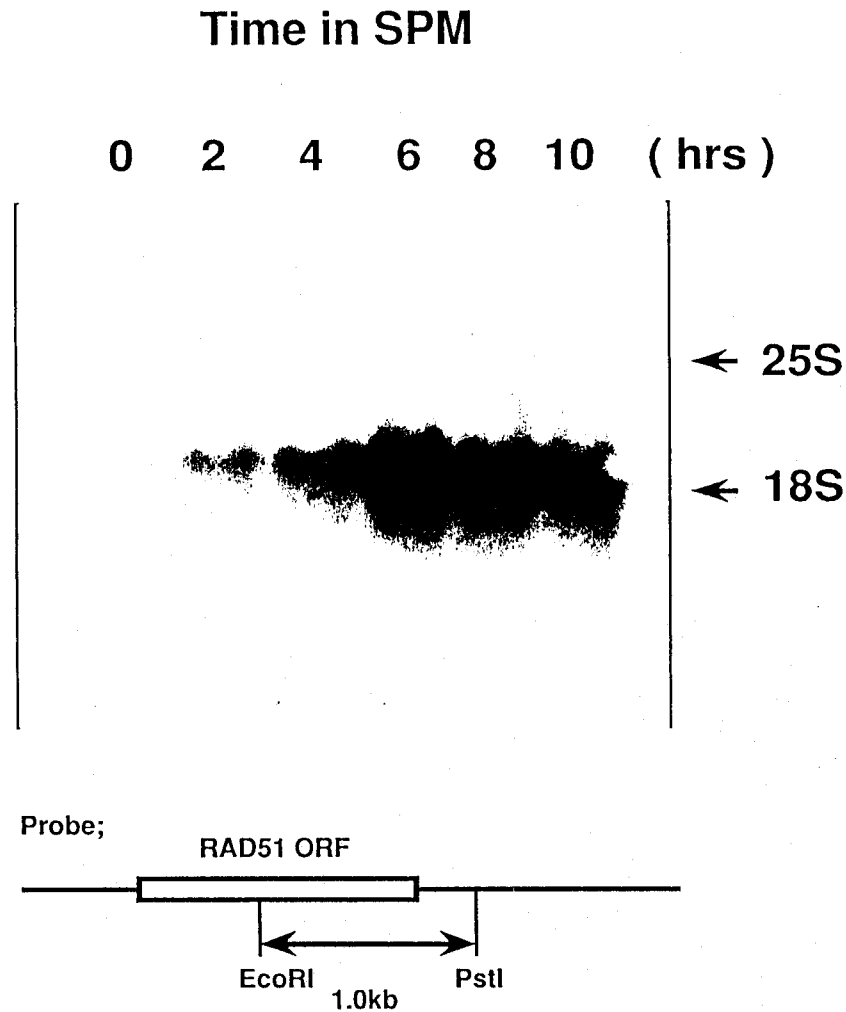


Figure 7. *RAD51* mRNA in Mitosis and Meiosis.

The equal amount of total RNA was probed with EcoRI-PstI, 0.9kb fragment. The cells, SKY501, were transferred to the sporulation medium at time 0. After a given time, sample was withdrawn and RNA were isolated. The measurement of meiotic recombination by monitoring his auxotroph shows the induction of the recombination initiated at 2, 3hrs after the transfer into SPM and reached to the maximum level around 9 or 10 hrs(Kuwahara et al., 1991). The lane at 0 time represents RNA in mitosis.

(6) The initiation site of mRNA synthesis for RAD51 gene

The analysis of MMS sensitivities of deletion mutants at 5' flanking region of *RAD51* gene revealed that the protein deleted 28 amino acids from 1st ATG can restore the MMS sensitivity of *rad51-1* mutant. Since this deletion mutant is thought to lack the promoter and the initiator sequence required for the transcription to produce RAD51 protein initiated from the 1st or 2nd ATGs (Figure. 2), the 5' end of RAD51 mRNA was analyzed by S1 mapping of the product which the complementary strand of the transcripts was synthesized by primer extension. The results revealed that the longest main band was mapped position at 561. It located at 83 bps upstream from 1st ATG, and the second was located at the position 707 just on the A of 2nd ATG. The third and fourth were positioned at 788 and 789, and located at 52 and 53 upstream from 3rd ATG, respectively. These results suggest that the longest mRNA encodes the protein initiating from 1st ATG and those of 2nd, 3rd and 4th mRNA would initiate from 3rd ATG. The possibility to initiate the transcription at position 561 was supported by the determination of molecular weight of the protein identified as RAD51 protein (see below).

(7). Identification of RAD51 protein by Western blotting experiments.

To identify the RAD51 protein produced in yeast cells, polyclonal anti-body was made to purified RAD51 protein as antigen synthesized in *E.coli* cells (see accompanying paper, Shinohara, et al.,). The RAD51 protein produced in *E. coli* cells consists of 400 amino acid residues, and was synthesized from 1st ATG. The molecular weight of the protein was determined as 43,000 by SDS polyacrylamide gel electrophoresis.

The protein synthesized in both mitotic and meiotic cells were analyzed the lysates with Western blott, and a protein of about 43,000 daltons was detected. The molecular weight of the protein was the same those of RAD51 protein purified from *E. coli* cells. Furthermore, the protein was induced by treatment with MMS in mitotic

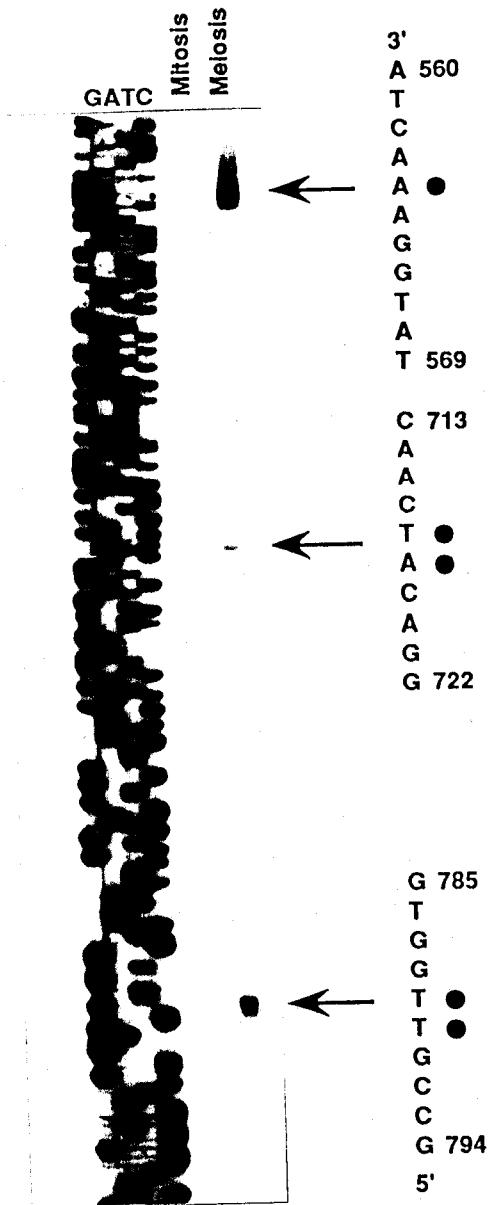


Figure 9 Primer extension of RAD51 gene

The primer extension was carried out as described in Materials and Methods. Each 10 μg of PolyA RNA from meiosis and mitosis was subject to the analysis. The sequence ladder was made using the same primer as in Primer extension analysis. Nucleotide sequences of bottom strand of RAD51 gene are shown to the left. The dots indicate the positions of the termination by primer extension.

cells and also induced in meiosis. These results were coincided well with the synthesis of transcripts of *RAD51* gene in mitosis and meiosis as described above. Thus, the initiation of transcription is determined at position 561. Three minor starting sites observed with S1 mapping are thought to be the degradation products of the longest one or the artifact products by pausing of reverse transcriptase.

(8) Characterization of *rad51* mutant in nucleotide binding consensus sequence

The importance of the A type consensus sequence in nucleotide binding was examined by introducing the base change in the motif using a site directed mutagenesis. One mutant had a substitution of 191 Lysine to Alanine, expecting complete loss of the function. The another had a homologous substitution of 191 Lysine to Arginine to observe the steric requirement of the positively charged residue. For RAD3 helicase in yeast and RecA protein in *E. coli*, the same mutations with the same amino acid substitutions in ATP binding sequence were made. The purified proteins of from the RAD3 and RecA mutants carrying the substitution of Lysine to Arginine bind to ATP, but not hydrolyze ATP *in vitro*, and deficient the ability to restore the UV resistance of *rad3* and *recA* mutants, respectively (Sung, et al., 1987; Kowalzkosky, 1990).

Both new mutants of *rad51* gene were defective the ability to restore the MMS resistance of *rad51-1* mutant as shown in Figure 10, although KR191 mutant was less sensitive than KA191 mutant at the high concentration of MMS. (compared with cell viabilities at the concentration of 0.005% and 0.01%). Biochemical studies of the proteins are necessary to elucidate whether KR191 mutant protein have the residual activity as seen in similar RAD3 and RecA mutant proteins.

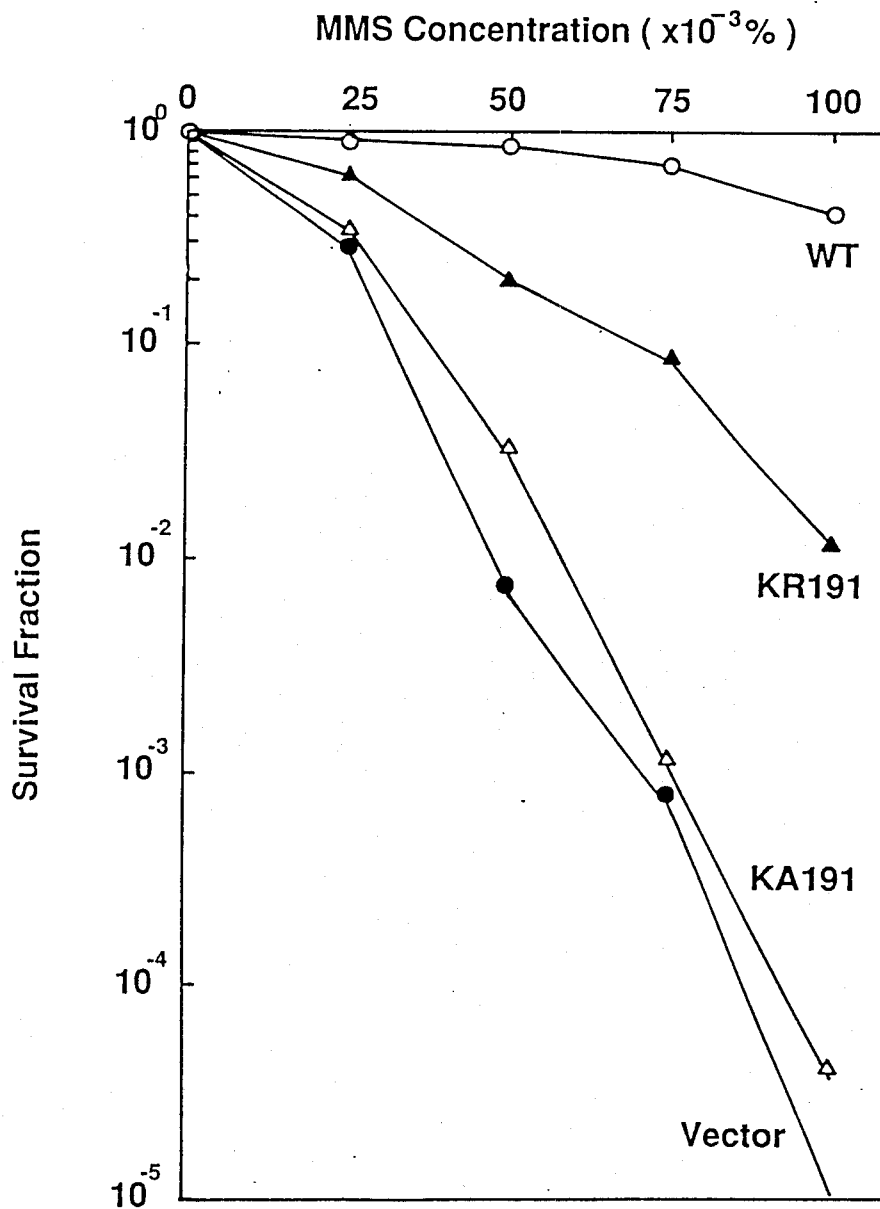


Figure 10 MMS sensitivity of the *rad51* mutants in nucleotide-binding sequences. XS3672-3C, *rad51-1* strain were transformed with pYRAD51, pRS316 based, CEN ARS vector. The transformants were grown to the stationary phase in SD lacking uracile. After aliquots dilution, aliquots were plated on SD plate containing various amount of MMS. The result shown is the *rad51-1* strain carrying RAD51 gene, wild type (○), KA191(△), KR191 and vector, pRS316(●). The mutations were created by the site-directed mutagenesis as described in Materials and Methods.

4. Discussion

Here, we classify the RAD52 epistatic group genes into three subgroups with their genetic characteristic properties and analyzed second subgroup consists of *RAD51*, *RAD52* and *RAD54* genes. And we try to analyzed the role of these genes in genetic recombination. Since the structure of *RAD52* gene was analyzed by Adzuma, et al., (1984) and the functions of the protein are analyzing by Shinohara, et al., (see accompanying paper). Therefore, we investigated here the *RAD51* gene of *S. cerevisiae*.

The nucleotide sequence of 3.7kb BamH1 fragment containing *RAD51* gene was determined and the reading frame for *RAD51* structural gene was identified. The results showed that the *RAD51* gene encodes 400 amino acid residues and its molecular weight is 43,000 daltons. The transcription of the gene and the synthesis of the protein were induced in meiotic state and also in mitotic cells treated with MMS.

(a) Some characteristic of the protein predicted from the primary structure.

Homology search of the amino acid sequence of the RAD51 protein showed that it contains an extensive homology to *E. coli* RecA protein (Figure 3). And the homology was located at the main domain of *E.coli* RecA protein containing ATP binding, ATP hydrosis and DNA binding sites. The significant homologies were found at A- and B-types ATP binding consensus sequences.

To test the importance for the RAD51 protein function of the A-type consensus sequence, the site directed mutagenesis at position 191 Lysine residue of RAD51 protein was carried out and the result obtained from the mutant having Alanine substitution showed that the Lysine residue was essential for the activity of RAD51 protein, while homologous substitution of the residue with Arginine showed for the modelate deficiency of the ability to restore MMS resistancy of *rad51-1* mutant. The abiliy to carry out meiotic recombination is remained to test.

Purified RAD51 protein produced in *E. coli* cells was found to have a ATP-dependent DNA binding activities (Shinohara, et al., in the accompanying papers). It

is necessarily to test the activity of these mutants protein *in vitro*.

Thus, the structural similarities of RAD51 with *E. coli* RecA protein indicates that RAD51 protein is the first protein having the same function with *E. coli* RecA protein found in Eukariotic cell.

Further analysis of the structure of the RAD51 protein showed that outside of the region from the 161th to 276th amino acid in RAD51 protein, there were few homology with RecA protein. And RAD51 protein had an extra long stretch of about 160 amino acid residues in the N-terminal region, while recA protein had a extra 100 amino acid stretch in C-terminal region. The region from 1st to 60th residue in the RAD51 protein is rich in hydrophilic amino acids; 11 amino acids are aspartic acid or glutamic acid, 11 for threonine or serine and 10 for glutamine or asparagine. This region also contains three potential glucosylation sites, Gln X Ser/Thr sequence ().

The analysis of the 5' end of the transcripts from *RAD51* gene and the molecular weight of RAD51 protein synthesized in yeast cells identified by Western blotts showed that the RAD51 protein is encoded from the 1st ATG codon. However, the genetic analysis of a series of 5' end deleted *RAD51* gene showed that the deletion mutant carrying 17 bases from the 1st ATG could restore the ability of MMS sensitivity of the rad51 mutant. Although this result dose not coincide with those of structural analysis as mentioned above, the largest coding frame is thought to be only an active RAD51 protein.

Recently, RAD51 protein produced in *E. coli* cells was found to have an ATP dependent ssDNA binding activity (Shinohara, et al., see accompanying paper). Therefore, the N-terminal negatively charged residue in the protein might resist against the phosphate backbone of DNA and be involved in the regulation of the interaction with DNA. Alternatively, this region might play role in the interaction with other proteins; such as RAD52 protein. Recently, we also have found that the purified RAD51 protein from *E. coli* cells has a strong affinity to RAD52 protein.

The homologous region with *E. coli* RecA protein is rich in hydrophobic amino

Amino acid sequence of Nucleotide binding for known RAD genes

	<u>A type NTP binding</u>	<u>B-type</u>
RecA (40- 75)	ISTGSLSLDIALGAGGLPMGRIVEIYGPESSGKTTL :: : : :: : :	VIVVDSVAALT (139-150) : :
RAD51 (159-193)	LTTGSKNLDTLG-GGYETGSITELFGEFRTGKSQL :: : : : : :	LIVVDSVMALY (276-287) :: :
RAD55 (18- 62)	LSSGITGLDEILN-LGFQARSIYEIFGPPGIGKTNF : : : :	LVIIDGFSQLV (131-143) ::: : :
RAD50 (8- 43)	SIQGI RSFDSNDRETI EFGKPLTLIVGMNGSGKTTI : : : ::: :	VIALDEPTNL (1229-1240) :::
RAD3 (34- 59)	KYIPEQTNYMCEIKKTLEVGGNSILEMPSGTGKTVS :: : : : :	IVIFDEAHNID (230-241)
RAD18 (334-369)	QLLSWDVSHINKTPQNSSNKGKISKLMIMKSNKGKSSS : : ::: :	: ::
RADH (9- 44)	LHLVSQLNTQQRAAALFDYTRGLQVIAGPGTGKTKV	HVLVDEFQDTN (246-257)
Consensus	G G G GKT S	XXXXD X;Hydrophobic

Figure 11 Sequence homology of nucleotide-binding consensus sequence among *RAD* genes.

Homologous alignment of A-type consensus sequence found in *RAD* genes of *S. cerevisiae* are shown. Bars and colons indicate identical and homologous amino acid, respectively. *RAD3*(Reynolds et al., 1985) and *RADH*(Aboussekha et al., 1989) are DNA helicase, although helicase activity of *RADH* is deduced from amino acid sequence. *RAD50*(Alani et al., 1989), *RAD18*(Jones et al.,1990), *RAD55* (Kuwahara et al.,1 991).

acids suggesting to involve in protein-protein mutual interaction by itself (Freitag and McEntee 1988; Horii, et al., 1991) and to involve the formation of filamentous structure by cooperative binding to DNA (Griffith et al., 1987). Therefore, The region of RAD51 protein might have the same role as the formation of the filament structure binding to DNA and/or formation of protein complexes by itself or with other proteins. Gel filtration analysis of purified RAD51 protein from *E. coli* revealed that the protein tended to form a large aggregate even in the high salt condition (Shinohara, et al., see accompanying paper), and also strong affinity to RAD52 protein which had an ATP-independent stranded transfer activity.

These results showed that the RAD51 protein likely played almost the same role with *E. coli* RecA protein in genetic recombination along with RAD52 protein, suggesting that the molecular mechanisms of the recombination reaction is quite different from that of RecA protein in *E. coli* cells.

(b) Control of the expression of the RAD51 gene.

Amount of *RAD51* mRNA is increased 10-20 folds similar to those of mRNA of other genes in the same *RAD52* epistasis groups, *RAD50*, *RAD52*, *RAD54* and *MRE11* (Cole et al. 1988, Nabetani and Ogawa to be published, Jouzuka K. and Ogawa, to be published, Wendy R. 1990).

While the expression of the genes involved in meiosis, such as *SPO11*, *SPO13*, *HOP1* and *MER1* is also induced in meiosis. These are categorized to three groups based on the pattern of the expression. The difference is that the repair genes are also transcribed in mitosis, while meiotic specific genes are not. Buckingham et al., (1990) suggested GGCGGCTA, URS sequence, is required in both the repression of meiotic specific genes in mitosis and the activation in meiosis. *RAD51* gene as well as other repair genes lacks this URS sequence, suggesting that the pathway of the induction is quite different. Some pathway is likely to exist in the activation of the genes required for the meiosis.

Comparison of the upstream region of *RAD51* with that of *RAD52* , 30 base

5. Materials and Methods

Strains and Media

For the cloning of the *RAD51* gene and the complementation test, XS3672-3C, *rad51*, *ura3*, *leu2*, *trp1*, *his4*, *lys2*, *ade2* was used. For the preparation of mRNA, SKY502(Kuwahara et.al. 1991) was used. Bacterial strains derivatives *E. coli* K12, AB1157recAdel(Ogawa and Shinohara,1991) and JM103recAdel(Shinohara and Ogawa, 1991) were used for plasmid preparation, dg98(Stratagene) for growth for ssDNA phage preparation and CJ286(Kunkel, 1988) for growth of the phagemid to incorporate uracile containing ssDNA. The plasmid vector used were pBluescript KS+(Stratagene), pRS316(Sikorski and Hiltter 1989) and YCP50(Rose et al., 1987). Liquid and solid yeast media were described else(Hartwell 1967, Wood 1982, Adzuma et al., 1984). Synthetic medium(SD) contained 0.67% yeast nitrogen base and 2% glucose, supplemented with the necessary amino acids. For the nutrient medium, YPD(1% yeast extract, 2% polypeptone, 2% glucose) or MYPD(0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% glucose) was used. L-broth and L-plate(Ogawa, 1975) were used for bacterial growth.

Plasmid construction

Plasmids were constructed by standard procedures(Maniatis et al., 1990). PY-RAD51, KS-RAD51 was constructed by the introduction of the BamHI 3.7 kb fragment containing RAD51 gene into the BamHI site of pRS316 and pBluescript KS+, respectively.

DNA sequencing

Serial deletion of 3.7kb BamHI fragment on pRS316 were constructed by ExoIII-S1 method(Heinoff 1982; Maniatis et al., 1990). After the transformation of dg98 with the serial deletion plasmid, single-stranded phage were recovered by the infection of helper phage VCSM13(Stratagene) and ssDNAs were prepared by PhOH extraction

of the phage particle precipitated with polyethylene glycol following isopropanol precipitation. Using the universal primer, the sequences of ssDNA were determined by dideoxy chain termination method(Sanger et al., 1977) with T7 DNA polymerase Sequenase version 2 according to the manufacture instruction(USB). Reaction products were analysed on 6% Acryl(19:1) 7M Urea buffer gradient sequencing gel(5X-0.5X Tris-borate). In some case sequence reaction were carried out by using Taq polymerase and dye primer and analyzed with ABI sequencer 372. Sequence of 3.7kb fragment was determined on the both direction, especially in BamHI-PstI 2.2kb fragment.

RNA isolation and Northern-blotting

RNA was isolated by using glassbead method(Carlson and Botstein, 1982). For mitotic RNA preparation, the culture of SK502 was grown to the log phase(1×10^7 cells/ml), harvested, suspended in ice-cooled water containing 100mg/ml Cycloheximide, put on ice for 20min, re-centrifuged, washed with ice-cold water and suspended in SDS-buffer(0.2M Tris-HCl(pH7.5), 1mM EDTA, 0.5M NaCl) following the addition of 10%(W/V) SDS to final 1%. Equal volume of 1:1 PhOH:CHCl₃ saturated SDS-buffer and equal volume of acid washed glassbeads ($\phi=0.5$ mm) were added and mixed vigorously with vortex mixer for 1min at the top speed following putting on ice for 1min. This cycle were repeated at least 5 times. After the centrifugation, upper layer was recovered, 1/10 volume of 3M NaOAc, 2.5X volume of ethanol and DEPC(diethylpyrocarbonate) were added, was kept at -20C for 1hr and centrifuged at 12,000g for 20min. The precipitate was washed with 70% Ethanol and dried up, suspended in 1/10 volume of Lithium buffer(10mM Tris-HCl(pH7.5), 1mM EDTA, 0.1M LiCl, 0.2% SDS). 1/3 volume of 5M LiCl was added and kept at -20C over night. After Lithium precipitation RNA was recovered by the centrifugation at 12,000g for 20min and dried up. Usually total RNA were stored as alcohol precipitation after the determination of the concentration by measuring the OD₂₆₀.

For polyA RNA preparation, dried RNA pellet after Lithium precipitation was suspended in Oligo-dT Loading buffer(20mM Tris-HCl(pH7.5), 1mM EDTA, 0.5M NaCl, 0.1% SDS), kept at 65 C for 10min and was applied quickly to 0.5ml of oligo-dT column(Pharmacia) equilibrated with the same buffer after washing with 0.2M NaOH and excess H₂O. Flow through fraction was collected, warmed again and re-applied to the column. This step was repeated at least three times. Poly-A RNA was eluted from the column by washing with washing buffer(20mM Tris-HCl(pH7.5), 1mM EDTA, 0.05% SDS) measuring OD₂₆₀, the eluate was subject to the ethanol precipitation for storage.

For RNA sample in meiosis, yeast was grown to the mid-log phase in YPA, harvested, washed thoroughly with H₂O transferred to SPM at the time when it is called 0 time in time course experiment. After a given time, aliquots are removed from the culture and RNA were prepared as described above.

The fractionation of RNA was carried out by the electrophoresis in 0.8% agarose containing 6% formaldehyde. After electrophoresis, the gel was washed in 0.25M NH₄CH₃COO(pH5.6) for 30 min while agitating. RNA was blotted onto the Nylon membrane(Immobilon N, Millipore) in Vacuum transfer system(Pharmacia). The membrane was baked at 80 C for 2 hrs in Vacuum oven. Pre-hybridization was performed in the prehybridization buffer(6X SSC(20X SSC contains 3M NaCl and 0.3M Trisodium citrate), 1x Denhardt(100X Denhardt contains 2% BSA, 2% Ficoll and 2% Polyvinylpyrrolidone), 0.1% SDS, 0.1mg/ml heat denatured calf thymus DNA) at 65 C for at least 2 hrs. Hybridization was in the same buffer containing 10⁵ cpm/ml probe DNA prepared by the random primer method. Incubating at 65C for 12hrs, the membrane was washed in 2XSSC while agitating at 65 C following extensive washing with 0.2X SSC and 0.1X SSC. The dried membrane was contacted with X-ray film(Konica) at -70 C over night.

Primer extension and S1 mapping

10 mg of polyA RNA was hybridized with 1X 10⁵ cpm of kinased primer DNA in 30ml

of hybridization buffer(20mM PIPES(pH6.4), 0.4M NaCl, 80% Formamide treated with AG501-X8 resin(Bio-Rad), 1mM EDTA) at 30 C for 12hrs. The sample was precipitated in ethanol, dried up and suspended in Reverse-buffer(50mM Tris-HCl(pH7.6), 60mM KCl, 10mM MgCl₂, 1mM dNTP, 1mM DTT, 1 units/ul RNguard (Pharmacia), 50 mg/ml Actinomycin D). 50 units of Reverse Transcriptase(from AMV, Pharmacia) was added into the mixture and incubated at 42 C for 2hrs. 1 ml of 0.5M EDTA and 1 ml of 5mg/ml RNAaseA were added to stop the reaction, following PhOH/CH₃Cl treatment and subsequently ethanol precipitation. The dried sample was suspended in the Loading buffer(80% Formamide, 10mM EDTA 1mg/ml BPB, 1mg/ml XC) was run in 6% Acryl-amide(19:1) 6M Urea denaturing gel along with sequenced sample made by using the same primer. The gel was dried and autoradiogramed.

Site-directed mutagenesis and MMS sensitivity test

Site directed mutagenesis was performed according to Kunkel's method(Kunkel, 1988). Uracile containing single stranded DNA was prepared from phage recovered by the infection of helper phage VCSM13(Stratagene) with CJ236 harboring Phagemid KS+-RAD51 following culturing in Super-broth supplemented with 20mg/ml Uracile at 37C for 12 hrs with shaking vigously. 1pmole of ssDNA was mixed with 1 pmole of the primer containg mutant sequence in 20 ml of the hybridization solution(20mM Tris-HCl(pH7.5), 0.5M NaCl, 20mM MgCl₂), heated at 80C for 10 min and cooled gradually at the speed of 1 C/min to ensure hybridization of the tempelate with the primer. At 30 C the reaction mixture was transferred on ice for further cooling. After the additon of 10 ml of Synthesis buffer(100mM Tris-HCl(pH7.6),50mM MgCl₂, 20mM DTT, 5mM dNTP's, 10mM ATP) and 1 units of T4 DNA polymerase and 200 units T4 Ligase, the mixture was kept on ice for 5 min, following the incubation at 37C for 1 hr. 90 ml of water was added to the mixture to stop the reaction.aliquots was used in the transformation of AB1157recAdel ung+ strain. Plasmid DNA was prepared by Alkaline-SDS method. Mutation was checked

by the direct sequencing of plasmid DNA. A portion of XbaI, BstEII fragment was cloned into shuttle vector pY-RAD51.

Yeast *rad51* strain was transformed with the pY-RAD51 bearing the mutant by LiCl method(Ito et al., 1983). Yeast were grown in SD lacking uracile to the stationary phase. After the adequte dilution, aliquots were plated on MYPD containing a given concentration of Methylmethanesulfonate. Incubating for 2-3 days, the number of colony was counted.

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Chapter II

Biochemical analysis of the RAD52 protein
The RAD52 protein carry out recombination
reaction *in vitro*.

Summary

The *RAD52* gene of *Saccharomysece cerevisiae* is required for the double strand break repair and the most of the recombination in yeast. *RAD52* protein was overproduced in *E. coli* and purified. *RAD52* protein bind to ssDNA stoichiometrically at the ratio of 10. *RAD52* protein can carry out strand transfer reaction using linear duplex DNA and homologous circular ssDNA as substrate. This reaction is dependent upon the homology between the substrate DNAs, but independent on ATP and SSB. Mg^{2+} ion stimulate this reaction. *RAD52* protein also has strong anealing activity which search the homology between ssDNAs. These results suggest *RAD52* protein is engaged in the search of homology between DNAs in genetic recombination *in vivo*.

Introduction

The genetic recombination is an important biological process that produce diversity of genetic materials, repair of damaged DNA and completion of meiosis. A lot of protein or protein complex are involved in this process. Little is known about the entities of protein or activities which have an important role in recombination, except for a few procaryotic system, *E. coli* and T4 phage(For review, Cox and Lehman, 1988). Especially the proteins which are thought to be involved in homology search of DNA strands and exchange of the materials, so-called recombinase, is likely to play a key role in recombination.

E.coli RecA protein, T4 uvsX proein and U. maydis Rec1 protein catalyze the exchange of DNAs *in vitro*, the defects of whose gene conferred the host cell to recombinationless phenotype. In Eukaryote the direct purification of the protein carrying out strand transfer reaction *in vitro* had led to the identification of the putative recombinase in yeast (Kolodner et al., 1988; Sugino et al., 1988; Dystraka et al., 1990), in Drosophila(Lowenhaupt et al., 1989) and in human(Heyer et al., 1988), though there is few genetical evidence *in vivo* that those protein are really involved in recombination.

In yeast *Saccharomysece cerevisiae*, simple eukaryote, many mutants have been isolated, which show defects in mitotic, meiotic recombinations and recombainatinal repair(For reviews, Hayens and Kunzt, 1982, Resnick,1988). Among them *rad52* mutant have been extensively studied. They show the dificiency in the most of the recombination in yeast including mitotic interchromosomal recombination(Prakash et al., 1980), intra-chromosomal recombination, mating type switching(Malone and Esposito, 1980), the integration of the linear plasmid into host genome(Orr-Weaver et al., 1981), repair of the DNA damages produced by X-ray, gamma-ray or alkylating agents like methylmethane sulfonate(Game and Mortimer, 1974) and meiotic recombiantion(Game et al.,1980; Prakash et al.,1980; Malone, 1983). Thus the *RAD52* gene is thought to play a central role in the common pathway in most type of

recombinations.

Recently the *RAD52* gene was cloned and sequenced(Adzuma et al., 1984). It enabled us to purify *RAD52* protein in *E. coli* and in yeast by using overexpression system. In this study we report the activities of *RAD52* protein from *E. coli in vitro*, especially that *RAD52* protein can exchange DNAs strands, depending on the homology of the substrate and not requiring ATP.

Results

Strand transfer activity of *RAD52* protein

RAD52 gene product of *S. cerevisiae* is involved in the most type of recombination in yeast. Several experiments have suggested that *RAD52* protein is likely to work on the homology search among DNAs. To address this possibility, We overexpressed *RAD52* protein in *E. coli* and purified it to nearly homogeneity. The overexpression was performed by constructing the plasmid in which *RAD52* gene from 3rd ATG(Adzuma et al., 1984) was synthesized under the control of *recA* promoter(See Materials and Methods). Purified *RAD52* protein crossreacted with anti-*RAD52*-b-Gal antiserum(Our unpublished result).

In order to test whether *RAD52* protein can search the homology between DNAs, conventional strand transfer reaction was examined using the circle ssDNA and the labeled homologous dsDNA as substrates. Two types of molecule are considered as the products of the reaction. One is the final product, Form II, open circular dsDNA, and the other is an intermediate, so-called joint molecule, in which one strand from linear duplex DNA pairs with complementary single-strand circular DNA(Figure 1). In the assay 1.3kb or 2.3kb or full length of 7.2kb M13mp18 dsDNA labeled in 3' end with Klenow fragment was used to avoid the contamination of ssDNA through the preparation of dsDNA(Svaer et al., 1987), which confer some difficulty to distinguish strand-transfer activity from annealing activity, since *RAD52* protein shows strong annealing activity (See below), while ssDNA was M13 circle viral ssDNA.

Figure 2 shows the result of the strand transfer reaction using 7.2kb dsDNA. The presence of *RAD52* protein in the reaction mixture produced the new band whose migration position corresponded to the products by *E. coli* RecA protein(Compare lane 7 and lane 8). The removal of ssDNA from the reaction eliminated the products. The addition of final 5mM MgCl₂ into the reaction increased the amount of the products. Judging from the position of heat denatured ssDNA, most of the products were thought to be joint molecule, since there was a little linear ssDNA

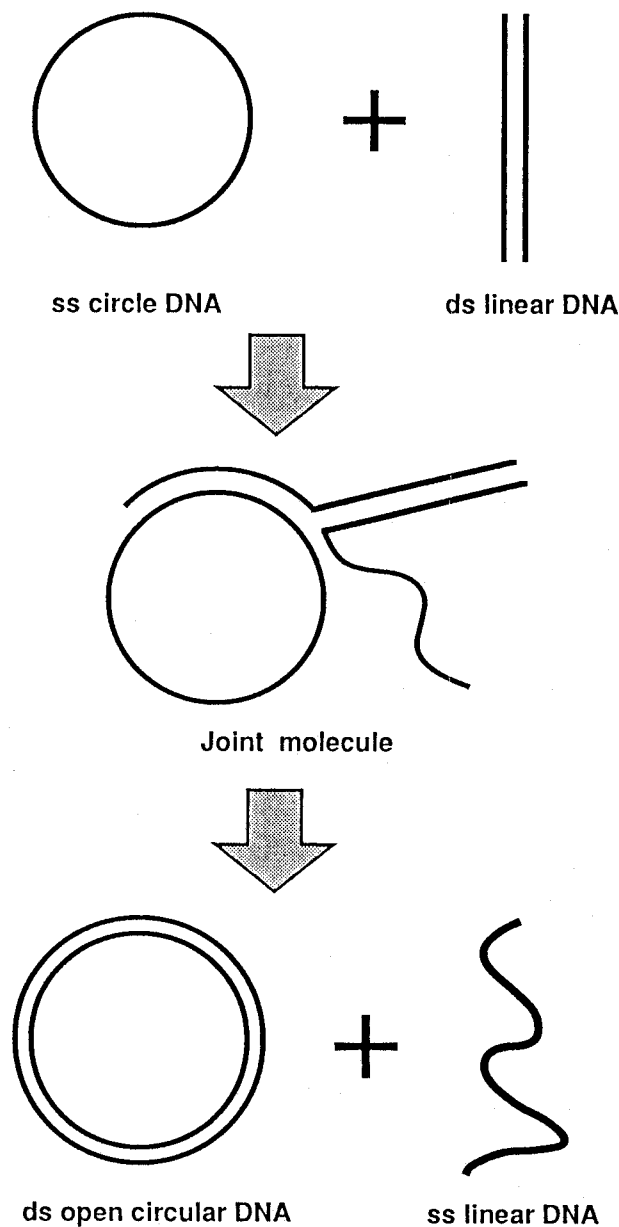


Figure 1. Diagram of strand transfer reaction. The strand transfer reaction are shown schematically The DNA substrates are circular M13 mp18 ssDNA(+ strand, 7250 nt) and full-length linear M13 mp18 dsDNA(7250 bp) that is ^{32}P labeled at both strands(+ and - strand). The a-form intermediate, called joint molecule, results from partial displacement of one strand of duplex molecule; it ha one dsDNA tail and one ssDNA tail attached to circelar DNA strand, which is base-paired for a region in length to that of the ssDNA tail. The final products is a nicked circular dsDNA and a linear ssDNA. When 1.3kb EcoRI, XmnI fragment and 2.3kb of SnaBI fragment from M13mp18 are used as dsDNA substrate, only -strand is ^{32}P labeled. The reaction products are joint molecule and final product. Released linear ssDNA is not detected due the lackeness of label at the end.

released as products. And also, the contamination of ssDNA in the dsDNA substrate was able to be ignored.

In the assay using the 1.3kb dsDNA fragment, the most of the character of the reaction was same as that using 7.2kb fragment except two points(Figure 3 A). First two slowly migrating products were detected. The upper band, called Product 1, seems to be either a joint molecule or aggregate of the joint molecules, since the size of the joint molecule is bigger than Form II DNA. The lower band, Product II, would be the final product, which consist of the ssDNA completely hybridized with short linear DNA from dsDNA. In this assay only - strand of dsDNA was labeled so that released linear + ssDNA could not be detected. In the kinetics of the strand transfer, Product I appeared first following the product II, indicating Product I was intermediate for product II(See below).

Secondly, about 50% of the substrate of 2.3kb dsDNA was converted into products while 5 to 10 % was utilized in the 7.2kb reaction. Little is known about the poor reactivity of long dsDNA. It might reflect the stability of the product in protein complex after or during the treatment with SDS and proteinase-K.

Kinetics of the strand transfer by *RAD52* protein.

Figure 4 show the time course of the strand transfer reaction by *RAD52* protein. At optimum concentration of *RAD52* protein the reaction proceeded relatively fast. The reaction completed for 15-20min in the assay with 7.2kb dsDNA, while 5-10 min with 1.3 kb or 2.3 kb dsDNA. Over 1hr, 50% of the input duplex was converted into the products with 2.3 or 1.3 kb dsDNA. On the other hand, only 10% of 7.2kb dsDNA became the product. The strand transfer reaction by *RAD52* protein might be an equilibrium one, depending on the length of homologous duplex since this reaction does not require high energy co-factor which direct the reaction to one direction as seen in RecA protein system. Some portion of dsDNA were converted into the product during the reaction, but in a way of reaction the reaction proceeded reversely so that the form II or joint molecule dissociated into duplex and circle

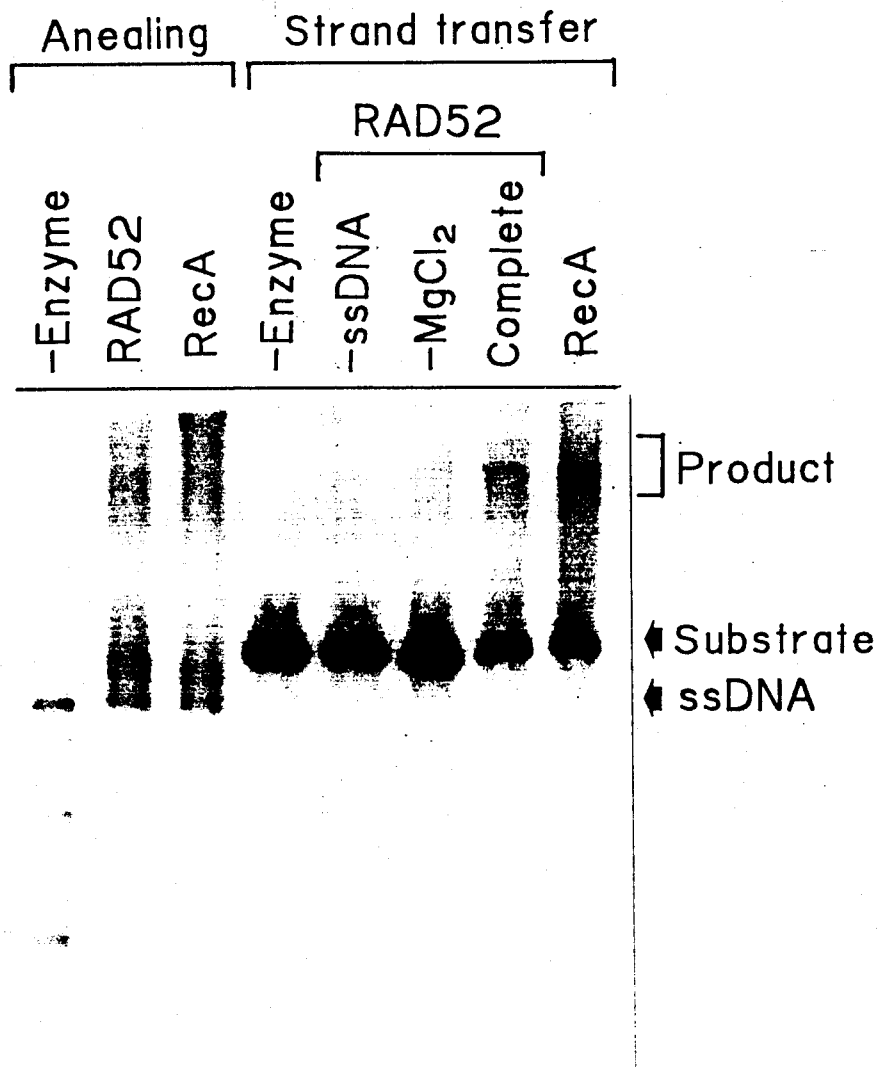


Figure 2. Property of *RAD52* strand transferase. Strand transfer reactions were carried out in following condition; 20mM Tris-HCl(pH7.6), 5mM MgCl₂, 40mM NaCl, 1mM DTT, 10μM M13 mp18 viral ssDNA, μM M13 mp18 linear duplex DNA, 1 μM *RAD52* protein. The reaction mixtures were incubated for 20 min at 37 C. SDS and Protease-K were added to final 1.0% and 1 mg/ml to terminate the reaction. The products were analyzed on 0.8% agarose gel electrophoresis. lane1, heated denatured m13 mp18 dsDNA as a marker; lane 2, the reaction without *RAD52* protein; lane 3, complete reaction without M13mp18 viral ssDNA; lane 4, complete reaction without NaCl; lane 5, complete reaction omitting MgCl₂; lane 6, complete reaction; lane 7, complete reaction with heterologous φX174 viral ssDNA instead of M13 ssDNA; lane 8 reaction with RecA protein and 2 mM ATP. Origin of agarose gel and the position of products are indicated.

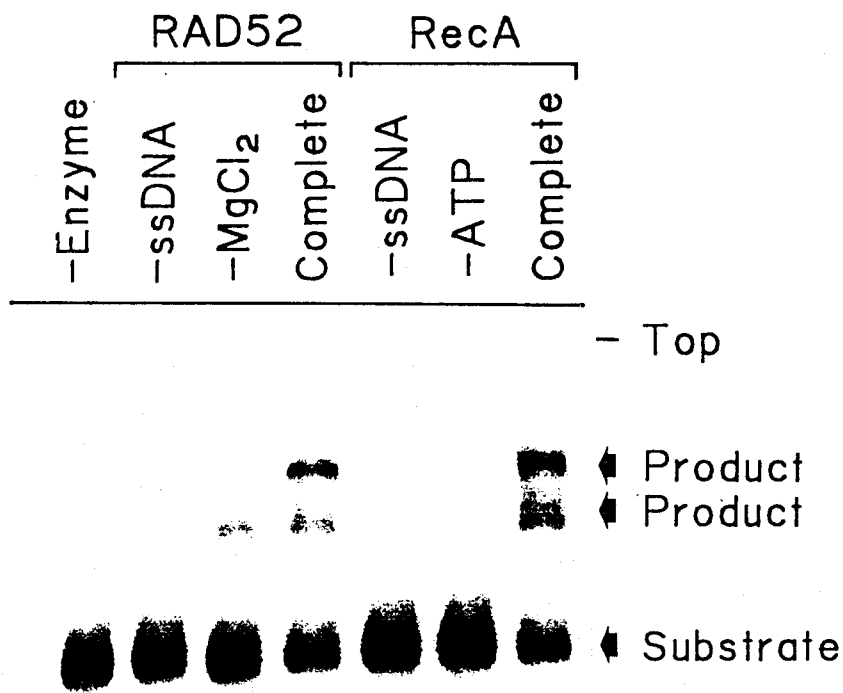
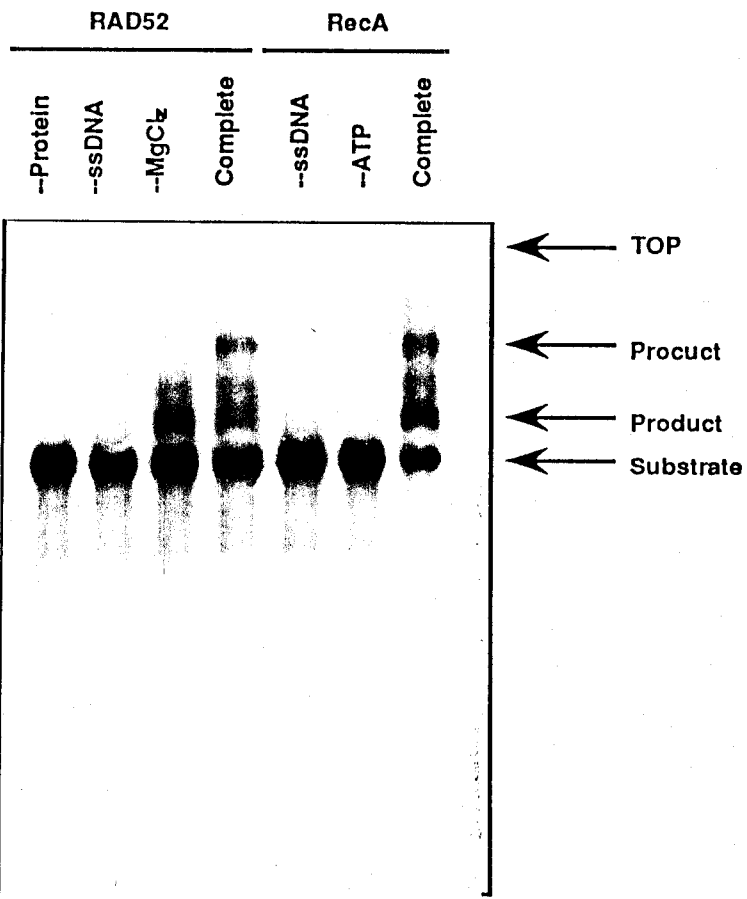


Figure 3. Strand transfer reaction with 1.3kb ds DNA. Strand transfer reactions were performed as described in Materials and methods or Figure legends of Figure 1. The products were analyzed on 0.8% agarose gel containing 0.5 mg/ml ethidium bromide. A. The reaction with 1.3 kb dsDNA fragment. *RAD52* (lane 2 to 4) and RecA protein (lane 5 to 7) were used. 2 mM ATP were added to the reaction with RecA protein. lane 1, the reaction without protein; lane 2, the reaction without m13 ssDNA; lane 3, the reaction omitting MgCl₂; lane 4, complete reaction; lane 5, reaction without ssDNA; lane 6, reaction without 2mM ATP; lane 7. complete reaction.



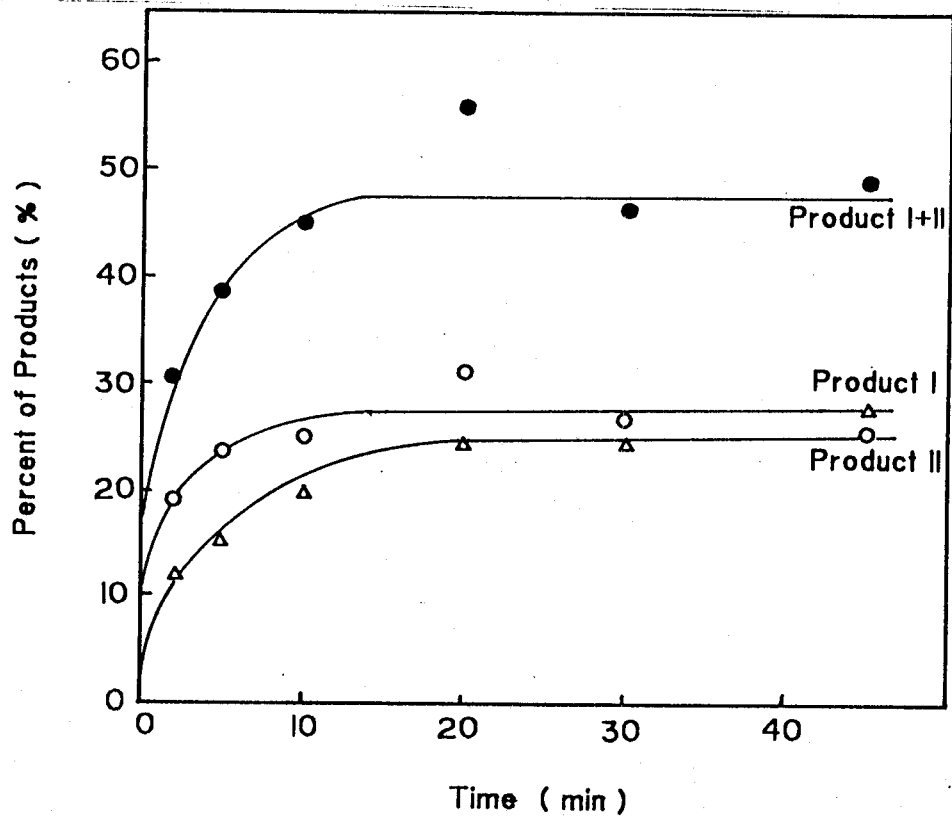


Figure 4. Kinetics of strand transfer reaction. Strand transfer reactions were carried out as described under "Materials and Methods". Reaction was analyzed on agarose gel, and then the gel was dried and autoradiographed. The density of the band on the autoradiographed film were quantified using Molecular Dynamics as under "Materials and Methods". Two products were detected in the reaction. The upper band is called Product I while the lower one Product II. For each product and both products, kinetics was determined.

ssDNA.

Effect of the ratio of *RAD52* to ssDNA on strand transfer

The effect of the protein:ssDNA ratio on the strand-transfer effected by *RAD52* protein was studied(Figure 5). When the ssDNA was exceeded *RAD52* protein, a little products was formed especially in the assay with 7.2kb, showing the stoichiometrical binding of the protein to ssDNA is crucial in this reaction and this reaction is not enzymatical. At the excess condition of the protein to ssDNA, a small amount of the inhibition of the reaction was observed. This might be due to the binding of *RAD52* protein to dsDNA in excess condition, which might prevent the interaction of the *RAD52*-ssDNA complex with dsDNA. Same curve were obtained both in the reaction with various amount of protein and constant amount of ssDNA and in one with various amount of ssDNA and constant of the *RAD52* protein. These results suggest that presynaptic phase; formation of *RAD52*-ssDNA complex is important for homology search step.

Effect of the ion or nucleotide on the strand transfer by *RAD52*

Some factors or condition are crucial to *in vitro* recombination reaction, seen in the other system. ATP, not ATP hydrolysis, is required for RecA reaction(Menetski et al., 1990; Rosselli et al., 1990). SSB or the aggregation of the DNAs is necessary to STP- α and - β (Hamatake et al 1988; Dykstra et al., 1990).

In the absence of Mg^{2+} ion, the strand transfer did proceeded in the *RAD52* reaction, although less than the presence of Mg^{2+} ion. 5-10mM Mg^{2+} ion accelated the reaction 2-3 folds, while more than 12mM inhibited the reaction drastically(Figure 6). Other bivalent ion such as Mn^{2+} , Ca^{2+} had no apparent effect on *RAD52* strand transfer activity. This property is clear difference from other syatem(Figure 7).

Nucleotide like ATP or GTP also didn't affect the strand transfer reaction. This is good agreement with that nucleotide binding sequence found in all of ATPase and GTPase was not observed in *RAD52* protein Same property is detected in other

In salt titration experiment, first the *RAD52*-eDNA complex was formed as described above and NaCl was added to the mixture monitoring at 400nm. The effect of the dilution by the addition of NaCl and increase of fluorescence of eDNA by salts was complemented by the calculation.

Annealing assay

Assay was carried out 10 μ l as described for the strand transfer assay. The reaction contained 20mM Tris-HCl(pH7.6), 1 mM DTT, 5mM MgCl₂, 5 μ M M13mp18DNA end labeled with ³²P with Klenow fragment, which had been denatured by boiling for 3 min and quenching on ice. The DNA was used immediately after the preparation. After the addition of the protein, reaction was incubated for 5 min at 30 C, unless otherwise stated.

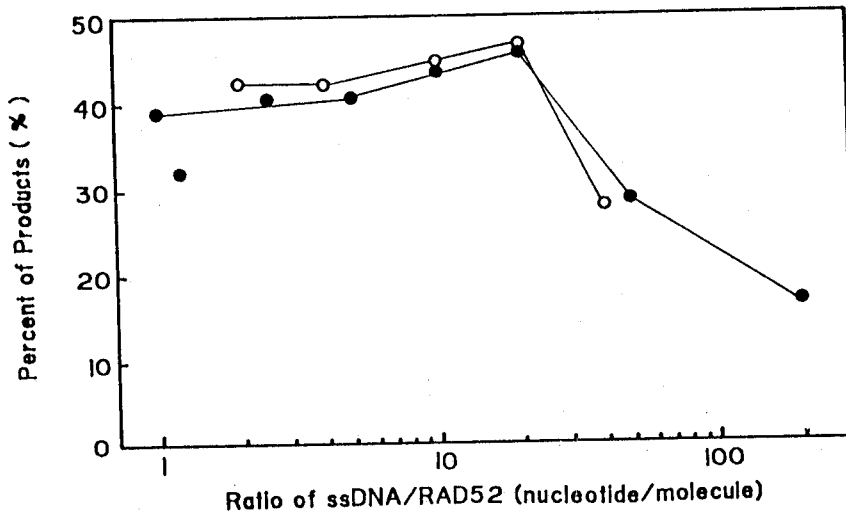
For analyzing products on agarose gel electrophoresis, 10mg/ml proteinase K and 10% SDS was added to 1mg/ml and 1%, respectively, and the reaction mixture was kept at room temperature for 20 min. Then 1/10 volumes of 0.25% bromophenol blue, 0.25% xylene cyanol, 50 mM EDTA, 20%(V/V) glycerol was added, and each samples were analysed by the electrophoresis through an 0.8% agarose slab gel run in buffer 1/20X Tris-borate and 0.5mg/ml ethidium bromide. After running at 1.5V/cm for 12hrs, the gel was dried and autoradiographed.

Aggregation Assay

The reaction was carried out the same condition as strand-transfer assay described above. After the incubation at 37 C for 5min, 30 μ l of reaction mixture was centrifuged at 16000g for 2min at room temperature. Two sequential aliquots of 8 μ l was taken from the supernatant and the pellet was suspended in 10 μ l of 1%SDS. The amount of labeled DNA in each aliquots of supernatant and the suspended materials was counted in liquid scintillation counter. The amount of the total supernatant (30 μ l) was estimated from the average counts of the first two aliquot of supernatant. In every experiment, the recover of labeled DNA in the supernatant and pellet was 85% or

greater, Residual count on the wall of the tube was less than 10%.

1. dsDNA:1.3kb RI-Xmnl



2. dsDNA:7.2kb RI

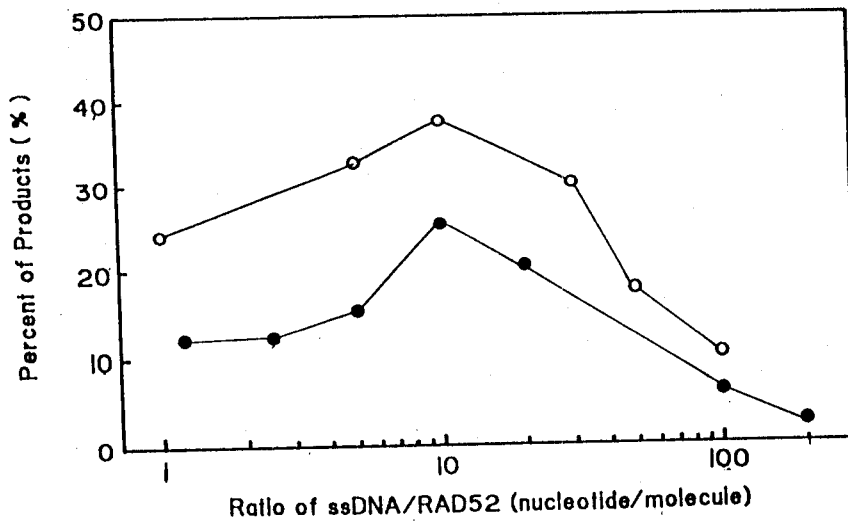


Figure 5. Effect of protein : ssDNA ratio on strand transfer. Experiments were performed varying the ssDNA and *RAD52* concentration. Assays were carried out in the presence of 1 μ M *RAD52* protein and variable amount of M13mp18 ssDNA (Open circle) and in the presence of 10 μ M M13 ssDNA and various concentration of *RAD52* (solid circle). Be careful that the logarithm represents logarithm of the ratio of nucleotide to *RAD52* concentration. A. the reaction with 1.3 kb dsDNA. B. The reaction with 7.2kb duplex DNA.

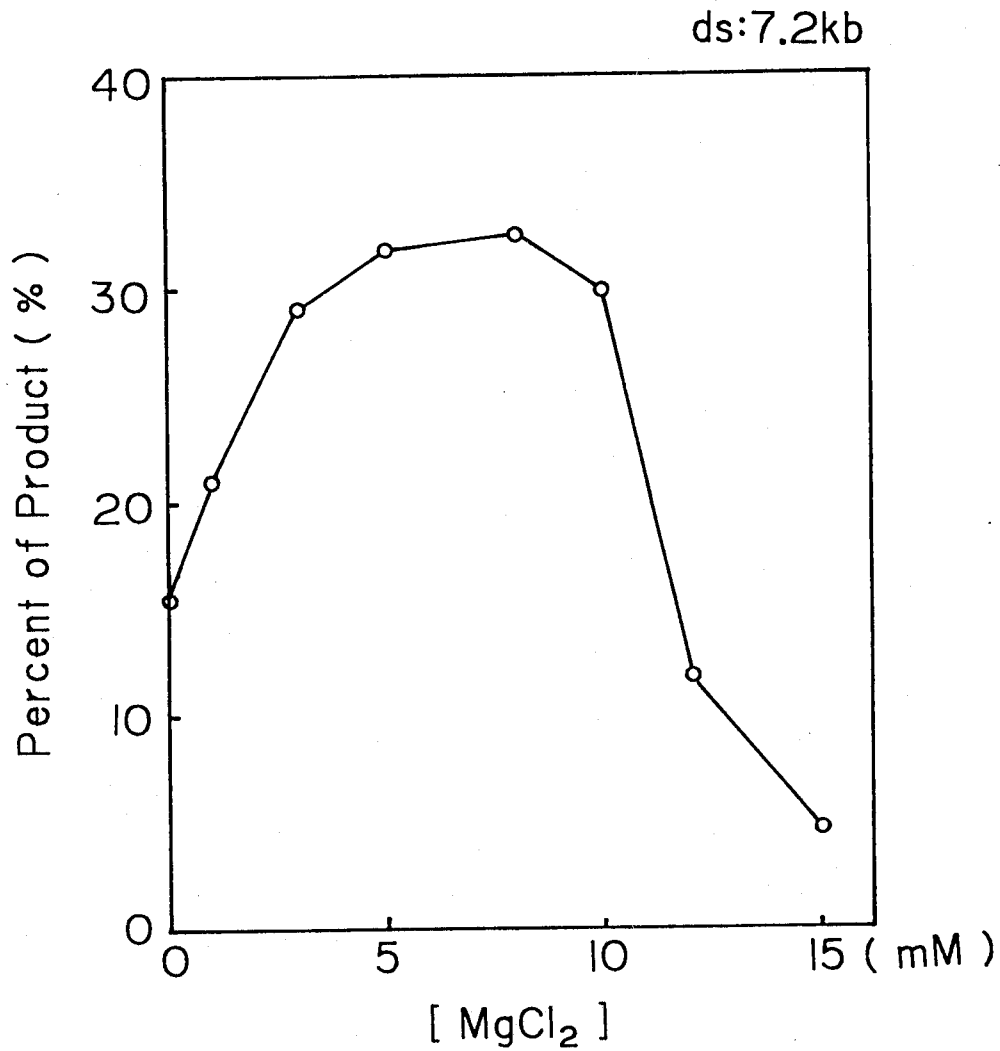


Figure 6. Effect of $MgCl_2$ on strand transfer. The assays were performed varying $MgCl_2$ concentration. The 10 μ l of reaction mixture contained 20mM Tris-HCl(pH7.6), 1mM DTT, 1 μ M *RAD52*, μ M M13 mp18 viral DNA, 0.5 μ M ^{32}P -labeled 7.2 kb of M13 duplex DNA and various concentration of $MgCl_2$. The percent of products against input DNA were determined measuring the density of the autodiagramed film with Molecular Dynamics.

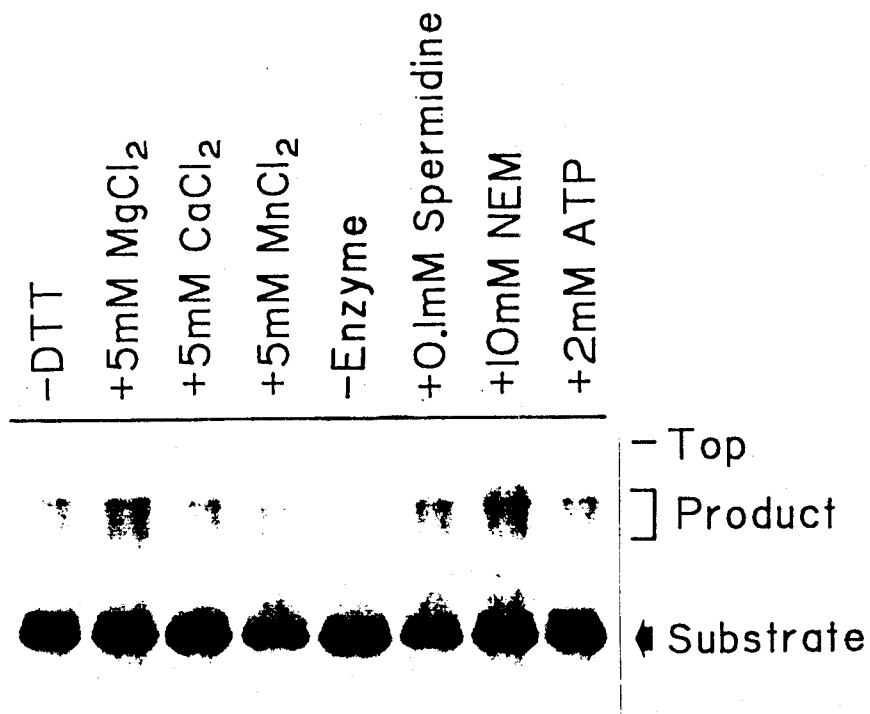


Figure 7. Property of strand transfer. Strand transfer reactions were carried out varying only the parameter indicated. Standard condition was described in lane 2. lane 1, the reaction without DTT; lane 2, complete reaction; lane 3, the reaction with 5mM CaCl₂ instead of MgCl₂; lane 4 reaction substituted 5mM MnCl₂ for MgCl₂; lane 5, the reaction with 5mM ZnSO₄ instead of MgCl₂; lane 6 the reaction supplemented with 0.1 mM spermidine; lane 7, the reaction containing 10 mM NEM; lane 8 the complete reaction containing 2 mM ATP.

eukaryotic strand-transfer system.

ssDNA binding property of *RAD52* protein - ϵ DNA binding assay-

RAD52 protein seems to interact with ss/dsDNA in the strand transfer reaction. To elucidate the protein complex with DNA, the ssDNA binding ability was monitored with ϵ DNA, which is the modified ssDNA that emit 400nm of fluorescence when excited by 300nm light. The intensity of the fluorescence of DNA varies depending upon the interaction with protein. For instance, the relative fluorescence increase to 2-3 folds when *E. coli* RecA protein binds to ϵ DNA (Silver and Fersht 1983; Menetski and Kowalczykoski 1985). The alternation of the fluorescence is thought to reflect the conformation of the bases in DNA-protein complex. More bases are stacking each other, more the fluorescence increase and vice versa.

When *RAD52* protein was mixed with ϵ DNA, the increase of the relative fluorescence was observed in proportion to the *RAD52* protein concentration (Figure 8). The increase reached to the plateau at a given concentration of *RAD52* protein, suggesting that the binding of *RAD52* protein to ϵ DNA is stoichiometrical. From the saturation point of the curve, the binding stoichiometry was calculated to 9.6 nucleotide per one *RAD52* protein. This value corresponded to the optimum ratio of *RAD52* protein to ssDNA in the strand transfer in which 10mM M13 viral DNA versus 1mM *RAD52* protein, indicating ssDNA complex not dsDNA complex is an important structure in the *RAD52* recombinase activity .

The presence of Mg^{2+} ion enhanced the relative fluorescence from 2.1 to 2.5 (Figure 9), but did not alter the binding stoichiometry. Thus Mg^{2+} ion might stimulate the conformational change of the *RAD52*- ϵ DNA complex so that bases of DNA are aligned more regularly

The stability of *RAD52*- ϵ DNA complex Salt titration experiment

To study the stability of *RAD52* - ϵ DNA complex, the salt, NaCl, was added to the reaction mixture after the formation of the complex. The decrease of the relative

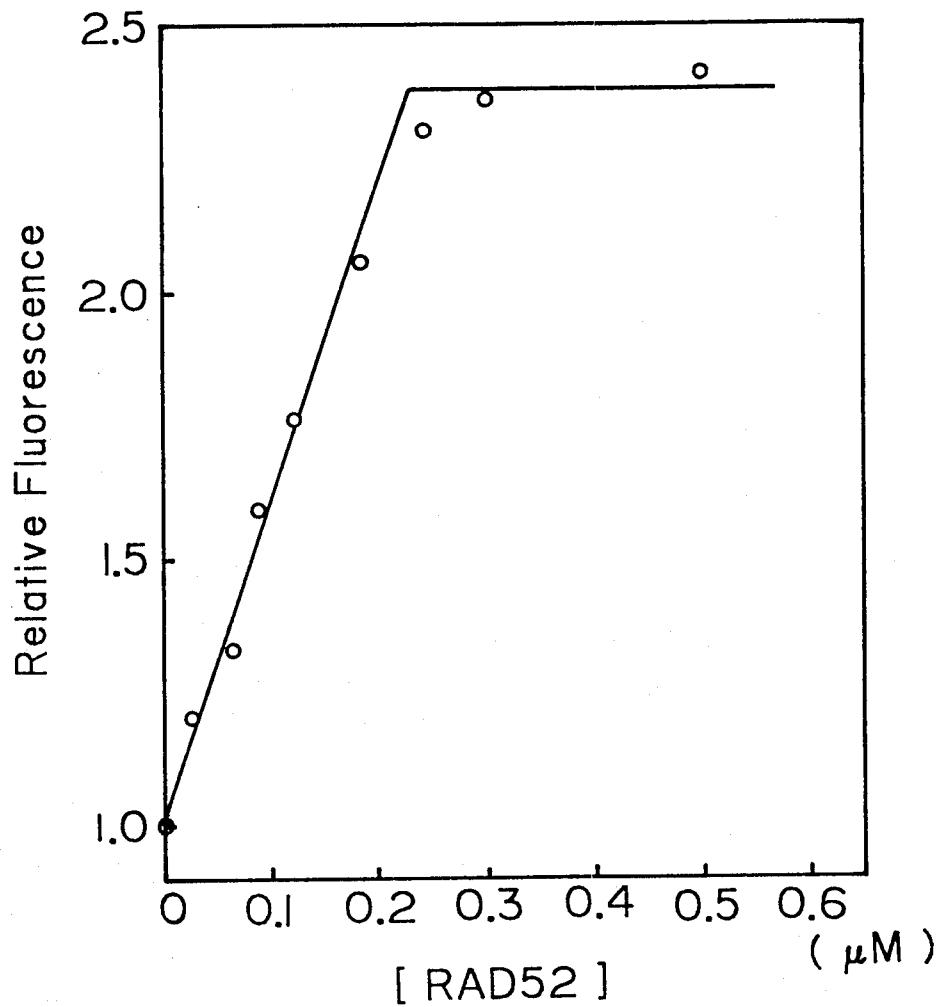


Figure 8. Titration of ϵ DNA by *RAD52* protein. 1 ml of reaction mixture contained 20mM Tris-HCl(pH7.6), 1mM DTT, 10mM MgCl_2 , 2.4 μM ϵ DNA and variable concentration of *RAD52* protein. The reaction was initiated by the addition of ϵ DNA. Until the increase of 400nm of fluorescence was saturated, the measurement continued, taking 30 seconds. The relative fluorescence was calculated by simply dividing the intensity in the presence of *RAD52* protein by that of the absence of the protein. Site size was calculated from the saturation point of the titration curve.

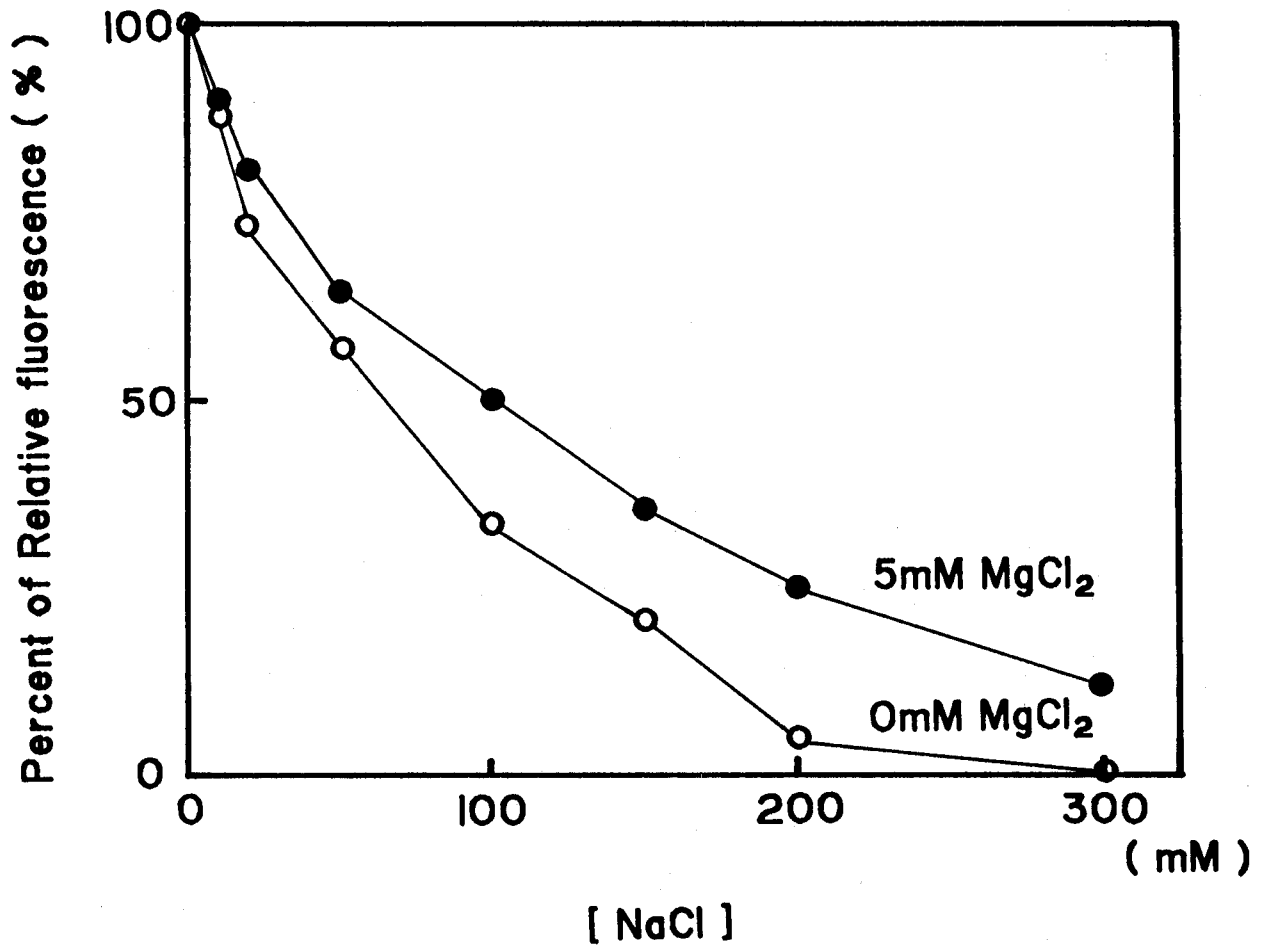


Figure 9. Salt titration experiment of *RAD52*- ϵ DNA complex. The stability of the *RAD52*- ϵ DNA complex was measured by the observing the decrease of the fluorescence in the presence of NaCl. The *RAD52*- ϵ DNA complex was formed at the ratio of 10. Various amount of NaCl was added to the reaction and the fluorescence was measured. The relative value was complemented by value of the increase of the fluorescence of ϵ DNA in the presence of various concentration of NaCl and the dilution by the addition of NaCl solution.

fluorescence was observed depending on the salt concentration(Figure 9). The titration midpoint of the complex was 80mM in the absence of MgCl₂ and 150mM NaCl in the presence of MgCl₂. Mg²⁺ ion stabilize the *RAD52*-εDNA complex, which might reflect the conformational change of *RAD52* protein by the ion(See above).

Renaturation of homologous complementary DNAs by *RAD52* protein

Dissociation(denaturation) of duplex DNA and reassociation are thought to be a part of pathway through strand transfer reaction. Most of the proteins known as strand transferase carry out annealing *in vitro*, which is association between ssDNAs; RecA(Bryant et al., 1989), UvsX(Yonesaki et al., 198), *SEPI*(Heyer et al., 1988) and the protein purified by Halbook and McEntee(1990).

The annealing reaction was examined for the *RAD52* protein. Heat denatured linear ³²P end-labeled M13mp18 was used as substrate. The reaction products were analysed on the agarose gel electrophoresis. Figure 10 shows kinetics experiments of annealing reaction.heat denatured ssDNA was detected as a broad bands on the gel due to the fragmentation by heat-up. In some experiments, they were too diffusible to detect. *RAD52* protein promoted the formation of products that didn't enter the gel and was trapped in the well of the gel since presumably they were large aggregates. Most of the DNAs in the products were thought to pair with between homologous sequence since the products were S1 nuclease resistant(Our unpublished results). Same product were produced by RecA protein. The formation of the products was rapid, completed within 10 minutes at 30 C. The intermediate, like full length duplex DNA, was not detected, which was observed in RecA mediated reaction(Bryant et al., 1989). It's thought that the lackness of the intermediate in this reaction is due to the variety of substrate rather than the characteristics of *RAD52* reaction. Another reaction containing heat denatured ssDNA and homologous circle ssDNA showed that the products entering the gel as diffusible bands were formed by *RAD52* protein(See Figure 2). Therefore several steps are considered to exist in the formation of large aggregates.

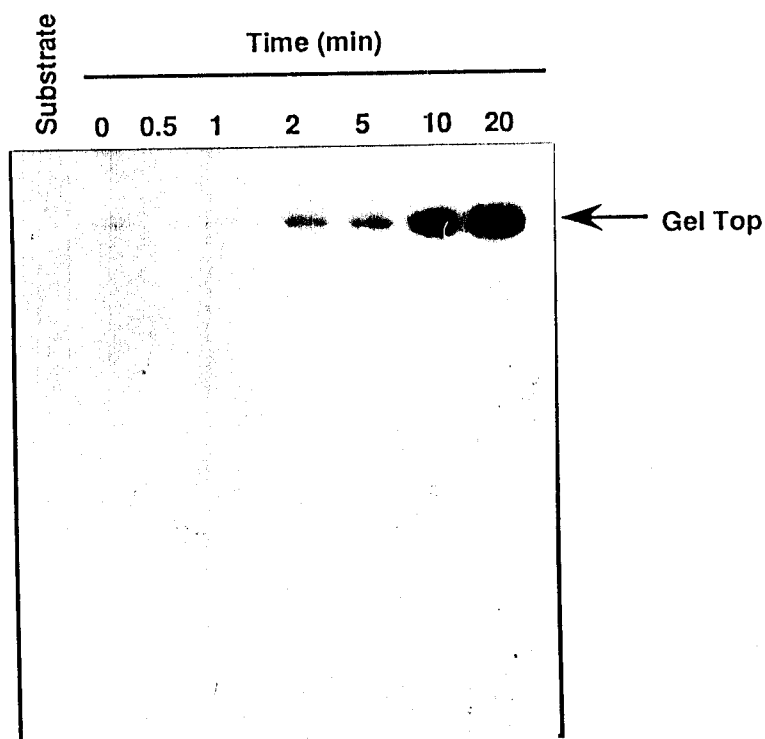


Figure 10. Agarose gel assay of the renaturation. Reactions were carried out as described under Materials and Methods. Reaction contained $1\mu\text{M}$ Heat denatured M13mp18 single-stranded DNA, $1\mu\text{M}$ *RAD52* protein, 20 mM Tris-HCl(pH7.6), 10 mM MgCl_2 , 1 mM DTT. Proteins were added last to initiate the reaction. At indicated time, 10 ml of aliquots were quenched with 1% SDS and 1 mg/ml proteinase-K and then subjected to electrophoresis through 0.8% agarose. lane1-8 are 0, 1, 2, 5, 7, 10, 15, 20 min reaction time points. The position of the well is indicated.

Aggregation of ss/dsDNAs by RAD52 protein

During the strand transfer reaction, *RAD52* protein is thought to interact with both ss and dsDNA. To detect *RAD52*-ss-ds DNA complex, the aggregation of DNAs by *RAD52* protein was examined, since *RAD52* protein forms large aggregate in itself; *RAD52* protein eluted in void fraction through Sephacry S-300 gel filtration even in the high salt condition with 1M NaCl(Unpublished result).

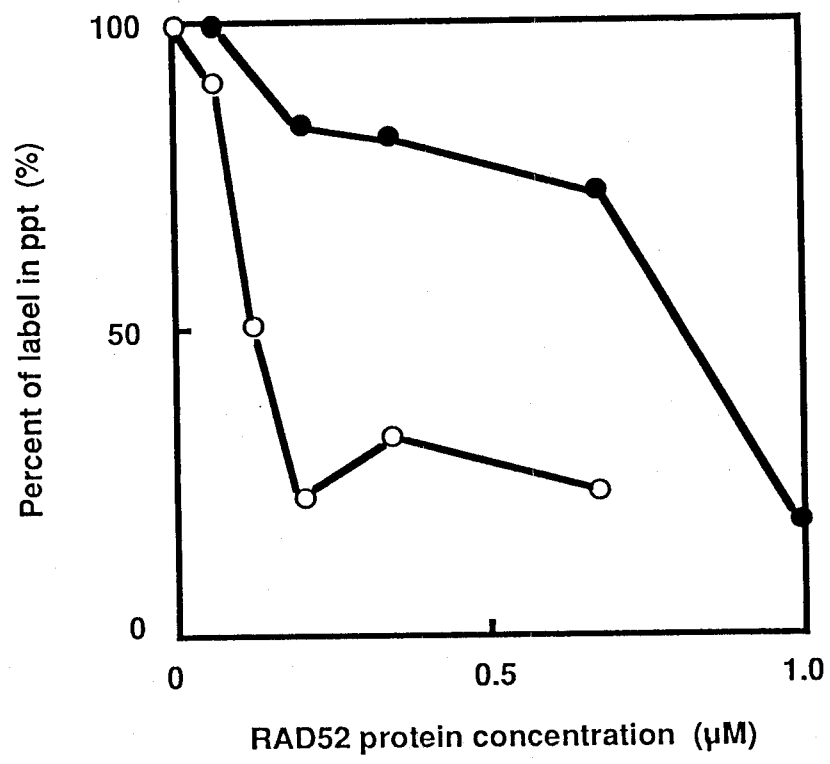
The aggregate was easily sedimented with brief high speed centrifugation as seen with RecA presynaptic filament(Tsang et al., 1985) and γ SSB(Hamatake et al., 1989).

When the *RAD52* protein was incubated with 32 P-labeled dsDNA, the dsDNA was recovered as pellet after the centrifugation even in the low concentration of the protein(Figure 11 A). When the ssDNA was added to the reaction, the complete uptake of dsDNA into the aggregate required more amount of *RAD52* protein than in the dsDNA alone. *RAD52* protein can associate with ssDNA more tightly than dsDNA in the presence of Mg^{2+} ion. It therefore is thought that *RAD52* protein primarily forms complex with ssDNA and later this complex(aggregate) uptake dsDNA in. For complete sedimentation of dsDNA, more than 0.8 mM of *RAD52* protein was required. The maximal strand transfer by *RAD52* protein proceeded in around 0.8-1.0 mM *RAD52* concentration in the same condition. It might be that the aggregation of dsDNA by *RAD52*-ssDNA complex is prerequisite for the optimum strand transfer.

The same tendency was observed in the assay using high $MgCl_2$ concentration(Figure 11 B). At 15mM of $MgCl_2$ more than 1 mM *RAD52* protein was necessary for complete precipitation of duplex DNA. In the same condition strand transfer was inhibited(See Figure).

The aggregation by *RAD52* protein didn't require the homology between ds and ssDNA. In Figure 11 B the ds DNA was linearized pBR322, heterologous to M13mp18 viral ssDNA while in Figure 11 A. dsDNA was homologous M13 mp18 duplex DNA.

A.



B.

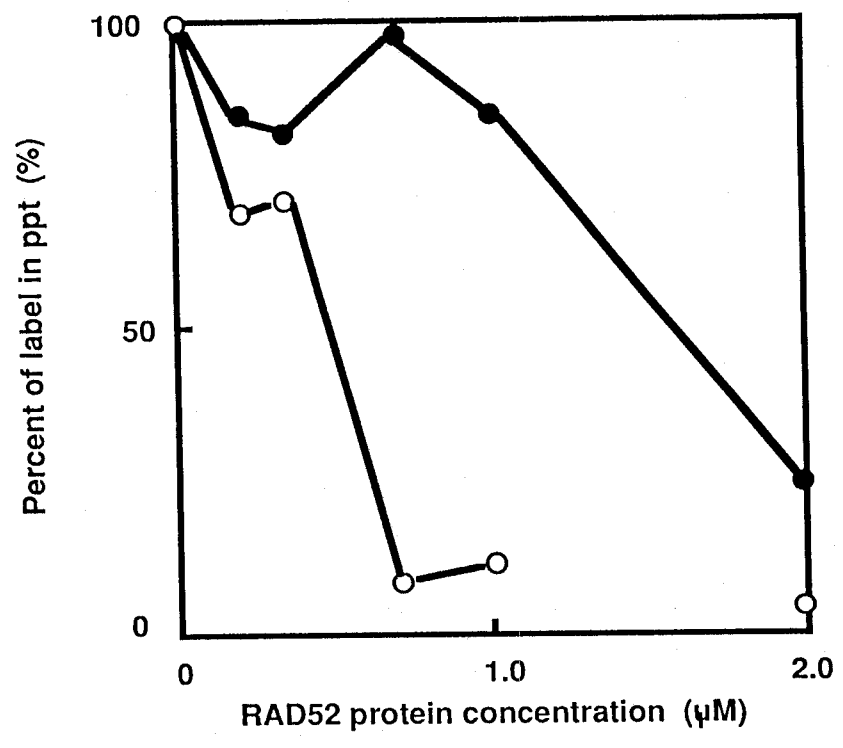


Figure 11. Aggregation of ss/dsDNA; effect of *RAD52* protein concentration. A), Aggregation of dsDNA. Reactions were performed in 0.5 μM ^{32}P -labeled M13 mp18 ds DNA under standard condition described under Materials and Methods in the presence(closed circle) or in the absence of(open circle) 10 μM of homologous M13mp18 viral ssDNA. The reaction mixtures were incubated for 10 min at 30 C and centrifugated for 3 min at 16,000rpm. The aliquots were withdrawn , put on the filter paper, dried, and the radioactivities were counted. B) Effect of MgCl_2 on aggregation of dsDNA. Reactions were performed with 10 μM M13mp18 viral ssDNA and 0.5 μM ^{32}P -labeled heterologous pBR322 dsDNA and various amount of *RAD52* protein. The concentration of MgCl_2 was 5 mM(open circle) and 15 mM(closed circle). The aggregate was assayed after the incubation for 10 min at 30 C.

Discussion

Here we describe the *in vitro* activities of the *RAD52* protein purified from *E. coli*. Most importantly, the *RAD52* protein performs the strand-transfer reaction and DNA-pairing reaction (annealing) *in vitro*, suggesting the *RAD52* protein is involved in the homology search between DNAs in genetic recombination. This is supported by genetical studies of *rad52* mutant.

The *rad52* mutant has shown to be defective in mitotic gene conversion, mitotic crossing-over, meiotic conversion, meiotic crossing-over, mating type switching and the repair of the double-strand break by X-ray or γ -ray. *In vitro* recombination analysis using yeast cell lysate has shown that the recombination between linear and circle duplex plasmid reduced 5-6 folds in the *rad52-1* mutant lysate (Symington et al., 1983).

Recent physical analysis of mating type switching, initiated by double strand break created by HO endonuclease, have indicated in *rad52* mutant the process stops at the intermediate stage after the processing of the double strand break (White et al., 1990). The authors suggest *RAD52* gene product works in the search of DNA homology or the repair synthesis of DNA. The former idea corresponds to our *in vitro* analysis of *RAD52* protein.

Neither ATP nor Mg^{2+} ion is required for the *RAD52* mediated strand transfer reaction. Mg^{2+} ion-independence simply reflects the fact that the *RAD52* protein binds ss/dsDNAs *in vitro* in the absence of the ion. The annealing activity by *RAD52* protein also doesn't require this divalent cation.

Some reports have indicated that there is significant residual recombinations in the absence of the *RAD52* gene (Resnick et al., 1986), suggesting either that another mechanism of the homology search exists in yeast, which does not require the *RAD52* gene or that the *RAD52* does not have a central role but a modified/regulatory role in genetic recombination. In *rad1 rad52* double mutant, ectopic recombination reduces synergistically than single mutations, supporting the idea that the major pathway in

yeast is dependent on the *RAD52*, the other minor one is not on the *RAD52* but on the *RAD1* gene. The *RAD1* protein has been cloned and sequenced, but there is little homology *RAD1* and *RAD52* or RecA protein(Reynolds, 1987).

Four strand-transferases have been identified from *S. cerevisiae*. All proteins don't require ATP in the reaction, but the reaction is dependent on Mg^{2+} ion. Molecular weight of *SEP1*(Koladner et al., 1988), *STP β* (Dystra et al., 1990) and the protein purified by Halbook and McEntee(1990) is 132k, 180k, 120k dalton, respectively, much larger than the predicted molecular weight of the *RAD52* protein(Adzuma et al., 1984). A smaller protein than the *RAD52* protein is *STP α* , whose molecular mass is 38kDa. *STP α* is different from the *RAD52* in DNA sequence as well as the requirement of strand transfer reaction(Sugino, personal communication). Therefore all strand transfer proteins are not *RAD52* gene product. The *RAD52* protein is the first strand transfer protein in eukaryote, which genetically has been proved to play a crucial role in the genetic recombination. These proteins are likely to be engaged in *RAD52*-independent pathway of recombination in yeast.

Is the strand transfer activity intrinsic to *RAD52* protein? Only 10-50% not 100%, input double strand DNA is converted to the product, while 100% input DNA is to the products in the other system. Since in this study *RAD52* protein has been purified from *E. coli*, some modification like glycosylation or phosphorylation in yeast is lacking for *RAD52* to perform complete functions. Or another protein is required for the reaction. Genetic analysis has indicated at least *rad51* and *rad54* mutant is deficient in the most type of recombination in yeast like *rad52* mutant. The similar situation was seen in the other system. For RecA and UvsX protein, the completion of strand transfer requires SSB and gp32, respectively(). Especially in T4 system UvsX, UvsY and gp32 behave like recombination machinery, forming the ternary complex(Yonesaki et al., 1989). Partially purified *RAD52* protein from yeast, although contaminated by exonuclease, carried out annealing reaction; homologous pairing of ssDNA(Unpublished result). At least DNA pairing activity is intrinsic to *RAD52* protein.

RAD52 protein bears strong annealing activity. The strand transfer activity of *RAD52*

protein might simply reflect on this annealing activity of this protein due to the contamination of exonuclease. But this is less possible as follows. When the *RAD52* protein is mixed with only dsDNA, neither denaturation of duplex DNA detected by the agarose gel electrophoresis nor any degradation of dsDNA whose ends were end-labeled to detect nuclease easily was observed in the same time range of strand transfer reaction. *RAD52* strand transfer activity is independent of Mg^{2+} ion, which is necessary for all known exonuclease action, including contaminating nuclease, whose activity is detected more than 5 hrs incubation.

Several common properties are seen between *RAD52* and RecA protein than other strand-transferase. Like RecA protein catalyzed reaction, the ratio of protein to ssDNA is crucial for the reaction. Other proteins are enzymatical rather than stoichiometrical. For example, only one molecule to one ssDNA is enough for STPa and STPb to carry out strand-transfer (Sugino et al 1988; Dykstra et al., 1990), while one *RAD52* to 10 nucleotide is necessary to perform the reaction at maximal. *RAD52* promote the aggregation of ssDNAs and the coaggregation of dsDNAs like RecA, SEP1 and the protein identified by Halbrook and McEntee (1990). For STP α and STP β the aggregation by γ SSB is essential to complete the reaction (Hamatake et al, 1989). The aggregation of DNAs might be an important step in the homology search since all known strand-transferase require the aggregation in the in vitro recombination reaction.

The most different characteristics of *RAD52* from RecA protein is the independence of ATP in the strand transfer. For RecA protein ATP hydrolysis is not essential for strand transfer (Menetski and Kowalczykowski 1990; Rosselli and Stasiak 1990), but RecA still requires ATP in the reaction to make the protein high affinity form to DNA. The DNA binding of *RAD52* protein is weak. The salt titration mid-point is 100mM and the binding is not cooperative. In yeast there are several SSBs (Jones et al., 1982) which bind DNAs tightly. How does *RAD52* protein overcome the competition by SSBs? Recent study of *RAD51* protein, which is thought to work on the same stage with *RAD52*, shows *RAD51* protein is homologous to RecA protein and binds

ss/dsDNA in the presence of ATP. Affinity chromatography of *RAD52* indicate this column retains *RAD51* protein, showing *RAD52* protein forms a complex with at least *RAD51* protein. High affinity state of *RAD52* protein to ssDNA might not be mediated by itself but through *RAD51* and other protein. The characterization of *RAD51-52* complex is necessary for the elucidation of the molecular mechanism of homology search between DNA.

Materials and Methods

Construction of plasmids

pRS52S1 is a pKH502(Shirakawa et al., 1984) based vector, in which the *RAD52* gene is under the control of *recA* promoter.

To locate 3rd ATG of *RAD52*(Adzuma et al.,1984) downstream of SD sequence, Synthetic DNA 64mer and 63mer which contained SD sequence and 3rd ATG to BstEII site of *RAD52* gene was replaced with partially digested BamHI, BstEII fragment of pRS52sz3, containing BstEII, BamHI kb fragment of *RAD52* gene and 3 kb fragment of LacZ gene from pMC871(Casadaban et al., 1984), fused in frame and inserted in BamHI site of pKH502.

pRS52S1 was made by the replacement of the BglII, Sall fragment of pRS52sz3 with the 1.8kb BglII, Sall fragment containing intact C-terminal portion and 3'-flanking sequence of *RAD52* gene from (Adzuma et al., 1984).

pRS52m5 was constructed by ligating the Sall-filled-in, PstI partially digested kb fragment containing *recA* promoter and *RAD52* gene from pRS52S1 and PstI, HaeIII digested 156 bps of fragment from pRS52S1.

Purification of *RAD52* protein from *E. coli*

594 harboring pRS52m5 were grown in 6 liters of L-broth containing 50mg/ml Ampicilline. At 4×10^8 cells/ml, Nalidixitic acid(10mg/ml in 0.1M NaOH) were added to final 50 μ g/ml and the culture continued further 3 hrs.

Cells were harvested and suspended in 50 ml of buffer A(25mM Tris-HCl(pH8.0), 1mM PMSF, 25%(W/V) Sucrose). When the cells were not used immediately, the cells were frozed in liquid N₂ and stored at -70C. All steps were carried out at 4 C otherwise mentioned. 10mg/ml Lysozyme were added to the cell suspension to final 0.6mg/ml for the cell lysis and the lysate was kept on ice for 20min for the complete lysis. The suspension was sonicated with Sonicator(), and centrifuged at 27k rpm for 30min in Beckman Type 30 rotor. The OD₂₆₀ of the supernatant was adjusted to

200 with the buffer-A. 10%(V/V) Polyethylene-imine(pH7.5 titrated with HCl) was added to the suspension to final 0.4% while stirring. The suspension was stirred for further 1 hr and centrifugated at 10K rpm for 10min. The supernatant was discarded and the pellet was suspended in buffer B(50mM Tris-HCl(pH7.5), 1mM EDTA, 1mM PMSF) containing 0.3M NaCl. The supernatant was recovered by the centrifugation at 10K rpm for 10min. Solid Ammonium sulfate were added to the supernatant to final 30% saturation(1.76 mg/ml) with stirring. After stirring for 1hr, the pellet was collected by the centrifugation at 13k rpm for 20min, dissolved in the P-buffer(20mM Phosphate(pH6.5), 1mM EDTA, 5mM β -mercapthaethanol, 10%(V/V) Glycerol) containing 0.2M NaCl and dialyzed against the same buffer for over-night with 3 times buffer exchange.

The dialysate was directly applied onto the Phospho-cellulose(P11 Whatman) pre-equilibrated with the P-buffer supplemented 0.2M NaCl. After the extensive washing, run at the speed of 24ml/hr, the column was washed with 0.2M-1.0M NaCl gradient. The *RAD52* protein was eluted from the column at 0.25-0.3M NaCl concentration. The fraction containing the *RAD52* protein were pooled. Ammonium sulfate was added to the fraction to final 50% saturation(3.13 mg/ml) with stirring. The pellet were collected by the centrifugation and dissolved in TEM-buffer containing 1M NaCl.

The solution was directly loaded onto Sephacryl S300 column(1.6cm X 90cm) previously equilibrated with the TEM-buffer containing 1 M NaCl. The column was run at the speed of 16ml/hr. The void fractions containing the *RAD52* protein was collected.

The pooled fractions were directly applied onto Hydroxy-apatite(Seikagaku-Kogo) column pre-equilibrated with P-buffer omitting EDTA. Washing extensively, the column was chromatographed with 20mM-300mM Phosphate buffer gradient. *RAD52* protein was eluted at 80-100mM Phosphate. The fraction was pooled and dialyzed against the Ammonium sulfate saturating buffer(50mM Tris-HCl(pH8.0), 1mM EDTA, 5mM β -mercapthaethanol, 5.6 mg/ml(80% saturation) Ammonium sulfate) for over-

night. The pellet of the protein was recovered by the centrifugation and dialyzed against the TEM-buffer containing 0.2 M NaCl for at least 5hrs with 3 times buffer exchange.

The dialysate was diluted with TEM-buffer to the concentration of NaCl 0.05M, and loaded onto the MonoQ HR5/5(Pharmacia) column pre-equilibrated with TEM-buffer at the speed of 0.5ml/min. The protein was eluted from the column with the gradient of 0.1-0.45M NaCl at 17.5mM Cl/ml. The *RAD52* protein was eluted from the column around 0.25M NaCl concentration. The fractions were subject to the ammonium precipitation. The pellet was dissolved with TEM-buffer containing 0.2 M NaCl, dialyzed against the same buffer and subsequently dialyzed against the stock buffer(50mM Tris-HCl(pH7.5), 1mM EDTA, 1mM DTT, 0.2M KCl, 50%(V/V) Glycerol).

The concentration of *RAD52* protein was determined by measuring the the extension coefficient. ϵ_{280} of *RAD52* protein was 22040.

Strand-transfer Assay

Strand-transfer assay was assayed as described(McCathy et al., 1988). ^{32}P labeled dsDNA was prepared by the end labeling with Klenow-fragment(TAKARA). The labeled DNA was purified either by the spun-column(Bio-Gel P100, Bio-Rad) or the agarose gel electrophoresis, following the PhOH/CH₃Cl treatment and subsequent alcohol precipitation.

Standard reaction was carried out in 10 μl volume in small siliconized Eppendorf-tube under the following condition; 20mM Tris-HCl(pH7.6), 1mM DTT, 5mM MgCl₂, 40mM NaCl, 10 μM M13mp18 viral DNA, 1 μM End-labeled dsDNA(7.3kb, EcoRI digested M13mp18, 2.3kb EcoRI, SnaBI digested DNA or 1.3kb EcoRI, XmnI digested DNA from M13mp18).

Reaction was started by the addition of the *RAD52* protein and incubated at 37 C for 20 min. Reactions were terminated with 1ml of 10%(W/V) SDS and 1 μl of 1mg/ml Protease-K(Merk). They were incubated for 30 min at room temperature.

The reaction was analyzed by 0.8% agarose gel electrophoresis. Gel was run at

15V for 16hrs or 30hrs, dependent on the length of dsDNA used in the reacton. After electrophoresis, the gel was soaked in 50%(V/V) Ethanol, following soaking in consecutive 70, 90 and 100% Ethanol for each 20min for fixation. The gel was dried and autoradiogramed.

Quantication was analysed by Lazer-sccaner Molecular-Dynamics(Tommy). The density of the bands on the film was measured. Percent of the products was calculated by simply dividing the value of the product with that of the sum of the substrate and the products.

Preparation of etheno-DNA

Heat denatued calf thymus DNA was reacted with chloroacetaldehyde by a method similar to that described by Kryzosiak(1981). The reaction mixture, consisting 2M chloroacetaldehyde, 0.5mg/ml heat denatued calf thymus DNA, 20mM NaCH₃COO(pH5.5), was allowed to react for 4.5hr at 40C and then dialysed against 10mM Tris-HCl(pH7.5),1mM EDTA at 4C overnight to mininimize the degradation. The ethenoDNA was further dialyzed against 10mM Tris-HCl(pH7.5), 1mM EDTA for 7 hrs at 50 C to mature the derivatized bases. After the dialysis, the DNA was precipitated with ethanol and dried up in vaccum evaporator and suspended in 10mM Tris-HCl(pH7.5), 1mM EDTA.

The concentration of ethenoDNA was detemined from the molecular extension coefficient; $E_{260} = 16000M^{-1}cm^{-1}$, and the titration by E. coli RecA protein; The ratio of RecA protein to ethenoDNA is 4.7(Menetski and Kowalczykowski, 1985).

Fluorescence titration

Titration data was obtained using Shimazu RF5000 spectrophotometor. The assay was carried out in 1ml of reaction mixture; 20mM Tris-HCl(pH7.6), 1mM DTT, 5mM MgCl₂, 1 μ M etheno calf thymus DNA at 25C. ethenoDNA fluorescence was measured by exciting at 300nm and monitoring at 400nm. The reaction was initiated by the additon of ϵ DNA.

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Chapter III

Biochemical analysis of the RAD51 protein and its interaction with RAD52 protein.

Summary

The *RAD51* gene in *Saccharomyces cerevisiae*, one of *RAD52* epistasis group genes, plays an important role in genetic recombination in yeast. The *RAD51* gene encodes the protein homologous to RecA protein. *RAD51* protein was overexpressed in *E. coli* and purified to nearly homogeneity to study the activities of this protein. Purified *RAD51* protein binds to both ss- and ds-DNA in the presence of ATP. The binding was stoichiometrical and cooperative. *RAD51* interacted with *RAD52* protein *in vitro*, which belongs to the same subgroup as *RAD51* in *RAD52* epistasis genes and bears DNA-pairing activity *in vitro*. The role of *RAD51-RAD52* complex in recombination, especially in homology search between DNAs, is discussed.

Introduction

Genetic recombination consists of several steps, in which many proteins are involved, initiation, the processing of the initiation signal, search for homology between DNAs, branch migration and the resolution of Holliday junction.

Initiation start from different pathway, like ssDNA nick or dsDNA break, while homology search or strand transfer is a crucial common pathway both in recombination and in repair. For instance, in *E. coli*, RecA protein, well-known strand transferase, is involved in the RecBCD pathway, the RecF pathway and repair of the DNA lesions (For review, Mahajan, 1988). Our understanding of the homology search has mainly come from the studies of RecA protein (For review, Cox and Lehman, 1987; Stasiak, 1988).

In eukaryote, the entity of the strand-transferase equivalent to RecA protein as well as the molecular mechanism of the homology search remains to be revealed. There has been many reports for the direct identification of the strand transferase from *S. cerevisiae* (Sugino et al., 1988; Koldner et al., 1987; Dykstra, et al., 1990), *Ustilago maydis* (Kmiec and Hollman, 1982), *Drosophila* (Louwenhaupt et al., 1990), lily (Hotta et al., 1984) and even mammalian cells (Heyer et al., 1988). However, genetical evidences that these proteins are really implicated in genetic recombination have been still missing.

Specific characteristics of recombination of eukaryote is that recombination occurs accompanying ultra-structures. Recombination nodule (RM) is the specific spherical structure seen on synaptonemal complex (SC), which is rod-like structure containing four sister chromatids, consisting of axial (lateral) element and central element in meiosis. Since the position of recombination nodule often coincides with the position of chiasma, which is the crossing-over point between homologous chromosome, recombination nodule is thought to play an important role in meiotic recombination (Carpenter, 1975). Components in SC have been identified recently; *HOP1* (Hollingsworth and Byer, 1990). However the protein in RM is obscure in

addition to its role.

Genetical studies have shed light on the genes as a candidate eukaryotic recombinase and a component of the structure in which recombination occurs. In *S. cerevisiae* *rad52* epistasis group genes have been shown to play an important role in the repair and recombination. Especially both *rad51* and *rad52* is defective in the most type of recombination in yeast, including mitotic, meiotic recombination, mating type switching and the repair of the double strand breaks produced by X-ray (For reviews, Game, 1984; Resnick, 1988). In addition to *RAD52* epistasis, *REC1* and *REC3* genes also are thought to be engaged in the common pathway of recombination (Esposito et al., 1990). In *Drosophila* there are a few candidate which might act on the homology search step, *mei9* and *rec1*, whose mutants show reduced recombination frequency through all chromosome (Baker and Carpenter, 1972; Grill, 1984).

The advent of molecular biology has unravelled the molecular figure of these genes. The *RAD51* gene and *RAD52* gene of *S. cerevisiae* has been cloned and sequenced (Adzuma et al., 1984). *RAD51* protein is significantly homologous to *RecA* protein through the long region spanning 200 amino acids. Also, the gene closely related to *RAD51* gene has been isolated by cloning the cDNA that is most abundant in meiosis (Bishop, personal communication). This *DMC1* gene is thought to work only in meiosis. *DMC1* and *RAD51* gene work in a different steps in meiosis since the phenotype of each mutant is different; *dmc1* mutant can neither enter meiosis first division nor produce spore, while *rad51* mutant produce spores but inviable ones.

In order to know the molecular role of *RAD51* protein in recombination, we studied the properties of the *RAD51* protein purified from *E. coli* in vitro. *RAD51* protein could bind ss/dsDNAs in the presence of ATP. The binding is stoichiometrical and cooperative. Furthermore several protein which interact with *RAD52* protein that has DNA-pairing activity in vitro were identified in yeast using *RAD52*-column. With the same column we have shown that *RAD51* protein binds to *RAD52* protein

directly. These results, in addition to another result, suggest that RAD51-52 complex containing some unidentified protein play important role in the homology search in recombination.

Results

Purification of RAD51 protein from *E. coli*

RAD51 protein was overexpressed in *E. coli* by using T7 promoter system. A low amount of expression of RAD51 protein was toxic to the cells, even though the transcription of the RAD51 gene was repressed by the expression of T7 Lysozyme from pLysS plasmid, which is a specific inhibitor of T7 RNA polymerase (Moffatt and Studier, 1987). Complete repression of T7 promoter by pLysE plasmid which provides more T7 Lysozyme than pLysS plasmid was enabled us to obtain stable transformant and high level expression of RAD51 protein. The protein was induced by the addition of IPTG to the culture of BL21(DE3) harboring pT7-RAD51 and pLysE, following an incubation for 5hrs at 30 C.

The RAD51 protein was purified by the three column chromatography combined with ammonium sulfate precipitation, DEAE, P-cell and Hydroxapatite (or MonoQ FPLC) to homogeneity 95%, not contaminating exonuclease.

The N-terminal amino acids sequence of purified RAD51 protein was determined, although 1st methionine was removed. The resultant sequence of 20 amino acid was completely corresponded to the predicted sequence (Our Unpublished result).

Characterization of RAD51 protein

ssDNA binding property of RAD51 protein

RAD51 protein is homologous to RecA protein. RecA protein interacts with both DNAs and ATP (Reviewed in Cox and Lehman, 1987). ssDNA binding of RAD51 protein was investigated, using two systems, nitrocellulose filter binding assay (McEntee et al., 198) and ethenoDNA fluorescence assay (Silver and Fersht, 1982).

In nitrocellulose filter binding, uniformly ³²P-labeled 960 nts of ssDNA synthesized in vitro was used as substrate. The ssDNA was mixed with RAD51 protein and after a given time the reaction mixture was applied onto alkaline treated nitrocellulose

filter. After washing filter, the label trapped on the membrane was counted.

Protein titration curve was illustrated in Figure 1 . The ssDNA was trapped on the filter by RAD51 protein in the presence of ATP, depending on the protein concentration. At low concentration of RAD51, the amount of the label captured on the filter was low and the titration curve is in sigmoidal fashion, suggesting the binding of RAD51 protein to ssDNA was cooperative. This was supported by the experiments utilizing short 20mer ssDNA as substrate, in which the oligonucleotide was not trapped on the membrane even in the presence of excess amount of the protein(Unpublished results). From the saturation point, the ratio of RAD51 protein to ssDNA was calculated to 1 nucleotide per one protein. In the absence of ATP there was few complex formed with ssDNA. Only high concentration, more than 100nM, the significant increase of the label trapped was observed, showing that RAD51 protein can interact with ssDNA without ATP but the complex is much less stable than that formed with ATP.

The binding mode was further in detail elucidated by the etheno-DNA fluorescence assay. etheno-DNA is a modified ssDNA containing 1, N⁶-etheno-adenosine and 3, N⁴-etheno-cytosine instead of adenine and cytosine, respectively, which emit 400nm of fluorescence when excited with 300nm. Magnitude of the fluorescence is affected by the binding of proteins. The magnitude is dependent upon the orientation of bases in etheno-DNA. It's known that RecA protein enhance the relative fluorescence(Silver and Fersht,1982; Menetski and Kowalczykowski, 1985). This property is also observed for RAD52 protein.

Figure 2 illustrates protein titration curve of relative fluorescence of etheno-DNA by RAD51 protein. In proportion to the concentration of the protein, the relative value of fluorescence increased irrespective of the presence of ATP, showing the binding of RAD51 to etheno-DNA is stoichiometrical whether with ATP. The saturation value of relative fluorescence was 1.8 in the absence of ATP while 2.9 in the presence of ATP. Also, RAD51 concentration required for the maximum saturation was identical in the both condition. From the saturation points value, calculated ratio of RAD51

protein to etheno-DNA was 1.8 per one RAD51 protein, similar value obtained from the filter-binding assay. ATP, therefore, is thought to induce a conformational change of RAD51-etheno-DNA complex, which might lead more base stacking in the DNA as through unwinding. Salt titration experiment for RAD51-etheno DNA complex formed in the presence of ATP shows the complex was stable (Figure 3). Titration mid-point was 300mM NaCl. Without ATP, the complex was much less stable. Even the addition of glycerol to 5% into the reaction eliminated the increase of fluorescence to the undetectable level (Data not shown).

The effect of nucleotide derivatives on the formation of RAD51-etheno-DNA complex was studied (Table 1). Among ATP, ADP, GTP and ATP- γ -S each in 50 μ M concentration, only ATP could augment the fluorescence. It's interesting ATP- γ -S had a small effect on the binding, contrary to ATP. It seems that steric requirement for the binding with nucleotide is strict so that γ -thio-moiety prevent the RAD51 from interacting with ATP- γ -S.

dsDNA binding property of RAD51

The association of RAD51 protein to dsDNA was examined in the filter binding assay. In this assay the substrate was 3.0kb 32 P-end labeled dsDNA (pBluescript KS+). Titration curve was shown in Figure 4. The binding of RAD51 protein was completely dependent on ATP. Without ATP no increased level of trapped DNA was observed above the basal level defined by the reaction without the protein. The binding curve was sigmoidal shaped and a few complex formation was examined at low concentration of the protein, showing that the interaction of RAD51 with dsDNA also is cooperative as seen in the interaction with ssDNA.

The formation of RAD51-dsDNA complex in the presence of salt was studied. Increase of NaCl concentration reduced the formation of complex (Figure 5). Titration mid-point was 100mM, less than ssDNA complex. The concentration of Mg^{2+} ion was critical in the binding to dsDNA (Figure 5). There was no complex formed in the reaction omitting $MgCl_2$. Optimum value for the binding was around

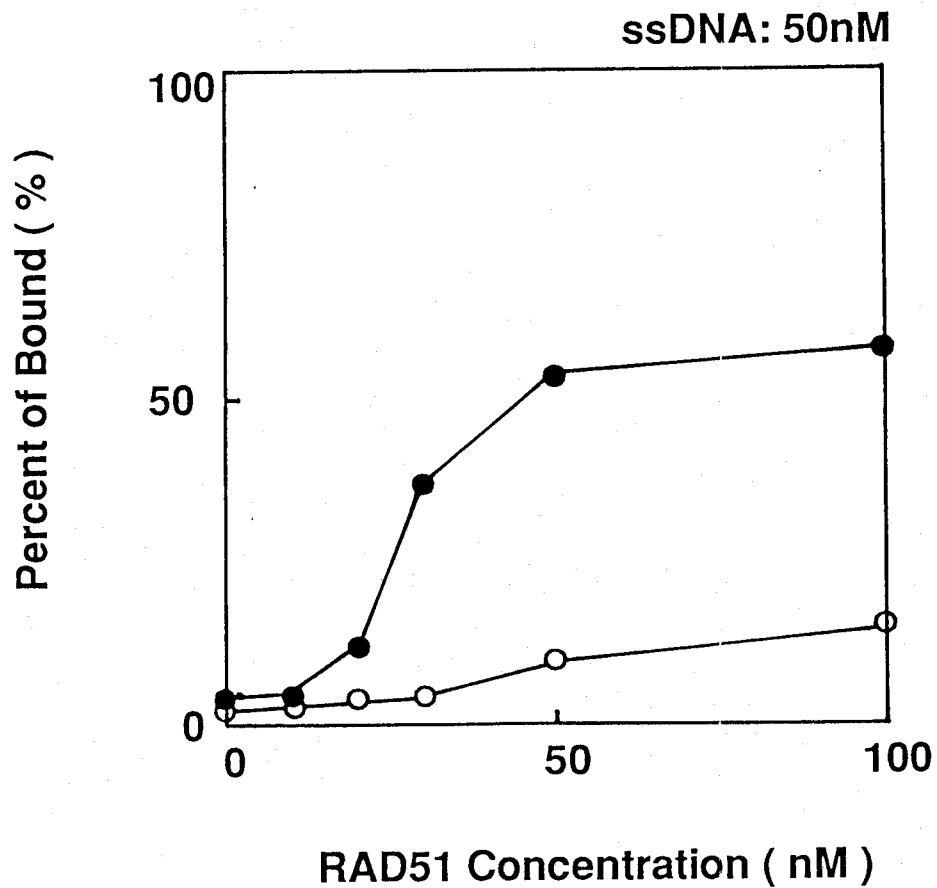


Figure 1. Stoichiometry in binding to ssDNA.

50 nM ^{32}P -labeled ssDNA was incubated with various amount of *RAD51* protein in standard reaction mixture; 20 mM Tris-HCl (pH 7.6), 5 mM MgCl_2 , 1 mM DTT, 20 mM KCl, 5% glycerol, 100mg/ml BSA. in the presence of 200mM ATP (closed circle) or in the absence of ATP (open circle). The mixture s were incubated at 30 C for 30 min, passed through the filters, and then dried up. The radioactivity of the trapped ssDNA was counted.

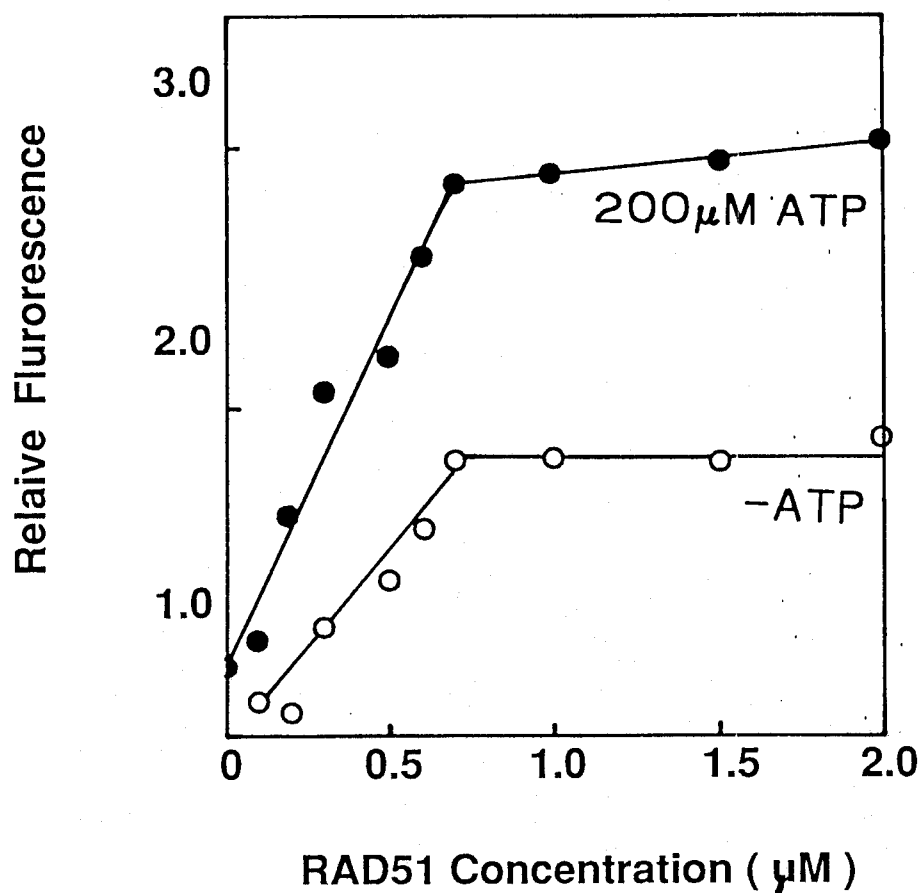


Figure 2. Titration of eDNA by *RAD51* protein.

The reaction mixture contained 20mM Tris-HCl(pH7.6), 5 mM MgCl₂, 1 M DTT, 20 mM KCl, 1 mM eDNA and various amount of *RAD51* protein in the presence of 0.2mM of ATP(closed circle) or in the absence of ATP(open circle). The reactions were started by the addition of the eDNA, incubate at 25 C while monitoring the 400nm emitted fluorescence when excited at 300nm. Monitoring continued until the absolute value of the fluorescence reached to the saturation, completed for 2 min. The relative value of the fluorescence was calculated by simply dividing the value obtained in the presence of the protein by that in the absence of the protein.

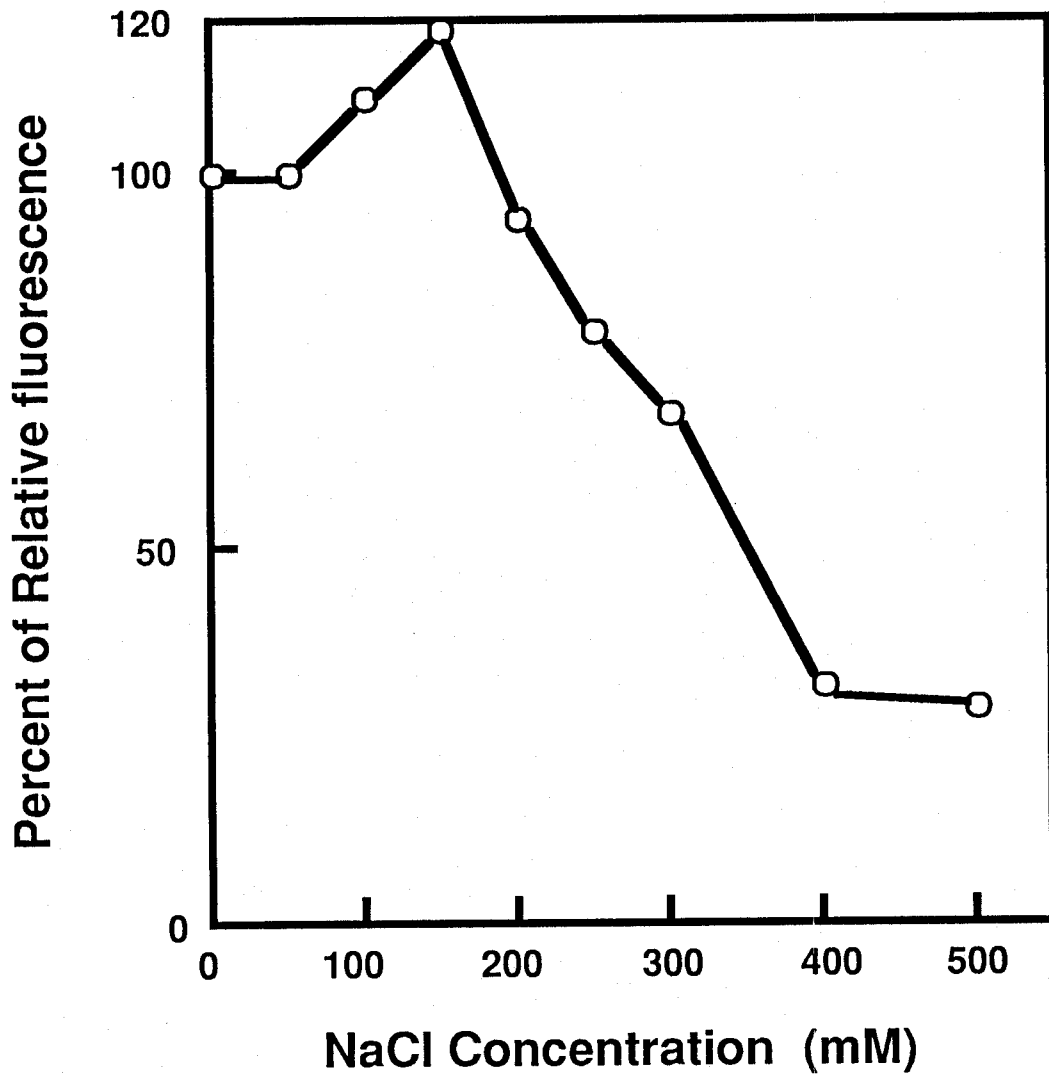


Figure 3. Salt titration of *RAD51* protein- ϵ DNA complex.

The stability of *RAD51* protein- ϵ DNA complex were monitored by observing the decrease of the fluorescence of ϵ DNA in the addition of NaCl. The *RAD51*- ϵ DNA complex was formed in the presence of 0.2 mM ATPas described in Materials and Methods or the legend of Figure 1, various amount of NaCl was added to the reaction and the decrease of the fluorescence were monitored. The absolute fluorescence of ϵ DNA varied depending on the concentration of NaCl. The difference by that were compensated to correct values.

7mM. Either more or less concentration of $MgCl_2$ decreased the binding to duplex DNA. More than 100mM ATP was necessary for the enough formation of the complex(Figure 6). Niether ATP, GTP nor AMP could support the binding(Data not shown).

Other acitivities of RAD51 protein

ATP hydrolysis into ADP and P_i by RAD51 protein was investigated in two assay system, Martin and Doty method and Enzyme coupling ssystem since RAD51 protein carry Nucleotide binding sequence and the binding assay showed RAD51 protein can interact with ATP. Weak activity was detected in both assay. The rate of the hydrolysis was 0.01 molecule hydrolysed per one minute per one RAD51 protein. This activity was stimulated by single stranded DNA and both ssDNA and RAD52 protein, 2.0 and 2.8 folds respectively. At this point it is hard to say that ATP hydrolysis activity is intrinsic to RAD51 protein. The weakness of ATP hydrolyzing acitivity might be either due to that the protein purified from *E. coli* lacks some modification for the activity or that specific condition like promoting factor is requiried.

Interaction between RAD51 and RAD52 protein

RAD52 affinity chromatography

Genetical studies have indicated *RAD51* and *RAD52* perhaps *RAD54* genes belong to the same subgroup among RAD52 epistasis genes; *RAD50*, *51*, *52*, *54*, *55*, *57* and *MRE11*(For review, Resnick, 1988). Mutants in these genes have shown similar phenotypes; defectiveness in most of recombination in yeast(See introduction). Therefore these gene products are assumed to work on the same stage of recombination and furthermore to interact each other or form complex. In T4 phage recombination system, *UvsX*, *UvsY* and *gp32* proteins behaves synergisitically in in vitro recombination reaction although true recombinase is *UvsX* protein(Yonesaki and Ninagawa 1989). Formosa et al.(1984) have shown that

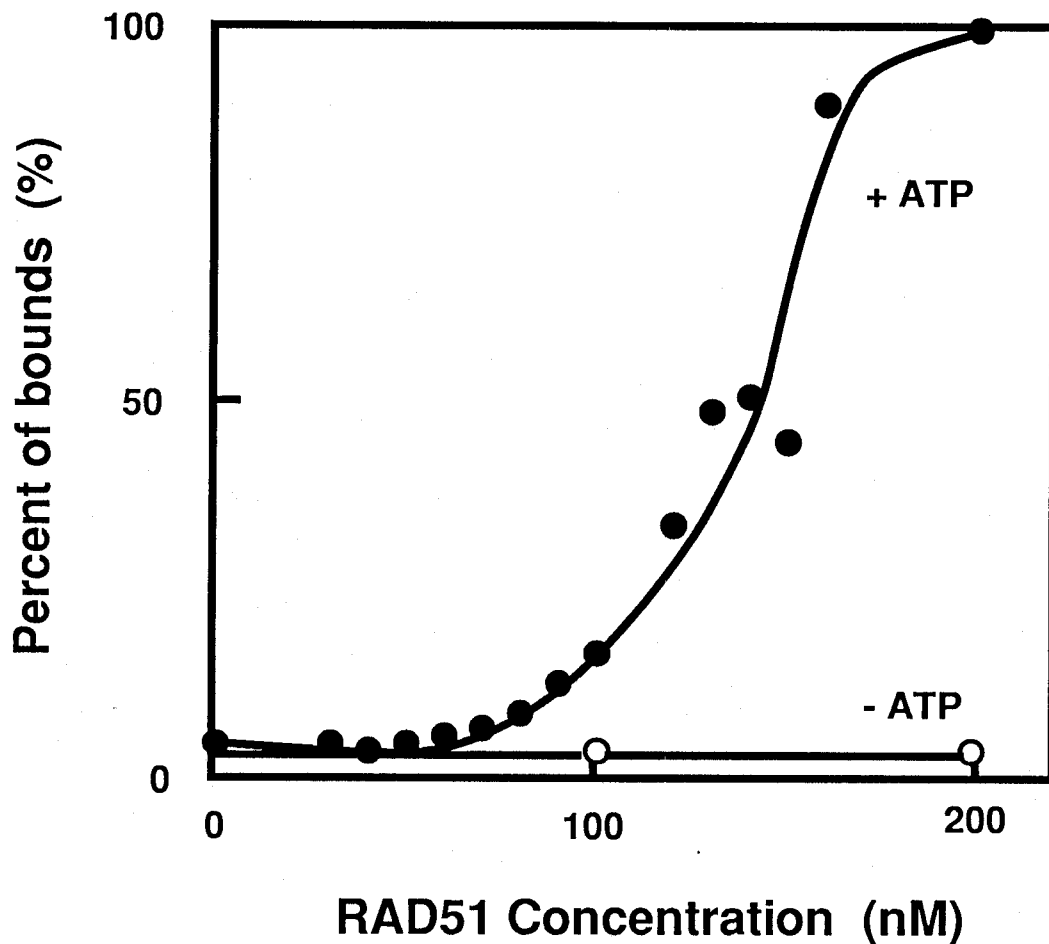


Figure 4. RAD51 protein titration on the binding to dsDNA.

1mM ^{32}P -labeled dsDNA(3.0 kb) was incubated with various amount of RAD51 protein in the standard reacton mixture; 20 mM Tris-HCl(pH 7.6), 5 mM MgCl_2 , 1 m M DTT, 20 mM KCl, 100 $\mu\text{g/ml}$ BSA in the absence of ATP(open circle) or in the presence of 0.2mM ATP(closed circle). The reaction mixtures wereincubated at 30 C for 30 min, filtrated on the nitrocellulose fiter, washed and then dried. The radioactivity retained on the membrane was counted.

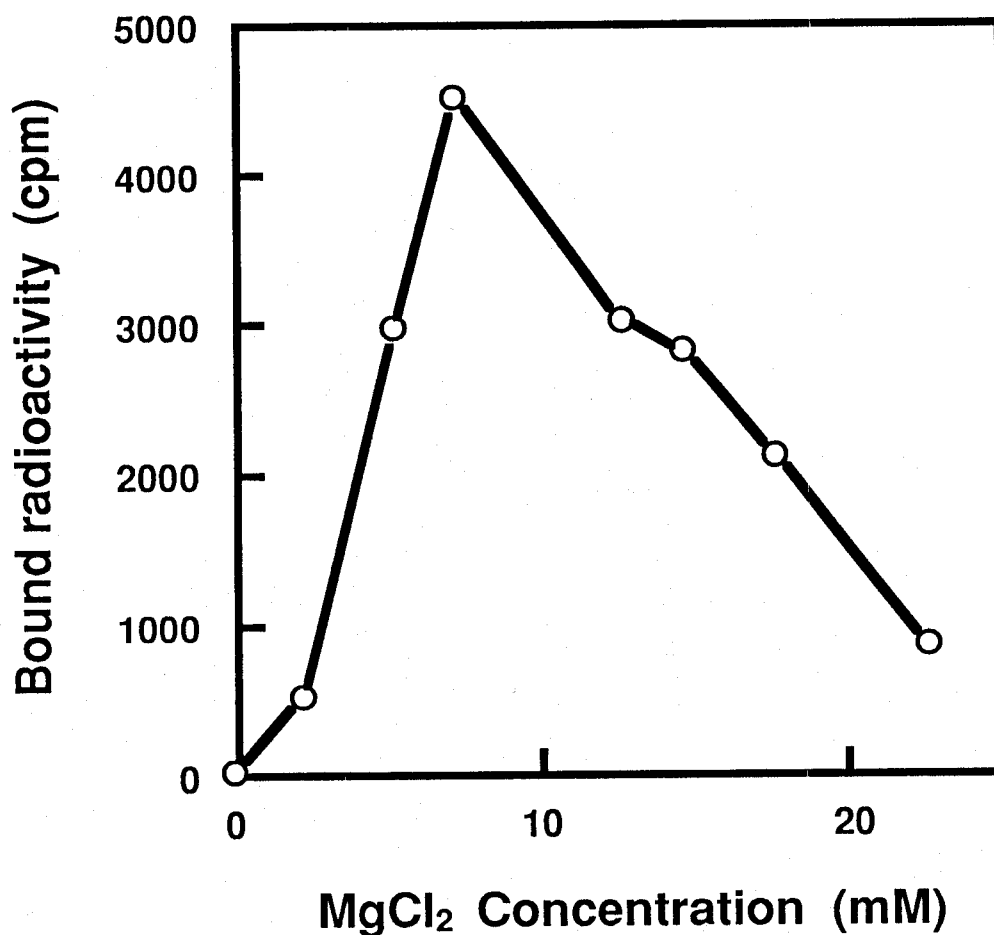


Figure 5. Effect of MgCl₂ concentration on the formation of RAD51-dsDNA complex. The reaction contained 20 mM Tris-HCl (pH 7.6), 1 mM DTT, 20 mM KCl, 200 nM RAD51 protein, 100 μg/ml BSA, 200 mM ATP, 1 μM ³²P-labeled dsDNA and various concentration of MgCl₂. The mixtures were incubated at 30 °C for 30 min, passed through the filter and dried up. The radioactivity trapped on the filter were counted in liquid scintillation counter.

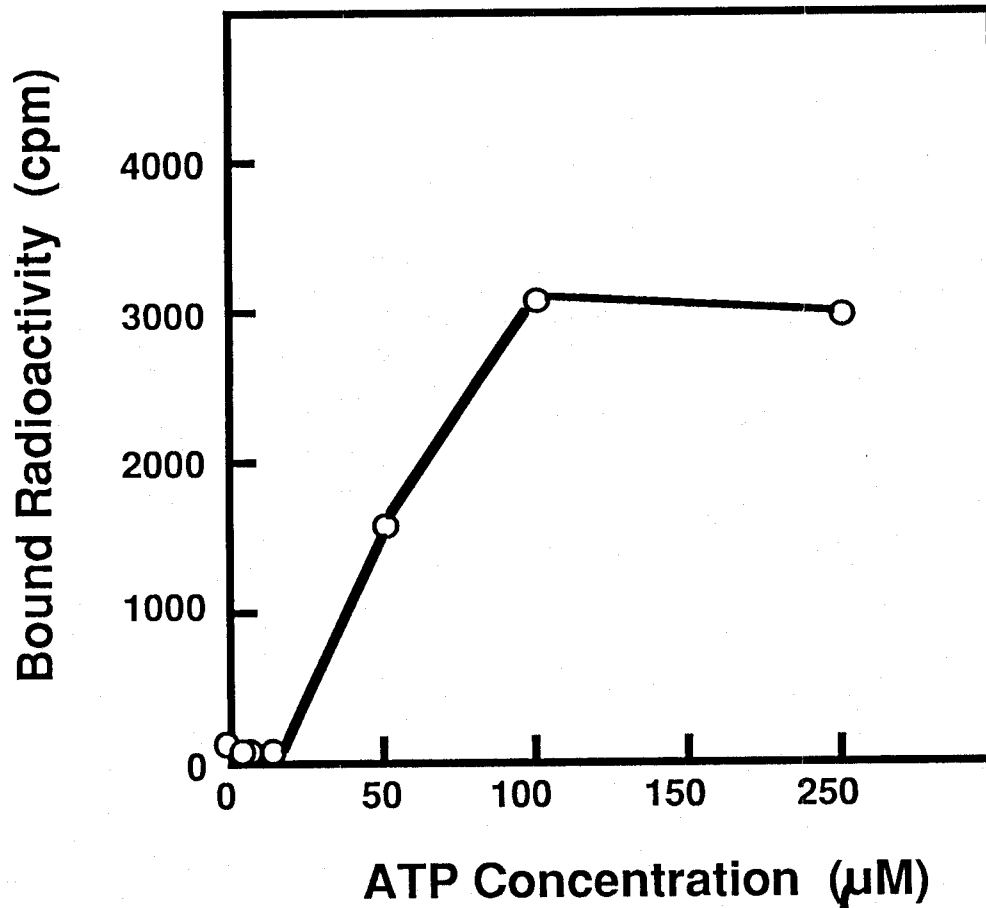


Figure 6 Effects of ATP on the formation of RAD51-dsDNA-ATP complex.

The assays were carried out in the solution; 20 mM Tris-HCl(pH7.6), 1 mM DTT, 5 mM MgCl_2 , 20 mM KCl, 200nM *RAD51* protein, 100 $\mu\text{g/ml}$ BSA, 1 μM ^{32}P -labeled dsDNA and various concentration of ATP. The reaction was initiated by the addition of the protein, incubated at 30 C for 30 min, filtrated onto the nitrocellulose filter. The radioactivity retained to the filer were counted.

these protein interact each other by using uvsX-Sepharose4B column, to which uvsY and gp32 protein and a few additional protein could bind.

We applied this method to yeast recombination system. By constructing RAD52-sepharose column, the proteins which interact directly with RAD52 protein or modulate RAD52 promoted strand transfer activity was searched.

First the interaction of RAD52 protein with RAD51 protein was studied, since genetical results suggest the protein that interact with RAD52 is most likely RAD51. The RAD52 protein was linked covalently to Sepharose 4B colum as described in Materials and Methods. Onto the column, purified RAD51, RAD52 , RecA and BSA proteins were loaded in low salt concentration. After wash in the buffer, the column was washed in the buffer containing 0.1 M NaCl following wash in 0.2 M, 0.5 M, 1.0M amd 2.0M NaCl(sometimes 4 M urea).

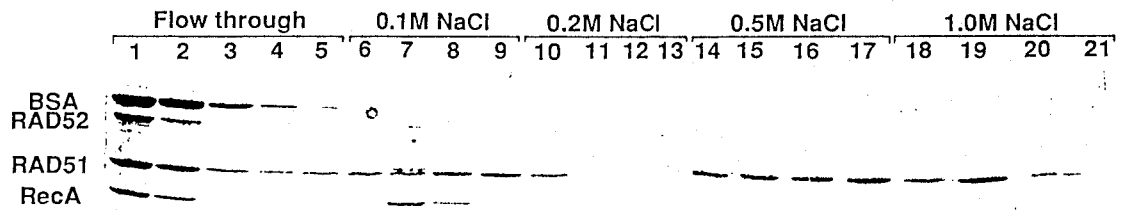
Figure 7 shows the result of the affinity chromatography. Obvioulsy RAD51 protein was retained on the RAD52 column, eluted twice in the buffer with 0.2M and 1.0M salts. This might reflect different modes of the interaction between two proteins. On contrary, BSA-column could not retain RAD51 protein, showing the interaction between RAD51 and RAD52 is not non-specific one. Most of RAD52 protein was recovered as the flow through fraction, although small fraction of RAD52 protein was trapped on the column. This is in a good agreement with the result of DNA-binding property of RAD52. The binding of RAD52 to DNAs is not cooperative.

RecA protein also binded to the column and eluted in 0.1M NaCl. This might reflect direct interaction of RAD52 with RecA protein, since RecA protein is homologous to RAD51 protein. Alternatively, it is due to the indirect interaction because RecA protein bears the hydrophobic suface for the self assembly(Feitag and McEntee, 1988), through which RecA protein indirectly interact with hydrphobic surface of RAD52 protein.

Yeast cell lysate was directly loaded onto RAD52-sepharose column and washed in the same fashion as described above(Figure 8 and 9). Although most of the proteins were eluted directly from the column as flow through fraction., several

protein were trapped on the column. While BAS-coumn as control retained few protein. Among the bound proteins, 18k, 43k and 75k dalton proteins were major ones. 43k is the same with the molecular weight of the predicted RAD51 protein; 42,943.

RAD52 -Sephrose



BSA-Sephrose

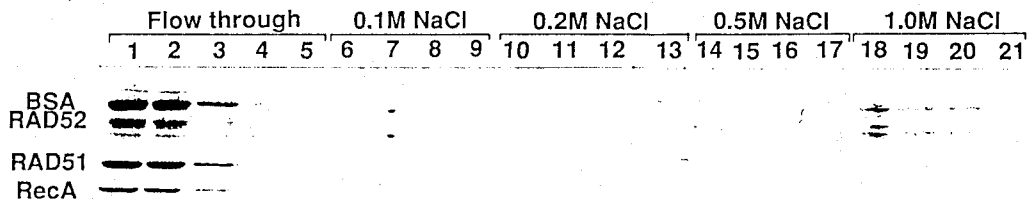


Figure 7. Analysis of SDS-PAGE of the proteins retained on *RAD52* affinity column and an albumin control column. The columns were loaded with 10µg of each purified *RAD51*, *RAD52*, RecA and BSA and eluted with 0.1M, 0.2 M, 0.5 M, 1.0 M and 2.0 M NaCl in affinity buffer(20 mM Tris-HCl(pH7.6), 5 mM MgCl₂, 1 mM DTT, 5% glycerol). The proteins were in each fraction were analysed by electrophoresis through 10% polyacrlamide gel, and visualized by Coomassie blue staining. Each proteins are indicated on the left margin.

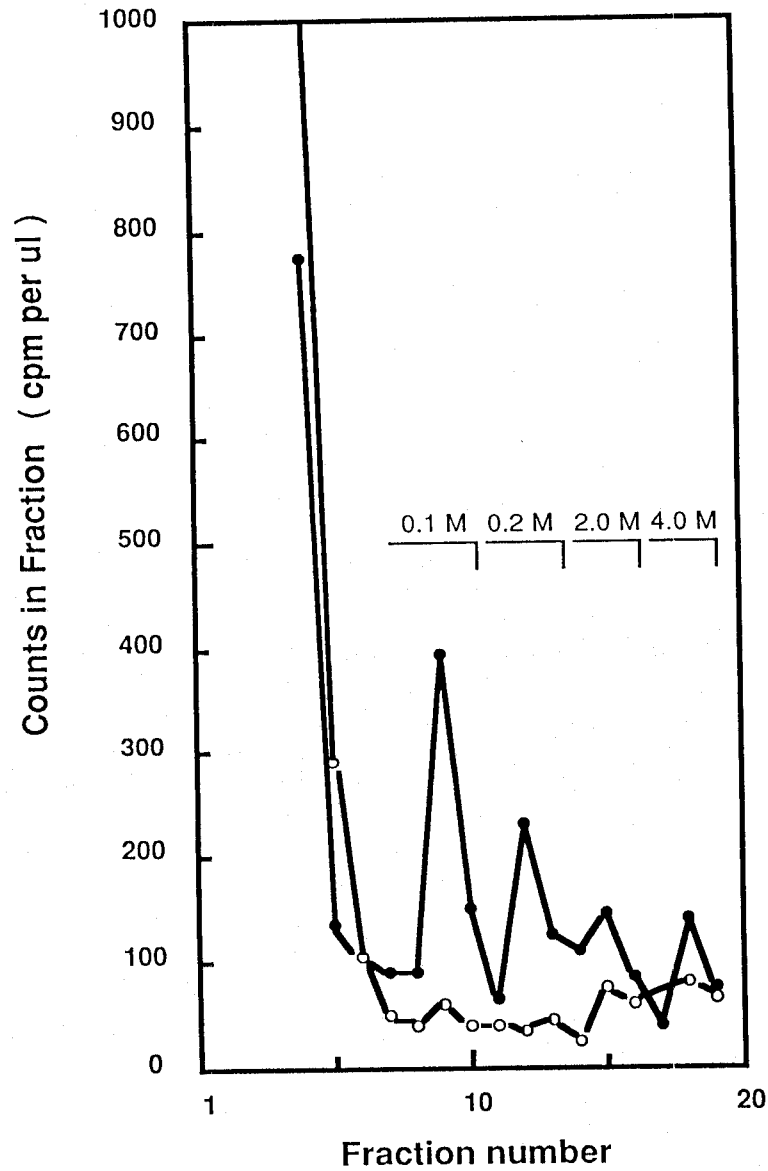


Figure 8. Elution profile comparing the radioactivity of the proteins eluted from a *RAD52*-affinity column and a BSA control column. In the experiment, 200 μ l of columns were used and 200 μ l of yeast 35 S labeled extracts were loaded into each column. The columns were eluted with 0.1 M, 0.2 M, 1.0 M NaCl and 4 M urea. The radioactivities in each fraction were determined by the measurement of 1/100 volume in liquid scintillation counter. The volume of the fraction was about 0.5 ml. Open circle and black circle indicate the profile of the elution from BSA and *RAD52* column, respectively.

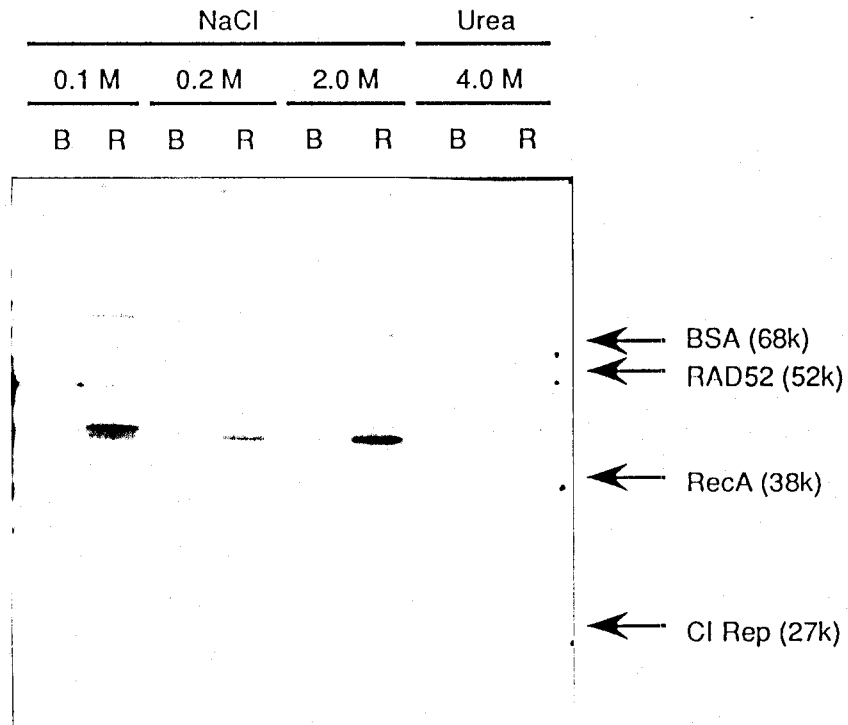


Figure 9. Analysis by SDS-PAGE of the proteins retained on *RAD52* affinity column and BSA control column. The columns were loaded with ^{35}S -labeled yeast extract and eluted with 0.1 M, 0.2 M, 1.0 M NaCl, and 4 M urea, as in Figure 8. The proteins in the peak fraction were resolved by electrophoresis through 10 % polyacrylamide gel, and fixed by Coomassie blue staining. The gel was soaked in Amptify, dried and fluorographed.

Discussion

The role of RAD51 protein in genetic recombination

Genetical studies have shown that RAD51 protein play an important role in most of recombination in yeast. Furthermore, molecular analysis have supported this idea; RAD51 protein is homologous to RecA protein. mRNA of RAD51 gene is induced at the period in meiosis when the meiotic recombination is induced 1000 folds.

Purified RAD51 protein from *E. coli* bind to ss/dsDNA. The binding is both stoichiometrical and cooperative. ATP stimulate the binding to ssDNA, while is essential for binding to dsDNA. This observation is prompted by RecA protein, to which RAD51 is homologous. RecA protein can associate with ssDNA and the interaction with ssDNA is tightened by ATP. While interaction with dsDNA requires both ATP and low pH around 6.5 or ATP- γ -S.

The main difference between RAD51 and RecA protein is that RAD51 protein lacks ATP hydrolysis and DNA pairing activities as seen in RecA protein. Very weak hydrolysis of ATP was observed in the fraction containing RAD51 protein. Prominent hydrolysis, thus, might be detected using different conditions. For example, ATP hydrolysis by RAD3 protein is seen only in low pH condition around 5.5(Sung et al., 1988).

In vivo there might be protein that promote the hydrolysis by RAD51 protein, as seen in other system. For GTP hydrolysis by ras protein, GAP protein is known to facilitate the GTP hydrolysis. ATP hydrolysis by MuB protein is stimulate by DNA and MuA protein. There is some candidate as RAD52 and RAD54 since these mutants show similar phenotypes as rad51 mutant. The observation that RAD52 interact directly with RAD51 protein is in good agreement with the above fact(See below).

Since RAD52 protein carry out DNA pairing reaction in vitro, RAD51 protein is likely to lack this activity despite of the homology with RecA protein. But RAD51 protein might support DNA exchange reaction by RAD52 protein through the

formation of RAD51-52 protein complex. This is a case since RAD52 strand transfer activity is weaker than known other strand transferase like RecA and uvsX protein.

Alternatively, the overexpression of the protein may hinder the activities. It's known that the overexpression of ras protein in *E. coli* reduce the k_{cat} of GTP hydrolysis and that SV40 T-antigen produced in *E. coli*, which is not phosphorylated, lacks some activity the protein purified from mammalian cells bears.

Purification of RAD51 protein from yeast is essential for further analysis of this protein.

The role of protein complex in genetic recombination

Many proteins involved in genetic recombination are thought to interact each other and/or form the complex as seen in replication machinery. For T4 phage recombination, uvsX, uvsY and gp32 confer the synergistic effects on in vitro recombination reaction (Yonesaki and Ninagawa, 1989). These proteins are shown to interact each other directly (Formosa and Alberts, 1984).

In yeast there are so many gene engaged in recombination. These gene products might communicate each other directly or indirectly. To identify the interaction in recombination machinery, we used affinity chromatography. Utilizing the RAD52-sepharose 4B column, there were several protein bound in yeast lysate. It seems that these bound proteins modulate the strand transfer activity by RAD52 protein. The fact that purified RAD51 protein also bind to this column is in a good agreement with the genetical result that rad51 and rad52 mutant show same phenotype. It's noteworthy that the size of one of the protein in yeast lysate bound to RAD52 column is 43 k, which is the same size to the predicted RAD51 protein. Use of antiserum and lysates of the deletion mutants for some genes will facilitate the identification of bound proteins.

It is known that specific protein complexes appears in meiosis. Among them recombination nodule (RM) carry important role in recombination since the positions of RM are coincident to the position of chiasma, crossing over point between

chromosome(Carpenter, 1972). Carpenter(1981) showed that there are two types of RM in meiosis, one emerges at zygotene and early pachytene, and the other at late pachytene. It is speculated that early RM is responsible for one type of recombination like gene conversion for the homology search between homologous chromosome and that late RM is for another like crossing over which ensure disjunction of homologues(Smithies and 1985; Carpenter, 198). Recent genetetical analysis have indicated that gene conversion and crossing-over are independent events at least in some points(Engerrchant and Roeder, 1990; Rockmill and Roeder,1990; Carpenter,1984), supporting the above idea

What is the component of RMs? It is considered that some component of RM is identical in both early and late ones and others are not but similar to carry out different roles. Similar but functionally different gene that is engaged in meiotic recombination are RAD51 and DMC1. These are 54% identical and 76% similar in protein sequence but have different role in recombination. dmc1 mutant do not undergo meiosis first division and no spore is formed in this mutant(Doug Bishop, personal communication). This pheotype is identical to that of recently identified new muatnt, rad50S mutant(Alani et al.,1990), in which SC(synaptonemal complex) isn't formed but SC precursores, so-called axial core elements, are formed completely to full length. dmc1 mutant also might lack SC. On the other hand, rad51 mutant make spore, but inviable ones(cited in Resnick, 1988; our unpublished results). Since rad52 mutant, similar mutant to rad51, make SC(cited in Resnick, 1988), rad51 might also form SC. Therefore DMC1 gene product likely to be one of tha components of early RM since early RM appears before and/or just on the assembly of SC. While, RAD51 protein might be one of the member, with RAD52, of late RM.

This idea is supported by the analysis of recombination in Drosophila. In Drosophila the mutants defective in meiotic recombination were well characterized(Reviewed in Carpenter, 1988). Some mutants like mei41 and mei218 is defective in meiotic recombiantion and in these mutants the distribution

of the crossing over points were altered. These gene, therefore, is thought to be involved in the determination of the points of the exchange of DNAs. Furthermore, these mutants have shown to carry morphologically altered late RM, supporting a classical idea that the RM decide the point of crossing-over. On the other hand, mutants like *mei9*, which is deficient in recombination through all chromosome, have no apparent effect on late RM morphology. *mei 9* and *mei 41* also are defective in DNA repair. Since the phenotype of *mei9* and *mei41* are similar to that of *rad51* and *rad52*, *RAD51* or *RAD52* might to be a member of late RM as seen in the role of *mei41* in *Drosophila*.

In *rad52* mutant a nuclease is missing (Chow and Resnick, 1988 and 1989) and immunologically same nuclease is also missing in *Drosophila mei41* mutant. This observation is consistent with that *mei41* gene and *RAD52* gene is functionally close though the sequence analysis is still missing for *mei41* gene. Further biochemical, genetical and cytological studies is necessary to dissect the role of *RAD51* and *DMC1* genes in genetic recombination, especially in the protein complex like RM and SC.

Materials and Methods

Plasmids

pT7-51B was constructed by the ligation of NdeI, BamHI digested fragment from pET3a (Rosenberg et al., 1987) and NdeI partially, BamHI cut 3.1kb fragment from pKS-51+, which contained the RAD51 from 1st ATG with 400 amino acid and 3' - flanking sequence.

To remove the 3' flanking sequence that inhibited the synthesis of the RAD51 protein in *E. coli*, pT7-51(1) was made by the replacement of BstEII, BamHI 1.9kb fragment of pT7-51B for the synthetic DNA fragment. The upper strand of the synthetic DNA was 5' GTGACCCCAGAGAAGAAGACGAGTAG-3' and the bottom strand was 5'-GATCGTACTCGTCTTCTTCTCTGGG-3', synthesized in ABI DNA synthesizer 372.

Purification of the RAD51 protein from *E. coli*.

The cells harboring both pT7-51(1) and pLysE (Moffat and Studier, 1987) was obtained by the transformation of BL21(DE3) (Studier and Moffat, 1986) with both plasmid simultaneously, selecting on the L-plate containing 50µg/ml ampicillin and 15µg/ml Chloramphenicol.

Fresh transformant were grown to the log phase in 2ml of L-broth supplemented with both antibiotics and further inoculated in 100ml of the same media for 8-12hrs. 20ml of the culture was inoculated into 2l of the media and the cell were grown to 2X10⁸ cells/ml. Then IPTG was added to the culture to final 50 mg/ml and culture was continued for further 3hrs at 37 C or 5hrs at 25 C. The cells were harvested by the centrifugation at 8000rpm for 10min. About 7 grams of the bacteria was recovered from 2l of the culture. Not used immediately, the cells were frozen in liquid N₂ and stored at -70 C.

All following procedures were carried out at 4C. 14g of the cells were suspended in 50ml of buffer-A (25mM Tris-HCl (pH7.8), 1mMEDTA, 1 mM PMSF, 25%(W/V) sucrose). For the complete lysis, the suspension was sonicated in Sonicator. The

cell debris were removed by the centrifugation at 27,000rpm in Beckman Type30 rotor for 30 min. The supernatant was recovered and OD260 of the supernatant was adjusted to 200 in the buffer A. Then 10%(V/V)Polyethyleneimine (pH7.6) to final 0.35% with stirring. After 1hr's stirring, the suspension was centrifugated at 10,000rpm for 10 min. The supernatant was discarded , the pellet was recovered and suspended in buffer-B(50mM Tris-HCl(pH7.5), 1mM EDTA, 1mM PMSF) containing 0.5 M NaCl. The suspension was centrifugated at 12,000rpm for 10min. The supernatant was collected and then solid ammonium sulfate was added to the solution to final 33% saturation(1.80 mg/ml) with stirring following continuous stirring for 1 hr. After the centrifugaion at 13,000rpm for 20min, the pellet was recoved, suspended in TEM buffer(50mM Tris-HCl(pH7.5), 1mM EDTA, 5mM β -mercapthethanol, 1mM PMSF, 10%(V/V) glycerol) including 0.2M NaCl, and dialyzed against the same buffer for at least 5 hrs with 3 times buffer exchange.

The dialysate was diluted to 0.05M NaCl concentration in TEM-buffer and applied onto 15ml of DEAE-cellulose(Brown)(1.6 X 7.5cm) previously equilibrated with the TEM-buffer. The protein was eluted from the column with the 0-0.5M NaCl gradient in the same buffer. The RAD51 protein was eluted at 0.25-0.33M NaCl concentration. The fraction containing RAD51 protein was pooled and precipitated by the addition of the solid ammonium-sulfate to final 50% saturation (3.13 mg/ml), following the 1 hr's stirring and the centrifugation at 12,00rpm for 20min. The pellet was dissolved in the P-buffer(20mM Phosphate(pH6.5),1mM EDTA, 5mM β -mercapthethanol, 1mM PMSF, 10%(V/V) glycerol) containing 0.2 M NaCl and dialyzed against the same buffer for 5 hrs. The dialysate was diluted to 0,05 M NaCl concentration in P-buffer and loaded onto the phospho-cellulose(P11, Whatman) pre-equilibrated with the same buffer. Run at 24ml/hr, the column was washed with the P-buffer. The flow-through fractions including RAD51 protein was recovered and directly applied onto hydroxyapatite(Seikagaku-kogyo) previously equilibrated with P-buffer omitting EDTA. The column was extensively washed in the same buffer following wash with 20-300mM phosphate(pH6.5) gradient in the

buffer. The RAD51 protein was eluted at 80mM phosphate. The fractions was collected, to which ammonium sulfate was added to final 50% saturation with mixing. The pellet was recovered by the centrifugaion, dissolved in TEM-buffer, dialyzed for 5 hrs following the dialysis against the stock buffer(50mM Tris-HCl(pH7.6), 1mM EDTA, 1mM DTT, 0.2M KCl, 50%(V/V) glycerol) for 12 hrs.

For further purification, the fraction after hydroxy-apatite was loaded onto MonoQ HR5/5 column (Pharmacia). The colum, run at 0.5ml/min, was washed with 0.2-0.55M NaCl gradient, 17.5mM Cl⁻/ml. The RAD51 protein was eluted at 0.41M NaCl concentration. The protein was stored as described above.

The concentration of RAD51 protein was determined from the extinction coefficient. ϵ_{280} of RAD51 protein was .

Nitrocellulose filter binding assay

Nitrocellulose membrane(Millipore PVDF) was prepared as follows. The filters were soaked in 0.4M KOH solution for 45 min at room temperature. After washing extensively in H₂O, the filters were stocked in 10mM Tris-HCl(pH7.6), 1mM EDTA.

Whole labeled ssDNA was synthesized on the + strand of pBluscripts anealed with 20mer primer DNA. with klenow fragment 2 mg of tempelate and 5 pmole of primer were incubated in the mixture(10 mM Tris-HCl(pH7.6), 10mM MgCl₂, 50mM NaCl) at 85 C for 5 min and following the incubation at 37 C for 30 min. 2 ml of 0.1 M DTT, 5ml of ³²P-a-dATP(3000Ci/mmole), 1 ml of 40 μ M dATP and 1 ml of 20mM dNTP without dATP were added. The reaction was atarted by the addition of 5 units of Klenow fragment (TAKARA-SHUZO, Japan) and incubated for 30 min at room temperature. futher 1 ml of 20mM dATP was added for the extension of the synthesis and incubated for further 20 min. The reaction was terminated by heating up at 65 C for 10 min. 1m NaCl was added to final 100mM. 10 units of BamHI was added to the digestion of DNA. After ethenol precipitaion, the products were analyzed on 6% Acryl amide 7M urea denaturation gel. 960nt of band were cut off the gel, crushed in 10mM Tris-HCl(pH7.6), 10 mM EDTA, incubated for 4 hrs with

visgous shaking. The supernatant was recovered and treated with PhOH/CH₃Cl following ethenol precipitation. After centrifugation at 16,000rpm for 10 min the pellet was recovered, dried up suspended in 10mM Tris-HCl(pH 7.6), 1 mM EDTA. The concentration of ssDNA was determined by counting the radioactivity of the solution.

The assay were carried out in 50ml of reaction mixture; 20mM Tris-HCl(pH7.6), 1mM DTT, 5mM MgCl₂, 50nM ssDNA, 200μM ATP. The reaction was initiated by the addition of the protein, incubated for 30min at 30 C and filterated through alkiline treated membrane. The membrane was wased in 1 ml of washing buffer(20mM Tris-HCl(pH7.6), 1mM DTT, 5mM MgCl₂), dried under the lamp for 30 min and the radioactivity on the filter were counted in liquid scintilation conter.

dsDNA end-labeld with ³²P was prepared with as described(Maniatis, 1990). Breifly, EcoRI digested pBluescript KS+ dsDNA was end filled with Klenow fragment in the presence of ³²P-α-dATP. The experimnts was performed as described above using the labeled dsDNA instead of ssDNA.

Construction of RAD52 affinity column

CNBr acitivated sepharose 4B(Pharmacia) was used as matrix of affinity chromaotgraphy. 1 gram of dried CNBr-sepharose 4B was suspended in 1mM HCl for 15min at room temperature for 5 min. The gel was washed on the glass-filter in 1mM HCl and then transfered to the coupling solution(0.1 M NaHCO₃(ph 8.3), 0.5M NaCl). 2mg of purified RAD52 protein from E.coli was added to the 1 ml of gel suspension immediately after the transfer of the regin to the coupling buffer. The gel and the protein were mixed in a test tube with mild agitation at 4 C for 12 hrs. The coupling was monitored by the determination of the protein concentration in the supernatant of the suspension, using Bradford mehods. The coupling of the protein with beads was terminated by the addition of 0.5 ml of 1 M ehanolamine(pH8.0) and following the contineous shaking at 4 C for 6hr for blocking the unreacted groups in the gel. Care should be paid since over-coupling sometimes denature

the protein. To remove unreacted protein, the matrix was washed on the glass-filter in coupling buffer following the washing in 0.1 M CH₃COOH (PH 4.0). The column was stored in the buffer(20mM Tris-HCl(pH7.6), 1 mM EDTA, 0.05mg/ml NaN₃) at 4 C.

RAD52 affinity chromatography

The column was made in 1ml of tip whose bottom was packed with siliconized glass-wool. 200 ml of the gel suspension was poured in to the tip and washed with affinity-buffer(20mM Tris-HCl(pH7.6), 1mM DTT, 5mM MgCl₂).

The ³⁵S labeled cell lysate was prepared as follows. The met strain was grown to the mid-log phase in 100ml of SD media supplemented with methionine and necessary amino acids. The cells were collected by the centrifugation at 3,000rpm, washed with SD omitting methionine and suspended in 100 ml SD without methionine. 1 mCi of ³⁵S-methionine was added to the culture. Growing for 12hrs at 30 C, cells were harvested by centrifugation, following washing in lysis buffer. Cells were disrupted by glassbeads-method. After centrifugation, supernatant was collected and used as the cell lysate.

300 µl of cell lysate was loaded onto the RAD52-sepharose column at the speed of 2ml/hr. Washing extensively in the affinity buffer, The column was washed with the affinity buffer containing 0.1 M NaCl, subsequent 0.2 M, 0.5 M, 1.0 M, 2.0 M NaCl and 4 M urea. 500ml of each fraction was precipitated by final 2% TCA with 10 mg of BSA as carrier protein, kept on ice for 30 min. The pellet was recovered by the centrifugation at 15,000rpm for 10min, washed with acetone, dried up and suspended in SDS-PAGE sample buffer. After boiling for 5 min the sample were loaded on 10% SDS-PAGE gel. After electrophoresis, gel was stained with Coomassie-brilliant blue B-250 with boiling for 8 min, destained in destained solution with boiling, soaked in the Amplify (Amersham) and dried up on filter-paper. The gel was fluorographed at -70C for 1 week.

To detect specific interaction with RAD52-column, purified protein was used as the

substrate for the affinity column. 200mg of RAD51, RAD52, RecA and BSA protein mixture in affinity-buffer was loaded on the 100ml of RAD52-sepharose 4B column previously equilibrated with the same buffer. Washing in the same buffer, the bound protein was eluted with step-wise elution as described above. The eluates were analyzed by the electrophoresis on 10% SDS-PAGE gel

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