Localization of RecA-like recombination proteins on chromosomes of the lily at various meiotic stages

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The Rad51 and Lim15 proteins of lily, which are homologs of the bacterial RecA protein, were found on chromosomes in various stages of meiotic prophase 1. The presence of both Rad51 and Lim15 proteins as discrete foci on leptotene and zygotene chromosomes and their colocalization suggest that meiotic recombination begins at the leptotene stage with the cooperation of these proteins and continues in zygotene. Localization of the foci on or adjacent to the chromosomes suggests that these proteins bind to the chromatin loops that extend from the axial cores. The proteins in these foci may participate in the searching and pairing of homologous DNA sequences, as the RecA protein does. The different pattern of localization of the Rad51 protein between the leptotene and pachytene stages and the absence of the Lim15 protein in the pachytene stage suggest that the Rad51 protein plays different roles in these stages.

[Key Words: Lily; Rad51; Lim15; meiotic recombination; chromosome synapses]

Received December 27, 1994; revised version accepted March 8, 1995.

Meiotic recombination is a fundamental mechanism by which the parental traits of eukaryotes are distributed to their offspring. During the prophase of meiosis 1, the homologous chromosomes interact and recombine with each other (Baker et al. 1976). Elaborate morphological changes occur in the chromosomes as they synapse sequentially during the leptotene, zygotene, pachytene, and diplotene stages. At the leptotene stage, each chromosome condenses from its interphase state to produce a long, thin thread with a proteinaceous central axis. Although each chromosome has replicated and consists of two sister chromatids, these chromatids are closely apposed and each chromosome therefore appears to be single. In the zygotene stage, synapsis between two homologs begins and their rope-like proteinaceous axes are brought together to form a synaptonemal complex. As soon as synapsis is complete all along the chromosomes, the cells enter the pachytene stage (for review, see von Wettstein et al. 1984). The chiasmata, which are the visible manifestations of crossover recombination, become observable in the diplotene stage.

Recombination nodules appear at intervals on the synaptonemal complexes of pachytene chromosomes. These nodules are thought to mediate chromosomal exchanges (Rasmussen and Holm 1978; Carpenter 1979; Zickler et al. 1992), which result in crossovers between two non-sister chromatids, one each of the two homologous chromosomes. Although invisible at pachytene, each such crossover will appear later as a chiasma. The nodules on chromosomes found from the pachytene through diplotene stages are believed to be the sites of enzymes for meiotic recombination (Carpenter 1975, 1988; Stack et al. 1989) and are called late nodules. Another class of nodules, called early nodules, is also observed. The early nodules are abundant and have been observed along axial elements at points of convergence between homologous chromosomes, suggesting that they play a role in the initial matching of homologous regions (Albini and Jones 1987; Anderson and Stack 1988). Early nodules are considered to be the points of convergence between homologous chromosomes.

The RAD51 and RAD52 genes in Saccharomyces cerevisiae play important roles in the repair of DNA damage and in recombination (for review, see Resnick 1987; Petes et al. 1991). In meiotic recombination, they participate in repair of double-strand breaks (DSBs) formed at meiosis-specific recombination hot spots (Shinohara et al. 1992; Ogawa et al. 1993b). The domain II region of the Rad51 gene product is structurally homologous to a region of the RecA protein of Escherichia coli. This region of the Rad51 and RecA proteins is involved in formation of the nucleoprotein filament, which is crucial for the searching and pairing of the homologous sequences (Ogawa et al. 1993a, b; Sung 1994) and is required for strand transfer in genetic recombination (for review, see Kowalczykowski and Eggleston 1994). The RAD51 homologs are widely distributed in different organisms, such as ascomycete fungus, fission yeast, clawed toad,
fruit fly, chicken, mouse, human (for review, see Kowalczykowski and Eggelston 1994; Akaboshi et al. 1994; Maeshima et al. 1995). Recently a RAD51 homolog was found in a plant, *Arabidopsis thaliana* (K. Smith and E. Signer, pers. comm.). It seems reasonable to consider that the Rad51 homologs in different organisms perform reactions that are analogous to those played by the RecA or yeast Rad51 protein.

Genes homologous to the RAD52 gene of *S. cerevisiae* (Adzuma et al. 1984) are also found widely in such organisms as fission yeast, chicken, mouse, and human (Bezzubova et al. 1993; Ostermann et al. 1993; Bendixen et al. 1994; Muris et al. 1994). The purified Rad52 protein of *S. cerevisiae* carries out a strand-transfer reaction (Ogawa et al. 1993b). It has been shown that the Rad51 and Rad52 proteins of *S. cerevisiae* are involved in a species-specific interaction with each other (Shinohara et al. 1992; Milne and Weaver 1993; Ogawa et al. 1993b, 1995; Donovan et al. 1994). These results suggest that these proteins are included in a functional complex in the recombination and the repair of DNA damage.

The *DMC1* gene of *S. cerevisiae* is specifically expressed in meiosis. It is involved in the repair of DSBs at recombination hot spots, in the formation of synaptonemal complexes, and in the progression of the meiotic cell cycle (Bishop et al. 1992). The Dmc1 protein is also structurally homologous to the Rad51 protein of *S. cerevisiae* and the RecA protein of *E. coli* (Story et al. 1993). The Rad51 and Dmc1 proteins are required for meiotic recombination in *S. cerevisiae* (Bishop et al. 1992; Shinohara et al. 1992). They have been localized to the same subnuclear sites in *S. cerevisiae*, although their exact locations on chromosomes and the timing of the meiotic stages have not been determined on the yeast chromosomes. However, the requirement of Rad51 protein for binding of the Dmc1 protein to the subnuclear sites suggests that these proteins may interact in the formation of a functional complex for meiotic recombination (Bishop 1994).

The *LIM15* gene of the lily plant, *Lilium longiflorum*, is expressed specifically in prophase I of the meiotic cells (Kobayashi et al. 1993, 1994) and is a homolog of the yeast *DMC1* gene (Shinohara et al. 1993). The Dmc1 protein has been shown to be highly homologous to eukaryotic Rad51 proteins (Ogawa et al. 1993b). In addition to the Lim15 protein, lily cells produce a protein that is immunologically highly homologous to the human Rad51 protein as described below. We call the protein specified by anti-human Rad51 antibody the lily Rad51 protein. Recently, both *RAD51* and *DMC1* genes have been cloned in *Arabidopsis*, suggesting that plants too have both recA-like genes (for *DMC1*, S. Sato and S. Taba, pers. comm.).

Identification of the Lim15 and Rad51 proteins in meiotic lily cells

The presence of the Lim15 protein in different stages of meiotic cells of the lily was examined by the Western blot analysis of cell lysates using SDS-PAGE. The polyclonal antibody was made against a KLH [keyhole limpet hemocyanin] conjugate with a peptide that consists of 18 amino acid residues from the amino terminus of the Lim15 protein (m.w. 38,270). The peptide is not homologous to any other known sequences expected for Dmc1 and Rad51 homologs nor any proteins in the data bases. A single species with a mobility consistent with the size predicted by the Lim15 coding region was found in the lysates prepared from microsporocytes in the leptotene, zygotene, and pachytene stages. This species was not detected in premeiotic and diplotene microsporocytes [Fig. 1A]. This stage, when the protein was found, coincides with the stages at which the *LIM15* mRNA has been known to be present (Kobayashi et al. 1994). These results show that the protein detected by the antibody is the Lim15 protein made in the specific meiotic stages.

Western blot analysis using the polyclonal antibody against the human Rad51 protein was used to determine the presence of the Rad51 protein in the same amount of cell lysates used for the detection of the Lim15 protein. One protein band was abundant in the lysates prepared from premeiotic and meiotic cells [Fig. 1B]. Because the mobility of this band coincides with the mouse Rad51 protein used as a molecular weight marker, the protein is considered to be the Rad51 homolog [Fig. 1B, lane 1].
Rad51 and Lim15 proteins on meiotic chromosomes

Figure 1. Western blot analysis of the Lim15 and Rad51 proteins of premeiotic and microsporocytes of the lily. Cell lysates were electrophoresed on a 12% SDS-polyacrylamide gel and Western blotted. Protein bands were visualized by using polyclonal rabbit anti-Lim15 IgG (A) or anti-Rad51 serum (B). (Lane 1) A lysate prepared from 8-week-old mouse testis used as a molecular weight marker (36,965); (lane 2) young anthers for premeiotic; (lanes 3-6) leptotene, zygotene, pachytene and diplotene microsporocytes, respectively.

Localization of the Rad51 and Lim15 proteins on meiotic nuclei

To examine the localization of the Rad51 or the Lim15 protein on meiotic nuclei in the microsporocytes during the leptotene, zygotene, and pachytene stages, the nuclei spread on a glass slide were treated first with the antihuman Rad51 or anti-Lim15 antibody and then stained with FITC-labeled anti-rabbit IgG donkey serum to detect these proteins. The preparations were then stained with 4',6-diamidino-2-phenylindole (DAPI) to observe the chromosomal DNA. Observation with a UV light microscope showed that the nuclei in the premeiotic, leptotene, zygotene, and pachytene stages were stained differently with the anti-Rad51 or anti-Lim15 antibody. In the leptotene and zygotene stages, the Rad51 and Lim15 proteins were observed as discrete foci on nuclei [Fig. 2A(a), A(b), B(a)]. In the pachytene stage, a whole nucleus was covered by staining with the anti-Rad51 antibody and had far fewer numbers of the Rad51 foci [Fig. 2A(c)]. No staining signal with the anti-Lim15 antibody was observed on pachytene nuclei [Fig. 2B(b)]. Staining was observed at none of these nuclei when the preimmune serum was used [Fig. 2C]. The stage-specific staining of nuclei with these antibodies probably reflects specific roles played by these proteins at different stages of meiotic recombination.

Localization of the Rad51 protein on meiotic chromosomes

To examine detailed localization of the Rad51 foci on the leptotene, zygotene, and pachytene chromosomes, the images of foci stained with the Rad51 antibody and the chromosomes stained with DAPI observed by a UV light microscope were recorded by a cooled CCD camera (Figs. 3, 4, 7, and 8). These images were then enhanced by a computer. To determine the location of Rad51 foci with respect to the chromosomes, the images of the chromosomes that were stained with the anti-Rad51 antibody were aligned with those of the same chromosomes stained with DAPI using a computer. To allow the quantitative comparison of the number of foci on the chromosomes at different stages, spreading of the chromosomes and fixing and staining of the protein were carried out under the same conditions. Although the efficiency of staining was dependent on the methods used for handling the samples, the relative numbers of foci at different stages were not changed significantly by the methods used. Therefore, we consider that the results presented here represent the values of relative abundance of the proteins on chromosomes at different stages.

Staining of meiotic chromosomes at each stage with
DAPI showed that the structures of chromosomes assigned as those at the zygotene, pachytene, and diplotene stages correspond to the meiotic structures of the lily assigned previously using silver staining [Stack et al. 1989]. Accordingly, the chromosomes stained as thin threads and thick bands with DAPI correspond to unsynapsed and synapsed regions of chromosomes, respectively.

In the leptotene stage, chromosomes were observed as unpaired long thin threads (Fig. 3A), and many foci stained with the anti-Rad51 antibody were present (Fig. 3B). Most foci were observed along the chromosomes (Fig. 3C). In early and late zygotene, portions of chromosomes were stained thickly with DAPI, indicating that chromosomes were closely paired at these sites (Figs. 3D,G). Regions of close pairing are likely to be regions where synapsis has occurred. When the same samples were stained with the anti-Rad51 antibody, many foci were observed (Figs. 3E,H) as seen in the leptotene stage (Fig. 3B), and they were located along both the synapsed and unsynapsed regions of chromosomes (Figs. 3F,I, also see Fig. 4, below). In the pachytene stage, synapsis was complete and the chromosomes were condensed, as seen for the chromosomes stained with DAPI (Fig. 3J). The number of the Rad51 foci on pachytene chromosomes (Fig. 3K,L) was far less than that on zygotene chromosomes, as described further below.

To show the location of the foci on the synapsed chromosomes in more detail, portions of the early zygotene chromosomes were stained with the anti-Rad51 antibody and DAPI and were photographed at a higher magnification (Fig. 4). In a region where homologous chromosomes were aligned, most foci were found on only one of homologous segments (Fig. 4A–F). The foci were

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**Figure 3.** Immunolocalization of the Rad51 protein on meiotic chromosomes. Photographs at left (A,D,G,J) show chromosomes stained with DAPI; those in the middle (B,E,H,K) show chromosomes stained with anti-human Rad51 IgG; photographs at right (C,F,I,L) are composite images prepared by alignment of the images of photographs at left and those in the middle. Chromosomes at right are pseudocolored in green. The spreads are from chromosomes at leptotene (A,B, and C); early zygotene (D,E, and F); late zygotene (G,H, and I); and pachytene (J,K, and L), respectively. Bars, 10 μm.
Rad51 and Lim15 proteins on meiotic chromosomes

Figure 4. Location of foci stained with anti-Rad51 antibody on early zygotene chromosomes. The chromosomes were stained with the anti-Rad51 antibody and DAPI. (A) Entire spread of chromosomes with the Rad51 foci located along both the synapsed and unsynapsed regions of chromosomes. (B–F) High magnification views of the foci on the chromosomes. (B–E) Most foci on only one of the synapsed homologous segments. Arrows in A, C, and F indicate examples of foci located adjacent at various distances from the chromosomes. Bars in A and D, 10 and 5 μm, respectively.

frequently separated from chromosomes by varying distances (Fig. 4A, C, F). These foci may correspond to chromatin loops that extend away from the axial cores and to which the Rad51 protein bound. As shown by staining of the late zygotene chromosomes with DAPI (Fig. 3G), the homologous chromosomes are aligned almost side by side.

In the pachytene stage, the number of Rad51 foci was decreased drastically (Fig. 3K). Instead, the whole chromosomes were stained with the anti-Rad51 antibody as shown in Fig. 2A(c). To examine the pattern of localization of the Rad51 protein in the chromosomes in more detail, early pachytene chromosomes stained with the anti-Rad51 antibody were sectioned (0.8 μm interval) and examined using a confocal microscope. In some regions of the sections, we found strongly stained bar-like bands in addition to closely aligned thin threads (Fig. 5).

On the other hand, when the zygotene chromosomes stained with the anti-Rad51 antibody were sectioned, only foci were observed [data not shown]. The structures seen in the pachytene chromosomes were not observed in the zygotene stage. The characteristic pattern of localization of the Rad51 protein on pachytene chromosomes shows the different roles of the protein during different stages in meiosis.

Figure 5. Localization of the Rad51 protein in a section of the early pachytene chromosomes. Staining for the Rad51 protein was carried out as described in Materials and methods. One of the sections (0.8 μm interval) of early pachytene chromosomes observed by a confocal microscopy is shown. Arrows and an arrowhead indicate examples of strongly stained bar-like bands and a thin thread-like staining, respectively. The location of chromosomes was detected by a light microscope [data not shown]. Bar, 10 μm.
**Number of Rad51 foci on the chromosomes**

The number of foci detected by staining with the anti-Rad51 antibody on the chromosomes of each stage of nuclei (20–30 nuclei) was determined. The result shows that the number differed depending on the meiotic stages of the nuclei. Their average numbers are ~900, 600, 100, and 0/mm² of the spread of the nucleus (size of nucleus, ~2–2.5 mm²) for leptotene, zygotene, pachytene, and diplotene nuclei, respectively (Fig. 6). On zygotene nuclei, the number of foci on the chromosomes decreased as the stage proceeded from early to late (see Fig. 3E,H).

**Localization of the Lim15 protein on meiotic chromosomes**

The protein on leptotene and zygotene chromosomes was stained with the anti-Lim15 specific antibody. The Lim15 foci were present in both leptotene (data not shown) and early zygotene (Fig. 7B,C) chromosomes in a manner very similar to that of the Rad51 foci (Fig. 3E,F). The number of foci was largest in the early zygotene stage; the average of zygotene stage was 400/mm². These results were in good agreement with the levels of proteins estimated by the Western blot analysis (Fig. 1A). To compare the pattern of localization of the Lim15 foci on the zygotene chromosomes with that of the Rad51 foci, a part of the zygotene chromosomes was examined at a higher magnification (Fig. 7D,E). No significant difference was found in the pattern of localization of the foci between the Lim15 and Rad51 proteins in the leptotene and zygotene stages.

In the pachytene stage, no staining was observed with the anti-Lim15 antibody (Fig. 2B[b]). Either the Lim15 protein was not present on the chromosomes, or the particular epitope that was used for preparation of the anti-Lim15 antibody was hidden in the structure of the pachytene chromosomes. This observation suggests the absence or alteration of roles of the Lim15 protein in pachytene stage.

**Localization of the Rad51 and Lim15 proteins at the same sites on the chromosomes**

Double staining of early zygotene chromosomes with the anti-Rad51 and anti-Lim15 antibodies was carried out to examine whether the two proteins were colocalized. The chromosomes were stained first with the anti-Lim15 antibody and then labeled with Texas Red-labeled anti-rabbit IgG donkey serum as described in Materials and methods. (A) Chromosomes stained with DAPI, (B) chromosomes stained with anti-Lim15 antibody, (C) a composite image of photographs in A and B aligned by a computer. (D,E) Portions of expanded pictures of C. Bars in A, B, and C, 10 μm; bars in D and E, 5 μm.

Double staining of early zygotene chromosomes with the anti-Rad51 and anti-Lim15 antibodies was carried out to examine whether the two proteins were colocalized. The chromosomes were stained first with the anti-Lim15 antibody and then labeled with Texas Red-labeled anti-rabbit IgG. Subsequently, the same sample was stained with the FITC-labeled anti-rabbit Rad51 antibody. The results show that 80% of the Rad51 and 70% of the Lim15 foci were stained with both antibodies (Fig. 8A) and that many of the foci were present in the regions on or adjacent to the chromosomes (Fig. 8B).

**Discussion**

Using lily cells, we identified the locations of the Rad51 and Lim15 proteins on chromosomes at different meiotic stages. The Rad51 and Lim15 proteins were abundant on leptotene and zygotene chromosomes, forming discrete foci. The number of foci of these proteins decreased drastically as the meiotic stage proceeded from early zygotene to pachytene. A large fraction of the Rad51 and
Rad51 and Lim15 proteins on meiotic chromosomes

Although the presence of homologs of the Rad51 and Lim15 proteins in yeast subnuclear sites has recently been shown (Bishop 1994), neither their exact chromosomal localization nor the meiotic stage of chromosomes to which they bind could be identified in the yeast system. The results summarized above show that, by taking advantage of the excellence of the cytological properties of the lily system, we could significantly advance our understanding of the process of meiotic recombination.

**Involvement of the Rad51 protein in early process in meiotic recombination**

It has been known that *S. cerevisiae* and human Rad51 proteins form a right-handed helical nucleoprotein filament similar to that formed by the RecA protein (Ogawa et al. 1993a; Benson et al. 1994) and that the *S. cerevisiae* protein has strand-transfer activity (Sung 1994). We think it reasonable to consider that the Rad51 protein of the lily carries out an analogous reaction during meiotic recombination. The Rad51 protein in the recombination complexes probably searches homologous DNA sequences and initiates their pairing in a manner similar to the presynaptic complexes formed by RecA and yeast Rad51 proteins. The Rad51 foci were rarely found on the corresponding regions of both homologous chromosomes. This finding supports the hypothesis that the formation of presynaptic complexes on only one of the homologous sequences is sufficient for searching of the homologous sequences that leads to their pairing, as is the case for the homology search by the RecA protein.

The presence of Rad51 on or adjacent to the DAPI-stained chromosomes suggests that the Rad51 protein binds to the DNA regions of the looped chromatin extended away from the axial cores of the chromosomes. On the basis of results of the three-dimensional reconstruction of a serial section of early zygotene chromosomes of the lily at the level of electron microscopy, it has been proposed that the synopsis of homologous chromosomes initiates at various places of the chromosomes and that a part of synaptonemal complex forms by attachment of the axial component after association of chromatin to the central region (Holm 1977). From these views, it is likely that the Rad51 and Lim15 foci on leptotene and zygotene chromosomes correspond to the site of initiation of recombination that begins at a stage before formation of synaptonemal complexes. These foci may correspond to early recombination nodules that have been observed along axial elements at points of convergence between homologous chromosomes.

**Involvement of the Rad51 protein in the late process of meiotic recombination**

On the pachytene chromosomes, the Rad51 protein was observed by confocal microscopy as strongly stained bands as well as two thin thread-like structures. It is evident that the pattern of localization of the Rad51 protein differs as the chromosome structure is changed. These results suggest different roles for this protein during different stages of meiotic recombination.
bands as well as two thin thread-like structures. Formation of this thin thread-like elongated structure along the chromosomes suggests that the Rad51 protein becomes a part of components of the lateral elements in synaptonemal complexes. The strongly stained bands may correspond to late recombination nodules, which have been implicated in the formation of chromosomal crossovers as precursors of chiasmata. The difference in the existence of the Rad51 protein in the early and late nodules and apparent absence of the Lim15 protein on the pachytene chromosomes show that these nodules formed in different stages may have different roles in the process of meiotic recombination.

We have shown the lily to be an excellent organism for studying the localization of the Rad51 and Lim15 proteins on meiotic chromosomes. The stage-specific colocalization of these proteins demonstrates the collaboration of these proteins in the formation of a functional structure. Formation of another stage-specific structure involving the Rad51 protein demonstrates a unique role played by the protein in a late process of meiotic recombination. In light of these results, our approach should be valuable for understanding behavior of various proteins in meiotic recombination.

Materials and methods

Preparation of antibodies

Anti-human Rad51 antibody The human Rad51 protein was overproduced in E. coli using the T7 promoter system (Studier et al. 1990). By using site-directed mutagenesis (Kunkel 1985), a Ncol or BamHI site was created in the first ATG site of the human Rad51 open reading frame (ORF) or just downstream of the ORF, respectively. Primers used were AGGACGGCTGTG-GACGGATCCAATGGAATCAGATC for the Ncol site and AGACTGAAGGATCCGTGTTTTTCTCTCG for the BamHI site. Plasmid pET–HsRad51 was constructed by cloning a Ncol-BamHI fragment containing the human Rad51 ORF into the Ncol–BamHI site of pET8a plasmid (Studier et al. 1990). After E. coli BL21(DE3) strain (Studier et al. 1990), harboring both pET–HsRad51 and pLYS5 plasmids, was grown to early log phase (1x 10^8 cells/ml) in the presence of ampicillin (50 μg/ml), the incubation was continued for 3 hr. The cells were collected, suspended in lysis buffer (20 mM Tris–HCl at pH 8.0), 1 mM EDTA, 5 mM β-mercaptoethanol, 20% (wt/vol) sucrose) with vigorous mixing using a vortex mixer. The lysates were then heated at 95°C for 5 min. An aliquot (1 μl) was electrophoresed in a 12% SDS–polyacrylamide gel using the Phast System (Pharmacia). The gel was stained with Coomassie brilliant blue R250, and the amount of protein in the designated bands was measured by a Laser Scanner (Molecular Dynamics Co. Ltd.).

The lysates containing an equal amount of protein were electrophoresed, and the gel was analyzed by Western blot using the anti-Rad51 or anti-Lim15 antibody.

Preparation of nuclei and chromosomes from microsporocytes

Microsporocytes were obtained from anthers of the lily, L. longiflorum, var. Hinomoto (Sakaguchi Farm, Wakayama, Japan). The meiotic stage of the cells (Erickson 1948) was determined by direct microscopic observation.

The microsporocytes were fixed in 4% (wt/vol) paraformaldehyde for 30–40 min and then placed on a polylysine-coated slide glass. The cells were treated further with 0.2% Triton X-100 for 2 min, and a coverslip was added. The samples were squashed carefully to spread the nuclei or chromosomes and then placed on a dry-ice block. The coverslips were removed from the frozen samples, and the samples were stained with an antibody.

Immunostaining

Chromosomes were first incubated in phosphate-buffered saline (PBS, Harlow and Lane 1988) with 3% (wt/vol) bovine serum albumin (BSA) for 30 min at 23°C (200 μl, without a coverslip). Then, 50 μl of PBS/BSA containing an appropriate dilution of a primary IgG (1:100) or the serum (1:2000 for Rad51, 1:1000 for Lim15) was placed on polylysine-coated slides, which were incubated for 3 hr at 23°C. The slides were washed by dipping them three times into PBS containing 0.05% Tween 20 each for 5 min. Staining with FITC or Texas Red-labeled anti-rabbit IgG donkey serum was carried out under the same incubation conditions used for the primary antibodies except that the incubation time was 60 min. After antibody staining, the chromosomes were stained with PBS containing 0.05 μg/ml of DAPI and mounted with 90% glycerol containing 1 mg/ml of p-phenylendiamine (antifade).

The Lim15–Rad51 sequential double staining was carried out as follows. Samples were stained with the anti-Lim15 antibody using the procedure described above. Then, unbound anti-rabbit donkey serum was saturated with unlabeled preimmune rabbit serum and washed with PBS. The slides were then stained with FITC-labeled anti-Rad51 IgG. Slides were then washed and stained with DAPI as described above.

For primary staining of chromosomes, purified anti-Rad51 IgG or anti-Lim15 IgG was used after appropriate dilution. The FITC or Texas Red-labeled anti-Rad51 IgG and FITC or Texas Red-labeled anti-rabbit IgG donkey serum (Amersham) were used also after dilution.

Western blot analysis of Rad51 and Lim15 proteins

The anthers with premeiocytes and microsporocytes were lysed in SDS lysis buffer (1% SDS, 5% β-mercaptoethanol, 30 mM Tris at pH 6.8) containing the same volume of glass beads (0.05 μm) with vigorous mixing using a vortex mixer. The lysates were then heated at 95°C for 5 min. An aliquot (1 μl) was electrophoresed in a 12% SDS–polyacrylamide gel using the Phast System (Pharmacia). The gel was stained with Coomassie brilliant blue R250, and the amount of protein in the designated bands was measured by a Laser Scanner (Molecular Dynamics Co. Ltd.).
Immunofluorescence microscopy

Images were taken with a Nikon epifluorescence microscope [Nikon Optiphot 2, 40× objectives with a numerical aperture of 0.84 or 100× objective with NA 1.25] equipped with a cooled charged coupled device (CCD) camera (PXL1400-C1-M) [Photometrics], which was controlled by a Macintosh Quadra 840 AV [Apple computer]. The images were recorded as monochrom data using the CCD image capture with IP Lab–PVCM software and Confocal-Deconvolution Software for image correction and enhancement [Signal Analysis Corporation]. Merging and pseudocoloring were accomplished using Adobe Photoshop software, and the images were printed out with a Pictorography 3000 [FUJIX].

For confocal microscopy, the chromosomes were stained with anti-human Rad51 antibody and then labeled with FITC-labeled anti-rabbit IgG donkey serum. The chromosomes were located under a light microscope, and the samples were viewed under an Olympus LSM-GB 100 confocal microscope equipped with an Argon laser. Each image was analyzed using Olympus LSM-GB software.

Acknowledgments

We thank Dr. Jun-ichi Tomizawa for valuable discussions during the research and for a critical reading of the manuscript. We thank to Drs. Joseph Inselburg and Douglas Bishop for comments and Dr. Satoshi Oghara, Mrs. Ichiro Nishii, and Masahiro Ueda for their kind help in manipulating a cooled CCD camera and confocal microscopy. This work was supported in part by a Grant-in-Aid for Specially Promoted Research (06101003) from The Ministry of Education, Science, and Culture of Japan, and by a Collaboration Research grant from National Institute of Genetics.

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*Genes Dev.* 1995, 9:
Access the most recent version at doi:10.1101/gad.9.8.925

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