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Characterization of Meiosis-Specific Strand Transfer Activities in Lily Microsporocytes

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Nuclear extracts of meiotic cells from lily microsporocytes were subjected to a search for strand transfer activities. In addition to the ATP-dependent strand transfer avtivity (m-rec) reported previously, another not requiring ATP (mAi-rec) was detected. Like m-rec, its activity was found to rise steeply during early prophase and reaches a peak at pachytene. During preliminary purification, it became evident that two separate fractions showing different requirement for Mg^{2+} concentration constitute the ATP-independent strand transfer activity. These results indicate the possibility that multiple enzymatic systems are involved in homologous recombination during meiotic prophase.

KEY WORDS: Strand transfer / Recombination / Meiosis

Introduction

The mechanisms of homologous recombination have been studied in both procaryotic and eucaryotic systems. For higher eucaryotes, an *in vitro* recombination assay, that can mimic some aspects of the *in vivo* recombination reaction, has proved a powerful tool for identifying recombination activities in cell extracts. So far, a number of proteins concerned with homologous recombination have thus been characterized and purified by using *in vivo* assay system, *e.g.* D-loop formation and strand transfer reaction (1-7).

Meiosis is known to be a process in which a high frequency of recombination between synapsed homologous chromosomes occurs. Intricate enzymatic systems are therefore expected to secure its fidelity. In previous studies, we concentrated on the involvement of an ATP-dependent recombination protein (m-rec) in lily microsporocytes (2), and on both ATP-dependent and -independent recombination (mAi-rec) activities in mouse spermatocytes (2,7). In the present manuscript, we describe a search for additional activities invoving meiotic homologous recombination in lily meiotic cells by means of the *in vitro* strand transfer assay.

Materials and Methods

Plant materials

Microsporocytes were prepared from anthers of lily, *Lilium longiflorum Thunb.* var

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Hinomoto according to the procedure in ref. (8) . Meiotic stage of the cells was estimated based on its correlation with bud length (9,10) after cytological examination. Microsporocytes were extruded, pooled and washed several times with White's medium, and stored at -20℃ in the medium containing 50% (v/v) glycerol as described elsewhere (10).

Preparation of crude extract of the nuclei

All the steps for preparation of cell extracts were carried out at 0-4℃. Lily microsporocytes stored in White's medium containing 50% (v/v) glycerol were collected by brief centrifugation and washed several times with buffer B (10% glycerol, 20mM Tris-HCl, pH7.5, 0.2mM EGTA, 0.1mM phenylmethylsulfonylfluoride (PMSF) , 1mM p-aminobenzamide, 0.5mM dithiothreitol (DTT) , 0.3M sucrose and 10mM $MgCl_2$). Cell pellets were homogenized with a mortar and a pestel precooled with liquid nitrogen, suspended in buffer B, and the nuclear fraction was precipitated by centrifugation at 3000 rpm for 15 min. After suspension and loading on buffer B containing 30% (v/v) Percoll (Pharmacia) followed by centrifugation at 5000 rpm for 10 min, pellets were washed twice with buffer B, and suspended in buffer A (10% glycerol, 20mM Tris-HCl, pH7.5, 0.2mM EGTA, 0.1mM PMSF, 1mM p-aminobenzamidine, and 0.5mM DTT) containing 0.5M NaCl. After incubation on ice for 30 min to 4hrs with occasional stirring, suspensions were centrifuged for 40000 rpm for 1hr and supernatants used for further analyses.

Single-stranded DNA column chromatography

Crude extract of meiotic nuclei was loaded onto a single-stranded DNA (Pharmacia) column equilibrated with buffer A. Following washing with buffer A containing 50mM NaCl, the fraction eluted with buffer A containing 500mM NaCl was collected with the single-stranded DNA binding proteins included.

Preparation of the cell extracts for strand-transfer assay

Proteins from the crude nuclear extracts were precipitated with ammonium sulfate (50% in w/v) and collected by centrifugation. Pellets were dissolved buffer C (10% glycerol, 10mM Tris-HCl, pH8.0, 1mM EDTA and 0.2mM PMSF) containing 50mM NaCl and dialyzed against the same buffer for several hours. The extracts were loaded onto DEAE-cellulose (Whatman, DE-52) columns equilibrated with buffer C with 50mM NaCl and the columns washed with 5 volumes of the same buffer. The proteins absorbed to resin were eluted stepwise with buffer C containing 0.1M to 1.0M NaCl.

Strand-transfer protein assay

Strand transfer activity was measured according to the procedure of McCarthy *et al.* (5). The reaction mixture (20 μl) consisted of 20mM Tris-HCl, pH7.5, 10mM $MgCl_2$, 1mM DTT, 5mM ATP, and 25 μg/ml of bovine serum albumin. 100 ng of the (+) strand of M13mp18 DNA and 2.5ng of the *Hin*dIII-*Hae*II 285-bp fragment from the *lac* region of pUC18, which had been labeled by ^{32}P at the 5' terminal of the *Hin*dIII site on the (-) strand, were added as substrates. Protein fractions were added indicated and the mixture was incubated at 23℃ for 30min. The reaction was terminated by adding 3 μl of a mixture containing 4% sucrose, 0.7% bromophenol blue, 4% sodium lauryl sulfate, 50mM EDTA, and 0.1mg/ml of proteinase K.

The resultant mixture was incubated at 45℃ for 20min and loaded onto 1.0% agarose gels. Following electrophoresis, X-ray films were exposed to at -80℃.

Two-dimensional (2-D) gel electrophoresis of proteins

Two-dimensional gel electrophoresis of single-stranded DNA binding proteins was performed according to the method of O'Farrell (11). The gels were stained by the procedure written in ref (12).

Results

Strand-transfer activities in meiotic cells

Strand transfer activity of the nuclear extract from microsporocytes was examined (Fig. 1). In the presence of meiotic nuclear extract, a clear band representing the DNA product resulting from the strand transfer reaction was observed. The same band appeared when when RecA protein of *Esherichia coli* was used in place of lily extract. However, no band was de-

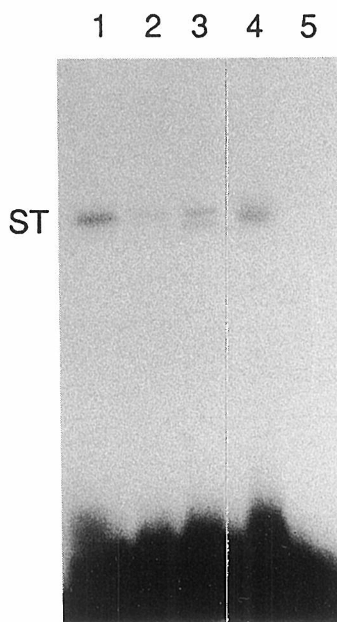

Fig. 1. *Strand tranfer assay of the nuclear extract of lily microsporocytes.*
The nuclear extract was prepared from microsporocytes at pachytene and the strand transfer activity was measured as described in Materials and Methods. Lane 1, RecA protein (0.5 μg) was used in place of lily nuclear extract; lane 2, all components used except for tissue extract; lane 3, same as in lane 2 but with lily extract (2 μg); lane 4, same as in lane 3 but in the absence of ATP; lane 5, same as in lane 3 but containing Φ X174 DNA in place of M13mp18 DNA. ST indicates the position of the reaction product.

tected detected when M13 single stranded DNA was replaced with Φ X174 DNA, indicating that the reaction depends on the sequence homology between the substrates.

The results illustrated in Fig. 1 show that extract still holds its activity in the absence of ATP. Previously, Hotta *et al.* (2) described a protein which catalyses the strand transfer reaction in the presence of ATP. It is therefore strongly suggested that multiple enzymes are involved in the homologous recombination during meiosis. To examine how much of the ATP-independent strand transfer activity (mAi-rec) observed in Fig. 1 takes part in meiotic recombination, a further characterization was carried out.

Characterization of strand-transfer activities

Nuclear extracts were prepared from microsporocytes at different stages of meiotic prophase and strand transfer activity measured in the absence of ATP. As shown in Fig. 2,

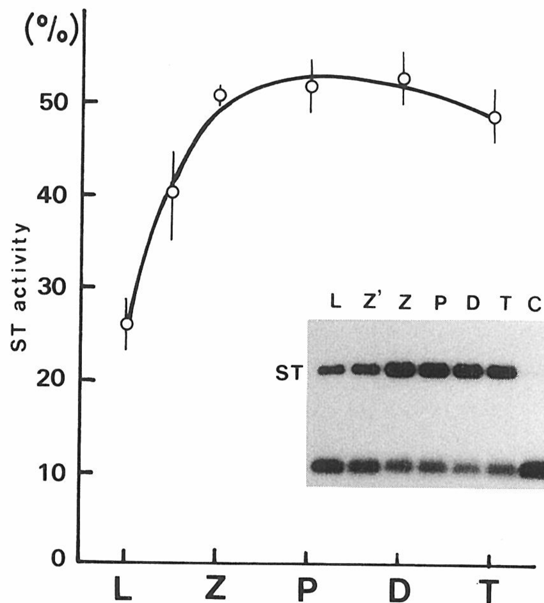

Fig. 2. *Profile of the strand transfer activity of mAi-rec during meiotic prophase.*
Nuclear extracts (5 μg) were prepared from microsporocytes at the indicated stages of meiotic prophase and the strand transfer assay carried out. The reaction products were quantitated by scanning the X-ray film by a density scanner. ST activity (%) indicates the ratio of the amount of the silver grains at the position of the reaction product among the sum of the grains corresponding to the product and the remaining substrate. L, leptotene; Z'. early zygotene; Z, zygotene; P, Pachytene; D, diplotene plus diakinesis; T, tetrads.

shifted bands resulting from the strand transfer reaction were clearly observed for all the fractions. According to quantitation of the reaction products by scanning of X-ray films, strand transfer activity rises steeply before reaching pachytene and gradually decreases subsequently. Pachytene is know to be the stage when recombination between the homologous chromosomes takes place (13.14). The result therefore suggests participation of mAi-rec activity in the homologous recombination reaction in meiosis.

To characterize mAi-rec activity in microsporocytes, nuclear extract was fractionated by ion-exchange column chromatography. It was loaded onto DEAE-cellulose columns and eluted stepwise with buffers containing increasing concentrations of NaCl, as described in the Materials and Methods. Aliquotes from each fraction were mixed with the reaction medium and the strand transfer assay carried out. Under the complete reaction condition including ATP, the major activity was detected in the fraction eluted by 0.2M NaCl [Fraction I] (Fig. 3). In the absence of ATP, a second peak emerged in the fraction eluted by 0.4M NaCl [Fraction II], while Fraction I was still active. Under condition lacking both ATP and Mg^{2+}, Fraction I still held its activity, whereas no or very little activity was detected in Fraction II. The results thus indicate that nuclear extract prepared under our condition, in addition to the

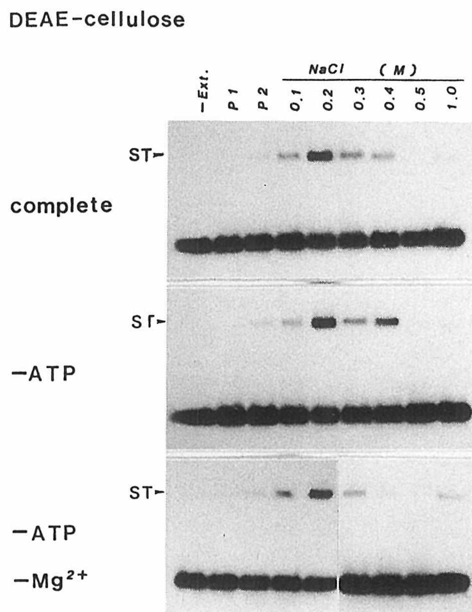

Fig. 3. *Fractionation of nuclear extract by DEAE-cellulose chromatography.* Nuclear extract from microsporocytes at pachytene was loaded onto a DEAE-cellulose column, and fractionated by stepwise elution as described in the Materials and Methods. Strand transfer activity was measured for each fraction under conditions as indicated in the figure. -Ext., assay without the nuclear extract; -P1, pass-through fraction; -P2, fraction eluted with buffer containing 50mM NaCl.

the previously reported ATP-dependent activity, contains at least two strand transfer activities with different requirements for Mg^{2+}.

The Mg^{2+} requirement of Fraction II was further examined by measuring strand transfer activity in the presence of various concentrations of $MgCl_2$. As shown in Fig. 4, a strand

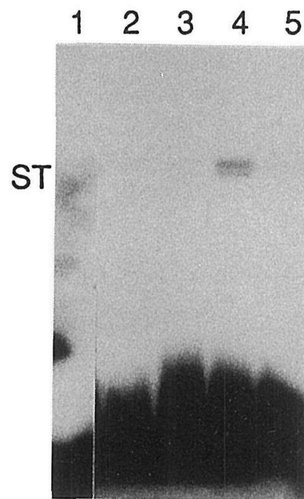

Fig. 4. *Mg^{2+} requirement for strand transfer activity of Fraction II*
Strand transfer activity of Fraction II (2 μg) assayed in the presence of various concentrations of $MgCl_2$. The reaction was carried out in the presence of lane; 1. all components except for Fraction II; lane 2, 0mM; lane 3, 1mM; lane 4, 10mM; and lane 5, 100mM of $MgCl_2$.

transfer reaction took place only at the concentration of 10mM and no reaction product was detected at 0mM, 1mM and 100mM of $MgCl_2$. The result clearly indicates a strict dependency of the strand transfer activity of Fraction II for Mg^{2+} ions.

Search for stage-specific proteins during meiotic prophase

Previous reports have indicated that the proteins which catalyze strand transfer and/or D-loop formation reactions exhibit binding to single-stranded DNA (2,7). As a preliminary experiment for tagging the recombination activities described above to corresponding polypeptides, an analysis of the proteins capable of binding to single-stranded DNA (ss DNA) was carried out. Nuclei prepared from microsporocytes at different stages of meiotic prophase were treated with buffer containing 0.5M NaCl. The soluble proteins were concentrated and loaded onto ss DNA cellulose columns, and the ss DNA binding fractions eluted with

0.5M NaCl pooled and resolved by 2-D gel electrophoresis as described in the Materials and Methods.

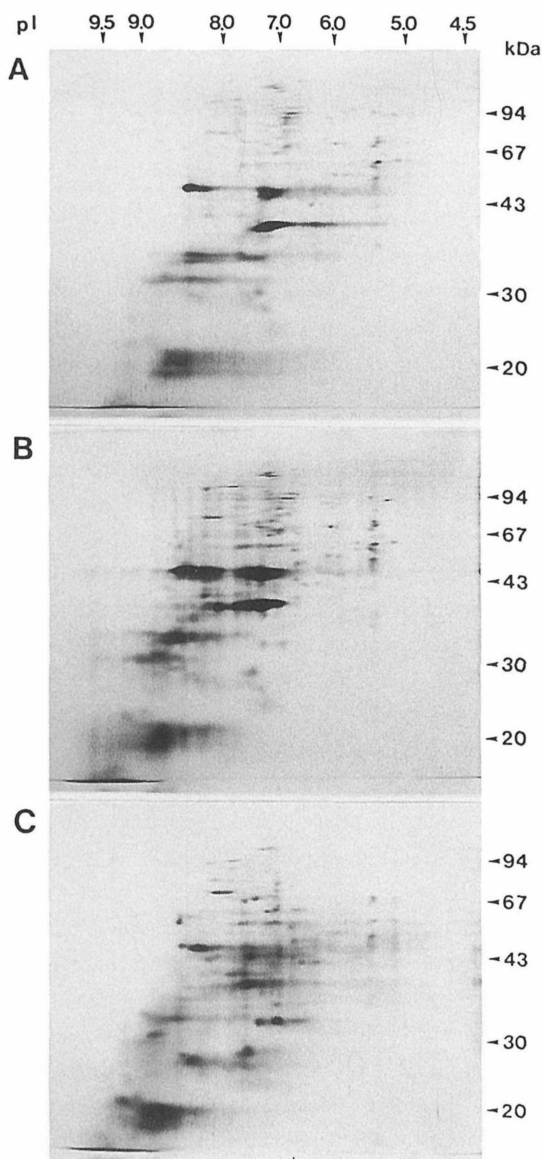

Fig. 5. *2-D analysis of the ss-binding proteins from nuclear extracts at various stages of meiotic prophase.*
The ss-DNA binding proteins were prepared from microsporocytes at various stages of prophase and separated on 2-D gels as described in the Materials and Methods. A, mixture of interphase and leptotene; B, mixture of zygotene and pachytene; C, mixture from diplotene to metaphase II. Approximately 40 μg of the proteins were loaded on the gels.

As shown in Fig. 5, drastic changes were observed in terms of both species and quantity of polypeptides which constitute the ss DNA binding fraction during meiotic prophase. Multiple polypeptides were detected as spots whose intensity increased as meiosis proceeded and decreased after the pachytene stage. Some of these spots should correspond to the polypeptides representing m-rec and mAi-rec, since their activities reach maxima in the pachytene stage ((2) and Fig. 2).

Discussion

In this report, we have described a novel strand transfer activity, mAi-rec, which is independent of ATP, in lily microsporocytes. The maximum activity of mAi-rec was detected at pachytene during the meiotic prophase, suggesting its relevance to meiotic recombination between homologous chromosomes. By preliminary purification, this activity was separated into two fractions, one of which is dependent on Mg^{2+} and the other is not.

Higashitani *et. al.* earlier described on ATP-independent recombination activity as well as ATP-dependent activity (m-rec) in mouse spermatocytes (7). In their study, it was shown that the mouse mAi-rec is meiosis-specific with a profile during meiosis showing only limited similarities to that of m-rec. The fact that the meiotic profile of mAi-rec in lily microsporocytes looks more like that of mAi-rec in the mouse than that of m-rec in lily (2) may indicate the occurrence of similar mechanisms for meiotic recombination in different species. Of the two fractions which were found in the present study to constitute the mAi-rec activity, Fraction II seems to correspond to mAi-rec in the mouse, since both require Mg^{2+} for catalyzing the strand transfer reaction.

There is evidence that histone H1 is able to mediate the strand transfer reaction in the absence of ATP and Mg^{2+} (15). We have also proved that, in lily, histone H1 alone can catalize the strand transfer reaction without ATP and Mg^{2+} (data not shown). Since Fraction I in mAi-rec in this study and histone H1 share the above characteristics in common, and judging from the behavior on the DEAE-cellulose chromatograohy, it is most probable that the mAi-rec in Fraction I is histone H1.

It was previously reported that mAi-rec in the mouse has ability to bind to ss-DNA (7). If both mAi-rec proteins of different origin share the same character, it is probable that lily mAi-rec would able to be recovered in fractions binding to ss-DNA. The result obtained from the present 2-D analysis of ssDNA binding proteins was too complex to specify mAi-rec to any corresponding polypeptide (s) for the moment. Nevertheless, several pachytene-specific spots were clearly observed, and it might be possible to further characterize mAi-rec and other proteins involved in recombination reactions by analyzing the polypeptides corresponding to these spots.

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