A DNA Exonuclease Induced during Meiosis of Schizosaccharomyces pombe*

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In meiotic cells of the fission yeast Schizosaccharomyces pombe, a DNA exonuclease activity increased approximately 5-fold after premeiotic S-phase and decreased to the initial level before the meiotic divisions. We have purified this activity, designated exonuclease I, to near homogeneity. The activity co-purified with a polypeptide with an apparent molecular weight of 36,000. With a linear double-stranded DNA substrate, exonuclease I degraded only the 5'-ended strand from each end to produce 3'-single-stranded tails. The enzyme also acted on nicked circular DNA with comparable affinity. The meiotic induction of exonuclease I and its mode of action, similar to that of recombination-promoting exonucleases from bacteria, suggest that exonuclease I is involved in meiotic homologous recombination in S. pombe.

Nucleases are believed to play important roles during breakjoin homologous recombination. Both endo- and exonucleases have been proposed to act at various steps in the pathways that lead from parental to recombinant chromosomes. In an early step of recombination, one or more participating DNA strands have to be broken. Such DNA breaks could occur as the result of DNA damage or its repair. Alternatively, specific endonucleases might introduce single or double strand breaks that lead to the initiation of genetic exchange. For example, purified RecBCD enzyme of Escherichia coli introduces a nick at a specific recombination-stimulating octanucleotide, Chi (Ponticelli et al., 1985); the single-stranded DNA product is then believed to initiate joint molecule formation (Smith et al., 1984).

DNA breakage is believed to be followed by the formation of hybrid DNA, the annealing of single strands from different parental chromosomes. This central step accounts for the homology dependence of genetic recombination. E. coli RecA protein (reviewed in Radding, 1982; Cox and Lehman, 1987) and homologous pairing activities from other organisms (fungi, Kolodner et al., 1987; Sugino et al., 1988; Halbrook and McEntee, 1989; Kmiec and Holloman, 1983; fruit flies, Eisen and Camerini-Otero, 1988; humans, reviewed in Kucherlapati and Moore, 1988) were most commonly detected by their ability to promote the formation of hybrid DNA between two homologous DNA molecules, one being single-stranded.

Purified RecA protein, in particular, requires at least partial single-strandedness of one DNA molecule for stable hybrid DNA formation.

The regions of single-stranded DNA that are the substrate for annealing may be produced by DNA unwinding enzymes (Taylor and Smith, 1980) or exonucleases that preferentially degrade only one strand of duplex DNA. Exonuclease VIII of E. coli and λ exonuclease both produce long 5'-single-stranded tails (Joseph and Kolodner, 1985; Little, 1987) and are required for the RecE and Red pathways of recombination, respectively (Gillen et al., 1981; Stahl, 1986). Bacteriophage T7 gene 6 exonuclease has properties similar to those of ExoVIII and λ exonuclease (Kerr and Sadowski, 1972) and has been implicated in the recombination of phage T7 DNA (Kerr and Sadowski, 1975). Mating type switching (White and Haber, 1990) and the processing of RAD50-dependent double strand breaks (Cao et al., 1990; Sun et al., 1991) in Saccharomyces cerevisiae, as well as plasmid recombination in Xenopus laevis oocytes (Maryon and Carroll, 1991), involve intermediates with long single-stranded tails presumably formed by exonucleases acting on double strand breaks.

In eukaryotes, recombination occurs in numerous cell types throughout the organism's life cycle. During meiosis, however, recombination is induced. For example, in Schizosaccharomyces pombe, intragenic and intergenic recombination are induced up to 200- and 1000-fold, respectively (Grossenbacher-Grunder, 1985). The degree of meiotic induction is variable from locus to locus. Similar values have been observed in S. cerevisiae (e.g. Menees and Roeder, 1989). This increase in recombination may be accounted for, at least in part, by the induction of meiosis-specific recombination factors. The background levels of recombination in mitotically dividing cells may be due to by-products of DNA damage repair (Resnick, 1979). Supporting the existence of meiosis-specific factors is the observation that of six mutants of S. pombe deficient in meiotic recombination (Ponticelli and Smith, 1989) none tested has a detectable effect on mitotic recombination. ¹ In S. cerevisiae, mutations in the genes HOPI, RED1, MER1, MER2, SP01, REC102, REC107, and, in certain cases, RAD50 affect recombination only in meiosis (e.g. Engebrecht et al., 1990, and references therein; Malone et al., 1991).

It is important for the biochemical analysis of meiotic recombination to have large quantities of cells undergoing synchronous meiosis. The conditional mutant pat1-114 of S. pombe undergoes meiosis at elevated temperature (Lino and Yamamoto, 1985a) and bypasses the requirements of nitrogen starvation and mating type heterozygosity for the initiation of meiosis (Beach et al., 1985). The pat1 gene encodes a protein kinase homologue (McLeod and Beach, 1986) and negatively regulates both conjugation of haploid cells and the

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¹ K. Larson and N. Hollingsworth, personal communication.

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initiation of meiosis (Nielsen and Egel, 1990; Beach et al., 1986). In pat1-114-induced meiosis, ade6 intragenic recombination frequencies are comparable to those observed in standard (pat1*) meiotic crosses, and the M26 meiotic hotspot has its full stimulatory effect (Gutz, 1971).2 Furthermore, in haploid pat1-114 cells at high temperature, recombination between an ade6 gene on a plasmid and its counterpart on the chromosome is detected in the 1% viable spores at a frequency comparable to that measured for plasmid-by-chromosome recombination in diploid meiosis (Ponticelli and Smith, 1989).2 Others have also observed high frequencies of recombination in pat1-114-induced meiosis (Lino and Yamamoto, 1985b; Bähler et al., 1991). We therefore conclude that the components of the meiotic recombination machinery are expressed during pat1-114-induced meiosis, even in haploid cells.

In this paper, we describe the purification of an exonuclease, called ExoI, from pat1-114 haploid cells induced to undergo meiosis. Its expression and mechanism of action suggest a role for ExoI during meiotic recombination in S. pombe.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Chemicals**—Restriction endonucleases and their buffers, E. coli DNA polymerase I large fragment, T4 polynucleotide kinase, S1 nuclease, and calf intestinal phosphatase were obtained from New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories. Unless otherwise indicated, chemicals were obtained from Sigma, J. T. Baker Chemical Co., and U. S. Biochemicals. Yeast RNA was purchased from BDH Biochemicals (Poole, Great Britain).

**Strains**—S. pombe GP535 is h− ade6-M26 pat1-114 end1-458; its genealogy is available on request. The mutations pat1-114 and end1-458 have been described (Lino and Yamamoto, 1985a; Uemura and Yanagida, 1984). S. pombe strain 458 (h− leu1-32 end1-458) was obtained from Mitsuhiro Yanagida. (This strain is GP61 in our collection.) E. coli B strain V1382 (thyk deo sup) was obtained from Richard Kolodner (Dana Farber Cancer Institute) as RDK1004. E. coli K12 strain V1453 was constructed as follows. From V1025 (lac-pro-hsdR hsdM λ rpsL F’ lac-pro lacI857), a gift from Stanley Brown, N. Yanagida, and K. Yamamoto (personal communication), spontaneous transconjugants were isolated on LB-H agar (0.5% yeast extract (Difco), 0.5% NaCl, 1% tryptone (Difco), adjusted to pH 7.0 with NaOH) containing trimethoprim (10 μg/ml) and thymine (50 μg/ml). Thereafter, a derivative, probably mutant in the deo operon, was selected for growth on a lower concentration of thymine (2 μg/ml).

**Cell Growth**—S. pombe strain GP535 was grown in YEL (0.5% yeast extract, 3% glucose), supplemented with adenine (80 pg/ml) and thymine (50 pg/ml). Thereafter, a derivative, probably mutant in the deo operon, was selected for growth on a lower concentration of thymine (2 μg/ml).

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phage T7 DNA, extracted twice with chloroform, and precipitated with ethanol. This protocol yielded approximately 1 mg of viral DNA and 150 μg of replicative form DNA at 5.5 × 10⁶ cpm/nmol of nucleotides.

**Nicked Plasmid DNA—**H-Labeled M13mp18 replicative form DNA (0.75 mg/ml), ethidium bromide (32 μg/ml), and the restriction endonuclease HindIII (100 units/ml) in 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT were incubated at room temperature for 1 h. The mixture was extracted twice with phenol, once with chloroform, and precipitated with ethanol. These conditions resulted in the formation of approximately 50% nicked circular DNA and less than 5% linear DNA as judged from an ethidium bromide-stained agarose gel. A portion of the nicked DNA was electrophoresed on and eluted from an agarose gel and linearized with the restriction enzyme BglII. On an alkaline agarose gel (Sambrook et al., 1989), the band corresponding to the linear strand was quantitatively converted into a discrete, faster migrating band (not shown), indicating that most of the molecules contained a single nick at the HindIII site.

**Internally 32P-Labeled DNA—**M13mp18 virion DNA (0.4 pmol of molecules, New England Biolabs) and 0.8 pmol of M13 universal primer (U. S. Biochemicals) in 12 μl of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT were heated to 65 °C and slowly cooled to room temperature. The complementary strand was synthesized with 2.5 units of E. coli DNA polymerase I large fragment for 30 min at room temperature, in the presence of 10 μCi of [α-32P]dCTP or [α-32P]dGTP (Du Pont-New England Nuclear, diluted to 80 Ci/mmol) and 600 pmol of each of the three unlabeled dNTPs. After enzyme inactivation at 65 °C for 15 min, the DNA was precipitated with ethanol, dissolved in 15 μl of water, and the counts measured were within 10% of each other.

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**Thin Layer Chromatography—**Reaction products were separated on a polyethyleneimine-cellulose plate with fluorescent indicator (J. T. Baker Chemical Co.) according to Randerath and Randerseth (1967). Prior to use, the plate was submerged in 1 M acetic acid for 2 min and then dried. Without this step, dCMP migrated as a smear at the front of the chromatogram. 20 nmol of nonradioactive nucleotides and 25% of the exonuclease digestion mixtures were spotted 2 cm from the edge of the plate, which was developed at room temperature in 1 M acetic acid for 2 cm. The plate was dried at 65 °C for 15 min, the DNA was digested with Smal restriction endonuclease, extracted with phenol and chloroform, and precipitated with ethanol.

**Other DNA Manipulations—**Linear DNA was labeled with 32P at the 5' or the 3' ends according to Sambrook et al. (1989). For 5'-end labeling, Sau3A-cleaved M13mp18 replicative form DNA was used with GATP, dGTP, TTP, and [α-32P]pGTP to form 5' ends. For 3'-end labeling, HaeIII-cleaved M13mp18 replicative form DNA was used. Approximately 10 6 cpm were incorporated per μg of DNA.

**Double-stranded DNA was analyzed on 1% agarose gels in TAE buffer (Sambrook et al., 1989) in the presence of ethidium bromide (0.5 mg/ml). Circular single-stranded virion DNA was separated on 1% agarose gels in 0.5 × TBE buffer (Sambrook et al., 1989) and stained afterwards with ethidium bromide (1 μg/ml).

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centrator (Amicon) and applied to a Supereose 12 column (HR10/30, FPLC system, Pharmacia) equilibrated in buffer B75/200. The column was run at 0.25 ml/min, and 0.5-ml fractions were collected. The fraction with the peak of enzyme activity (fraction VI, 0.5 ml) was dialyzed overnight against buffer C, and aliquots were frozen in liquid nitrogen and stored at -70 °C.

Small scale preparations of crude extracts from 0.8 liter of culture harvested during a meiotic time course (see above) were done as follows. Cells were thawed and transferred into a 2-ml screw cap tube (Sarstedt) containing 1 ml (packed volume) of glass beads. The tube was filled with buffer A and closed avoiding air bubbles. With the Mini-Bead-Beater (Biospec Products), cells were broken during 10 pulses of 30 s at 5 °C with 30-s intervals on ice. The liquid was removed, and the glass beads were rinsed with 1 ml of buffer A. The lysate was spun at 100,000 × g in a Beckman 70.1 Ti rotor for 30 min. The supernatant was dialyzed and assayed for exonuclease activity as described above.

Protein Concentrations—Protein concentrations were determined according to Bradford (1976) using the protein assay dye purchased from Bio-Rad and bovine serum albumin as a standard. Protein samples were separated by NaDodSO4-polyacrylamide gel electrophoresis (Laemmli, 1970) on a 0.75-mm gel in a Mighty Small apparatus (Hoefer) and stained with Coomassie Brilliant Blue R-250. Samples in large volumes were concentrated by precipitation in 10% trichloroacetic acid.

**RESULTS**

**An Exonuclease Activity Induced during Meiosis of a patl-114 Strain**—We used a haploid S. pombe strain (GP535) carrying the temperature-sensitive patl-114 mutation to obtain synchronous meiotic cultures, as described under “Experimental Procedures.” A strong DNA endonuclease activity is present in extracts from both meiotic and mitotic cells of S. pombe. A mutation (endl-458) abolishing this activity has been described (Uemura and Yanagida, 1984); endl-458 does not affect meiotic recombination.2 We used this mutation in our strains in order to more readily detect exonuclease activity, measured as the production of acid-soluble nucleotides from linear duplex DNA (see "Experimental Procedures").

**patl-114** cells efficiently enter meiotic differentiation when first grown in minimal medium (EMM2*) to a maximal density of approximately 5 × 10^6 cells/ml (B5 et al., 1985). To synchronize large cultures of GP535 prior to the shift to the restrictive temperature and the following onset of meiosis, the cells were first grown to saturation in yeast extract medium (YEL), transferred into minimal medium, and allowed to grow for approximately one generation (see below). This amount of growth in minimal medium was sufficient for the cells to exit from starvation arrest and still allowed reasonable synchrony as judged by monitoring DNA synthesis and meiotic divisions (Fig. 1). To analyze DNA synthesis, a culture of GP535 was allowed to undergo meiosis in the presence of [3H] uracil, as described under “Experimental Procedures.” Total "H incorporated into DNA during premeiotic S-phase was measured at various times in acid-soluble, alkaline-resistant material (Fig. 1, squares). To ensure that only DNA and not RNA synthesis was measured, a parallel experiment was carried out with hydroxyurea, an inhibitor of DNA synthesis (Mitchison, 1974) added to 50 mM 30 min after heat induction. In this case, no significant incorporation of "H was measured during 6 h (not shown). To determine the number of cells with four nuclei, resulting from the two sequential meiotic divisions, cells were removed from a heat-induced culture of GP535 at various times, stained with the fluorescent DNA dye DAPI and observed under a fluorescent microscope (Fig. 1, triangles). The bulk of DNA synthesis occurred between 1.5 and 3 h after shift to the restrictive temperature. Because we observed only a single burst of "H incorporation into DNA soon after the temperature was shifted to 33 °C, we infer that at time zero (Fig. 1) most of the cells were in G1 phase. As observed for starvation-induced meiosis of patl" strains, temperature-induced meiosis of patl-114 strains also starts in G1 (Beach et al., 1985). The percentage of cells with four nuclei rapidly increased between 5 and 6 h after shift to the restrictive temperature. Up to 90% of the cells had four nuclei after 7 h (not shown).

From samples taken during this meiotic time course, we measured exonuclease activity on uniformly "H-labeled T7 DNA. We found that maximal activity required a high concentration of DNA ends (see below) and therefore cleaved the [3H]T7 DNA with the restriction endonuclease HaeIII into 69 fragments with an average size of 600 base pairs. Exonuclease activity was induced about 5-fold, peaking between 4.5 and 5 h (Fig. 1, closed circles). Hence, the activity was maximal between premeiotic S-phase and the meiotic divisions, a time span in which meiotic recombination is believed to occur (Borts et al., 1984; Resnick et al., 1984). To rule out the possibility that the induction of exonuclease activity was a response to heat shock, the same growth protocol was applied to a patl" strain (458). In this case, no induction of exonuclease activity was observed (Fig. 1, open circles). We conclude that the induction of exonuclease activity is patl-114-dependent and, therefore, is a true meiotic event. Using a diploid strain homozygous for the patl-114 mutation, we measured the same timing and degree of induction of exonuclease activity (not shown).

**Purification of a Meiotic Exonuclease**—Large quantities of S. pombe strain GP535 were grown and induced to undergo meiosis for 4.75 h as described under “Experimental Procedures.” The purification scheme, summarized in Table I, yielded a polypeptide of about 36 kDa, as estimated by NaDodSO4-polyacrylamide gel electrophoresis (Fig. 2a). The protein was purified approximately 10,000-fold to near homogeneity. The faint additional band around 60 kDa, enriched in contaminating with keratin during handling of the sample. From the Mono Q column, aliquots of fractions including and surrounding the peak of enzyme activity were analyzed by gel electrophoresis; the amount of enzyme activity coincided with the intensity of the 36-kDa polypeptide (Fig. 2b). The same result was obtained with fractions from the Superose 12 column (not shown). By calibrating the Superose 12 column, the molecular weight of the enzyme was estimated at about 20,000 (not shown). This suggests that the native enzyme is...
S. pombe Meiotic Exonuclease

**TABLE I**

| Step | Total protein | Volume | Total activity | Specific activity | Purification (total/step) | Yield (total/step) |
|------|---------------|--------|---------------|------------------|--------------------------|-------------------|
|      | mg            | ml     | units         | units/mg         | -fold                    | %                 |
| I (crude extract) | 6,500 | 650 | 1,400 | 0.21 | 1 | 100 |
| II (polyethyleneimine/(NH₄)₂SO₄) | 3,500 | 150 | 1,200 | 0.34 | 1.6 | 88 |
| III (DNA-cellulose) | 37 | 41 | 460 | 12 | 59/36 | 33/38 |
| IV (S-Sepharose) | 1.5 | 4.9 | 160 | 110 | 500/8.6 | 12/35 |
| V (Mono Q) | 0.05 | 0.96 | 86 | 1,400 | 6,500/13 | 6.3/54 |
| VI (Superose 12) | ND* | 0.9 | 7 | ND | ND | 0.5/8.1 |

*ND, not determined, due to insufficient protein.

**FIG. 2. Electrophoretic separation of purified protein samples.** a, aliquots from fractions I to VI (see “Experimental Procedures,” Table I) were run on a 12% polyacrylamide gel as indicated. The following amounts were loaded. I, 50 µg (0.01 enzyme unit); II, 46 µg (0.02 unit); III, 30 µg (0.3 unit); IV, 5 µg (0.5 unit); V, 1.8 µg (3 units); VI, 3 units, M, size standards (low range, Bio-Rad), 0.5 µg each. The molecular sizes (in kilodaltons) of the size standards are indicated. b, 2 µl of 50-fold dilutions of fractions 46 to 54 from the Mono Q column were assayed for exonuclease activity (bar graph). The molecular size markers as in a. Column fractions 49 and 50 were pooled to give fraction V (Table I). TCA, trichloroacetic acid.

Exonuclease I is a Nonprocessive Double-stranded DNA Exonuclease—The cofactor requirements of ExoI are summarized in Table II. A divalent cation was essential for activity; Mg²⁺ was more effective than Mn²⁺. As counterion OAc⁻ was slightly favored over Cl⁻. Maximal activity was obtained between 2 and 5 mM Mg(OAc)₂, with higher concentrations being slightly inhibitory (50% activity at 20 mM; not shown). Both Ca²⁺ and Zn²⁺ inhibited the activity to 40% at 5 mM and to 2% at 0.1 mM, respectively. The addition of ATP to 1 mM had no detectable influence. In experiments with crude extracts, we did not observe any ATP-dependent nuclease in meiotic or mitotic extracts of S. pombe (not shown). Enzyme activity was slightly salt-sensitive, being reduced to 65% at 100 mM NaCl. The optimal pH was between 7 and 7.5, with approximately 20% as much activity at pH 6 or 8 (not shown). The temperature optimum of ExoI was 36 °C, with 80% as much activity at our standard reaction temperature of 31 °C (not shown). Yeast RNA (1 µg, which provided at least a 10-fold excess of RNA ends over DNA ends as calculated from the average length of the RNA molecules; not shown) did not compete significantly with the substrate DNA. Therefore, Exol is DNA-specific.

To compare the activity of ExoI on double-stranded and single-stranded DNA, we used a 5' ⁴°P-labeled single-stranded T7 DNA, 0.5 nmol, and a 36 kDa protein, we estimate from the activity in the crude extract that there are about 1,000 molecules of ExoI per induced cell.

**TABLE II**

| Reaction condition | Relative activity |
|--------------------|------------------|
| Standard reaction* | 100 |
| -Mg(OAc)₂ | 0 |
| -Mg(OAc)₂, +Mn(OAc)₃ at 5 mM | 42 |
| -Mg(OAc)₂, +MgCl₂ at 5 mM | 59 |
| -DTT* | 85 |
| -BSA | 97 |
| +Ca(OAc)₂ at 1 mM | 78 |
| +Ca(OAc)₂ at 5 mM | 40 |
| +Zn(OAc)₂ at 0.01 mM | 2 |
| +Zn(OAc)₂ at 0.1 mM | 2 |
| +NaCl at 50 mM | 78 |
| +ATP at 1 mM | 63 |
| +RNA | 97 |
| +ATP at 1 mM | 97 |
| +dNMPs, 0.125 nmol each | 87 |

*Reactions as described under “Experimental Procedures” with 36 × 10⁶ units of fraction VI.

*ExoI diluted in buffer lacking DTT.
To some samples, replicative form DNA linearized with SmaI was reacted with ExoI before unlabeled DNA also reduced the production of acid-soluble or distributive. The experiment in Fig. 1975; not shown). We also determined whether digestion by ExoI is processive or distributive. The experiment in Fig. 4 used reactions to which a 5-fold excess of ends from unlabeled DNA was added to the reactions at different times. The addition of unlabeled DNA before ExoI reduced the production of acid-soluble label to the same extent as that of the control reaction (closed circles). When added 3 min after ExoI, the unlabeled DNA also reduced the production of acid-soluble label to the same extent (open circles). This implies that ExoI has a low degree of processivity and dissociates from the DNA after hydrolyzing a limited number of phosphodiester bonds.

In this experiment, linearized [3H]M13 DNA was used so that the molecules were not completely hydrolyzed during the time of the reaction (see below for rates). ExoI has no detectable activity on supercoiled plasmid DNA or circular single-stranded M13 virion DNA as determined by agarose gel electrophoresis (not shown). The enzyme acts only exonucleolytically.

Exonuclease I Degradates Only One Strand from a Double-stranded DNA End—To further characterize the mode of degradation by ExoI, linear blunt-ended DNA labeled at either the 3' or the 5' end with 32P was mixed with the uniformly 3H-labeled substrate DNA. ExoI produced acid-soluble 32P label from the 5' end (Fig. 5b, open squares), but not from the 3' end (Fig. 5a, open circles). Comparable overall activity of ExoI in both experiments was confirmed by measuring the release of the uniform 3H label (Fig. 5, a and b, filled symbols). This result suggests that only the 5'-ended strand is detectably degraded from a given double strand end.

In the following experiment, we determined that ExoI indeed produces single-stranded DNA by sparring the 3' ends. We tested this notion with standard exonuclease reactions followed by digestion with the single strand specific nuclease S1 (Wiegand et al., 1975). Fig. 6a shows that, depending on the extent of digestion with ExoI (hatched bars), a nearly equal amount of DNA was rendered acid-soluble by a subsequent incubation with nuclease S1 (black bars). Reactions

![Fig. 3. Double strand specificity of digestion by ExoI. 1 nmol of HaeIII-cleaved [3H]T7 DNA, either double-stranded (O) or heat-denatured (C), was reacted with ExoI (3.6 x 10^-3 units or 0.25 μl of fraction VI) for the times indicated. There was approximately a 300-fold excess of DNA ends over enzyme molecules (assuming approximately 200 ng of protein to be in the ExoI band in Fig. 2a, lane VI). TCA, trichloroacetic acid.](image)

![Fig. 4. Distributive action of ExoI. 6 nmol of HaeIII-cleaved [3H]M13mp18 replicative form DNA linearized with Smal was reacted with ExoI (3.6 x 10^-3 units or 0.25 μl of fraction VI) for the times indicated (O). To some samples, 0.5 μg of unlabeled HaeIII-cleaved pBR322 DNA (5-fold excess of double strand ends from unlabeled DNA) was added before (■) or 3 min after (O) addition of ExoI. TCA, trichloroacetic acid.](image)

![Fig. 5. Polarity of digestion by ExoI. 1 nmol of HaeIII-cleaved [3H]T7 DNA was reacted with ExoI (3.6 x 10^-3 units or 0.25 μl of fraction VI) for the times indicated. a, these reactions contained in addition 0.5 pmol of blunt-ended DNA labeled with 32P at the 3' end (see "Experimental Procedures"). Radioactivity was determined by double label scintillation counting. b, these reactions contained in addition 0.5 pmol of blunt-ended DNA labeled with 32P at the 5' end. TCA, trichloroacetic acid.](image)

![Fig. 6. Production of single-stranded DNA during digestion by ExoI. a, 1 nmol of HaeIII-cleaved [3H]T7 DNA was reacted with ExoI (3.6 x 10^-3 units or 0.25 μl of fraction VI) for the times indicated in triplicate, as standard reactions (hatched bars) or standard reactions followed by incubation with nuclease S1 buffer alone (gray bars) or with S1 nuclease (black bars). b, 1 nmol (O) or 0.5 nmol (C) of HaeIII-cleaved [3H]T7 DNA was reacted with ExoI (3.6 x 10^-3 units or 2.5 μl of fraction VI) for the times indicated at a ratio of DNA ends to enzyme molecules of 30 and 15, respectively. The change in the production of trichloroacetic acid (TCA)-soluble counts upon addition at 15 min of another aliquot of substrate or at 60 min of another aliquot of enzyme is shown by a dashed line and a dotted line, respectively. For further explanation, see text.](image)
continued with low pH, Zn$^{2+}$-containing S1 buffer but without S1 (gray bars) inhibited further ExoI activity and did not yield additional acid-soluble material.

Since ExoI had a strong preference for double-stranded DNA (Fig. 3), these results suggest that 50% of the input substrate, i.e. the single-stranded product, should be resistant to extensive digestion. Fig. 6b shows an experiment in which 1 nmol (closed circles) or 0.5 nmol (open circles) of HaeIII-cleaved [$^3$H]T7 DNA was digested to the limit by ExoI. 60% of the DNA mass was rendered acid-soluble independent of its amount present in the reaction mixture. That the remaining 40% of the DNA mass were resistant to digestion (e.g. by having been rendered single-stranded) rather than the enzyme loosing its activity was confirmed by two control experiments. First, we added an additional, equal amount of substrate to one sample after 15 min; 50% of this DNA was digested (dashed line). Second, an additional, equal amount of enzyme was added to one sample after 60 min of reaction, and no further digestion was observed (dotted line). In Table II (bottom), we show that neither reaction product, single-stranded DNA or deoxyribonucleoside monophosphates, inhibited enzyme activity when they were added at about twice the concentrations produced in the above experiment. This rules out product inhibition in the experiment shown in Fig. 6b. The fact that 60% rather than 50% of the substrate was digested could have several explanations; for example, as Fig. 3 shows, ExoI has a weak activity on single-stranded DNA; and nicks accumulated during substrate preparation might produce short acid-soluble oligonucleotides when rendered single-stranded.

Estimating the concentration of ExoI in fraction VI (see above) and knowing that only one strand is degraded at a time, we calculated that in the experiment shown in Fig. 3 one ExoI molecule cleaves 750 phosphodiester bonds per min on the average (see also product analysis below).

**Exonuclease I Produces 5'-Mononucleotides**—We wished to determine the nature of the acid-soluble products of digestion by ExoI. We addressed two questions. (i) Is the acid-soluble material composed of nucleoside monophosphates or short oligonucleotides? (ii) Does cleavage of the DNA backbone by ExoI produce 5' or 3' phosphates? The two questions were addressed by the experiment shown in Fig. 7 with the following rationale. A double-stranded DNA substrate containing internal $^{32}$P label was constructed by synthesizing the complementary strand of M13 single-stranded DNA with DNA polymerase and the four deoxyribonucleoside triphosphates, one being $^{32}$P-labeled at the α position. If cleavage by ExoI produces 5’-phosphates, then the $^{32}$P atoms would be released with the same nucleotide used for the synthesis. If, on the other hand, ExoI produces 3’ phosphates, then the $^{32}$P atoms would be released with the nucleotides positioned on the 5’ sides of the nucleotide used for the synthesis.

Fig. 7 shows that the $^{32}$P label was always released as TMP when TTP was the labeled nucleotide and as dCMP when dCTP was the labeled nucleotide. At least 25% of the released label should have been released as 3’-dGMP (estimated from the published sequence of M13mp18, Yanisch-Perron et al., 1985) if 3’-phosphates were produced by ExoI. If dinucleotides were released, those products, of heterogeneous composition, would be expected to migrate slower, as judged from the d(pGpO) marker. The reaction products were quantitated in two ways. First, an aliquot of the reaction was precipitated with trichloroacetic acid (as in standard exonuclease reactions) and radioactivity of the soluble and insoluble fractions was determined by Cerenkov counting. Second, from the thin layer chromatography plate, the spots with the products and the origin were excised, and radioactivity was determined. The two measurements were in agreement and yielded 5% acid-soluble counts and 5% mononucleotides, respectively. This argues against ExoI producing acid-soluble short oligonucleotides that remain at the origin of the chromatogram.

We conclude that ExoI releases 5’-nucleoside monophosphates.

**Nature of the 5’-Ends Required for Digestion by Exonuclease I**—In order to elucidate the role of exonuclease I in vivo, we measured its activity on different kinds of DNA breaks. First, we determined the $K_v$ value for double strand ends, our standard substrate. Reactions were carried out with varying amounts of HaeIII-cleaved or SmaI-cleaved [3H]M13mp18 DNA such that the concentration of 5’ ends was between 0.7 and 35 nM. Fig. 8 shows the results displayed in a Lineweaver-Burk plot. The deduced $K_v$ values are 8.3 nM 5’ ends for the SmaI-cleaved DNA and 10 nM 5’ ends for the HaeIII-cleaved DNA (see legend to Fig. 8). Due to the different average substrate length in the two determinations, the ratio of 5’ ends to DNA mass differed by a factor of 15. Nevertheless, the $K_v$ values are in close agreement, indicating that ExoI interacts preferentially with DNA ends. The deduced $V_{max}$ values are 9 μmol and 12 μmol of nucleotides rendered acid-soluble per min per mg of ExoI protein for the HaeIII- and SmaI-cleaved DNA, respectively.

We then tested the ability of ExoI to act on nicked plasmid DNA. The DNA preparation used contained approximately 50% nicked circular DNA (see “Experimental Procedures”). The residual supercoiled DNA is unlikely to interfere with ExoI since 2 μg of supercoiled DNA do not interfere with
The enzyme, exonuclease I, degraded only one strand from a given double strand end in the 5' → 3' direction, producing 3'-single-stranded tails. The enzyme also acted on nicks with comparable affinity.

We looked for such an activity because we are interested in studying the meiotic recombination machinery of *S. pombe*. To obtain synchronous meiotic cultures, we have utilized the conditional mutation *pat1-114* from *S. pombe*. By all criteria analyzed, these cells undergo a true meiosis at the restrictive temperature (see introduction; Iino and Yamamoto, 1985b; Beach et al., 1985).^2^ *S. pombe* pat1-114 cells were synchronized by starvation in stationary phase prior to heat induction of meiosis. These cultures then underwent synchronous meiosis as we deduce from the timing of premeiotic S-phase and the meiotic divisions. We were interested in determining whether activities proposed to act during recombination were induced in these meiotic cultures. Extracts from cells of such a time course show the sharp induction of a double-stranded DNA exonuclease activity immediately followed by a rapid decrease. We did not investigate whether exonuclease activity before heat induction of meiosis can be attributed to a basal level of the alternative activity or whether other, mitotic exonucleases are present. Therefore, the induction of ExoI might be part of DNA metabolic processes such as DNA double-stranded DNA and homologous single-stranded DNA.

Exonuclease activity peaks shortly before the meiotic divisions. The exact time when meiotic recombination takes place in *S. pombe* has not been determined yet. It would be of great interest, for example, to monitor the appearance of a recombinant restriction fragment as has been done in *S. cerevisiae* (Borts et al., 1984) and to correlate the result with the time of exonuclease induction.

It is likely that the enzyme designated ExoI is indeed the same activity showing meiotic induction in crude extracts. All column chromatography steps used during purification showed a single peak of double strand exonuclease activity (not shown). In addition, pilot purification experiments showed a 5' → 3' polarity of digestion at earlier steps of purification (not shown). The construction of a mutant lacking ExoI will give the final answer.

Our results show that ExoI acts in the 5' → 3' direction on double strand ends, and we presume that its action on nicked circular DNA proceeds with the same polarity. Such an activity might act either in the formation of 3'-single-stranded tails from double strand breaks or in the excision of a strand starting at a nick to form a single strand gap. Both functions could be part of DNA metabolic processes such as DNA damage or mismatch repair and recombination.

We could not detect any endonucleolytic activity of ExoI and therefore postulate the existence of an endonuclease that acts prior to ExoI. Unfractionated extracts from strains used in this study have no detectable endonucleolytic activity on supercoiled plasmid DNA (not shown). Such an activity might be tightly regulated by low abundance, dependence on a certain chromatin configuration, or, perhaps, sequence specificity.

The weak activity of ExoI on single-stranded DNA implies an additional function such as the digestion of single strand loops formed by strand invasion. Such a function would also require the prior action of a single strand endonuclease. Alternatively, ExoI could process 5'-single-stranded tails into flush ends thereby creating its own favorable substrate. The origin of such 5'-single-stranded tails is, however, unclear.

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*S. pombe* Meiotic Exonuclease

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ExoI requires a 5'-phosphate to initiate degradation. This implies that the enzyme acts only on a subset of possible DNA breaks, for example, those introduced by a specific endonuclease. Other breaks, with 5'-hydroxyl groups, possibly caused by some DNA-damaging agent, would not be processed by ExoI and would, therefore, lead into a different pathway of double strand break repair.

The properties of ExoI suggest a role for it during meiotic homologous recombination in S. pombe. Because ExoI acts on both nicks and double strand breaks, we envision two models for its function. Multiple pathways might contribute to meiotic recombination in S. pombe. Indeed, temporally separated pathways of meiotic recombination have been proposed (Carpenter, 1987; Engebrecht et al., 1990); an early pathway that initiates homologue alignment by gene conversion and a later one involving reciprocal exchange to ensure proper disjunction of homologues.

One possibility is that an endonuclease introduces a nick in double-stranded DNA. This break is then processed by ExoI into a single strand gap which would serve as a recipient for an invading complementary single strand from the homologue. Strathern et al. (1991) have demonstrated that the phage fI gene II product, when expressed in S. cerevisiae, stimulates mitotic recombination in conjunction with the fI nicking site artificially integrated into the genome. The chromosome containing the fI nicking site acts predominantly as the recipient of genetic information with a bias to the 5' side of the postulated nick.

A second possibility is a double strand break introduced by a specific endonuclease. ExoI would process the ends into 3' overhangs which then serve as a substrate for activities that promote homologous pairing and strand exchange. If the 3' ends were partially degraded by other nucleases, the broken chromosome would act predominantly as a recipient of genetic information in the region of the initial double strand break.

S. pombe has approximately 50 exchanges per meiotic genome (Munz et al., 1989) and a nuclear diameter of roughly 2 microns (Robinow and Hyams, 1989). Assuming initiation of meiotic recombination by double strand breaks, one per exchange, the concentration of double strand ends would be 4 \times 10^{-4} M or 5 times the Km for ExoI. We have estimated that there are 1,000 ExoI molecules per cell; if they are all nuclear, the concentration of ExoI would be 4 \times 10^{-7} M or 10 times the postulated concentration of double strand ends. Thus, both ExoI and its presumed substrate are present at concentrations above those required for maximal activity in vitro.

Both double strand breaks and single-stranded tails have been reported as recombination intermediates in S. cerevisiae and X. laevis (see introduction; White and Haber, 1990; Cao et al., 1990; Sun et al., 1991; Maryon and Carroll, 1991). We expect that activities similar to ExoI exist in those and other organisms. Indeed, an activity very similar to ExoI has recently been described from mitotic cells of S. cerevisiae (Dörlberg et al., 1991). Its expression during meiosis, though, has not been reported yet. The formation of single-stranded DNA as a prerequisite for hybrid DNA formation might be ubiquitous in all recombining creatures. Therefore, enzymes like ExoI might play a crucial role in eukaryotic homologous recombination.

Chow and Resnick (1988) and Resnick et al. (1984) have described a nuclease activity from S. cerevisiae that is induced during meiosis and strongly reduced in rad52 mutants. Cells lacking the RAD52 function exhibit increased sensitivity to x-rays and are deficient in various aspects of recombination (reviewed in Malone et al., 1988). In particular, the RAD52 function has been implicated in the repair of double strand breaks. The purified enzyme has endonuclease activity on single-stranded DNA and exonuclease activity on double-stranded DNA. 32P label is readily released from the 5'-end of double-stranded DNA (Chow and Resnick, 1987). Unfortunately, 3'-end labeled DNA was not analyzed, and it is therefore unclear whether this activity is related to ExoI from S. pombe.

Two exonucleases involved in recombination, lambda exonuclease from phage lambda and exonuclease VIII from a cryptic lambda-like prophage rac of E. coli, have properties very similar to those of ExoI (see introduction; Little, 1967; Joseph and Kolodner, 1983). Mutations in the gene coding for lambda exonuclease abolish the recombination system of phage lambda (Shulman et al., 1970). The E. coli sbcA mutations allow expression of ExoVIII and thereby suppress mutations in recB and recC, to restore recombination proficiency during conjugation and transduction (Barbour et al., 1970; Gillen et al., 1981).

We have purified another exonuclease from S. pombe, ExoII, which is not induced during meiosis. ExoII specifically degrades single-stranded DNA with a 5' to 3' polarity, similar to that of the E. coli recJ gene product (Lovett and Kolodner, 1989). We suggest that ExoII does not act in a rate-limiting step of meiotic recombination or that it functions in other processes, such as mitotic recombination and DNA damage repair.

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