Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 1327–1331, February 1997
Genetics

Evidence for a role for DNA polymerase β in mammalian meiosis

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Communicated by Charles M. Radding, Yale University, New Haven, CT, December 17, 1996 (received for review August 26, 1996)

ABSTRACT **DNA polymerase β (pol β) is an enzyme possessing both polymerase and deoxyribose phophatase activities. Although pol β is not believed to participate in the replication of genomic DNA, several studies have indicated a role for pol β in DNA repair. The high level of expression of pol β in mouse and rat testes raises the possibility that pol β participates in mammalian meiosis. Using antibody localization, we detect foci that stain with pol β antisera at discrete sites along homologous chromosomes as they synapse and progress through prophase of meiosis I. These data suggest that pol β participates in meiotic events associated with synapsis and recombination.**

At least five mammalian DNA polymerases have been identified. DNA polymerases α, δ, and ε most likely function in DNA replication of the nuclear genome, whereas polymerase γ replicates the mitochrondrial DNA (1–4). Polymerase β (pol β), a 39-kDa protein with polymerase and deoxyribose phosphatase activities, does not seem to play a direct role in cellular DNA replication (2, 5). However, pol β is able to substitute for DNA polymerase I (pol I) of *Escherichia coli* in lagging-strand DNA replication and in the conversion of single-stranded to double-stranded DNA in *Xenopus* oocyte extracts (6, 7). A role for pol β in base excision repair is supported by evidence demonstrating that cells deleted of both copies of the pol β gene do not support base excision repair *in vitro* and that rat pol β dominant-negative mutants inhibit base excision repair in *Sacharomyces cerevisiae* (8, 9). Pol β may also function in nucleotide excision repair in *Xenopus* oocytes (10).

Pol β is highly expressed in mouse and rat testis and its putative *S. cerevisiae* homolog, pol IV, is induced in cells undergoing meiosis, suggesting that pol β participates in meiosis or in a process associated with maturation of germ cells (11–15). In mammals, meiosis consists of two successive divisions resulting in the production of gametes. Reduction of the chromosome number is accomplished by synapsis, recombination, and segregation of homologous chromosomes. Several meiosis-specific structures are found in meiotic prophase nuclei. These include the synaptonemal complex (SC), the proteinaceous structure that forms between homologs, and both "early" and "late" recombination nodules (16, 17), which are electron dense nodular structures positioned along the SC. Recombination nodules are believed to be structures involved in synapsis and recombination (18, 19).

Because pol β is highly expressed in cells undergoing meiosis, we investigated the possibility that pol β participates in mammalian meiosis. Using antibody localization, we detect foci that stain with pol β antisera at discrete sites along homologous chromosomes as they synapse and progress through prophase of meiosis I. To our knowledge, these data are the first to localize a DNA polymerase to mammalian meiotic chromosomes during meiosis, and they suggest that pol β participates in this process.

MATERIALS AND METHODS

Antigen and Antiserum Preparation. The rat pol β cDNA was subcloned into pPR977 (New England Biolabs) to generate pMBP-β. This construct carries the pol β cDNA fused to the maltose binding protein (MBP) gene with a linker in between the two that encodes a thrombin cleavage site. The production of a pol β–MBP fusion protein is under control of the *tac* promoter, rendering its expression inducible in the presence of isopropyl β-D-thiogalactopyranoside. The *E. coli* strain used for expression of the pol β-MBP fusion protein was BL21 (Novagen) and has the genotype F^- *ompT* r_{B^-} m_{B^-}.

The pol β antigen was purified according to the following scheme. Cells carrying pMBP-β were grown to OD_{600} of 0.5, and overexpression of the fusion protein was induced by the addition of 0.3 mM isopropyl β-D-thiogalactopyranoside and continued incubation of the cells for 2 hr. Cells were harvested, resuspended in column buffer containing 10 mM Tris·Cl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethyl sulfonylfluoride, 1 μg/ml pepstatin A, and 2 mg/ml sodium bisulfite, and lysed by 1 cycle of freezing and thawing. The lysed cells were centrifuged at 10,000 × *g* for 30 min to produce the soluble crude extract. Amylose resin (New England Biolabs) was added to the supernatant, and this suspension was shaken gently for 2 hr at 4°C. The slurry was poured into a column, allowed to settle, and washed with 8 column volumes of column buffer. The fusion protein was eluted with column buffer containing 10 mM maltose and 15% glycerol. This fraction was resolved on a polyacrylamide gel containing SDS (SDS/PAGE gel) and was shown to consist of ≈90% fusion protein, detected as an 84-kDa band (J.B.S., unpublished data). The 84-kDa band was excised from the gel, pulverized, and used to prepare rabbit polyclonal antiserum. The antiserum was further purified on immobilized protein A-Sepharose (Pharmacia) to yield a pool of IgG molecules.

Preparation of Pol β IgG Fraction Depleted for Pol β-Specific Antibodies. Purified IgG fraction from pol β-specific antiserum was added to an amylose-Sepharose (New England Biolabs) column coupled to pol β–MBP fusion protein. IgG molecules that do not bind this column were collected in the flow-through and reapplied to a fresh affinity column three times. All three flow-through fractions were collected, concentrated, and stored at −80°C.

Preparation of Nuclei from Mouse Testes. Six- to 12-week-old male mice were euthanized by cervical dislocation, testicles were removed and placed in ice cold PBS (pH 7.5) containing 1 mM EDTA, and protease inhibitor cocktail (0.1 μg/ml pepstatin A/0.1 μg/ml chymostatin/0.1 μg/ml antipain/0.1 μg/ml leupeptin/10 μg/ml aprotinin), and tubular contents

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Abbreviations: pol β, polymerase β; SC, synaptonemal complex; MBP, maltose binding protein.
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were isolated by manual disruption of tubule followed by removal of tubular remnants. Whole nuclei preparation was as described with the following modifications: all buffers contained 1 mM EDTA and protease inhibitor cocktail, and the final extraction and dialysis steps were omitted (20). The resulting fraction (containing whole nuclei) was divided into small aliquots and quick frozen in a dry ice/ethanol bath and then stored at −80°C. Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce) and was normally between 3–4 mg/ml.

Western Blotting. Whole nuclei (6 μg) isolated from mouse testes as described above were resolved on a 10% SDS/PAGE gel with the buffer system described by Laemmli (21) and the proteins were electro-blotted onto nitrocellulose. The membrane was incubated in blocking buffer (10 mM Tris·Cl, pH 8.0/0.3 M NaCl/0.1% Tween 20/5% BSA), washed three times in washing buffer (10 mM Tris·Cl, pH 8.0/0.4 M NaCl/0.1% Tween 20), then incubated with the anti-pol β primary IgG at a 1:300 dilution in blocking buffer. Immunoreactive bands were visualized with SuperSignal ULTRA chemiluminescent substrate (Pierce), after incubation with goat anti-rabbit horseradish peroxidase (1:20,000) and exposure to x-ray film.

Activity Gel Analysis. Nuclei from mouse spermatocytes (50 μg) were assayed for pol β activity with modifications for renaturation of pol β as described (22–24).

Immunohistochemical Staining of Mouse Testes. Testes from normal 3-week-old C57BL/6 mice were fixed in 10% neutral buffered formalin and paraffin embedded by standard procedures. Sections (6 μm) were deparaffinized and rehydrated through a graded alcohol series. Endogenous peroxidase activity was quenched using 2% hydrogen peroxide in methanol for 30 min at room temperature. Slides were first incubated with dilute normal goat serum at room temperature for 20 min and then incubated with primary antibody as indicated in the figure. Slides were washed with 1% Triton/PBS, washed with PBS, incubated with biotin-conjugated goat anti-rabbit IgG (6 μg/ml) at room temperature, and then washed with PBS. Immunoperoxide staining was carried out using the Vectastin ABC Elite and diaminobenzidine (DAB) peroxidase substrate kits (Vector Laboratories) following the manufacturer's protocol. Slides were washed in distilled water and counterstained slightly with Harris haematoxillin solution (Sigma) and mounted. Pictures were taken at ×400 magnification.

Immunofluorescence Studies. Mice were from the C57BL/6 inbred strain. Nuclei were imaged from young males (17 days to 3 months). In addition, nuclei were imaged from oocytes from 16- to 18-day old fetuses taken from pregnant females. Surface spreads from both spermatocytes and oocytes were prepared using the method of Speed (25), as modified by Antoine Peters (personal communication). Antibody incubation and detection was a modification of that of Moens *et al.* (26) as described by Ashley *et al.* (27). Because the pol β antiserum was raised in rabbit, the double-labeling experiments were accomplished by using polyclonal antibody raised in mouse directed against the Cor1 protein from Syrian hamster; CorI antiserum was used at a dilution of 1:600 (supplied by Peter Moens) (28). Cor1 was isolated as a component of the axial/lateral elements of the SC, and the antibody has been shown to be specific for these components (28). Pol β IgG was diluted 1:100 for images from mid- and late pachynema and 1:50 for images from early pachynema. Pol β was detected with goat anti-rabbit IgG conjugated with fluorescein isothiocynante (FITC) from (Pierce), and Cor1 was detected with goat anti-mouse IgG conjugated with rhodamine (Pierce). Dilution of the secondary antibodies was (1:100) in ADB (10% goat serum/3% BSA in PBS). Following completion of the detection steps, the preparations were counterstained in 4′,6-diamidino-2-phenylindole (DAPI) at 200 ng/ml and mounted in antifade [2% 1,4-diazobicyclo-(2,2 2)-octane (DABCO) in 20 mM Tris, pH 8.0/90% glycerol]. Preparations were examined and digitally imaged on a Zeiss Axiskop (×63, 1.2 NA Plan Neofluar oil-immersion objective). Each fluorochrome image was captured separately as an 8-bit source image using a computer-assisted cooled charge coupling device camera (Photometrics CH 220) and enhanced with the Adobe PHOTOSHOP image-processing program. The monofluor (rhodamine and FITC) images were merged and pseudocolored with custom software developed by Tim Rand (29). Although DAPI images were captured for each nucleus, for simplicity of interpretation, these were not included in the final merge.

RESULTS

Characterization of the Antisera to Pol β. To study the *in situ* localization of DNA pol β during meiosis, we raised a specific rabbit antiserum to a recombinant pol β–MBP fusion protein. To determine if our IgG fraction specifically reacted with DNA pol β, we performed a western blotting experiment on partially purified (>90%) recombinant rat pol β. In addition, to determine whether DNA pol β is present in mouse testes, we utilized Western blotting analysis on whole nuclei isolated from mouse testes. The pol β IgG recognizes two intense bands of approximate molecular weights of 39 kDa and 84 kDa present within a partially purified fraction of recom-

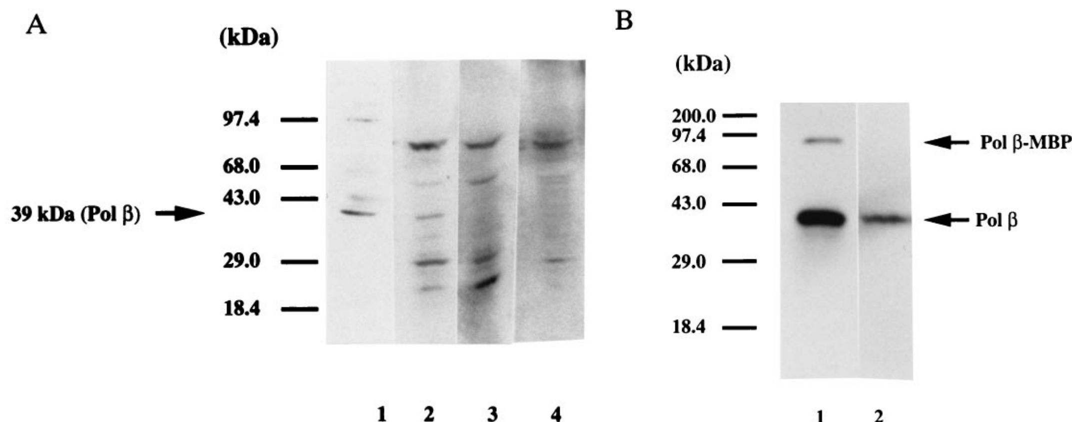

FIG. 1. The antiserum raised against a pol β–MBP fusion protein specifically recognizes pol β. (*A*) Western blot. Lane 1, 100 ng of a partially purified fraction (from *E. coli*) of recombinant rat DNA pol β detected with pol β antiserum at 1:300 (larger band is residual uncleaved pol β-MBP). Lanes 2–4, whole testicular nuclei (6 μg). Lane 2, pol β antiserum at 1:300; lane 3, pol β-depleted serum at 1:50; lane 4, preimmune serum at 1:50. (*B*) Activity gel analysis. Lane 1, partially purified recombinant rat DNA pol β (100 ng) from *E. coli*; lane 2, testicular nuclei (50 μg).

binant pol β protein (Fig. 1*A*, lane 1). The 39-kDa band corresponds to the size of the pol β protein (30); the 84-kDa band is the size expected from residual uncleaved pol β–MBP fusion protein. In addition, a band with a molecular weight of ≈45 kDa and of lesser intensity than the pol β band is also recognized by the pol β IgG and most likely corresponds to free MBP (31). In whole nuclei from mouse testes our pol β IgG recognizes a 39-kDa band corresponding in size to pol β protein (lane 2). This IgG fraction also recognizes a few other nonspecific bands. These nonspecific bands are also recognized by the pol β-depleted serum or the preimmune serum (lanes 3 and 4, respectively). The 39-kDa band is not recognized by the pol β-depleted serum or the preimmune serum. Therefore, the Western blotting experiments demonstrate that our pol β antiserum contains an activity that is specifically depleted by incubation of the antiserum with purified pol β protein.

To determine if active pol β protein was present in mouse testis tubules, we used activity gel analysis (22–24). This assay measures enzymatic activity of the DNA polymerase while it is embedded within a gel matrix. We observe a band of polymerase activity in our partially purified pol β preparation in lane 1 of Fig. 1*B*; this band of activity corresponds to the 39-kDa pol β protein. We also observe a 39-kDa band of pol β activity in our mouse tubule nuclei, as seen in lane 2 of Fig. 1*B*. This shows that pol β is not only present, but active, in mouse tubular contents.

Pol β Antisera Stains Differentiating Spermatocytes in Mouse Testes. An earlier study using chemical inhibitors indicated that pol β was active in rat spermatogenic cells leading us to suspect that our pol β IgG might recognize a protein in testicular cells (12). To perform our study, we initially utilized immunohistochemical staining techniques on mouse testicular sections from normal 3-week-old mice. As seen in Fig. 2*A*, differentiating spermatocytes, as well as spermatids, and possibly spermatagonia and spermatogenic epithelium, stain with our pol β IgG, while an equivalent testicular section incubated with IgG specifically depleted for pol β IgG molecules (see *Materials and Methods*) does not stain (Fig. 2*B*). This result indicates that pol β is present in differentiating spermatocytes; the Rad 51 and MLH 1 proteins are also present in differentiating spermatocytes (refs. 32 and 33; E.S., unpublished results). These data suggested to us that pol β might directly participate in meiosis, the primary activity of differentiating spermatocytes.

Pol β Antisera Detect Foci on SCs. We next used immunofluorescence microscopy and antibody colocalization to more precisely determine pol β localization in meiotic nuclei. To provide a spacial and temporal framework for interpreting the pol β localization, we immunolabeled the axial/lateral elements of the SC with a mouse polyclonal antibody raised to the Syrian hamster Cor1 protein (28) As shown in Fig. 3, several meiotic nuclear reactions were observed.

The first substage at which pol β foci are detected is zygonema. In late zygotene nuclei, most pol β foci were observed over the Cor1-labeled axes on both asynapsed axes and fully synapsed synaptomenal complexes, although some nonaxial foci were also present in these nuclei (Fig. 3*A* and *B*). Some pairing forks had an unusually high concentration of pol β foci near the pairing fork ("Y" junction) (arrows, Fig. 3*A* and *B*), while others did not (arrowhead, Fig. 3*B*). While some fully synapsed bivalents had numerous foci along their lengths, others had only one or two. The presence of pol β on asynapsed and synapsed bivalents during zygonema and early pachynema suggests it is involved in synaptic initiation. Some bivalents have faint pol β foci at each end, and occasionally a terminal signal can be detected on unsynapsed axes (bivalent, lower right, Fig. 3*A* and *B*).

By mid-pachynema most of the foci along the axes are no longer evident, although a few foci may still be found (arrowhead, Fig. 3*C*). In addition comet-shaped streamers of pol β signal are visible on one, or both sides of several bivalents (large arrows, Fig. 3*C*). In addition a single round "bright body" is often apparent near the XY bivalent. Also at this stage, a pol β signal becomes evident at each end of both the autosomal bivalents and the axes of the X and Y (small arrows, Fig. 3*C*). It is interesting to note that the appearance of this terminal pol β signal precedes the thickening of the axial elements and increased concentration of Cor1 signal that occurs later in pachynema.

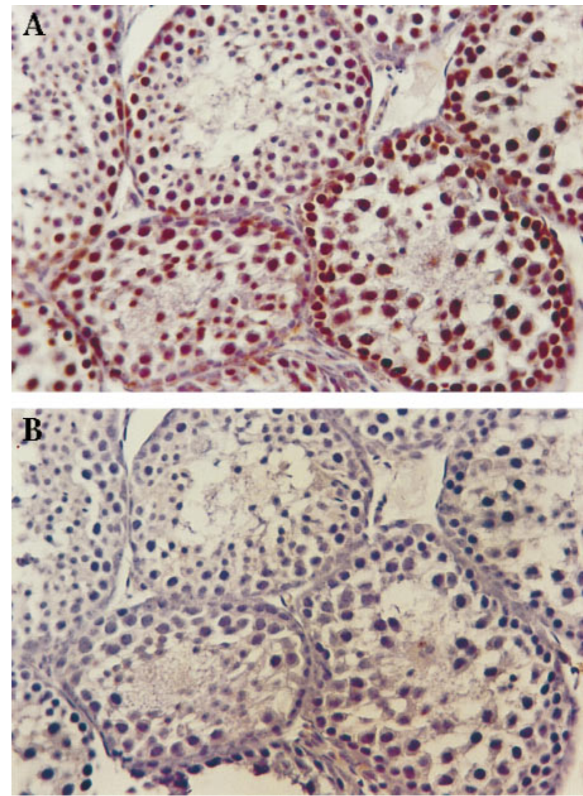

FIG. 2. Differentiating mouse spermatocytes stain with antiserum to pol β. (*A*) Immunohistochemical staining of seminiferous tubule of a normal 3-week-old mouse labeled with pol β IgG (60 μg/ml). Mouse spermatocytes show significant brown staining with the pol β IgG, while the interstitial cells and Sertoli cells do not stain brown. (*B*) Immunohistochemical staining of seminiferous tubule from a normal 3-week-old mouse incubated with pol β-depleted IgG (60 μg/ml). No brown staining was detected.

As nuclei proceed into diplonema, the terminal pol β signals persist, as do the pol β positive "streamers" associated with some bivalents and round "bright-body" that remains in the vicinity of the XY bivalent (Fig. 3*D*). As a control, at each stage, spread meiotic nuclei were also incubated in parallel with preimmune serum and pol β-depleted serum; in both cases, no staining was detected (data not shown).

DISCUSSION

DNA pol β is known to be highly expressed in mouse and rat testes (11–13). We initially found that differentiating spermatocytes in sections from mouse testis specifically stain with pol β antiserum. These results prompted us to examine nuclei from mouse spermatocytes and oocytes for the presence of pol β during meiotic prophase on synaptomenal complexes. We observed foci that stain with pol β arrayed along SCs during meiotic prophase in both spermatocytes and oocytes. The simplest interpretation of our results is that the foci that stain with pol β antisera represent sites of pol β activity along the paired homologous chromosomes during meiotic prophase. In

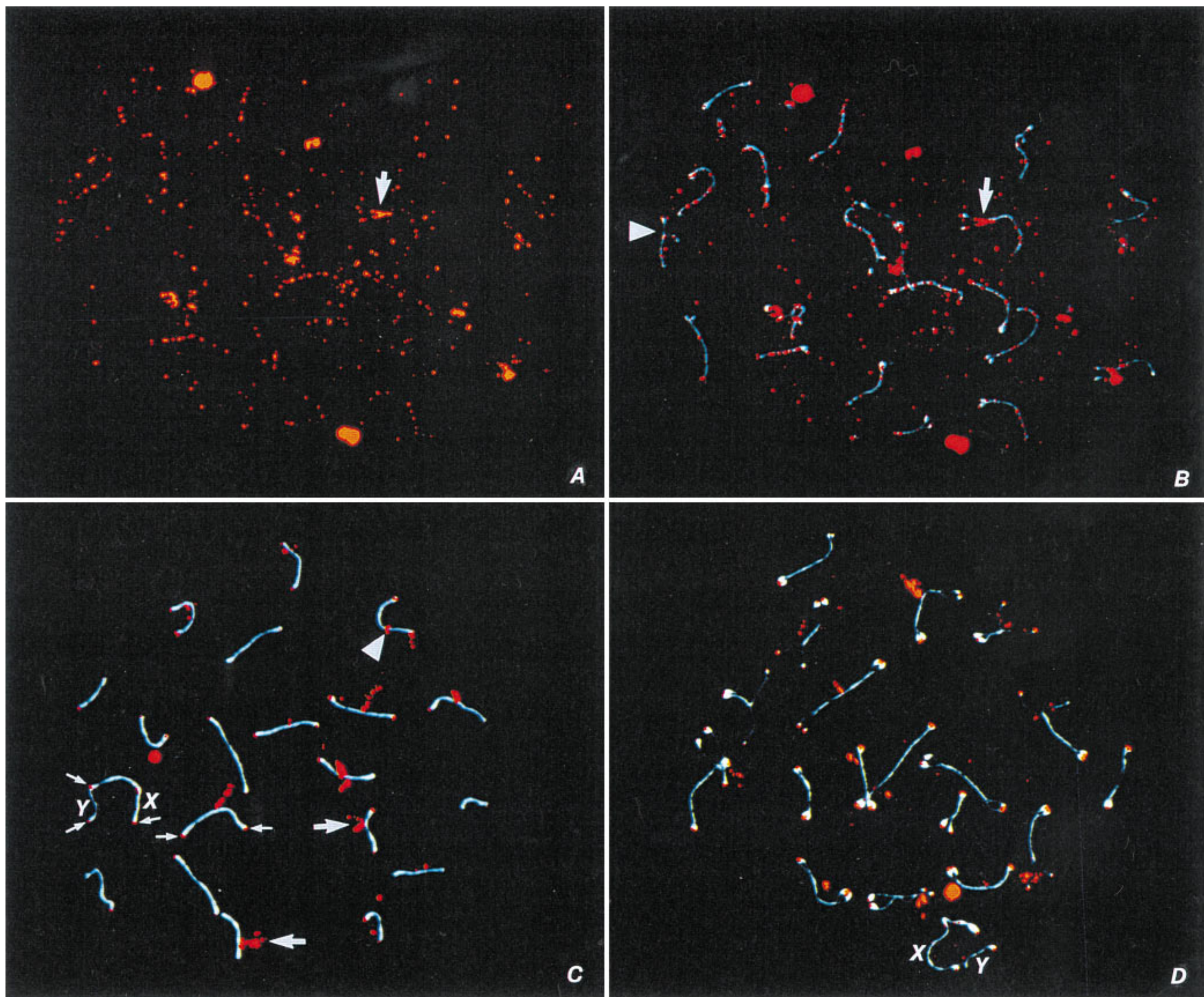

FIG. 3. Pol β IgG stains discrete foci on mouse chromosome homologs during prophase I of meiosis in mouse spermatocytes. Mouse spermatocytes in early prophase. (*A* and *B*) Nucleus in late zygonema. Pol β-stained nucleus (red) is on the left; pol β and Cor I-stained nucleus (white) is on the right. (*C*) Nucleus in mid-pachynema. Merged image of pol β and Cor I-stained nucleus. (*D*) Nucleus in late pachynema and proceeding into diplonema. Merged image of pol β and Cor I-stained nucleus. Control experiments done in parallel with equivalent chromosomal spreads using preimmune serum or pol β-depleted IgG fractions yielded no detectable staining (data not shown).

addition, our data add to the growing list of proteins that have been localized to meiotic chromosomes; these include Rad 51, DMC 1, LIM 1, MLH 1, PMS 2, and UBC 9 (32, 33, 35–39).

We and others have presented evidence that pol β participates in base excision repair in somatic cells (8, 9). *In vitro* studies demonstrate that pol β catalyzes the release of 5′-terminal deoxyribose phosphate residues from incised apurinic-apyrimidinic sites and that pol β fills in the small gap resulting from excision of damaged bases (5, 8). The presence of pol β at discrete sites on homologous chromosomes throughout zygonema and the early stages of pachynema during meiosis is striking in that it suggests that some form of DNA synthesis takes place at these sites. Because pol β is known to fill small gaps during base excision repair, our data imply that a type of repair synthesis takes place throughout the early stages of meiosis. However, Stern and coworkers (12, 40) have proposed that there are two intervals of DNA synthesis after pre-meiotic S-phase; one interval involves semiconservative DNA synthesis during zygonema and the other interval is during pachynema and resembles repair synthesis. Because pol β is present at multiple sites during both zygonema and pachynema, and not earlier during premeiotic DNA synthesis, our data are consistent with a role for pol β in both semiconservative DNA synthesis during zygonema and in repair synthesis during pachynema and imply that pol β may function during synapsis and recombination. It is tempting to speculate that pol β may be a component of recombination nodules in view of the fact that DNA synthesis occurs at nodules (41). Alternatively, pol β may function in some form of DNA repair during meiosis that is not directly related to synapsis or recombination.

It is interesting that we observe pol β foci at the ends of bivalents. It is known that there is a high frequency of recombination near the ends of chromosomes in male eutherian mammals (42). Thus, the presence of pol β at the ends of bivalents might suggest a role for this enzyme in a recombination pathway (42). Alternatively, pol β may participate in DNA synthesis or repair of terminal sequences in germ cells.

The number of pol β clusters we observe at the ends of bivalents and the comet shape of these clusters in early pachytene nuclei is similar to the reported number and shape of nucleolar organizing regions in this strain (43). The similarity of shape (round) and proximity to the sex body of the pol β "bright body" and the previously reported "dense" body is intriguing (34). Dresser and Moses (34) have presented evidence that the "dense body" contains nucleolar proteins. No

previously described property of pol β accounts for its presence at these sites. Thus, our cytological localization of pol β in meiotic prophase nuclei of the mouse suggests that it plays roles in synapsis and recombination and raises the possibility of involvement in processes which are yet to be uncovered.

We thank Drs. Peter Moens and Barbara Spyropoulos for the mouse Cor1 antibody; Dr. Charles Radding for the rabbit Rad51 antibody; Drs. Lawrence Loeb, Sean Baker, Ann Blank, and David Ward for helpful discussions; and Dr. Barry M. Kacinski for his support. This work was supported by National Institutes of Health Grant GM-49799 to T.A and National Cancer Institute Grant CA-64134 to J.B.S. C.A.C. was supported by a National Research Service Award Postdoctoral Fellowship CA-68764.

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