Journal of Cell Science 113, 663-672 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS0891

Expression and nuclear localization of BLM, a chromosome stability protein mutated in Bloom's syndrome, suggest a role in recombination during meiotic prophase

Peter B. Moens[1,*], Raimundo Freire[2,‡], Madalina Tarsounas[1,§], Barbara Spyropoulos[1] and Stephen P. Jackson[2]

[1]Department of Biology, York University, Toronto, Ontario, M3J 1P3, Canada
[2] The Wellcome Trust and Cancer Research Campaign Institute of Cancer and Developmental Biology, and Department of Zoology, Cambridge University, Tennis Court Road, Cambridge, CB2 1QR, UK
*Author for correspondence (e-mail: moens@yorku.ca)
‡Present address: Unidad de Investigaçion, Hospital Universitario de Canarias, 38320, Tenerife, Spain
§Present address: Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts, EN6 3LD, UK

Accepted 3 December 1999; published on WWW 31 January 2000

SUMMARY

Bloom's syndrome (BS) is a recessive human genetic disorder characterized by short stature, immunodeficiency and elevated risk of malignancy. BS cells have genomic instability and an increased frequency of sister chromatid exchange. The gene mutated in BS, *BLM*, encodes a 3′-5′ helicase (BLM) with homology to bacterial recombination factor, RecQ. Human males homozygous for *BLM* mutations are infertile and heterozygous individuals display increased frequencies of structural chromosome abnormalities in their spermatozoa. Also, mutations in the *Saccharomyces cerevisiae* homolog of *BLM*, *Sgs1*, cause a delay in meiotic nuclear division and a reduction in spore viability. These observations suggest that BLM may play a role during meiosis. Our antibodies raised against the C terminus of the human protein specifically recognize both mouse and human BLM in western blots of cell lines and in successive developmental stages of spermatocytes, but fail to detect BLM protein in a cell line with a C-terminally truncated protein. BLM protein expression and location are detected by immunofluorescence and immunoelectron microscopy as discrete foci that are sparsely present on early meiotic prophase chromosome cores, later found abundantly on synapsed cores, frequently in combination with the recombinases RAD51 and DMC1, and eventually as pure BLM foci. The colocalization of RAD51/DMC1 with BLM and the statistically significant excess of BLM signals in the synapsed pseudoautosomal region of the X-Y chromosomes, which is a recombinational hot spot, provide indications that BLM protein may function in the meiotic recombination process.

Key words: Bloom's syndrome, BLM, DMC1, RAD51, RecQ, Recombination, Checkpoint control, Synaptonemal complex, Meiosis, Immunofluorescence, Immunoelectron microscopy

INTRODUCTION

Bloom's syndrome (BS) is a rare autosomal recessive human genetic disorder characterized by a number of abnormalities including short stature, immunodeficiency, sun sensitivity, male sterility and cancer predisposition (reviewed by German, 1993). In contrast with most other human cancer predisposition syndromes, BS patients have an enhanced risk of developing a wide variety of cancer types, probably linked to the genomic instability observed in BS cells. Various types of chromosome aberrations have been observed in BS cells at a higher frequency than in normal cells, including chromosome gaps, breaks, sister chromatid exchanges (SCE) and exchanges between homologous chromosomes. This genomic instability is also reflected at the molecular level in increased rates of somatic mutation at various loci (German, 1993).

The gene mutated in BS, *BLM*, encodes a 1417-amino-acid-residue protein with a central domain that is homologous to a subfamily of helicases, including *Escherichia coli* RecQ, which have a characteristic DEXH box in one of the helicase motifs (Ellis et al., 1995). The N- and C- terminal domains have little homology to other known proteins, as is the case with other members of the RecQ helicase subfamily including WRN, the human protein defective in Werner's syndrome (WS), which is characterized by premature aging and cancer predisposition (Yu et al., 1996). Also in this subfamily is *Schizosaccharomyces pombe* Rqh1, which is required for effective recovery from S-phase arrest and cell cycle checkpoint control (Stewart et al., 1997; Davey et al., 1998), and *S. cerevisiae* Sgs1p (see below). Recently, both BLM and WRN proteins have been shown to possess 3′-5′ helicase activity (Karow et al., 1997; Gray et al., 1997). Despite detailed investigations into BLM and BS cells, the precise physiological function(s) for BLM in humans is currently unclear.

In recent years, it has become increasingly evident that fundamental mechanisms of genomic maintenance are, in general, highly conserved throughout eukaryotic evolution. This raises the possibility that BLM functions in a similar manner to its yeast counterparts. Consistent with this premise, *S. cerevisiae sgs1* null mutants share with BS cells the phenotypes of genomic instability and hyper-recombination (Watt et al., 1995, 1996). Moreover, the hyper-recombination defects of *sgs1* mutant yeast cells can be partially restored by the expression of the human BLM and WRN proteins (Yamagata et al., 1998). Sgs1p interacts physically with topoisomerases II and III in yeast (Watt et al., 1995; Gangloff et al., 1994). In addition to genomic instability in mitotically dividing cells, mutations in *S. cerevisiae Sgs1* lead to chromosomal missegregation during meiosis (Watt et al., 1995), although in contrast to the situation in mitotic cells, these mutations have not been reported to confer heightened rates of meiotic recombination (Watt et al., 1996). Meiotic functions of BLM are also implied by high levels of the *BLM* transcript in testes (Chester et al., 1998; Seki et al., 1998) and by the fact that BS homozygous males are infertile, have abnormally small testes and no mature sperm (German, 1993) and BS females display problems in menstruation, although in some cases they are fertile (German, 1993).

One attractive route to assess whether BLM plays a role during meiosis in mammals is to study meiosis in animals rendered defective in BLM function by targeted gene disruption. Unfortunately, disruption of the *BLM* gene in mice causes embryonic death, precluding studies on gametogenesis (Chester et al., 1998). As an alternative approach to investigating the potential meiotic functions of BLM, we have studied the time course of BLM protein expression during mouse spermatogenesis and the association of the protein with meiotic chromosomes, the synaptonemal complexes and the RecA-type recombinases, RAD51 and DMC1. The association of BLM protein with the recombinases and with a specific crossover site supports a role for this protein in meiotic recombination.

MATERIALS AND METHODS

Anti-BLM antibody production and purification

The 3′ cDNA corresponding to amino acid residues 1041-1416 was cloned fused to the hexahistidine tag of the vector pQE-30 (Qiagen). The protein was expressed and soluble protein was purified according to the manufacturer's instructions. Although the expected molecular size of the recombinant protein is approximately 43 kDa, a product of ~33 kDa was purified, suggesting that the very C terminus of the recombinant protein was lost. To test that possibility the recombinant protein was transferred to PVDF membrane and sequenced by the Protein and Nucleic Acid Chemistry Facility of the Department of Biochemistry, University of Cambridge, UK. The sequence obtained corresponded to the expected leader sequence and first BLM amino acids of the construct. Moreover, the insert of the plasmid DNA was sequenced from the 5′ end and it corresponded to the expected sequence. Antibodies against the recombinant protein were raised in rabbits by standard procedures (Harlow and Lane, 1988). The recombinant C terminus of the BLM protein was attached to Sulfo-Link Coupling Gel (Pierce, USA) according to the manufacturer's instructions and was used to immunoaffinity purify anti-BLM antibodies from crude rabbit serum, as described previously (Freire et al., 1998). To remove antibodies to the hexahistidine tag, the purified antibody was incubated with a sulfo-link column with a hexahistidine hRAD1 attached to it and the flow-through was collected for the experiment in Fig. 1E. Western blotting was performed as described previously (Harlow and Lane, 1988) and the blots were developed by the 'Enhance' chemiluminescence reagent (Amersham), according to the manufacturer's instructions. I. D. Hickson provided the full-length recombinant BLM protein with a C-terminal hexahistidine tag (Karow et al., 1997).

Other antibodies

Anti-COR1 antibodies were generated in mice (8L2, 12RB) against a fusion protein from *E. coli* containing an expression vector incorporating the gene for the 32 kDa protein of the hamster meiotic chromosome core (Dobson et al., 1994). Anti-synaptic protein, SYN1, antibody was used to enhance the immune staining of synapsed regions of the chromosome cores (Dobson et al., 1994). The mouse anti-DMC1 antibody was described previously (Freire et al., 1998). This antibody will crossreact with RAD51 unless immunodepleted for crossreaction epitopes. In a study with epitope-specific, immunodepleted DMC1 and RAD15, we found that the two completely colocalize when observed with immunofluorescence and with electron microscopy (Tarsounas et al., 1999). Therefore, in this report, DMC1 detection is synonymous with DMC1/RAD51 detection. The CREST anti-centromere serum was obtained from a patient with CREST syndrome (Dobson et al., 1994). Anti-β actin antibody was purchased from Sigma (UK). The anti-WRN antibody was prepared by R. Freire and S. P. Jackson and will be reported separately.

Immunocytology methods

Mouse testicular cells were surface-spread on 0.1 M NaCl, attached to glass slides, fixed in 2% paraformaldehyde with 0.03% SDS, washed in 0.4% Kodak Photo-Flo 200 and dried briefly. After washes in 0.1% Triton X-100 and 10% antibody dilution buffer (ADB: 10% goat serum, 3% BSA, 0.05% Triton X-100) in PBS, nuclei were incubated in primary antibody at dilutions of 1:500-1:1000 in ADB in PBS for 2 hours at 37°C or overnight at room temperature. After washes, nuclei were incubated in FITC and/or rhodamine-conjugated secondary antibody for 1 hour at 37°C. After washing and drying, the slides were mounted in ProLong antifade (Molecular Probes, Eugene, OR, USA) with 4 μg/ml DAPI.

Immunofluorescence images were recorded in colour on Fujichrome ASA 400 film or in black and white with Kodak T-Max 400. Multi-colour images were obtained by exposing a single colour-positive frame with two or three types of fluorescence by changing the filters. The images shown in this report were scanned from colour-positive slides or black and white prints and printed with Adobe PhotoDeluxe on an Epson Stylus Photo 700 printer. This procedure avoids artefacts introduced by digitally collected images that require multiple background reductions, reconstituted images with reduce visibility and false colouring.

For electron microscopy, the glass slides were coated with 0.55% Falcon Petri dish plastic in chloroform. After immune-staining with 5, 10 and 15 nm gold grains, the plastic film was floated off and transferred to nickel EM grids. Electron density of the chromosome cores and SCs was increased by staining with 1% osmium tetroxide.

The immunofluorescent BLM foci in spermatocytes could be suppressed completely by immunodepletion of the antibody with the recombinant C-terminal BLM protein. Staining with antibodies generated against DMC1 protein could also be blocked entirely by immunodepletion with DMC1 protein. We used immunofluorescent foci of the RecA homologs, RAD51/ DMC1, as reference points for presumptive recombination-associated, double-strand breaks and strand exchange.

RESULTS

Generation of polyclonal anti-BLM antibodies

To study the possible roles of BLM in meiosis and in particular its potential association with meiotic chromosomes, we raised antibodies against the C terminus of the protein. The portion of the human *BLM* cDNA that encodes the last 380 amino acid residues of BLM outside its central helicase domain was amplified by PCR from a human cDNA library and cloned in a bacterial expression vector. The resulting recombinant N-terminally hexahistidine-tagged protein was expressed in *E. coli*, purified to homogeneity (see Materials and Methods) and used to immunize two rabbits. Both antisera recognized a protein with an apparent molecular mass of approximately 180 kDa on western blots of HeLa whole cell extracts (Fig. 1A), a size that is in accord with previous immunological analysis of the BLM protein (Karow et al., 1997).

The anti-BLM antibodies were subsequently affinity-purified on a column containing the immobilized recombinant C-terminal BLM protein, resulting in improved binding specificity (Fig. 1A). This affinity-purified antibody detected sub-nanogram amounts of the recombinant protein (Fig. 1B). To further test the specificity of the antibody and to confirm that the ~180 kDa polypeptide that it recognized was indeed BLM, we used extracts of BS cells containing a homozygous *BLM* mutation predicted to encode a prematurely terminated 737 residue protein (approx. 80 kDa) lacking the C-terminal region against which our antibodies were raised (Kaneko et al., 1997). As shown in Fig. 1B, whereas the antibody efficiently detected the approx. 180 kDa protein in western blots of extracts of normal cells with no mutation in BLM (lanes 4 and 6), this protein was not detected in BS cells (lane 5). In one of the control cell lines, the antibody recognized an additional protein species of approx. 160 kDa that could correspond to proteolytic degradation of BLM or to a splicing or polymorphic variant of BLM in this particular cell type.

For further tests of the antibodies, western blot analysis was performed with purified recombinant full-length protein (Fig. 1D,E) expressed in *S. cerevisiae* (Karow et al., 1997). The antibody was able to recognize the full-length protein, although it presented a different mobility from the BLM protein detected in extracts, probably due to the N-terminal and C-terminal additions in the recombinant protein (Karow et al., 1997). The C-terminal full-length recombinant protein contains a hexahistidine tag that could potentially be recognized by the BLM antibody as the C-terminal BLM recombinant protein is an N terminus his-tagged protein. To exclude that possibility, we incubated the antibodies with a recombinant 6× his-tagged to a specific protein (hRad1; Freire et al., 1998; see Materials and Methods) and immunodepleted the anti-BLM antibodies from antibodies against the hexahistidine tag. Fig. 1E demonstrates that the BLM antibody was still able to recognize

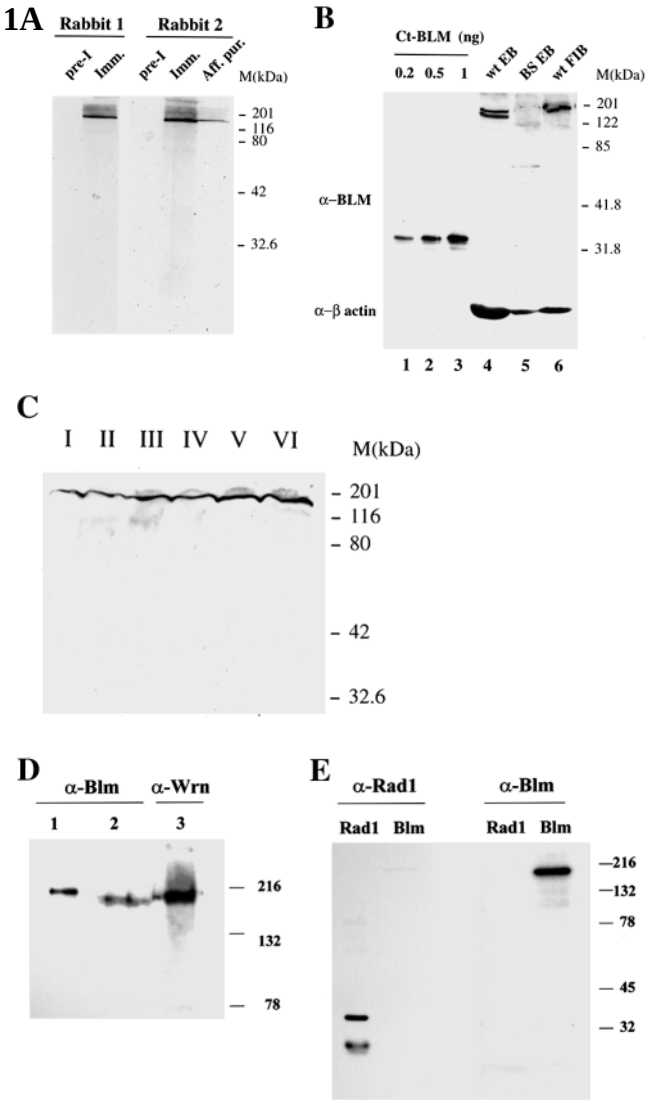

Fig. 1. Generation of anti-BLM antibodies and distribution of BLM in spermatocyte development. (A) Western blot with two different antisera raised against recombinant BLM C terminus. Each lane contains 70 ng of HeLa whole-cell extract. The two antisera recognize a polypeptide of approx. 180 kDa. (B) Affinity-purified antibody #2 was used to probe western blots of the indicated amounts of recombinant-tagged, bacterially expressed BLM (lanes 1-3) and whole-cell extract from EB-transformed cell lines from normal individuals (lane 4), BS patients with a premature termination mutation (lane 5) and normal primary fibroblast (lane 6). (C) Western blot of testicular cells from 30-day-old rats separated by centrifugal elutriation. All the lanes contain nuclear extracts normalized with respect to the number of cells (around 5×10^5 cells per lane). Fraction I, small cells, zygotene and early pachytene; fraction II, cells in leptotene, zygotene and some pachytene; fraction III, mostly full pachytene; fraction IV, late pachytene and some diplotene cells; fractions V and VI, late pachytene and post-pachytene cells. (D) Western blot evidence that the anti-BLM antibody does not recognise the WRN helicase of similar but not identical molecular mass. Lane 1, 4 ng of full length recombinant BLM. Lanes 2 and 3, 293T whole cell extracts (30 μg). Lanes 1 and 2 were probed with anti-BLM antibody and lane 3 was probed with anti-WRN antibody. (E) Western blot evidence that the anti-BLM antibody that was prepared against a his-tagged recombinant protein recognises the BLM antigen rather than the his-tag. Anti-RAD1 and anti-BLM were immunodepleted of possible anti his-tag epitopes and tested on 4 ng of Rad1 antigen (30 kDa) and 4 ng of BLM full-length recombinant protein (160 kDa). The first panel shows that the anti-RAD1 antibody does not react with his-tagged BLM protein. The second panel demonstrates that anti-BLM antibody recognizes the BLM protein but not the his-tag of the RAD1 protein.

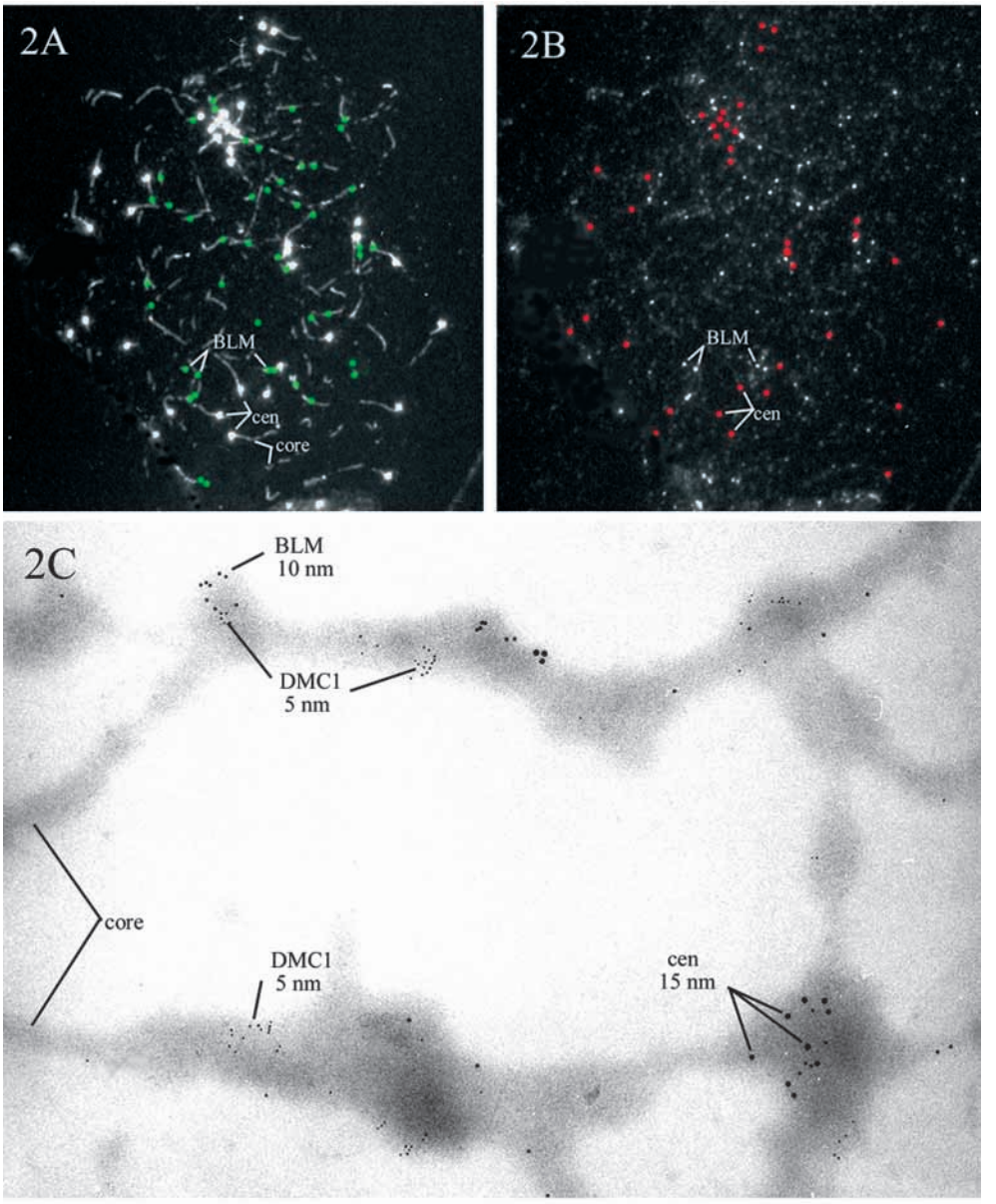

Fig. 2. BLM expression and location prior to chromosome synapsis at the leptotene stage of early meiotic prophase. (A) A whole-mount leptotene nucleus with 40 single, unpaired centromeres (cen) stained with human CREST anti-centromere serum. At this stage, there are the earliest chromosome core segments (core) stained with mouse anti-COR1 antibody. To demonstrate the association between the leptotene cores and BLM, the positions of the BLM foci in Fig. 2B have been copied to 2A and they are in green colour (BLM). (B) The same nucleus as in Fig. 2A with the centromeres (cen) artificially coloured red to assist with orientation. The bright BLM foci (BLM) were stained with rabbit anti-BLM serum. At this stage, there are few BLM foci (about 50 here) relative to the large number of RAD51/DMC1 foci (about 200-250) per leptotene nucleus (not shown). (C) Electron micrograph of a similar leptotene nucleus where the early chromosome cores are the electron-dense strands, which are not well organized at this stage (core). The centromeres are labelled with 15 nm immunogold grains (cen), the BLM antigen with 10 nm grains (BLM) and RAD51/DMC1 nodes with 5 nm grains (DMC1). There are few BLM nodes, more RAD51/DMC1 nodes and the three antigens can be detected together in some of the nodes which are associated with the chromosome cores.

the full-length recombinant protein after removal of the putative antibodies to the hexahistidine epitope and it did not recognize the hRad1 protein.

To exclude the possibility that the anti-BLM antibody was recognizing WRN protein, a western blot was performed with the same extracts and incubated with both antibodies (anti-WRN; R. Freire and S. P. Jackson, to be published elsewhere). Fig. 1D shows the different mobility between the products on a PAGE gel, suggesting that each antibody recognizes a different protein.

BLM protein levels are regulated during spermatogenesis

BLM mRNA levels are very high in mouse testis when compared with other tissues (Chester et al., 1998; Seki et al., 1998) and are regulated during spermatogenesis (Seki et al., 1998). To test the ability of our antibody to detect BLM in rodent tissues and to assess the levels of BLM protein levels during prophase I of spermatogenesis, rat spermatocytes were sorted by size with centrifugal elutriation into six consecutive developmental stages (Freire et al., 1998) and extracts of these fractions were analysed by western blotting (described in the legend to Fig. 1C). The anti-BLM serum detected BLM at all stages in somewhat increasing amounts from early stages (leptotene/zygotene) to late stages (pachytene and diplotene) (Fig. 1C). As shown below, at early stages the protein is present mostly in core/SC-associated foci (Figs 2, 3), and at later stages BLM also accumulates in the chromatin and strongly in the sex vesicle (Figs 4, 5B,D).

Visualization of BLM expression and localization at meiotic prophase

(A) Technical

In addition to western blot analysis of BLM protein expression at consecutive meiotic prophase stages (Fig. 1C), the expression and location of the BLM antigen was monitored with rabbit anti-BLM serum and FITC-tagged secondary antibody for green immunofluorescence and with secondary antibody conjugated to 5 or 10 nm gold spheres for electron microscopy. Detailed localization was obtained by the simultaneous application of one

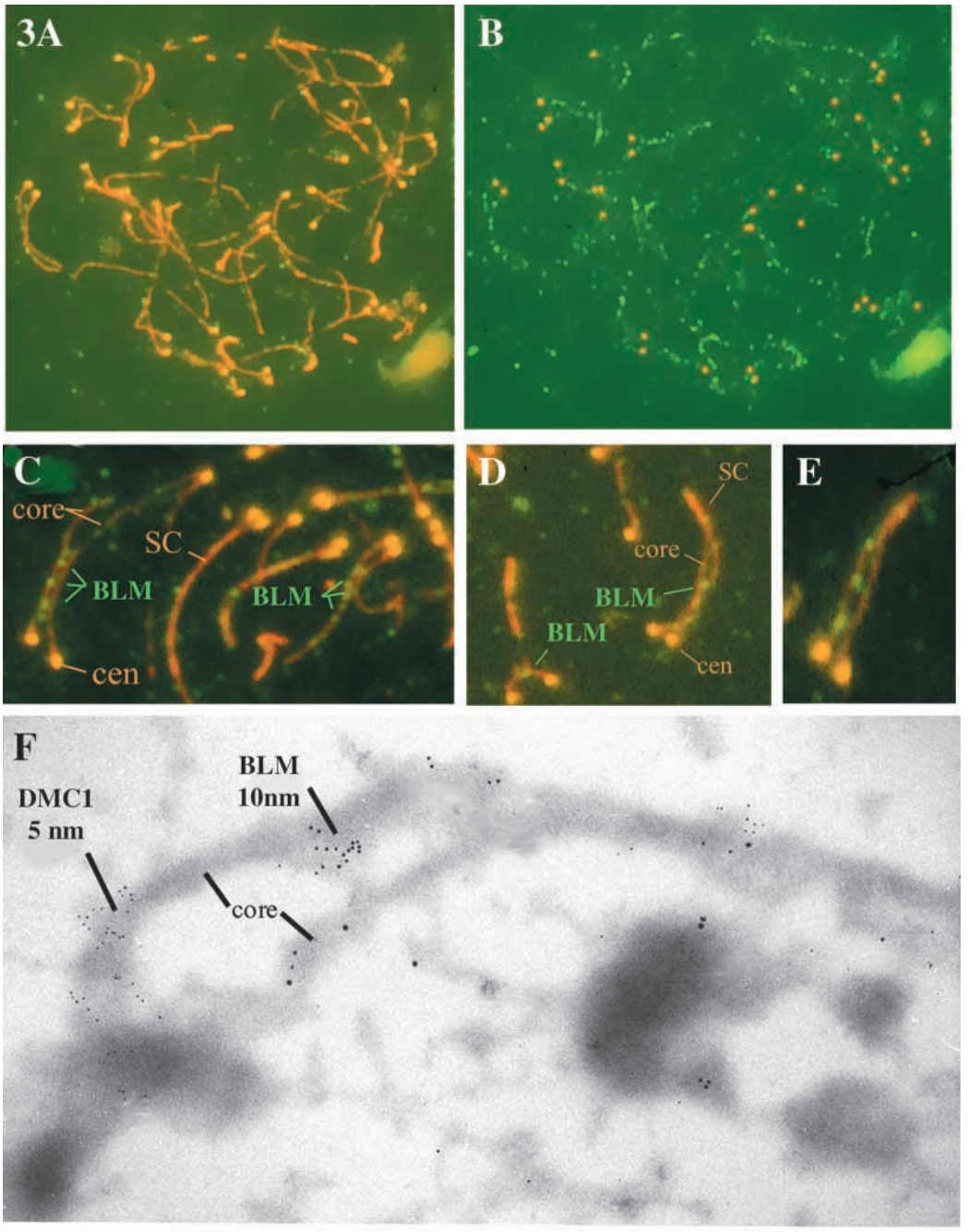

Fig. 3. Numbers and locations of BLM foci during synapsis of the chromosome cores, the zygotene stage of meiosis. (A) A whole-mount zygotene nucleus with single and partially synapsed chromosome cores/SCs and centromeres in orange and BLM foci in green. (B) The same nucleus without the cores/SCs showing only the immune-stained BLM foci in green and centromeres in orange (artificially coloured from 3A). The centromeres serve to correlate the two images. There are approximately 200 BLM foci and the double exposure in Fig. 3A shows that most of the foci are associated with the unpaired cores and with the SCs. The sperm nucleus in the right bottom corner has such an intense blue DAPI fluorescence that it penetrates the rhodamine and DAPI filters. (C-E) Higher magnifications of the BLM protein (BLM) at the sites of core alignment (core) and synapsis (SC). (F) Electron micrograph of a pair of homologous cores (core) in the process of synapsis. There is a BLM node marked by 10 nm immunogold grains (BLM) at the site of the aligned cores. Also present is a RAD51/DMC1 node marked by 5 nm gold particles (DMC1).

or more additional primary antibodies. Chromosome cores and SCs were visualized with mouse anti-COR1 antibody, mouse anti-SYN1 to accentuate the synapsed regions, mouse anti-DMC1 to identify the foci that contain the RAD51 and DMC1 proteins and human anti-centromere antibodies for general identification purposes (Dobson et al., 1994). The secondary antibodies were conjugated with fluorochromes or gold grain sizes that contrasted with the BLM detection.

DMC1 and RAD51 share epitopes and our mouse anti-DMC1 serum recognizes both antigens unless immunodepleted for RAD51 cross-reacting antibodies. With such immunodepleted antibodies, we have found that DMC1 and RAD51 colocalize to immunofluorescent foci and both are present in electron microscope images of the foci (Tarsounas et al., 1999). We therefore used the mouse anti-DMC1 to detect the RAD51/DMC1 foci. Colocation of RAD51 and DMC1 was reported earlier in the yeast, *S. cerevisiae* (Bishop, 1994), and the partial colocalization by Dresser et al. (1997) and Shinohara et al. (1997).

(B) BLM at the onset of meiotic prophase

The development of chromosome cores is initiated at the onset of meiotic prophase following meiotic S phase. In such leptotene nuclei, cores and centromeres are mostly unpaired (Fig. 2A, BLM foci have been transferred artificially from 2B to 2A and they are coloured green). There are relatively few bright BLM foci (Fig. 2B. For identification purposes, the centromeres from 2A have been transferred artificially to 2B and they are coloured red). Most of the roughly 50 BLM foci are associated with chromosome core segments and a few are off the cores. BLM antigen in association with the unpaired chromosome core is illustrated at high resolution by a group of 10 nm immunogold grains in Fig. 2C. The figure also demonstrates the simultaneous presence of groups of 5 nm gold

grains that mark the sites of DMC1 antigen. Five DMC1 clusters of 5 nm grains do not contain detectable amounts of BLM antigen whereas the group of 10 nm grains is in close contact with a few 5 nm grains, establishing the colocation of BLM with RAD51 and DMC1 (also demonstrated in Figs 6, 7B). The higher number of RAD51/DMC1 foci relative to BLM foci corresponds to the reported presence of about 250 RAD51/DMC1 foci per nucleus at the leptotene stage of meiotic prophase (Moens et al., 1997). These observations indicate that BLM is moderately expressed early in meiotic prophase, that it is frequently associated with chromosome cores, that the BLM foci are fewer than the RAD51/DMC1 foci and that BLM can colocalize with RAD51 and DMC1.

(C) BLM accumulation at chromosome core synaptic sites

Increasing levels of BLM expression observed in western blots of successive meiotic prophase stages (Fig. 1C) are reflected in the proliferation of BLM foci in nuclei that enter the stage of chromosome pairing and synapsis (Fig. 3A,B; green foci are BLM; cores and centromeres are orange. These orange centromeres in B have been digitally enhanced to facilitate comparisons between A and B). The numbers of foci associated with cores/SCs rise to between 100 and 200 per nucleus and they are particularly noticeable in the regions where pairing and synapsis are in progress (Fig. 3C-E). Mouse oocytes at zygotene and pachytene appear to have fewer core/SC-associated BLM foci, a maximum of about 50 bright foci per nucleus (counted in 12 nuclei). Observations of synaptic regions at high resolution also demonstrate the presence of BLM antigen at sites where chromosome cores are undergoing or have completed synapsis (Fig. 3F, BLM, 10 nm immunogold). The numbers of DMC1 foci (Fig. 3F, DMC1, 5 nm) have declined at this stage to about half the numbers at leptotene and they can occur in combination with BLM antigen (Figs 6, 7A,B).

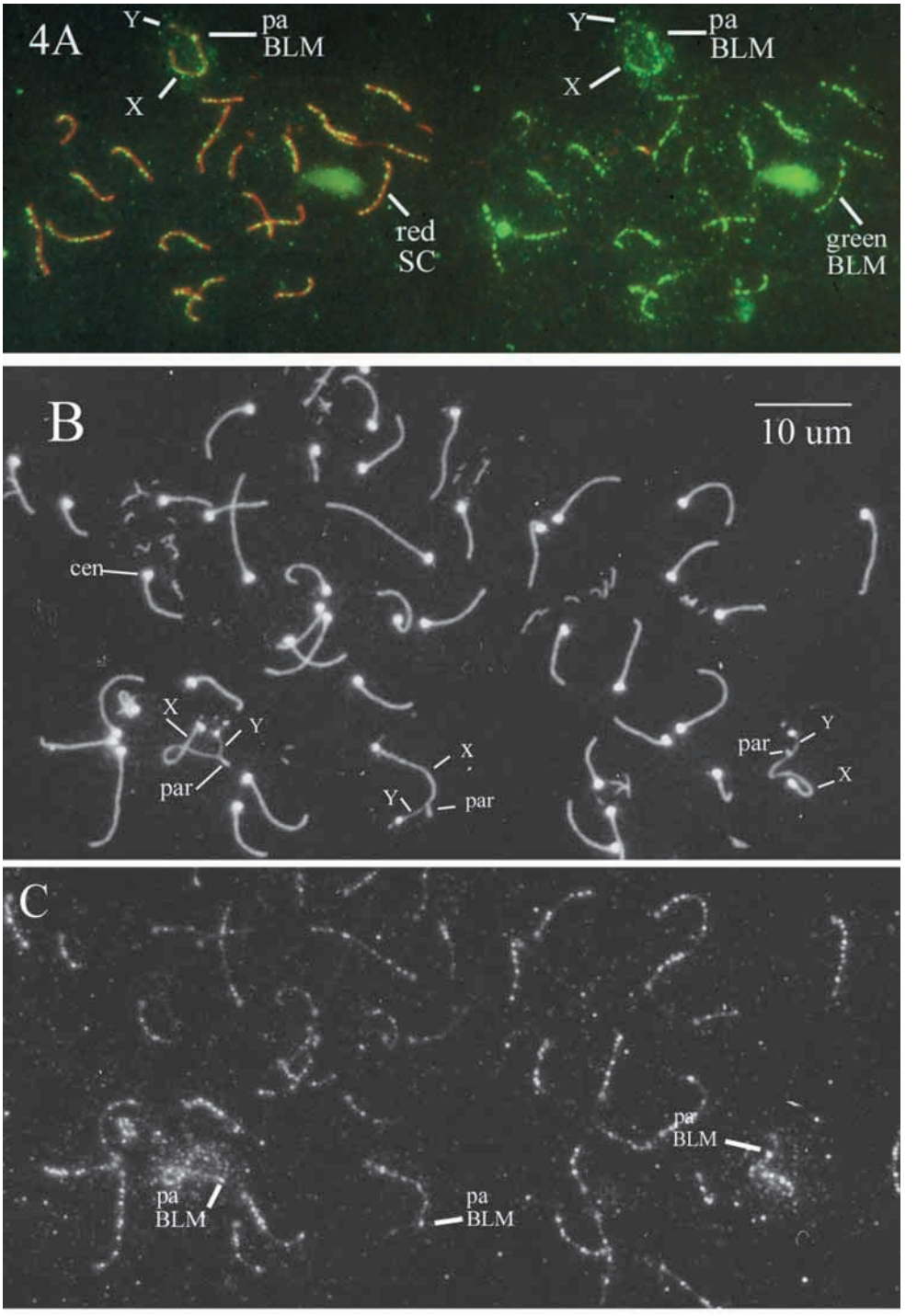

Fig. 4. The association of BLM protein with the pseudoautosomal (pa) region of the X and Y chromosomes. (A) Two images of one whole-mount nucleus with fully synapsed chromosomes in the pachytene stage of meiotic prophase. The X and Y cores (X and Y) and the synaptonemal complexes (SC) are stained red with rhodamine while the BLM foci are green fluorescent with FITC (BLM). The figure illustrates the bright BLM signal (pa BLM) at the pseudoautosomal region of the X and Y chromosomes, a region that is known to have one or more crossovers. The oval body is a bright blue fluorescent sperm nucleus that shines through the FITC filter. (B) Parts of three pachytene nuclei with well-defined SCs and X and Y chromosomes (X and Y) and their pseudoautosomal regions (par). (C) The same three nuclei stained with anti-BLM antibody confirm the regular presence of a BLM signal in the pseudoautosomal region of the X and Y chromosomes (pa BLM) suggesting a correlation with crossover sites.

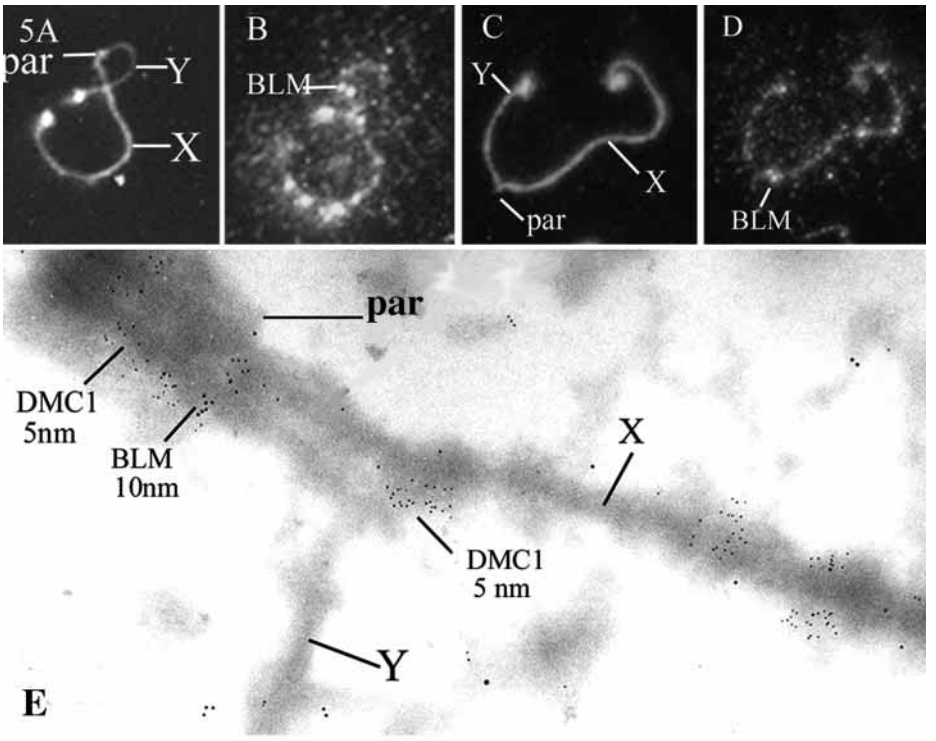

Fig. 5. Details of the pseudoautosomal associated BLM signal. (A,C) The X and Y chromosome cores and the pa region (par) in a late-stage, end-to-end arrangement of the X and Y chromosome cores. (B,D) The same X-Y chromosomes stained with anti-BLM antibodies reveal two appressed BLM signals in the partially detached pa region (par). (E) An electron micrograph of the X and Y chromosomes and the pa region. The X chromosome (X) has several RAD51/DMC1 foci which are marked with 5 nm gold spheres (DMC1). There is a group of 10 nm gold grains that marks the location of a BLM (BLM) node within the pa region (par).

(D) BLM association with the X-Y pseudoautosomal region

Casual observation of pachytene nuclei suggest that the pseudoautosomal, pa, region of the X-Y chromosomes frequently contains one or two BLM foci. This observation might be of significance with respect to the presence of one or more reciprocal crossovers in the pa region. To quantify the occurrence of BLM foci in the pa region, all micrographs, colour and black/white fluorescence and electron microscopy, which had been recorded in the absence of a bias for the presence or absence of BLM foci in the pa region, were examined. The criterion for fluorescence micrographs was the presence in the surface-spread nucleus of a well-defined immunostained X-Y pair (Fig. 4). The EM images were selected for a pa region at low magnification where the gold grains are not visible and then recorded at high magnification (Fig. 5E). Of 27 optically recorded nuclei with a well defined X-Y pair, 22 contained one or two pa-associated BLM foci and of 9 EM pa images, seven contained BLM antigen. Given that on the average 1 μm of SC length has less than one BLM focus and that in the pachytene nucleus the pa region is about 0.5 μm, the frequent presence of a BLM focus in the pa region suggests a biased distribution. When comparing the distribution of all BLM foci in the 22 nuclei with either an even distribution of foci or a random distribution of foci along all SCs with the Poisson statistic the observed distribution is highly significantly different from the expected. The implication is that there is a preferential allocation of BLM foci to the pa region. In 8 out 22 cases, the unusual brightness of the pa-BLM focus was found to be the result of two closely appressed signals (Fig. 5A-D), possibly a correlation with the occurrence of double crossovers and a lack of interference in the pa region (Soriano et al., 1987).

BLM foci share the peculiarity of RAD51/DMC1 foci that they are present along the single, unpaired core of the X-chromosomes but not the Y chromosome (Moens et al., 1997). Potentially, the X chromatids might engage in sister chromatid exchanges and BLM might suppress that tendency but insufficient observations have been reported to speculate on the functions. The foci of checkpoint proteins hRAD1 and ATR, on the other hand, occur on both the X and the Y chromosome cores at meiosis (Freire et al., 1998; Moens et al., 1999), supporting the concept of different functions for recombination versus checkpoint proteins at meiosis.

(E) Spatial and temporal distribution of BLM relative to RAD51/DMC1 foci

Immunofluorescence observations on spermatocyte nuclei at successive stages of meiotic prophase that are doubly labelled with green fluorescent FITC for the BLM antigen and rhodamine red for DMC1 antigen permit an analysis of the spacial and temporal occurrence of the two proteins relative to each other. For example, in an early pachytene nucleus (Fig. 6, right side, recorded first with an FITC filter and then re-exposed with a rhodamine filter), there are red foci that contain DMC1 antigen and little or no BLM antigen. The few green foci, conversely, have the BLM antigen with little or no DMC1 antigen. The majority of the foci are orange, representing a green and a red focus close together or one focus with both antigens. That the two antigens can occur together is regularly seen in electron micrographs (Figs 2C, 5E). In a later stage nucleus on the left of Fig. 6, the green BLM foci predominate with only a very few red or orange foci remaining. The transition implies that RAD51 and DMC1 antigen is progressively lost from these foci. When the numbers of foci are recorded separately for the two antigens, it is apparent that the RAD51/DMC1 foci assemble and reach their maximum number at early prophase while BLM foci assemble later and peak later at the end of zygotene and the beginning of pachytene (Fig. 7A, summarized from 20 completely analysed nuclei immunostained with rabbit anti-BLM and mouse anti-DMC1 serum). Because many of the foci contain all three antigens, a more accurate analysis differentiates between the three types of foci, RAD51/DMC1, BLM and mixed foci (Fig. 7B). From the bar graph in Fig. 7B and from Fig. 6, it appears

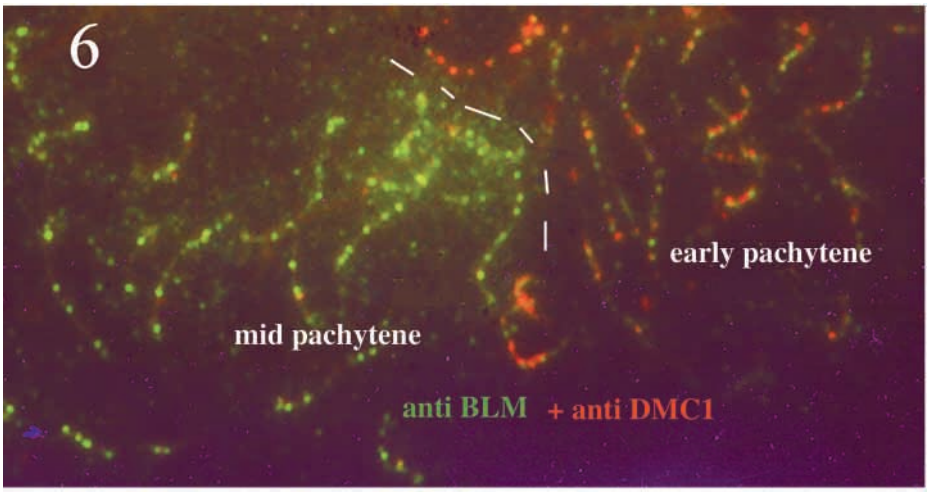

Fig. 6. Transition from mixed BLM/RAD51/DMC1 foci to BLM foci during the pachytene stage of meiotic prophase. Shown are the three types of foci that are associated with the SCs in two whole-mount nuclei at early (right) and later (left) pachytene stage of meiotic prophase. The earlier nucleus has SCs with RAD51/DMC1 in red, mixed BLM/RAD51/DMC1 foci in orange and BLM foci in green. The later nucleus on the right has a similar number of foci but most of the RAD51/DMC1 component is absent. It is speculated that the transition marks the development from early to late recombinant functions of the foci.

that BLM becomes associated with RAD51/DMC1 foci at late leptotene (yellow segment of the bar) and at later times, the amount of RAD51/DMC1 antigen is reduced in the mixed foci until BLM foci predominate.

DISCUSSION

BLM is an autosomal recessive disorder characterized, among other symptoms, by a high risk of cancer, which may be related to the prevalence of chromosome abnormalities in BS cells. These abnormalities include a high frequency of sister strand exchanges and increased exchanges between homologous chromosomes, revealing a role for BLM protein in mitotic genomic stability in humans. It has been found in mammals that a number of proteins associated with somatic cell-induced DNA-damage signalling and repair are present constitutively at the chromosome cores/SCs during prophase of meiosis: HRAD1 (Freire et al., 1998), MLH1 (Anderson et al., 1999), ATR (Moens et al., 1999) and RAD51 (Barlow, A. L. et al., 1997; Moens et al., 1997). To these reports, we add the observation that the BLM protein colocates in a stage-dependent manner with RecA-type recombination complexes at the meiotic prophase chromosome cores/SCs. RAD51 and DMC1 are mammalian homologs of the *E. coli* protein, RecA, which enhances the ability of single-stranded DNA to search homologous double-stranded DNA and participates in heteroduplex formation and strand-exchange processes in both DNA repair and recombination reactions (Kowalczykowski et al., 1994; Baumann et al., 1996). The early events of meiotic recombination in the yeast, *S. cerevisiae*, include the induction of double strand breaks by a complex that includes the *Spo11* gene product followed by the RecA functions in homology search and strand exchange (Keeney et al., 1997; for reviews, see Roeder, 1997; Haber, 1998). The protein complexes of these homologous recombination events have been defined by in vitro and in vivo interaction in a number of cell lines and phylogenetically distant organisms (reviewed by Thacker, 1999). The established sites of potential recombination, the joint molecules, can later be resolved by topoisomerase activity (Stahl, 1996) or by Holliday junction resolution (Bennet and West, 1995).

Immunocytology

The time course and localization at meiotic prophase of the recombinases and damage/repair complexes have been monitored by immunocytology for those proteins that are sufficiently abundant to be detected with the relevant antibodies. Thus, in mammalian spermatocytes, some members of the recombination complex such as RAD51 and DMC1 have been visualized but others such as SPO11, RAD52 and ligases are not obvious (P. M., personal observation). Similarly, putative checkpoint control proteins, ATR and HRAD1, have been observed in association with cores/SCs but ATM and HRAD17 are not well defined (Barlow C. et al., 1997; P. M., personal observation). Members of the two classes of these functionally diverse complexes, recombinases versus checkpoint control proteins, occur separately along the cores/SCs such that the recombinases RAD51 and DMC1 colocate in 100 nm nodules that are separate from ATR and HRAD1 foci. Similarly RAD51/DMC1 foci are found along the core of the single X chromosome but not the core of the Y chromosome, whereas ATR and HRAD1 occur along both the X and the Y cores. Since the function of BLM at meiotic prophase was not known, it could not be predicted whether the protein would be immunologically detectable at meiotic prophase and, if detected, how it would relate to previously observed recombinases or checkpoint proteins. This report shows that the BLM protein colocalizes with the RAD51/DMC1 complex, which suggests involvement with recombination. The late appearance of BLM relative to RAD51 and DMC1 and its association with a recombination hot spot suggest a role in late recombinant events.

BLM and crossing over

The relationship between immunocytologically observed recombination-related foci and crossing over remain tenuous. Several correlations can be indicative of a functional interaction. For example, the *mutL* homolog, *MLH1*, is involved in crossing over (Baker et al., 1996) and the numbers and positions of the MLH1 foci that are associated with SCs resemble the distribution of crossovers (Anderson et al., 1999). However, the numbers of immunofluorescent foci of several presumptive recombination related proteins, RAD51, DMC1, ATR, RPA, HRAD1 and in the present case, BLM, tend to be

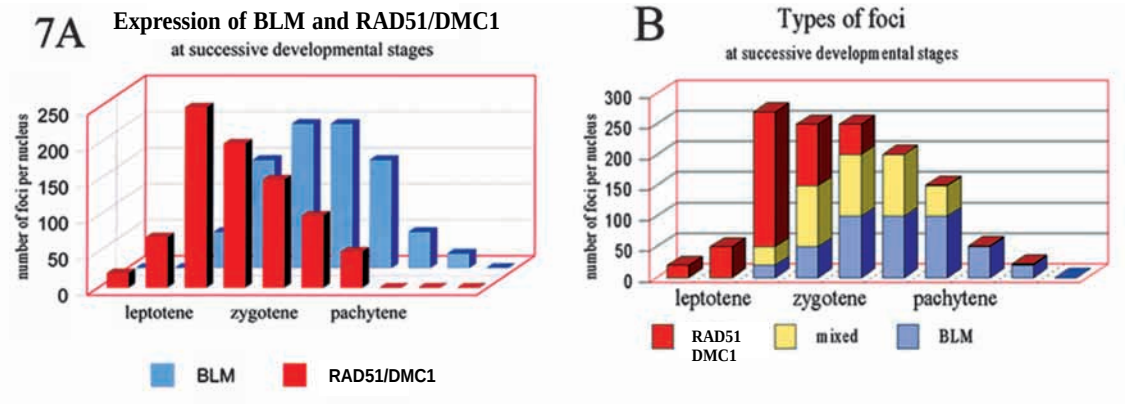

Fig. 7. Average numbers of RAD51/DMC1, BLM and mixed foci per nucleus that are associated with chromosome cores/SCs at successive developmental stages. (A) Numbers of RAD51/DMC1-containing foci depicted by red bars and the numbers of BLM-containing foci in blue, based on 20 completely analysed nuclei immunostained with anti-DMC1 and anti-BLM serum. The numbers of RAD51/DMC1-containing foci per nucleus increase rapidly at early prophase stages and then decline during zygotene and pachytene. There are fewer BLM-containing foci per nucleus at early prophase stages and the numbers reach their maximum at the beginning of the pachytene stage. (B) The same data set as in A, illustrating that a proportion of the foci contain all three proteins represented by the yellow segments of the bars.

in great excess of the number of cytologically or genetically determined recombinant events per nucleus. Evidently not all of those protein complexes are activated to produce a mature recombinant event and only a select subsample may be triggered. Given the observation that the BLM protein normally stabilizes chromosome structure and prevents sister chromatid exchanges in somatic cells, it may be that BLM also has a limiting function in the initiation meiotic recombination.

The involvement of the optically/EM observable protein complexes in recombination can potentially be supported if they are found to be associated with recombination nodules, RNs. EM-defined 'late recombination nodules' have been shown to correlate with reciprocal recombination positions in animals and plants where closely related species differ in the position of chiasmata, localized versus non-localized (Bernelot-Moens and Moens, 1986; Albini and Jones, 1988). However it has not been possible to show a direct correlation between RNs and foci in the mouse because of the poor visibility of late recombination nodules. Alternatively, if the position of a given crossover is highly localized, then the recombination-associated proteins can be expected to be present at that position. The male mouse has at least one crossover in a predictable position, that is the short pseudoautosomal, pa, region of the X-Y sex chromosomes (Soriano et al., 1987). This study detects the regular occurrence of one or two BLM foci in the pa region (Figs 4, 5). The probability that 29 of the 36 observed pa regions have one or two BLM foci due to chance alone is calculated to be less than 1%. It therefore appears that the BLM-pa foci have a statistically highly significant correlation with the site of a reciprocal recombination event. In addition, the regular presence of two BLM foci in the short pa region correlates well with the genetic evidence of double crossovers in the pa region where genetic interference is absent (Soriano et al., 1987).

Walpita et al. (1999) in their analysis of BLM foci in mouse spermatocytes did not report BLM foci in the pa region. Because of this and because of differences with MLH1 focal patterns, a function of BLM in reciprocal recombination was not considered. However, our fluorescent and EM images (Fig. 4, 5) demonstrate unequivocally the presence of BLM foci in the pa region and we show that the distribution has a statistically highly significant bias towards the pa region.

Stage-dependent colocalization of BLM with RAD51 and DMC1

At early meiotic prophase, the number of RAD51/DMC1 foci located on the single unpaired chromosome cores increases from zero to about 250 per nucleus (Fig. 7A). The BLM foci that are connected to the cores appear somewhat later and reach a maximum of only 50 at leptotene (Figs 2A,B, 7A). With double staining, it is evident from fluorescent and EM images that a fraction of the foci contain three antigens (Figs 2C, 6, 7B). Subsequently, while chromosome synapsis is in progress, the numbers of RAD51/DMC1 foci decline while the BLM foci increase in numbers to about 200 (Figs 3, 7A), half of which are mixed with RAD51/DMC1 protein (Fig. 7B). Eventually, the mixed foci lose most of the RAD51/DMC1 components (Figs 6, 7B). With the caveat that only a few of the proteins of the recombination complex are being visualized, it can be speculated that the observed change in composition of the recombination complex might signal a change from early to late recombination functions of the foci.

One potential role for BLM in recombination and one that would match its dynamics and distribution profiles during meiosis, is a function in crossover control. In this regard, it is notable that it has been hypothesized previously that the bimolecular intermediates formed in the later stages of homologous recombination might be resolved by a topoisomerase/resolvase enzyme (Stahl, 1996; Smith and Nicolas, 1998). Since the yeast homolog of BLM, Sgs1p, associates with topoisomerases II and III, an attractive model is that BLM also associates with topoisomerase(s) and is involved at late meiotic prophase stages in resolving recombination events. To address this and related issues, it will clearly be of great interest to conduct further biochemical and immunological studies on BLM and other proteins that are associated with meiotic recombination. Our observations differ in several details from Walpita et al. (1999) who report that there are no BLM foci on the early single cores or in initial SC formation and that BLM foci are found on only some zygotene

SCs. Quite to the contrary, we demonstrate with fluorescent and EM images that the BLM foci are evident on the early unpaired cores (Fig. 2), on single cores at zygotene (Fig. 3A,B,F), at the initial SC formation (Fig. 3C-E) and that all SC segments have BLM foci (Fig. 3B).

Particular thanks to Hideo Kaneko from Gifu University, Japan, for providing the Bloom's cell line extracts, Nadine Kolas from York University for providing mouse oocytes, and Carol Featherstone from the Wellcome Trust and CRC Institute for comments. The recombinant full-length BLM protein was kindly provided by Dr Ian Hickson, ICRF laboratories, Institute of Molecular Medicine, Oxford, UK. Funding for R. Freire and S. P. Jackson from the Cancer Research Campaign and the A-T Medical Research Trust. Funding for M. Tarsounas, B. Spyropoulos and P. B. Moens was from NSERC of Canada.

REFERENCES

Albini, S. M. and Jones, G. H. (1988). Synaptonemal complex spreading in *Allium cepa* and *Allium fistulosum*. II. Pachytene observations. The SC karyotype and correspondence of late recombination nodules and chiasmata. *Genome* **30**, 399-410.

Anderson, L. K., Reeves, A., Webb, L. M. and Ashley, T. (1999). Distribution of crossing over on mouse synaptonemal complexes using immunofluorescent localization of MLH1 protein. *Genetics* **151**, 1569-1579.

Baker, S. M., Plug, A. W., Prolla, T. A., Bronner, C. E., Harris, A. C., Yao, X., Christie, D.-M., Monell, C., Arnheim, N., Bradley, A., Ashley, T. and Liskay, R. M. (1996). Involvement of mouse *Mlh1* in DNA mismatch repair and meiotic crossing over. *Nature Genet.* **13**, 336-342.

Barlow, A. L., Benson, F. E., West, S. C. and Hultén, M. A. (1997). Distribution of the Rad51 recombinase in human and mouse spermatocytes. *EMBO J.* **16**, 5207-5215.

Barlow, C., Liyanage, M., Moens, P. B., Deng, C. X., Reid, T. and Wynshaw-Boris, A. (1997). Partial rescue of the prophase I defects of *ATM*-deficient mice by *p53* and *p21* null alleles. *Nature Genet.* **17**, 462-466.

Baumann, P., Benson, F. E. and West, S. C. (1996). Human Rad51 promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. *Cell* **87**, 757–766.

Bennet, R. J. and West, S. C. (1995). RuvC protein resolved Holliday junctions via cleavage of the continuous (non crossover) strands. *Proc. Natl. Acad. Sci. USA* **92**, 5639.

Bernelot-Moens, C. and Moens, P. B. (1986). Recombination nodules and chiasma localization in two Orthoptera. *Chromosoma* **93**, 220-226.

Bishop, D. K. (1994). RecA homologs Dmc1 and Rad51 interact to form multiple nuclear complexes prior to meiotic chromosome synapsis. *Cell* **79**, 1081-1092.

Chester, N., Kuo, F., Kozak, C., O'Hara, C. D. and Leder, P. (1998). Stage-specific apoptosis, developmental delay, and embryonic lethality in mice homozygous for a targeted disruption in the murine Bloom's syndrome gene. *Genes Dev.* **12**, 3382-3393.

Davey, S., Han, C., Ramer, S., Klassen, J., Jacobson, A., Eisenber, A., Hopkins, K., Lieberman, H. and Freyer, G. (1998). Fission yeast rad12$^+$ regulates cell cycle checkpoint control and is homologous to the Bloom's syndrome disease gene. *Mol. Cell. Biol.* **18**, 2721-2728.

Dobson, M. J., Pearlman, R. E., Karaiskakis, A., Spyropoulos, B. and Moens, P. B. (1994). Synaptonemal complex proteins, occurrence, epitope mapping and chromosome disjunction. *J. Cell Sci.* **107**, 2749-2760.

Dresser, M. E., Ewing, D. J., Conrad, M. N., Dominguez, A. M., Barstead, R, Jiang, H. and Kodalek, T. (1997). *DMC1* functions in a *Saccharomyces cerevisiae* meiotic pathway that is largely independent of the *Rad51* pathway. *Genetics* **147**, 533-544.

Ellis, N. A., Groden, J., Ye, T.-Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M. and German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* **83**, 655-666.

Freire, R., Margay, J. R., Tarsounas, M., Lowndes, N. A., Moens, P. B. and Jackson, S. P. (1998). Human and mouse homologs of *Schizosaccharomyces pombe* rad1$^+$ and *Saccharomyces cerevisiae* RAD17, linkage to checkpoint control and mammalian meiosis. *Genes Dev.* **12**, 2560-2573.

Gangloff, S., McDonald, J., Bendixen, C., Arthur, L. and Rothstein, R. (1994). The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog, a potential eukaryotic reverse gyrase. *Mol. Cell. Biol.* **14**, 8391-8398.

German, J. (1993). Bloom Syndrome, a Mendelian prototype of somatic mutational disease. *Medicine* **71**, 393-406.

Gray, M., Chen, J., Kamath-Loeb, A., Blank, A., Sopher, B., Martin, G., Oshima, J. and Loeb, L. (1997). The Werner syndrome protein is a DNA helicase. *Nature Genet.* **17**, 100-103.

Haber, J. E. (1998). The many interfaces of Mre11. *Cell* **95**, 583-586.

Harlow, E. and Lane, D. (1988). In *Antibodies, A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor.

Kaneko, H., Inoue, R., Fukao, T., Kasahara, K., Tashita, H., Teramoto, T. and Kondo, N. (1997). Two Japanese siblings with Bloom syndrome gene mutation and B-cell lymphoma *Leuk. Lymphoma*. **27**, 539-542.

Karow, J., Chakraverty, R. and Hickson, I. D. (1997). The Bloom's syndrome gene product is a 3′-5′ DNA helicase. *J. Biol. Chem.* **272**, 30611-30614.

Keeney, S., Giroux, C. and Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalysed by Spo11, a member of a widely conserved protein family. *Cell* **88**, 375–384.

Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. and Rehrauer, W. M. (1994). Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* **58**, 401-465.

Moens, P. B., Chen D. J., Shen, Z., Kolas, N., Tarsounas, M., Heng, H. H. Q. and Spyropoulos, B. (1997). Rad51 immunocytology in rat and mouse spermatocytes and oocytes. *Chromosoma* **106**, 207-215.

Moens, P. B., Tarsounas, M., Morita, T., Habu, T., Rottinghaus, S. T., Freire, R., Jackson, S. P., Barlow, C. and Wynshaw-Boris, A. (1999). The association of ATR protein with mouse meiotic chromosome cores. *Chromosoma* **108**, 95-102.

Roeder, G. S. (1997). Meiotic chromosomes, it takes two to tango. *Genes Dev.* **11**, 2600-2620.

Seki, T., Wang, W.-S., Okumura, N., Seki, M., Katada, T. and Enomoto, T. (1998). cDNA cloning of mouse BLM gene, the homologue to human Bloom's syndrome gene which is highly expressed in the testis at the mRNA level. *Biochim. Biophys. Acta* **1398**, 377-381.

Shinohara, A., Gasior, S., Ogawa, T., Kleckner, N. and Bishop, D. K. (1997). *Saccharomyces cerevisiae* recA homologues RAD51 and DMC1 have both distinct and overlapping roles in meiotic recombination. *Genes Cells* **10**, 615-629.

Smith, K. N. and Nicolas, A. (1998). Recombination at work for meiosis. *Curr. Opin. Genet. Dev.* **8**, 200-211.

Soriano, P., Keitges, E. A., Schorderet, D. F., Harbers, K., Gartler, S. M. and Jaenisch, R. (1987). High rate of recombination and double crossovers in the mouse pseudoautosomal region during male meiosis. *Proc. Natl. Acad. Sci. USA* **84**, 7218-7220.

Stahl, F. (1996). Meiotic recombination in yeast. Coronation of the double-strand break repair model. *Cell* **87**, 985-968.

Stewart, E., Chapman, C., Al-Khodairy, F., Carr, A. and Enoch, T. (1997). rqh1$^+$, a fission yeast gene related to the Bloom's and Werner's syndrome genes, is required for reversible S phase arrest. *EMBO J.* **16**, 2682-2692.

Tarsounas, M., Morita, T., Pearlman, R. E. and Moens, P. B. (1999). RAD51 and DMC1 form mixed complexes associated with mouse meiotic chromosome cores and synaptonemal complexes. *J. Cell Biol.* **147**, 207-220.

Thacker, J. (1999). A surfeit of *RAD51*-like genes? *Trends Genet.* **15**, 166-168.

Walpita, D., Plug, A. W., Neff, N. A., German, J. and Ashley, T. (1999). Bloom's syndrome protein, BLM, colocalizes with replication protein A in meiotic prophase nuclei of mammalian spermatocytes. *Proc. Natl. Acad. Sci. USA* **96**, 5622–5627.

Watt, P., Louis, E., Borts, R. and Hickson, I. (1995). Sgs1, a eukaryotic homolog of *E. coli* RecQ that interacts with topoisomerase II in vivo and is required for faithful chromosome segregation. *Cell* **81**, 253-260.

Watt, P. M., Hickson, I. D., Borts, R. H. and Louis, E. J. (1996). *SGS1*, a homologue of the Bloom's and Werner's Syndrome genes, is required for maintenance of genome stability in *Saccharomyces cerevisiae*. *Genetics* **144**, 935-945.

Yamagata, K., Kato, J.-I., Shimamoto, A., Goto, M., Furuichi, Y. and Ikeda, H. (1998). Bloom's and Werner's syndrome genes suppress hyper recombination in yeast *sgs1* mutant, Implication for genomic instability in human diseases. *Proc. Natl. Acad. Sci. USA* **95**, 8733-8738.

Yu, C., Oshima, J., Fu, Y.-H., Wijsman, E., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G., Mulligan, J. and Schellenberg, G. (1996). Positional cloning of the Werner's syndrome gene. *Science* **272**, 258-262.