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Mammalian ubiquitin-conjugating enzyme Ubc9 interacts with Rad51 recombination protein and localizes in synaptonemal complexes

(ubiquitination/homologous recombination/meiosis/spermatocytes)

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ABSTRACT ***Hsubc9*, a human gene encoding a ubiquitin-conjugating enzyme, has been cloned. The 18-kDa HsUbc9 protein is homologous to the ubiquitin-conjugating enzymes Hus5 of *Schizosaccharomyces pombe* and Ubc9 of *Saccharomyces cerevisiae*. The *Hsubc9* gene complements a *ubc9* mutation of *S. cerevisiae*. It has been mapped to chromosome 16p13.3 and is expressed in many human tissues, with the highest levels in testis and thymus. According to the Gal4 two-hybrid system analysis, HsUbc9 protein interacts with human recombination protein Rad51. A mouse homolog, *Mmubc9*, encodes an amino acid sequence that is identical to the human protein. In mouse spermatocytes, MmUbc9 protein, like Rad51 protein, localizes in synaptonemal complexes, which suggests that Ubc9 protein plays a regulatory role in meiosis.**

Genetic and biochemical studies have shown that ubiquitination is involved in regulation of gene expression, DNA repair, cellular stress response, cell cycle progression, signal transduction, programmed cell death, and other important cellular processes (1–3). The degradation of many key proteins is mediated by ubiquitination, including mitotic cyclins, oncoproteins, the tumor suppressor protein p53, transcriptional regulators, and cell surface receptors. In the ubiquitination reaction, ubiquitin, a highly conserved protein of 76 amino acids, is first activated by an activation enzyme, E1. It is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2 enzyme). The E2 enzyme, either alone or together with a ubiquitin-protein ligase, E3, catalyzes transfer of ubiquitin to a lysine residue of a target protein. Multiply ubiquitinated proteins are finally recognized and degraded by a multisubunit 26S protease complex (3). Ubiquitination of some proteins, such as calmodulin, histones H2A and H2B, actin, and certain membrane receptors, may serve a regulatory function without targeting them for cytosolic degradation (4).

In yeast, at least 10 different E2 enzymes have been identified (5). Among them, the recently discovered *Saccharomyces cerevisiae* Ubc9 protein and its *Schizosaccharomyces pombe* homolog, Hus5, are essential for cell viability. Mutations in the *hus5* gene lead to sensitivity to UV light, ionizing radiation, and hydroxyurea. Following meiosis, haploid segregants with a disrupted *hus5* gene display multiple mitotic abnormalities and soon cease dividing (6). In *S. cerevisiae*, depletion of Ubc9 protein results in cell cycle arrest in G_2/M phase. In this case, Ubc9 protein has been implicated in degradation of mitotic B-type cyclins, Clb5 and Clb2, which have dramatically increased stability in a *ubc9* mutant (7).

Several human homologs of the yeast ubiquitin-conjugating enzymes (UBCs) have been cloned, including those of Ubc2 (Rad6), Ubc3 (CDC34), Ubc4, and Ubc8 enzymes. We describe the isolation of a human homolog of yeast Ubc9 protein, named HsUbc9, which was discovered by virtue of its interaction with HsRad51 recombinase, a human homolog of RecA protein. Recent observations on several species have revealed the association of members of the RecA/Rad51 family of recombination proteins with synaptonemal complexes (8–11). Experiments described here show that Ubc9 protein is also found in synaptonemal complexes.¶

MATERIALS AND METHODS

DNA Sequencing. The nucleotide sequences were determined by the dideoxyribonucleotide chain-termination procedure (12), using modified T7 DNA polymerase (Sequenase 2.0; United States Biochemical). In some cases, sequencing was done on an Applied Biosystem 373A DNA sequencer with multiple fluorophores from the DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems).

GAL4 Two-Hybrid System. The GAL4 two-hybrid technique (13) was used to detect protein–protein interactions. The pGBT9 expression vector [$Gal4_{(1-147)}$ DNA-binding domain; *TRP1*; Amp^r] was used to generate a fusion of the Gal4 DNA-binding domain with target protein *X*. Another expression vector, pGAD424 [$GAL4_{(768-881)}$ activation domain; *LEU2*; Amp^r) was used to generate a fusion of the Gal4 activation domain with protein Y, potentially interacting with the target protein *X* (14). To detect protein–protein interaction, two fusion constructs were introduced into *S. cerevisiae* HF7c strain (15) carrying *HIS3* and *lacZ* reporter genes under control of different Gal4-responsive elements, and into SFY526 strain (16) carrying the *lacZ* reporter. HF7c transformants were plated on two selective synthetic dextrose (SD) media (17): (*i*) lacking leucine and/or tryptophan and (*ii*) lacking histidine as well. The ratio of cells that grew under these two conditions (*ii*:*i*) was taken as the relative frequency of His^+ transformants. Activation of the *lacZ* reporter in HF7c was detected by a filter assay for β-galactosidase activity (14), and the activity was quantitated in strain SFY526 by a liquid assay (18).

The following constructs were used in the two-hybrid assay. Plasmids pEG918 and pEG960 were made by ligating the

Abbreviation: UBC, ubiquitin-conjugating enzyme.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U31933 and U31934).

entire coding sequence of the human *rad51* gene into vectors pGBT9 and pGAD424, respectively. Plasmid pEG947 isolated from the screen of a human cDNA library (see *Results*) contains an in-frame fusion of the Gal4-activation domain with cDNA of human *ubc9* gene in vector pGAD GH (Clontech). Plasmid pEG959 was made by subcloning the coding sequence of *Hsubc9* gene from pEG947 into pGBT9. Plasmid pCL1 expresses the full-length Gal4 protein (19). Plasmids pLAM5′ and pVA3, both derived from vector pGBT9, encode human lamin $C_{(66-230)}$– and murine $p53_{(72-390)}$–Gal4 DNA-binding domain fusions, respectively (14, 20). Plasmid pTD1 encodes a simian virus 40 large T $antigen_{(84-708)}$–Gal4 activation domain hybrid in pGAD3F (21).

To identify proteins interacting with human recombination protein HsRad51, we used a library of human cDNA fragments (isolated from HeLa S3 cells) fused to the Gal4 activation domain in pGAD GH vector (Clontech). The library was screened by using the HsRad51–Gal4 protein fusion expressed from plasmid pEG918 as a bait.

Primers for Amplification of a Mouse Homolog of *ubc9* cDNA. Amplification by reverse transcription–PCR of a mouse homolog of *ubc9* cDNA was achieved by using primers derived from the sequence of *Hsubc9* cDNA. The forward primer was homologous to a sequence of 16 nt located just upstream of the ATG initiator codon plus the ATG codon itself. The reverse primer was complementary to a stretch of 20 nt which started 25 nt downstream of the TAA stop codon of *Hsubc9*.

Complementation Analysis. Complementation analysis of *Hsubc9* gene in yeast was done in the *S. cerevisiae* strains YWO2a (*MAT***a** *his3 leu2 lys2 trp1 ura3*) and YWO102a (*MAT***a** *ubc9*Δ::*TRP1 ubc9-1*^{ts}::*LEU2 his3 leu2 lys2 trp1 ura3*) obtained from B. Futcher (7). The coding sequence of the *Hsubc9* gene was subcloned from pEG947 into vector YEplac195GAL (22) to yield the plasmid pEG954. Transformants were selected for growth at 23°C on synthetic complete medium lacking uracil. Viability of transformants was examined after streaking out cells on either 1% yeast extract/2% peptone/2% dextrose (YPD) medium (with glucose) (17) or YPGal [the same as YPD except with 2% (wt/vol) galactose instead of glucose] and incubating cultures at the indicated temperatures for 2–3 days.

Northern Blot Analysis. A Northern blot containing approximately 2 μg of poly(A)$^+$ RNA per lane from various human tissues (Clontech) was used to analyze expression of the human *ubc9* gene homolog. The whole *Hsubc9* cDNA insert from pEG947 was taken as a probe. It was labeled with [α-^{32}P]dCTP by random priming (22). For comparison, the blot was reprobed with a human γ-actin probe.

Isolation of a Genomic Clone of *Hsubc9*. A human genomic DNA library in λ phage DASH II (Stratagene) was used for isolation of a genomic clone of the human *ubc9* homolog. The phage (≈10^6 plaque-forming units) were plated on *Escherichia coli* XLI-Blu MRA (P2) (Stratagene) and plaques were transferred onto Hybond-N membrane (Amersham). The entire *Hsubc9* cDNA insert from plasmid pEG947 was used as a probe. Positive λ clones were tested with probes representing two nonoverlapping fragments of *Hsubc9* cDNA. One clone, λ13, was obtained whose DNA hybridized to both probes. Amplification by PCR with three pairs of primers derived from the cDNA sequence of *Hsubc9* confirmed that the λ13 clone carries at least part of the *Hsubc9* gene.

Mapping the *Hsubc9* Gene on the Human Chromosome. DNA from λ13 was labeled by nick-translation with biotin-11-UTP. Digoxigenin-labeled oligomer GM-009 was used as an *Alu*-banding probe (23). Metaphase spreads were prepared from peripheral blood cultures of a normal individual according to standard cytological procedures. Hybridization was carried out as described (24). The biotin-labeled λ13 probe was detected with rhodamine-avidin and a digoxygenin-labeled *Alu* probe was detected with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibodies. Chromosomes and cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were taken and processed as described (10, 25).

Purification of HsUbc9 Protein. The coding sequence of the *Hsubc9* gene was subcloned from pEG947 plasmid into expression vector pTrcHis B (Invitrogen) as a translational fusion with six consecutive histidine residues at the N terminus. The fusion protein was expressed in *E. coli* DH10B (Life Technologies, Grand Island, NY) and purified by nickel-chelate chromatography. The isolated protein, which was more than 95% pure, was used for preparation of mouse polyclonal antibody (26).

Nontagged HsUbc9 protein was expressed in *E. coli* BL21(DE3) (27) from the plasmid pEG957 containing the coding sequence of the *Hsubc9* gene in pRSET B vector (Invitrogen). (Details of the purification are available on request.)

Immunocytology. Cytological preparations of spermatocytes from male mice 17–21 days old were prepared and treated with antibodies as described (11). The primary antibodies were polyclonal rabbit antibodies directed against HsRad51 protein and polyclonal mouse antibodies directed against HsUbc9 protein. Antiserum against HsRad51 was used at dilutions of 1:100–1:250, and anti-HsUbc9 was diluted 1:200–1:500. Goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) from Sigma was the secondary antibody used to visualize anti-HsRad51, and goat anti-mouse IgG conjugated with rhodamine (Pierce) was the secondary antibody used to visualize anti-HsUbc9. Specimens were examined and digitally imaged on a Zeiss Axioskop (63×, 1.2 numerical aperture Plan Neufluar oil-immersion objective), as described by Ried *et al.* (25).

RESULTS

Isolation and Sequence Analysis of Human and Mouse *ubc9* Gene Homologs. Using the yeast Gal4 two-hybrid system (13) to search for proteins that interact with human recombination protein HsRad51, we obtained a clone, designated pEG947, that contained a 1171-bp human cDNA insert (see *Materials and Methods*). The insert has a single open reading frame (nt position 119–595) coding for a protein of 158 amino acids with predicted M_r of 18,000. A polyadenylylation signal is present beginning at position 1133, and a poly(A)$^+$ tail starts at position 1150. The encoded protein, designated HsUbc9, is homologous to proteins Hus5 of *S. pombe* and Ubc9 of *S. cerevisiae*, which are members of the family of E2 ubiquitin-conjugating enzymes (Fig. 1). HsUbc9 and Hus5 protein are 66% identical and 81% similar at the amino acid level; HsUbc9 and ScUbc9 protein are 56% identical and 75% similar. The two yeast proteins Hus5 and Ubc9 are 61% identical and 78% similar at the amino acid level.

Using primers described in *Materials and Methods*, we amplified the coding sequence of a mouse homolog of *ubc9* (*Mmubc9*) by reverse transcription–PCR from total mouse testis RNA. The nucleotide sequence of the *Mmubc9* open reading frame is 89% identical to that of *Hsubc9*, and the derived amino acid sequence is identical to the sequence of the human homolog.

Further Characterization of the Human *ubc9* Gene. A DNA fragment encoding full-length HsUbc9 protein was placed under control of the strong galactose inducible *GAL1* promoter in a high copy number vector, YEplac195GAL (yielding plasmid pEG954) and introduced into both a *ubc9* mutant strain and a congenic strain which carries a wild-type *UBC9* allele. A mutant *ubc9* yeast strain that carries plasmid pEG954 was able to grow at 37°C on medium containing galactose, whereas the nontransformed *ubc9* strain was not. Control congenic wild-type strains carrying either plasmid pEG954 or

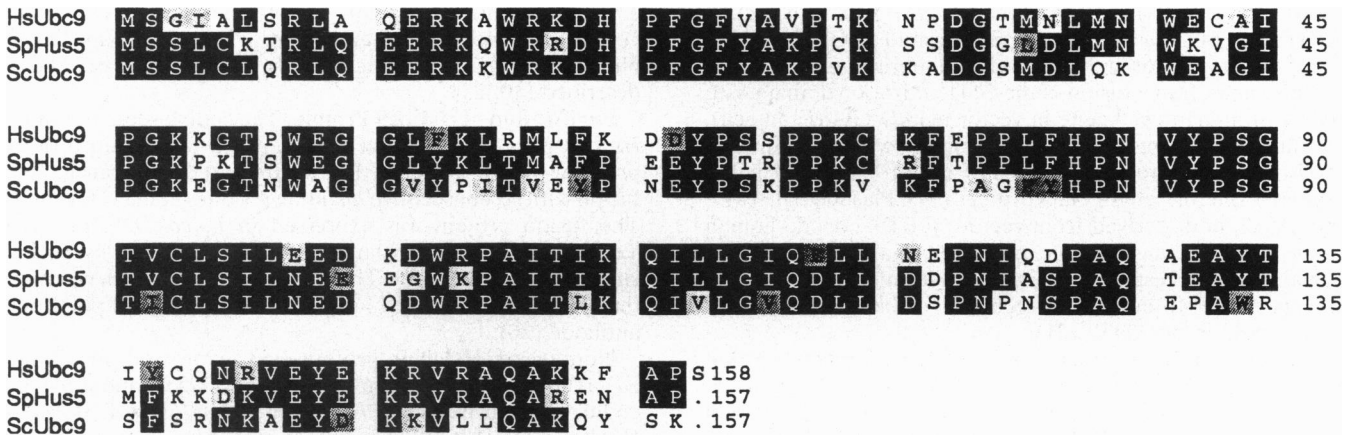

FIG. 1. Sequence alignment of HsUbc9 protein with *S. pombe* Hus5 and *S. cerevisiae* Ubc9 proteins. Alignment was done by using the PILEUP program of the Wisconsin Package (Genetics Computer Group). Identical residues are highlighted in black; similar residues are in gray.

the parent vector YEplac195GAL grew at 37°C. Moreover, rescue of the *ubc9* mutant by plasmid pEG954 was dependent on galactose, confirming that expression of the human *ubc9* was necessary for complementation. The *ubc9* cells that were rescued by the presence of *Hsubc9*, as well as congenic wild-type cells expressing *Hsubc9*, were indistinguishable from the wild-type strain, as judged by microscopic inspection (data not shown). Thus, the isolated human *ubc9* gene homolog encodes an active protein that is functionally related to the yeast Ubc9 protein.

Using a genomic clone of the human *ubc9* gene in λ phage as a probe, we carried out fluorescent *in situ* hybridization (FISH) and mapped the *Hsubc9* gene to human chromosome site 16p13.3 (data not shown).

To determine the expression pattern of the *Hsubc9* gene in different human organs, a probe derived from the cDNA sequence was used for Northern blot analysis. For comparison, the blot was reprobed with human γ-actin probe. *Hsubc9* mRNA was expressed in all human organs and tissues analyzed, which included spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. The strongest expression was observed in testis and thymus (Fig. 2).

Using polyclonal Ubc9 antisera raised in the mouse, we detected Ubc9 protein in transformed human kidney 293 cells and in mouse spermatocytes. The antibody detects a single band whose position corresponds to the position of full-length HsUbc9 protein isolated from a strain of *E. coli* carrying overproducing plasmid pEG957 (Fig. 3).

Interaction of HsUbc9 and HsRad51 Proteins *in Vivo*. To confirm the interaction of HsRad51 protein and HsUbc9 protein that led to the discovery of the latter, we introduced the plasmid pEG947 into *S. cerevisiae* HF7c and SFY526 cells, either alone or in combination with several other plasmids (Table 1).

The untransformed HF7c and SFY526 strains or the strains transformed with either pEG918 or pEG947 alone did not show any detectable activity of the *HIS3* or *lacZ* reporter genes (Table 1, lines 1, 2, and 4). Plasmid pEG947 also did not activate the Gal4 reporters in combination with the pGBT9 vector encoding the Gal4 DNA-binding domain (Table 1, line 6) or with pLam5′ plasmid encoding the fusion of human lamin C with the Gal4 DNA-binding domain (Table 1, line 7). On the other hand, cells carrying both pEG918 and pEG947 did show activity of the reporter genes (Table 1, line 8) comparable to that of a positive control consisting of the combination of two strongly interacting proteins p53 and simian virus 40 large T antigen (line 11).

HsUbc9 protein also showed a strong homotypic interaction (Table 1, line 9; compare with lines 3 and 4). The same was true for HsRad51 protein (Table 1, line 10; compare with lines 2 and 5). The self-association of yeast Rad51 protein in the same version of the two-hybrid system was reported earlier (28).

Localization of HsUbc9 Protein in Synaptonemal Complexes. Using antibodies, we have previously observed a dynamic pattern of association of Rad51 protein with synaptonemal complexes. The protein appears in premeiotic S phase and persists into metaphase I, observations which indicate that

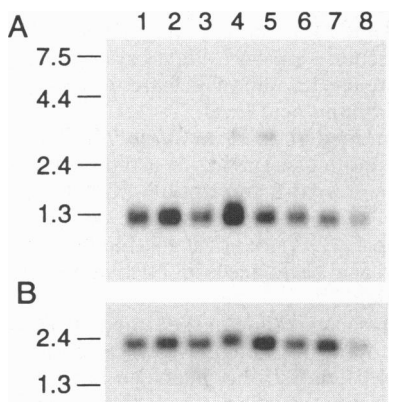

FIG. 2. Autoradiogram of a Northern blot of mRNA from human tissues hybridized with *Hsubc9* cDNA. Lanes 1–8 contain, in order, RNA from spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. The positions of RNA size markers (in kb) are indicated on the left. (*A*) Hybridization with *Hsubc9* cDNA probe. (*B*) Hybridization with human γ-actin probe.

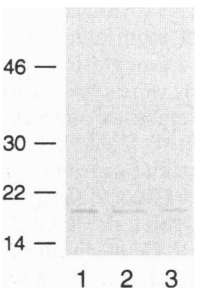

FIG. 3. Western blot analysis of Ubc9 protein. Total cell extracts were analyzed by SDS/12% PAGE and Western blotting with polyclonal mouse anti-HsUbc9 serum. Lane 1, HsUbc9 protein (wild-type sequence) purified from *E. coli* BL21(DE3)/pEG957; lane 2, extract of human 293 cells; and lane 3, extract of mouse seminiferous cells. Positions of protein molecular mass standards (in kDa) are indicated on the left.

Table 1. Transcriptional activation by hybrid Gal4 proteins

	DNA-binding domain plasmid	Activation domain plasmid	Combination of protein hybrids	β-galactosidase activity, units	Relative frequency of His$^+$ transformants, %
1.	None	None	None	<1	NA
2.	pEG918	None	dbRad51/none	<1	<0.1
3.	pEG959	None	dbUbc9/none	<1	<0.1
4.	None	pEG947	none/adUbc9	<1	<0.1
5.	None	pEG960	none/adRad51	<1	<0.1
6.	pGBT9	pEG947	dbGal4/adUbc9	ND	<0.1
7.	pLam5′	pEG947	dbLaminC/adUbc9	<1	<0.1
8.	pEG918	pEG947	dbRad51/adUbc9	4.0×10^1	57
9.	pEG959	pEG947	dbUbc9/adUbc9	1.3×10^2	47
10.	pEG918	pEG960	dbRad51/adRad51	1.0×10^2	31
11.	pVA3	pTD1	dbp53/adT antigen	1.1×10^2	69
12.	pCL1	pCL1	Gal4	2.6×10^3	87

pCL1 expresses full-length Gal4 protein. db stands for fusion with the DNA-binding domain of Gal4; ad stands for fusion with the activation domain of Gal4; ND, not determined; NA, not applicable.

Rad51 may play a role in the initial pairing of homologous chromosomes, as well as in subsequent crossing-over (11). The interaction of Ubc9 protein with Rad51 protein, as detected by the two-hybrid assay, prompted experiments on the association of Ubc9 protein with synaptonemal complexes.

When antibodies against Ubc9 and Rad51 proteins were incubated with mouse prophase spermatocytes, the Rad51 immunolabel identified the boundaries of the synaptonemal complexes. During early to midpachynema, after homologous chromosomes had paired and the synaptonemal complexes had fully formed, the Ubc9 antibody faintly stained the axes of the autosomal complexes (Fig. 4). The antibody more heavily stained unsynapsed axes of the X and Y chromosomes (Fig. 4 *B* and *D*). Staining of both the autosomes and sex chromosomes by anti-Ubc9 tended to concentrate in foci, whereas staining by anti-Rad51 in pachytene was more uniform. However, later in pachynema, both anti-Ubc9 and anti-Rad51 prominently stained both ends of the synaptonemal complexes (Fig. 4 *C* and *D*).

A round "bright body," generally located in the vicinity of the sex chromosomes, was present throughout pachynema: it may correspond to the "dense body" seen by electron microscopy which is thought to be related to nucleoli (29).

Staining by preimmune serum produced a diffuse background but did not illumine any of the specific structures stained by antibody to Ubc9 or Rad51.

DISCUSSION

We have identified a new member of the family of ubiquitin-conjugating enzymes, a human homolog of the yeast ubiquitin-conjugating enzyme Ubc9. The human homolog encodes a protein that is functionally related to the yeast enzyme since *Hsubc9* can complement the temperature sensitivity of a yeast *ubc9ts* mutant.

The observed interaction between HsUbc9 and HsRad51 protein is intriguing. Rad51 protein, a eukaryotic homolog of RecA protein, has been implicated in homologous recombination and recombinational repair of DNA damage (30–32). Purified Rad51 protein from yeast and humans is able to form a RecA-like nucleoprotein filament on DNA and catalyze DNA strand exchange reactions *in vitro* (33–35), (R. Gupta and C.M.R., unpublished observations). Nuclear foci of Rad51 protein are induced in human cells by DNA damage, and the homologous mouse protein localizes to synaptonemal complexes during meiosis (10, 11).

Recently, interactions *in vivo* of yeast Rad51 with the members of the *RAD52* epistasis group Rad52 and Rad55 proteins, as well as homotypic Rad51 interactions, were demonstrated by the two-hybrid system (28, 36–38). The interaction reported here appears to be of a different type but also has precedents: In yeast, the *RAD6* gene encodes a ubiquitin-conjugating enzyme Ubc2 which is involved in DNA repair, induced mutagenesis, and protein degradation by the N-end rule pathway. Rad6 protein forms a tight complex *in vitro* with Rad18 protein, which is a member of the *RAD6* epistasis group and is thought to mediate the error-free pathway of postreplication repair of UV-damaged DNA (39). Bailly *et al.* (39) suggested that the Rad6/Rad18 interaction might target Rad6 to the sites of DNA damage, where it could function in specific protein degradation.

The homotypic interaction of human Ubc9 protein also has precedents. Several UBCs were shown to form homo- and heterodimers, including Ubc3/Ubc3, Ubc4/Ubc4, Ubc6/Ubc7, Ubc7/Ubc7, and others. Purification of HsUbc9 protein expressed in *E. coli* also indicates the presence of Ubc9 dimers, as well as higher order complexes (data not shown). The role of homo- and heterotypic interactions of UBCs is not understood, although it was suggested to play some role in defining the range of substrate specificities (40).

There are several possible explanations for the role of the Rad51/Ubc9 protein interaction. First, Ubc9 might be directly involved in ubiquitin-dependent degradation of Rad51 protein. Second, by analogy with the hypothesis on the interaction of yeast Rad6 protein with Rad18 protein (39), HsRad51 protein might target HsUbc9 protein to the sites where recombination and/or DNA repair take place. At sites such as those in synaptonemal complexes, Ubc9 protein might cause degradation or modification (such as monoubiquitination) of other proteins required for interactions of homologous chromosomes.

A regulatory role of monoubiquitination has recently been suggested for yeast Cbf2 protein, a subunit of the key centromere protein Cbf3, which is thought to be the target of CDC34 (Ubc3) enzyme (41). Such a modification of certain proteins could be important for homologous chromosome separation in metaphase I or at a later stage for sister-chromatid separation in metaphase II (42, 43).

The pattern of staining of synaptonemal complex by anti-HsUbc9 is complex. There is intense staining of the unpaired sex chromosome axes by both anti-Ubc9 and anti-Rad51. By contrast, anti-Ubc9 stains the axes of autosomes more faintly, and this staining is most evident as foci that coincide with the chromosomal axes but do not correspond exactly to the more uniform distribution of Rad51. This pattern shifts, however, in late pachytene, when both Rad51 and Ubc9 colocalize at the ends of synaptonemal complexes. More detailed experiments are required to elucidate these relationships.

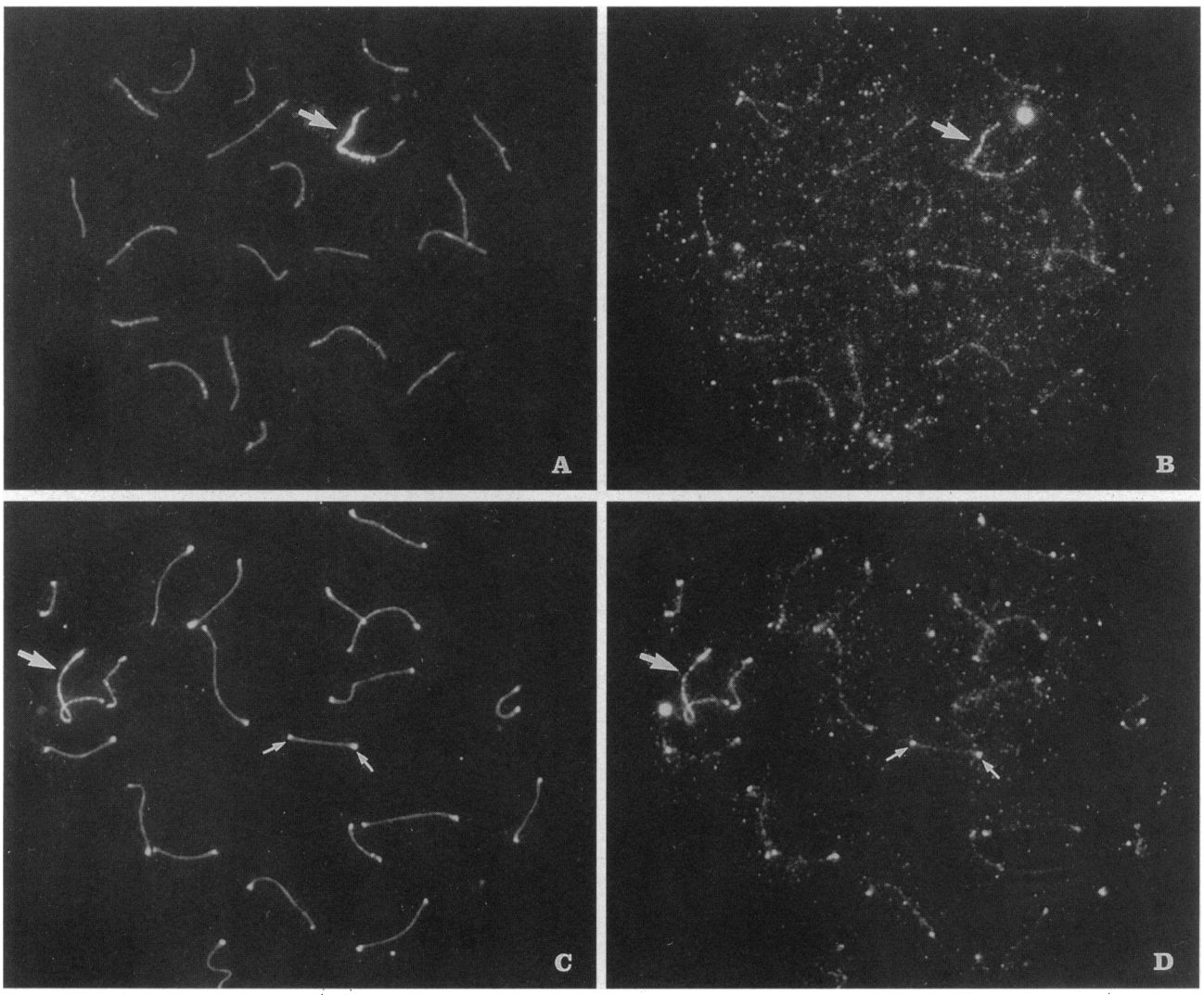

FIG. 4. Immunolocalization of MmUbc9 protein in synaptonemal complexes. (*A* and *B*) Mouse spermatocytes in early to midpachynema. (*A*) Stained with anti-Rad51 antibody. (*B*) The same cell as in *A* except stained with anti-Ubc9. (*C* and *D*) Late pachynema. (*C*) Stained with anti-Rad51 antibody. (*D*) The same cell as in *C* stained with anti-Ubc9. Large arrows, X and Y chromosome axes; small arrows, ends of bivalents.

As just indicated, interactions of Ubc9 protein with other proteins in the synaptonemal complex are not excluded, but the location of both Ubc9 and Rad51 in synaptonemal complexes adds credence to the view that the interaction of Rad51 and Ubc9, as detected by the two-hybrid assay, is biologically significant.

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