Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3264–3268, April 1996
Developmental Biology

Targeted gene disruption of *Hsp70-2* results in failed meiosis, germ cell apoptosis, and male infertility

(gametogenesis/heat shock protein/programmed cell death/spermatogenesis/stress protein)

DAVID J. DIX*†‡, JAMES W. ALLEN§, BARBARA W. COLLINS§, CHISATO MORI¶, NORIKO NAKAMURA¶, PATRICIA POORMAN-ALLEN||, EUGENIA H. GOULDING†, AND E. M. EDDY†

*Gamete and Early Embryo Biology Branch, Reproductive Toxicology Division, and §Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; †Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709; ¶Department of Anatomy, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan; and ||Division of Medicines Safety Evaluation, Glaxo Wellcome, Inc., Research Triangle Park, NC 27709

Communicated by George E. Seidel, Jr., Colorado State University, Fort Collins, CO, December 11, 1995 (received for review November 6, 1995)

ABSTRACT **In addition to the five 70-kDa heat shock proteins (HSP70) common to germ cells and somatic tissues of mammals, spermatogenic cells synthesize HSP70-2 during meiosis. To determine if this unique stress protein has a critical role in meiosis, we used gene-targeting techniques to disrupt *Hsp70-2* in mice. Male mice homozygous for the mutant allele (*Hsp70-2*$^{-/-}$) did not synthesize HSP70-2, lacked postmeiotic spermatids and mature sperm, and were infertile. However, neither meiosis nor fertility was affected in female *Hsp70-2*$^{-/-}$ mice. We previously found that HSP70-2 is associated with synaptonemal complexes in the nucleus of meiotic spermatocytes from mice and hamsters. While synaptonemal complexes assembled in *Hsp70-2*$^{-/-}$ spermatocytes, structural abnormalities became apparent in these cells by late prophase, and development rarely progressed to the meiotic divisions. Furthermore, analysis of nuclei and genomic DNA indicated that the failure of meiosis in *Hsp70-2*$^{-/-}$ mice was coincident with a dramatic increase in spermatocyte apoptosis. These results suggest that HSP70-2 participates in synaptonemal complex function during meiosis in male germ cells and is linked to mechanisms that inhibit apoptosis.**

Members of the 70-kDa heat shock protein (HSP70) family are chaperones that assist folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum (1). In mice, the HSP70 family contains at least seven different proteins, including the constitutively expressed 70-kDa heat shock cognate (HSC70) (2) and 75-kDa and 78-kDa glucose-regulated proteins GRP75 (3) and GRP78 (4). In addition, environmental or physiological stress induces the expression of two additional HSP70s, HSP70-1 and HSP70-3 (5), which protect cells and help them recover from stress-induced damage. Two additional unique members of the HSP70 family are expressed during spermatogenesis. Spermatocyte-specific HSP70-2 is expressed at high levels in pachytene spermatocytes during the meiotic phase of spermatogenesis (6, 7), and testis-specific HSC70 (HSC70t) is expressed in postmeiotic spermatids (8, 9).

The developmentally regulated expression of HSP70-2 during spermatogenesis implies that it performs a specialized function during meiosis that cannot be accomplished by other HSP70s. Processes that occur during meiotic prophase in spermatocytes include chromosome condensation, pairing of homologous chromosomes, formation of the synaptonemal complexes (SC), and genetic recombination (10). SC form in conjunction with homologue synapsis and are thought to function in recombination processes during meiotic prophase. We have identified HSP70-2 as a component of the SC in prophase nuclei of spermatogenic cells from mice and hamsters (11). These observations led to the hypothesis that HSP70-2 is required for spermatogenesis to proceed beyond meiotic prophase, through the transition from prophase into metaphase. To test this hypothesis, we used gene-targeting techniques to generate mice bearing a null-allele of *Hsp70-2.*

MATERIALS AND METHODS

Targeting the *Hsp70-2* Gene. The targeting construct contains genomic DNA cloned from a B6/CBA mouse genomic library (Stratagene Cloning Systems) inserted into the pIC19R/MC1-TK vector (12), along with a Pgk-1/*neo* expression unit (13) and an additional thymidine kinase (TK) gene (see Fig. 1 *A–C*). Embryonic stem cell stocks (E14TG2a/BK4, gift of B. Koeller, University of North Carolina at Chapel Hill) were targeted by electroporation with 5 nM of targeting vector linearized with *Sca* II at 360 V from a 125-μF capacitor (14). Embryonic stem cells were grown on primary cultures of embryonic feeder cells derived from transgenic mice carrying the neomycin-resistance gene *neo*; neomycin-resistance (Neo^r) mice were also the gift of B. Koeller. Targeted cells were selected with G418 and gancyclovir (a gift from Syntex, Palo Alto, CA) and genotyped by PCR and Southern blot; cells from one line injected into C57BL/6 blastocysts produced two male chimeras that transmitted the mutant *Hsp70-2* allele to their offspring.

Fertility and Fecundity of Mice. Six groups of five 8-week-old male mice were test-mated for 2 months. If no pregnancy resulted during the first month, the control female was replaced. The six groups were *Hsp70-2*$^{-/-}$, *Hsp70-2*$^{+/-}$, *Hsp70-2*$^{+/+}$ mice of either C57BL/6 or 129Sv/Ev genetic background; all animals were either second or third generation backcross. At the end of the mating regimen, these same mice were then harvested for analysis of reproductive organ weights, sperm counts, histology, and immunohistochemistry, detection of apoptosis, or SC analysis. Four groups of five female mice were also test-mated for 2 months; the groups were composed of *Hsp70-2*$^{-/-}$ and *Hsp70-2*$^{+/+}$ mice of either C57BL/6 or 129Sv/Ev genetic background.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GRP75 and GRP78, 75-kDa and 78-kDa glucose-regulated proteins; HSC70, 70-kDa heat shock cognate; HSC70t, testis-specific HSC70; HSP70, 70-kDa heat shock proteins; HSP70-1 and HSP70-3, inducible HSP70s; HSP70-2, spermatocyte-specific HSP70; *Hsp70-2*, gene encoding mouse HSP70-2; SC, synaptonemal complexes; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling; TK, thymidine kinase; *neo*, neomycin-resistance gene.

‡To whom reprint requests should be addressed at: Mail Drop 72, Reproductive Toxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

Downloaded at Palestinian Territory, occupied on December 20, 2021

Histology and Immunohistochemistry. Testes of 16-week-old mice were immersed in Bouin's fixative, paraffin-embedded, sectioned, either stained with hematoxylin and eosin or immunostained with a rabbit polyclonal antibody for HSP70-2 (15), which was detected with Elite ABC-peroxidase (avidin–biotinylated horseradish peroxidase complex; Vector Laboratories). Meiosis I and II spermatocytes were counted by identifying metaphase or anaphase nuclei in sections.

Detection of Apoptotic Germ Cells. The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) method was used for *in situ* visualization of DNA fragmentation indicative of programmed cell death (16). Testes were fixed in 4% paraformaldehyde for 16 hr at 4°C, embedded in paraffin, and sectioned; sections were placed on silanized slides. After proteinase K treatment, 3′-OH DNA ends were labeled with biotin-dUTP and detected with avidin-conjugated fluorescein isothiocyanate (FITC). Internucleosomal cleavage of genomic DNA was confirmed by end-labeling with [^{32}P]dCTP and Klenow polymerase, followed by agarose gel separation, transfer to nylon membrane, and detection by autoradiography.

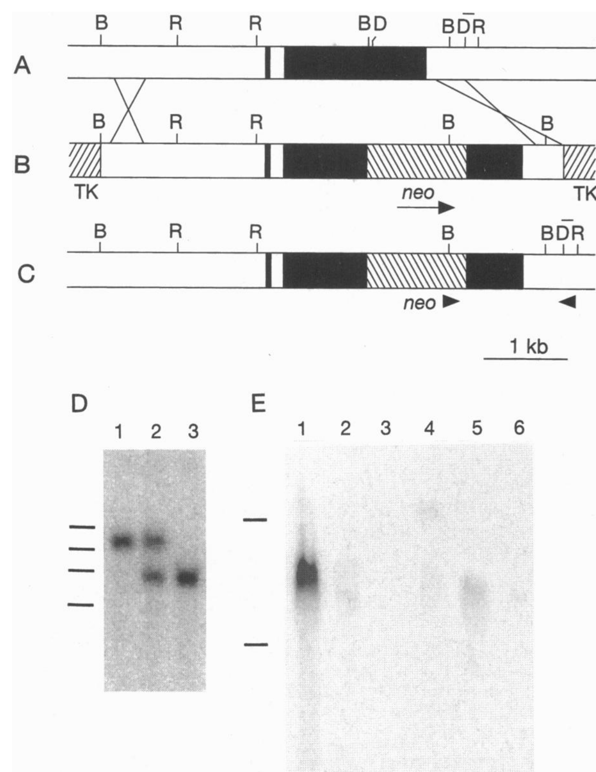

FIG. 1. Targeted disruption of *Hsp70-2.* (*A*) The two exons of the endogenous *Hsp70-2* locus are indicated by filled bars. The small line overhead indicates the DNA probe used in Southern blot hybridizations. *Bam*HI (B), *Eco*RI (R), and *Dra* III (D) restriction enzyme sites are indicated. (*B*) The *Hsp70-2/neo* targeting vector. Orientation of the neomycin-resistance (*neo*) and thymidine kinase (TK) genes are indicated by the arrow. A short deletion (15 bp) and the insertion of the *neo* gene interrupts the second exon of *Hsp70-2* at the *Bam*HI and *Dra* III sites. (*C*) Homologous recombination results in replacement of the wild-type allele with *Hsp70-2/neo* on the embryonic stem cell chromosome. Diagnostic PCR primers used to provisionally identify targeted cell lines are indicated by arrowheads. (*D*) Southern blot of genomic DNA from *Hsp70-2/neo* mice. Hybridization with DNA probe indicated above (*A* and *C*) detects a 5.5-kb *Hsp70-2/neo EcoRI* fragment and a 3.8-kb *Hsp70-2 EcoRI* fragment. Lanes: 1, DNA from a *Hsp70-2*$^{-/-}$ mouse; 2, DNA from a *Hsp70-2*$^{+/-}$ mouse; 3, DNA from a *Hsp70-2*$^{+/+}$ mouse. Lines to the left of the blot indicate positions of 3-, 4-, 5-, and 6-kb DNA markers, respectively. (*E*) Northern blot of total RNA hybridized with a probe specific for the 3′ untranslated region of Hsp70-2 mRNA (21). Lane 1 contains testis RNA from a *Hsp70-2*$^{+/+}$ animal, in which the 2.7-kb Hsp70-2 mRNA is abundant. Only trace amounts of Hsp70-2 mRNA were detected in brain (lane 2) or muscle (lane 3) from this animal. Little to no Hsp70-2 mRNA was detected from the testis, brain, or muscle of an *Hsp70-2*$^{-/-}$ animal (lanes 4–6, respectively). Faint bands in lanes 2 and 4–6 could be due to trace amounts of Hsp70-2 mRNA or to cross-hybridization of the probe to other Hsp70 mRNAs. A faint 4.5-kb band is present in lane 4 and may represent chimeric Hsp70-2/neo mRNA. Positions of 18S and 28S ribosomal RNAs are indicated by lines to the left of the blot.

SC Analysis. Surface-spread SC from testes of 16-week-old mice were analyzed by phase-contrast microscopy and by immunofluorescent staining (17). HSP70-2 was detected with rabbit antiserum 2A (15) and goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. As a positive control for SC immunostaining, human autoimmune serum (CREST) was used (Chemicon International). Suspended cell metaphase preparations from whole testes (18) were used to score metaphase I and II cells.

RESULTS AND DISCUSSION

Spermatocytes synthesize large amounts of HSP70-2 (6, 7) in addition to the five HSP70s found in mouse somatic tissues (2–5). *Hsp70-2* expression is regulated developmentally during the meiotic phase of mammalian spermatogenesis (15, 19, 20), and ≈300 bp of DNA sequence upstream of the transcription start site is sufficient for transcription in transgenic mice (21). While inducible HSP70s (e.g., HSP70-1 and HSP70-3) protect somatic cells from exposure to heat shock and other stressors, the function of HSP70-2 in gamete development remains unclear. To test the hypothesis that HSP70-2 is essential for meiosis, we introduced a mutation at the *Hsp70-2* locus in embryonic stem cells (Fig. 1 *A–C*) and used these cells to generate mice bearing the mutated gene (Fig. 1*D*). Expression of Hsp70-2 mRNA was detectable in the testes of wild-type *Hsp70-2*$^{+/+}$ mice (Fig. 1*E*, lane 1). However, *Hsp70-2*$^{-/-}$ mice had no detectable Hsp70-2 mRNA in their testes (Fig. 1*E*, lane 4).

Lack of *Hsp70-2* expression in males resulted in infertility when the null mutation was present on either 129Sv/Ev or C57BL/6 genetic backgrounds (Table 1). When test-mated, all *Hsp70-2*$^{-/-}$ males were infertile, while all *Hsp70-2*$^{+/-}$ and *Hsp70-2*$^{+/+}$ males were fertile and of similar fecundity. Testes of adult *Hsp70-2*$^{-/-}$ mice were significantly lower in weight than the testes of *Hsp70-2*$^{+/-}$ or *Hsp70-2*$^{+/+}$ mice, but epididymal and seminal vesicle weights were comparable (Table 2). Since *Hsp70-2*$^{-/-}$ males also had typical secondary sex characteristics and scrotal testes, it appears unlikely that

Table 1. Infertility phenotype of *Hsp70-2*$^{-/-}$ male mice

		Test-matings to assess fertility and fecundity				
Sex	*Hsp70-2* genotype	Genetic Bkg	No. mated	No. of litters	Litter size*	No. fertile
Male	+/+	C57BL/6	5	11	6.7 ± 1.4	5
	+/+	129Sv/Ev	4	8	5.6 ± 1.3	4
	+/−	C57BL/6	5	12	7.4 ± 3.1	5
	+/−	129Sv/Ev	5	12	3.4 ± 2.0	5
	−/−	C57BL/6	5	0	n/a	0
	−/−	129Sv/Ev	5	0	n/a	0
Female	+/+	C57BL/6	5	6	5.7 ± 0.9	5
	+/+	129Sv/Ev	5	6	7.3 ± 1.4	5
	−/−	C57BL/6	4	6	6.2 ± 2.9	4
	−/−	129Sv/Ev	5	12	6.5 ± 1.9	5

Mice were mated for 2 months with appropriate wild-type controls (see *Materials and Methods*). No significant differences between litter sizes were recognized between wild-type and heterozygous male mice within the same genetic background (Bkg). For comparison, offspring from mating heterozygous males by heterozygous females were produced in 1:2:1 Mendelian proportions and averaged 6.9 pups per litter.
*Mean ± SD.

Downloaded at Palestinian Territory, occupied on December 20, 2021

Table 2. Reproductive organ weights of *Hsp70-2* mutant mice

Hsp70-2 genotype	Testis, g	Epididymis/ vas deferens, g	Seminal vesicle, g	Body weight, g
+/+				
(n = 9)	0.11 ± 0.018	0.05 ± 0.005	0.23 ± 0.050	28.3 ± 4.16
+/−				
(n = 6)	0.11 ± 0.026	0.05 ± 0.010	0.33 ± 0.102	28.5 ± 3.23
−/−				
(n = 11)	0.04 ± 0.005*	0.04 ± 0.008	0.32 ± 0.143	27.6 ± 3.00

Male mice were 16 weeks of age, harvested at the end of test-matings (see *Materials and Methods*). Each group (e.g., +/+) was composed of mice from both C57BL/6 and 129Sv/Ev genetic backgrounds. All values are means ± SD.

*Significantly different from both +/+ and +/− groups ($P < 0.001$ by Student's t test).

endocrine dysfunction causes their infertility. In contrast, all *Hsp70-2*$^{-/-}$ and *Hsp70-2*$^{+/+}$ females test-mated were fertile and of similar fecundity, consistent with our findings that *Hsp70-2* is not expressed in pachytene oocytes (11). Furthermore, no conclusive evidence of embryonic or neonatal lethality was observed. Although spermatocytes contain constitutively expressed GRP75, GRP78, and HSC70 (6, 15), it appears that neither constitutive nor inducible HSP70 compensate for the deficiency of HSP70-2 in spermatocytes.

The seminiferous tubules of *Hsp70-2*$^{+/+}$ mice contain spermatogenic cells in mitotic (spermatogonia), meiotic (spermatocytes), and postmeiotic (spermatids) phases of development (Fig. 2*A*). HSP70-2 protein is synthesized in spermatocytes of *Hsp70-2*$^{+/+}$ mice throughout meiosis (22, 23) and remains in spermatids and spermatozoa (Fig. 2*B*). However, *Hsp70-2*$^{-/-}$ mice were deficient in postmeiotic spermatids (Fig. 2*C*), had no HSP70-2 in their seminiferous tubules (Fig. 2*D*), and lacked spermatozoa in the epididymis. While some tubules were highly vacuolized, others were relatively intact but still lacked appreciable levels of postmeiotic cells. Most seminiferous tubules in *Hsp70-2*$^{-/-}$ mice contained pachytene spermatocytes with condensed nuclei, some of which appeared fragmented and apoptotic. These observations suggested that in the absence of HSP70-2, meiosis fails in male germ cells and programmed cell death is triggered.

The TUNEL method was subsequently used to label genomic DNA (16) and to show that, whereas apoptotic germ cells were rare in adult *Hsp70-2*$^{+/+}$ mice (Fig. 3*A*), most of the seminiferous tubules in adult *Hsp70-2*$^{-/-}$ mice contained apoptotic cells (Fig. 3*B*). In histological sections from the testes of *Hsp70-2*$^{-/-}$ mice, many pachytene spermatocytes with abnormal morphology were observed, and most TUNEL-positive nuclei were pachytene spermatocytes. To confirm that this germ cell death was due to apoptosis, genomic DNA isolated from the testes of *Hsp70-2*$^{+/+}$ and *Hsp70-2*$^{-/-}$ mice was examined for fragmentation. The presence of fragmented DNA that increased by 180-bp increments from *Hsp70-2*$^{-/-}$ mice was indicative of internucleosomal endonuclease activity typical of apoptosis (Fig. 3*C*).

Previous studies have shown that heat shock and other stressors trigger apoptosis in somatic cells and that pretreatment with antisense oligomers to decrease *Hsp70* expression enhances the initiation of apoptosis (24). Conversely, condi-

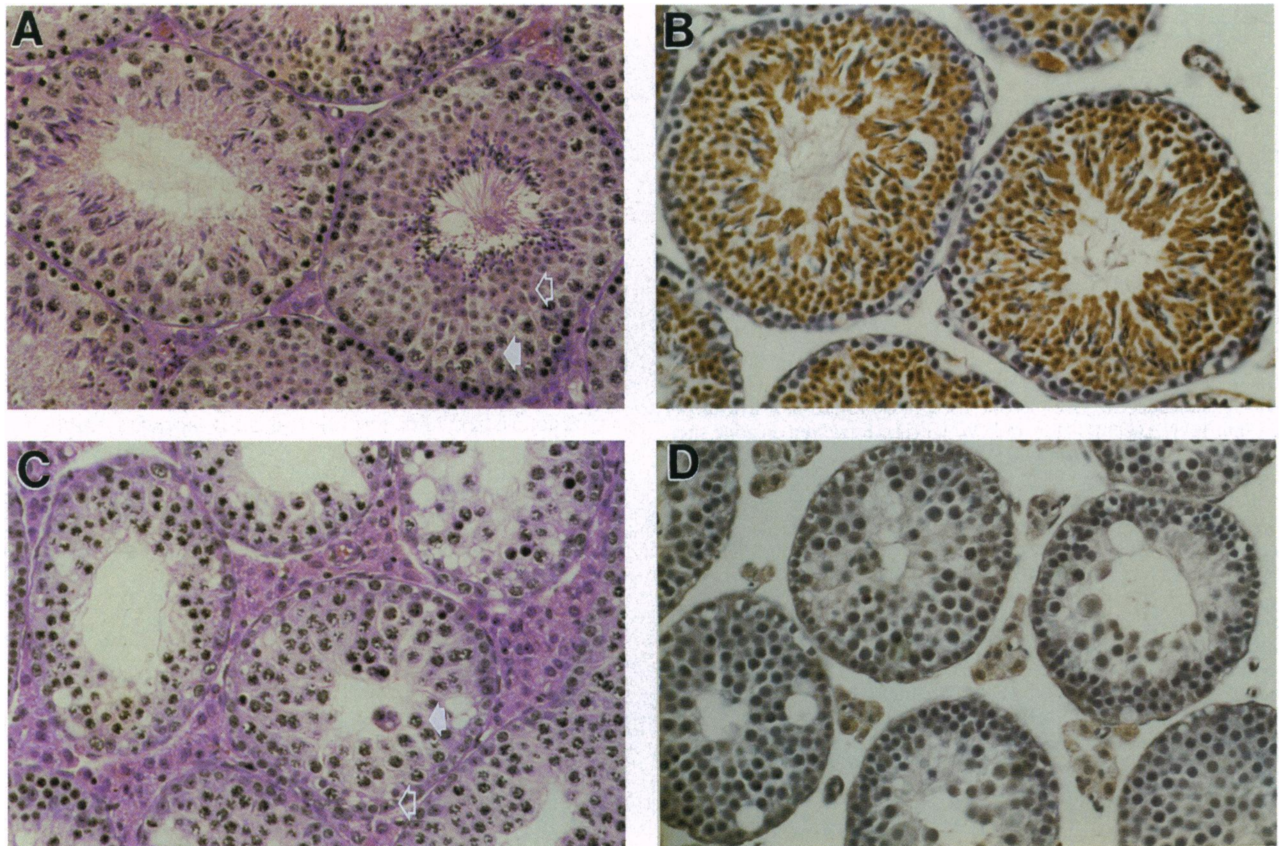

FIG. 2. Testes from *Hsp70-2*$^{-/-}$ mice lack postmeiotic germ cells. (*A*) Histological section of testis from a *Hsp70-2*$^{+/+}$ mouse stained with hematoxylin and eosin. Spermatogenesis progresses from mitotic spermatogonia at the periphery of seminiferous tubules to meiotic spermatocytes (filled arrow) and then postmeiotic spermatids (open arrow) in the adluminal region and spermatozoa in the lumen. (*B*) Immunoperoxidase staining for HSP70-2 in spermatogenic cells of a *Hsp70-2*$^{+/+}$ mouse reveals significant amounts of HSP70-2 from early meiosis through the postmeiotic phase. (*C*) Seminiferous tubules from a *Hsp70-2*$^{-/-}$ mouse contain mitotic spermatogonia (open arrow) and meiotic spermatocytes (filled arrow) but no postmeiotic spermatids or spermatozoa. (*D*) Immunoperoxidase staining was negative for HSP70-2 on sections from *Hsp70-2*$^{-/-}$ mice. (×160.)

Downloaded at Palestinian Territory, occupied on December 20, 2021

tioning of cells with mild thermal stress induces *Hsp70* gene expression and limits apoptosis (25). These results are consistent with increased frequency of apoptotic spermatocytes in *Hsp70-2*$^{-/-}$ mice and with the possibility that HSP70-2 protects pachytene spermatocytes from apoptosis. However, what triggers apoptosis in the absence of HSP70-2? One key feature of meiosis is that DNA strand breaks must occur to allow for recombination (26). It is known that DNA strand breaks can trigger p53-dependent apoptosis (27), and relatively high levels of p53 are expressed in pachytene spermatocytes (28). Although spermatocytes appear poised to undergo apoptosis triggered by DNA damage, they also contain relatively high levels of poly(ADP-ribose) polymerase (29) and Rad51 (30), which are involved in DNA repair and may reduce susceptibility to apoptosis (31). Thus, in the absence of HSP70-2, proteins involved in DNA repair or recombination that require HSP70-2 chaperone activity may be incorrectly folded, transported, or assembled, thereby disrupting the balance between inhibitors and inducers of apoptosis and leading to germ cell death.

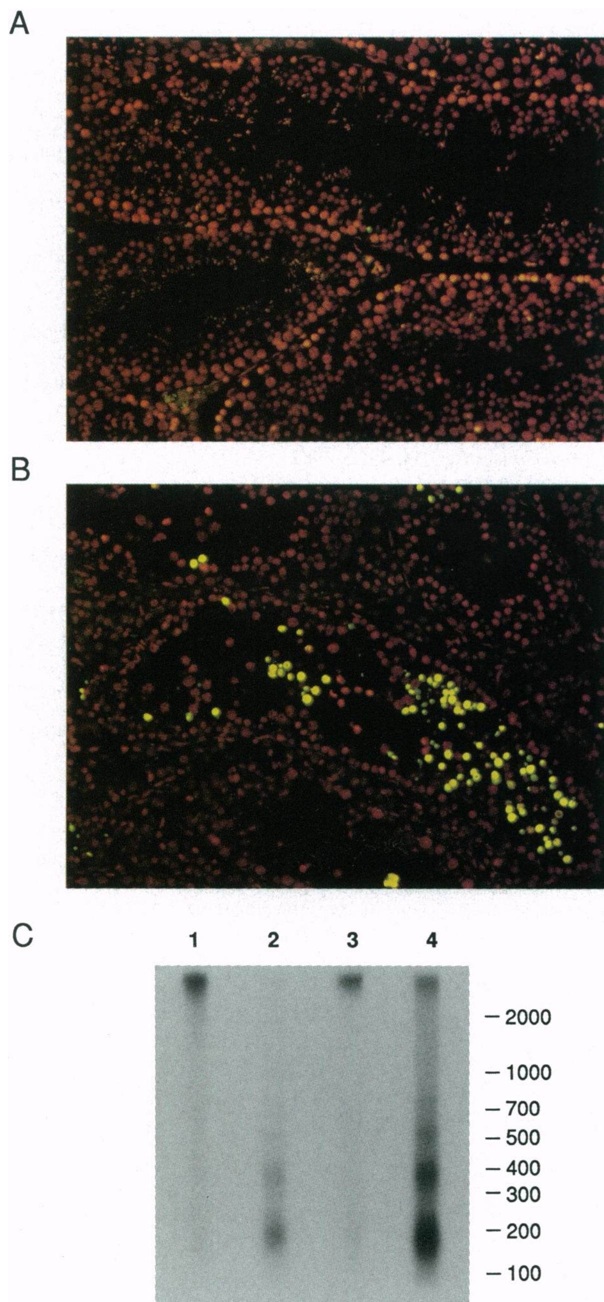

FIG. 3. Spermatocytes of *Hsp70-2*$^{-/-}$ mice are apoptotic. (*A*) The TUNEL method was used on histological sections to detect free 3′-hydroxy ends of genomic DNA. Spermatogenic cells in testis from a *Hsp70-2*$^{+/+}$ mouse were negative for fluorescent TUNEL staining. All nuclei are counterstained red with propidium iodide. (×130.) (*B*) Many spermatocyte nuclei in a testis from a *Hsp70-2*$^{-/-}$ mouse are positive for fluorescent yellow TUNEL staining indicative of DNA fragmentation and apoptosis. (×130.) (*C*) Genomic DNA (5 μg per lane) from the testes of *Hsp70-2*$^{-/-}$ mice (lanes 2 and 4) but not from *Hsp70-2*$^{+/+}$ (lane 1) or *Hsp70-2*$^{+/-}$ (lane 3) mice show evidence of internucleosomal degradation and apoptosis. Position and size (bp) of DNA markers are indicated to right of gel.

SC form with the pairing of meiotic chromosomes and represent the aligned axes of homologues during synapsis (32). SC are believed to function in meiotic chromosome recombination and segregation (10). We have used a highly specific antiserum to show that HSP70-2 is associated with SC in pachytene spermatocytes from *Hsp70-2*$^{+/+}$ mice (ref. 11, and Fig. 4*A*). As expected, HSP70-2 was not detected in nuclei of pachytene spermatocytes from *Hsp70-2*$^{-/-}$ mice (Fig. 4*B*). However, by using the CREST antiserum to label kinetochores and lateral elements (17), we observed typical-appearing SC in pachytene cells from *Hsp70-2*$^{-/-}$ mice (Fig. 4*C*). While SC assembled in *Hsp70-2*$^{-/-}$ mice, SC development beyond the middle to late pachytene stages was not observed. Many late pachytene SC were fragmented (Fig. 4*D*), and electron microscopic evaluation also revealed fragmented SC. This contrasts with normally desynapsing lateral elements in diplotene SC from *Hsp70-2*$^{+/+}$ mice, wherein the lateral elements maintain their longitudinal integrity until extensive desynapsis has oc-

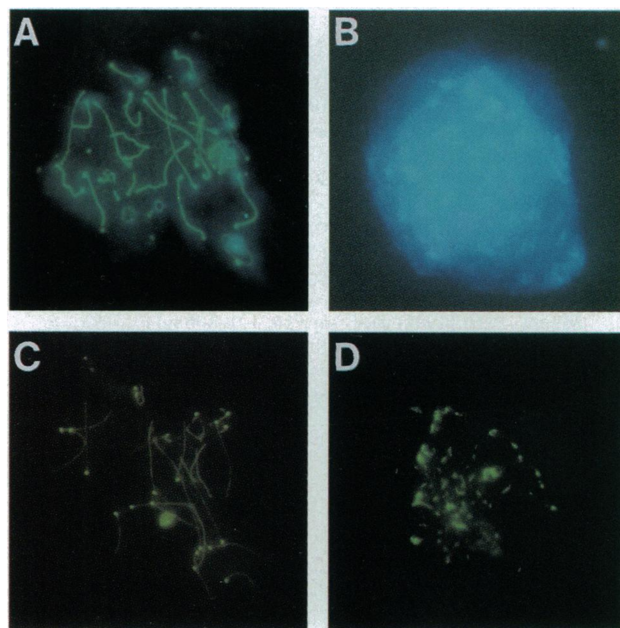

FIG. 4. The synaptonemal complexes (SC) of pachytene spermatocytes assemble in the absence of HSP70-2, but SC development beyond the middle to late pachytene stages was abnormal and few cells made the transition to metaphase. (*A*) Immunofluorescent staining of HSP70-2 (green) labels SC in a pachytene spermatocyte from a *Hsp70-2*$^{+/+}$ mouse. Chromatin is stained blue with 4,6-diamidino-2-phenylindole (DAPI). (*B*) No HSP70-2 is detectable by immunofluorescence in pachytene nucleus from a *Hsp70-2*$^{-/-}$ mouse. Even after photographic overexposure, only the DAPI staining is visible. (*C*) Immunofluorescent staining (green) of SC from a *Hsp70-2*$^{-/-}$ mouse with a human autoimmune serum (CREST). At this stage of pachytene development, the SC appear normal, despite the lack of HSP70-2. No DAPI was applied to this section. (*D*) Fragmented SC from a *Hsp70-2*$^{-/-}$ mouse immunofluorescently stained (green) with the CREST serum. No normal diplotene SC with desynapsing but intact lateral elements were observed in *Hsp70-2*$^{-/-}$ mice. (×3250.)

Downloaded at Palestinian Territory, occupied on December 20, 2021

curred. We were unable to identify any normally desynapsing diplotene SC, and there were very few spermatocytes that had progressed to the meiotic divisions. As noted before, the SC of pachytene oocytes do not contain HSP70-2, and *Hsp70-2*$^{-/-}$ female mice were fertile.

Localization of HSP70-2 to SC in pachytene spermatocytes, abnormalities of SC from *Hsp70-2*$^{-/-}$ mice, and the inability of spermatocytes lacking HSP70-2 to progress to metaphase suggest that HSP70-2 is not required for SC assembly in spermatocytes but is necessary for functions during synapsis, which allow progression to the subsequent meiotic divisions. Thus HSP70-2 may participate in processes that occur during the lengthy meiotic prophase of spermatogenic cells such as mismatch repair (33), chromosome recombination (34), or SC disassembly and G_2/M-phase transition (35). The exact role of HSP70-2 in SC function and the relationship between disruption of meiosis and triggering of apoptosis in spermatocytes of *Hsp70-2*$^{-/-}$ mice will require further studies. However, the present results indicate that a previously unrecognized relationship exists between a heat shock protein, meiosis, and apoptosis.

We thank Kathy Bobseine, Donna Bunch, Rita Ingram, Shawn Jeter, Alex Merrick, Fred Mitchell, Lisa Padilla-Burgos, Clyde Rogers, Mike Rosenberg, Oliver Smithies, Cheri Barrett, Jeff Welch, John Wiley, and Bill Willis for their help.

1. Georgopoulos, C. & Welch, W. J. (1993) *Annu. Rev. Cell Biol.* **9,** 601–634.
2. Giebel, L. B., Dworniczak, B. P. & Bautz, E. K. (1988) *Dev. Biol.* **125,** 200–207.
3. Domanico, S. Z., Denagel, D. C., Dahlseid, J. N., Green, J. M. & Pierce, S. K. (1993) *Mol. Cell. Biol.* **13,** 3598–3610.
4. Kozutsumi, Y., Normington, K., Press, E., Slaughter, C., Sambrook, J. & Gething, M.-J. (1989) *J. Cell Sci. Suppl.* **11,** 115–137.
5. Hunt, C. R., Gasser, D. L., Chaplin, D. D., Pierce, J. C. & Kozak, C. A. (1993) *Genomics* **16,** 193–198.
6. Allen, R. L., O'Brien, D. A. & Eddy, E. M. (1988) *Mol. Cell. Biol.* **8,** 828–832.
7. Zakeri, Z. F., Wolgemuth, D. J. & Hunt, C. R. (1988) *Mol. Cell. Biol.* **8,** 2925–2932.
8. Matsumoto, M. & Fukimoto, H. (1990) *Biochem. Biophys. Res. Commun.* **166,** 43–49.
9. Maekawa, M., O'Brien, D. A., Allen, R. L. & Eddy, E. M. (1989) *Biol. Reprod.* **40,** 843–852.
10. Moens, P. B. (1994) *BioEssays* **16,** 101–106.
11. Allen, J. W., Dix, D. J., Collins, B. W., Merrick, B. A., He, C., Selkirk, J. K., Poorman-Allen, P., Dresser, M. E. & Eddy, E. M. (1996) *Chromosoma*, in press.
12. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336,** 348–352.
13. Adra, C. N., Boer, P. H. & McBurney, M. W. (1987) *Gene* **60,** 65–74.
14. Smithies, O. & Kim, H.-S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3612–3615.
15. Rosario, M. O., Perkins, S. L. & O'Brien, D. A., Allen, R. L. & Eddy, E. M. (1992) *Dev. Biol.* **150,** 1–11.
16. Mori, C., Nakamura, N., Kimura, S., Irie, H., Takigawa, T. & Shiota, K. (1995) *Anat. Rec.* **242,** 103–110.
17. Dresser, M. E., Pisetsky, D., Warren, R., McCarty, G. & Moses, M. (1987) *J. Immunol. Methods* **104,** 111–121.
18. Evans, E. P., Breckon, G. & Ford, C. E. (1964) *Cytogenetics* **3,** 289–294.
19. Bonnycastle, L. L. C., Yu, C.-E., Hunt, C. R., Trask, B. J., Clancy, K. P., Weber, J. L., Patterson, D. & Schellenberg, G. D. (1994) *Genomics* **23,** 85–93.
20. Wisniewski, J., Kordula, T. & Krawczyk, Z. (1990) *Biochim. Biophys. Acta* **1048,** 93–99.
21. Dix, D. J., Rosario-Herrle, M., Gotoh, H., Mori, C., Goulding, E. H., Barrett, C. V. & Eddy, E. M. (1996) *Dev. Biol.*, in press.
22. O'Brien, D. A. (1987) *Biol. Reprod.* **37,** 147–157.
23. Allen, R. L., O'Brien, D. A., Jones, C. C., Rockett, D. L. & Eddy, E. M. (1988) *Mol. Cell. Biol.* **8,** 3260–3266.
24. Wei, Y. Q., Zhao, X., Kariya, Y., Teshigawara, K. & Uchida, A. (1995) *Cancer Immunol. Immunother.* **40,** 73–78.
25. Wei, Y.-Q., Zhao, X., Kariya, Y., Teshigawara, K. & Uchida, A. (1994) *Cancer Res.* **54,** 4952–4957.
26. Stern, H. (1993) in *Cell and Molecular Biology of the Testis*, eds. Desjardins, C. & Ewing, L. L. (Oxford Univ. Press, Oxford), pp. 296–316.
27. Clarke, A. R., Gledhill, S., Hooper, M. L., Bird, C. C. & Wyllie, A. H. (1994) *Oncogene* **9,** 1767–1773.
28. Schwartz, D., Goldfinger, N. & Rotter, V. (1993) *Oncogene* **8,** 1487–1494.
29. Ogura, T., Takenouchi, N., Yamaguchi, M., Matsukage, A., Sugimura, T. & Esumi, H. (1990) *Biochem. Biophys. Res. Commun.* **172,** 377–384.
30. Haaf, T., Golub, E. I., Reddy, G., Radding, C. M. & Ward, D. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2298–2302.
31. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. & Earnshaw, W. C. (1994) *Nature (London)* **371,** 346–347.
32. Moses, M. J. (1968) *Annu. Rev. Genet.* **2,** 363–412.
33. Baker, S. M., Bronner, C. E., Zhang, L., Plug, A. W., Robatzek, M., Warren, G., Elliott, E. A., Yu, J., Ashley, T., Arnheim, N., Flavell, R. A. & Liskay, R. M. (1995) *Cell* **82,** 309–319.
34. Hawley, R. S. & Arbel, T. (1993) *Cell* **72,** 301–303.
35. Wiltshire, T., Park, C., Caldwell, K. A. & Handel, M. A. (1995) *Dev. Biol.* **169,** 557–567.

Downloaded at Palestinian Territory, occupied on December 20, 2021