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Expression of Heat Shock Proteins by Isolated Mouse Spermatogenic Cells

RANDY L. ALLEN,* DEBORAH A. O'BRIEN, CAROL C. JONES, DOUGLAS L. ROCKETT, AND E. M. EDDY

Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Received 1 March 1988/Accepted 3 May 1988

Proteins of the hsp70 family are abundant in mouse spermatogenic cells. These cells also synthesize relatively large amounts of a 70,000-molecular-weight protein (P70) that appears to be a cell-specific isoform of hsp70, the major heat-inducible protein (R. L. Allen, D. A. O'Brien, and E. M. Eddy, Mol. Cell. Biol. 8:828–832, 1988). In this study, proteins of unstressed and heat-stressed spermatogenic cells consisting of purified preparations of preleptotene, leptotene-zygotene, pachytene spermatocytes, and round spermatids were analyzed by two-dimensional polyacrylamide gel electrophoresis. Unstressed preleptotene and leptotene-zygotene spermatocytes contained little P70, whereas relatively large amounts of P70 were present in pachytene spermatocytes and round spermatids. Labeling studies showed that P70 was synthesized primarily in pachytene spermatocytes and that little synthesis occurred in round spermatids or in preleptotene and leptotene-zygotene stages of spermatogenesis. Synthesis of hsp70 was not detectable in unstressed cells but was induced in all stages of isolated germ cells following heat stress. These results indicate that P70 is expressed in a stage-specific manner during cell differentiation, whereas hsp70 is synthesized in response to stress in all populations of isolated spermatogenic cells examined.

Cells exposed to elevated temperatures or other environmental stresses respond by synthesizing heat shock proteins (7, 12, 27, 37). The most prominent heat shock protein in all organisms has a molecular weight of approximately 70,000 (hsp70) and has been highly conserved throughout evolution (37). In eucaryotic cells, the gene for hsp70 is a member of a multigene family (13, 19). Other products of this gene family in mice are two heat shock cognate proteins with molecular weights of approximately 71,000 (hsc71) and 74,000 (hsc74) which are synthesized in unstressed cells (28, 33).

Members of the hsp70 family have been shown to be related by nucleic acid hybridization (28), sequence homology (8), and immunological reactivity (1, 24). Both hsp70 and hsc71 bind ATP (8, 25, 42) and the fatty acids palmitate and stearate (16). The ATP-binding property has been used in the purification of these proteins (42). Although hsp70 and related proteins appear to protect cells against adverse environmental conditions (26, 34, 35), their mode of action within stressed cells remains unclear.

Heat shock proteins are also synthesized during normal development, which suggests that they serve a role in cell differentiation as well as in response to stress. Proteins related to hsp70 are developmentally regulated in unicellular, invertebrate, and vertebrate organisms (3–5, 17, 24). A similar pattern of heat shock protein expression occurs during ascospore formation in *Saccharomyces cerevisiae* and during *Drosophila* oogenesis, suggesting a highly conserved role for these proteins in cellular differentiation (24). High levels of heat shock proteins also are expressed in mouse embryonal carcinoma cells and embryonic ectoderm cells in the absence of stress (4, 14). Differentiation induced in embryonal carcinoma cells by retinoic acid or dibutyryl cyclic AMP is accompanied by a decrease in the abundance of some hsp70 family proteins (4).

Spermatogenesis involves a series of differentiative events that transform spermatogonial stem cells into structurally and functionally unique and haploid spermatozoa. Recent reports have identified abundant hsp70-related transcripts in mouse spermatogenic cells in the absence of stress (21–23, 44). We have shown that a cell-specific hsp70-like protein (P70) and products of the hsp70 gene family are abundant in mixed preparations of mouse spermatogenic cells and that hsp70 is induced in mixed germ cells following heat stress (1). However, the process of spermatogenesis is sensitive to even slight elevations of temperature and to many toxic agents (9, 29). One possibility is that heat shock proteins have a developmental role during spermatogenesis and do not protect germ cells against adverse environmental effects. Another possibility is that synthesis of hsp70 and other heat shock proteins cannot be induced during certain stages of spermatogenesis, leaving these stages particularly vulnerable to stress. To distinguish between these possibilities, the present study examines the synthesis of hsp70 family members in isolated preleptotene spermatocytes, leptotene-zygotene spermatocytes, pachytene spermatocytes, and round spermatids before and after heat stress.

MATERIALS AND METHODS

Cell preparation and culture. Germ cell suspensions were prepared from CD-1 mice by enzymatic dissociation of testes as previously described (31). Germ cells at defined stages of differentiation were isolated from mixed cell suspensions by unit gravity sedimentation (2, 36). Preleptotene spermatocytes, a population containing both leptotene and zygotene spermatocytes (leptotene-zygotene), and pachytene spermatocytes were isolated from 17-day-old mice. Pachytene spermatocytes and round spermatids were isolated from adult mice. Criteria for purity have been described elsewhere (2).

Purified germ cells (4×10^6 cells per ml) were cultured at 32°C with 5% CO_2 in 60-mm-diameter petri dishes containing 5 ml of methionine-free minimal essential medium supplemented with 10 μM methionine–6 mM sodium L-lactate–1 mM sodium pyruvate–100 U of penicillin per ml–100 μg of streptomycin per ml (31). Methionine-free minimal essential

* Corresponding author.

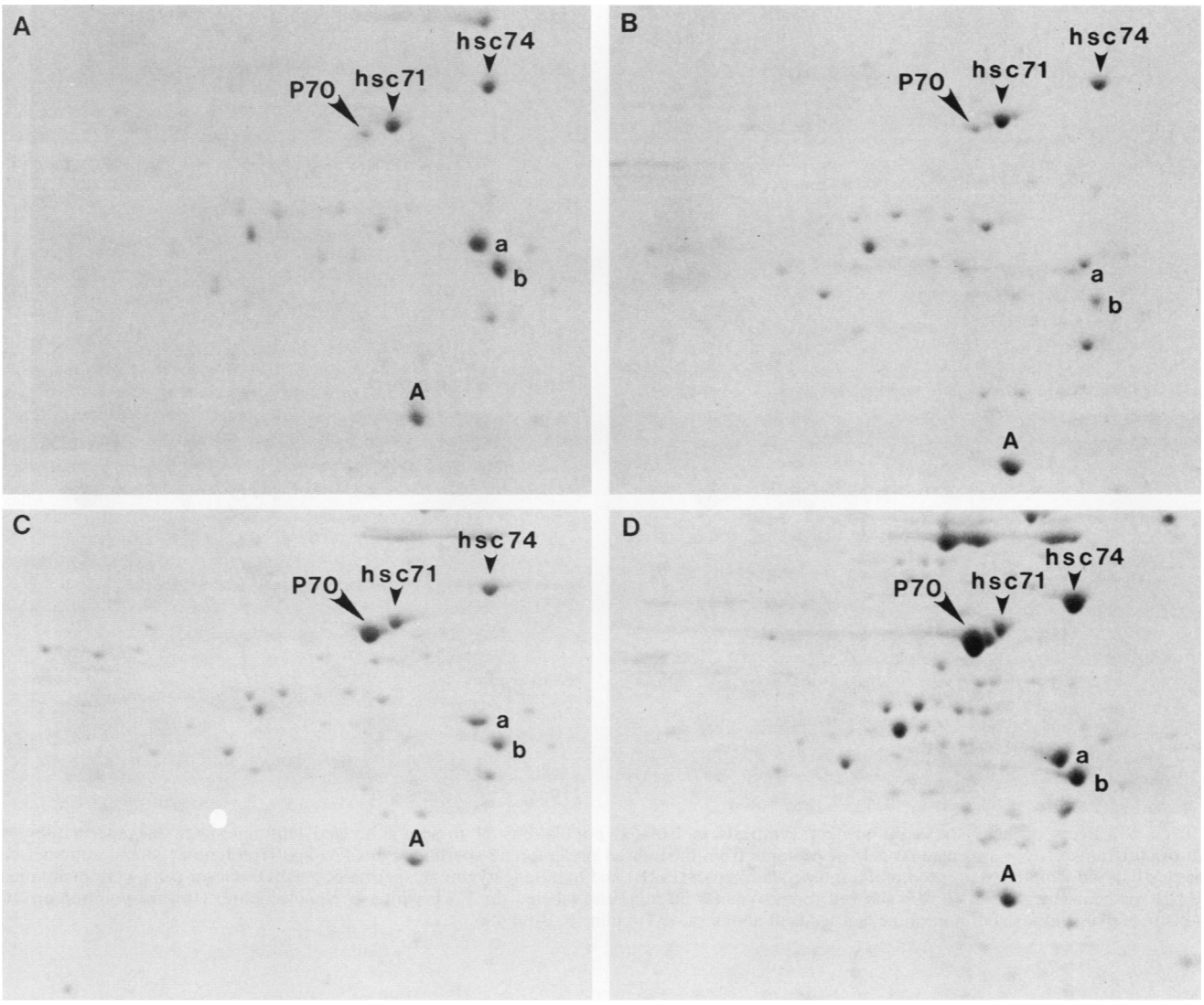

FIG. 1. Comparison of polypeptides from unstressed spermatogenic cells. Proteins from preparations of purified preleptotene spermatocytes (A), leptotene-zygotene spermatocytes (B), adult pachytene spermatocytes (C), and round spermatids (D) were separated by two-dimensional PAGE and stained with Coomassie blue. Spots: a, α-tubulin; b, β-tubulin; A, actin.

medium was obtained from Flow Laboratories, Inc.; all other tissue culture reagents were from GIBCO Laboratories.

Heat shock conditions. Culture dishes containing isolated populations of germ cells were maintained at 32°C or sealed and immersed for 10 min in a water bath at 42.5°C. Cells were allowed to recover for 30 min, and the proteins were labeled biosynthetically for 5.5 h by adding to the medium 100 μCi of L-[^{35}S]methionine per ml (specific activity, >1,000 Ci/mmol; Dupont Co.-New England Nuclear Corp.). Labeled cells were harvested and washed three times in ice-cold phosphate-buffered saline and frozen at −70°C.

Two-dimensional PAGE and immunoblot analysis. L-[^{35}S]methionine-labeled cells were sonicated in 2 mM Tris–1 mM $MgCl_2$ (pH 7.4) containing pancreatic RNase (50 μg/ml; Sigma Chemical Co.). DNase (20 μg/ml; Sigma) was added before addition of solid urea and O'Farrell sample buffer as previously described (32). Two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed as described by O'Farrell (32), by using pH 3.5 to 10 Ampholines (LKB Instruments, Inc.) and 10% acrylamide gels for the second dimension. Gels were stained with Coomassie blue, dried, and exposed to Kodak X-Omat AR film at −70°C.

Proteins from adult pachytene spermatocytes maintained at 32°C and heat stressed at 42.5°C were separated by two-dimensional PAGE and transferred electrophoretically to nitrocellulose (38). The blots were blocked for 2 h in phosphate-buffered saline containing 5% nonfat dry milk (20) and incubated for 1 h with a polyclonal antibody (diluted 1:100 in blocking solution) that has been shown to be specific for heat-inducible hsp70 (41). The blots were rinsed three times in blocking solution (10 min per wash) and incubated for 1 h with a 1:500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Kirkegaard and Perry Laboratories). After being rinsed twice in blocking solution and once in phosphate-buffered saline, the blots were reacted with 4-chloro-1-naphthol (18).

One-dimensional peptide mapping. Adult pachytene spermatocytes and round spermatids were heat stressed as described above. Mouse NIH 3T3 cells were heat stressed at

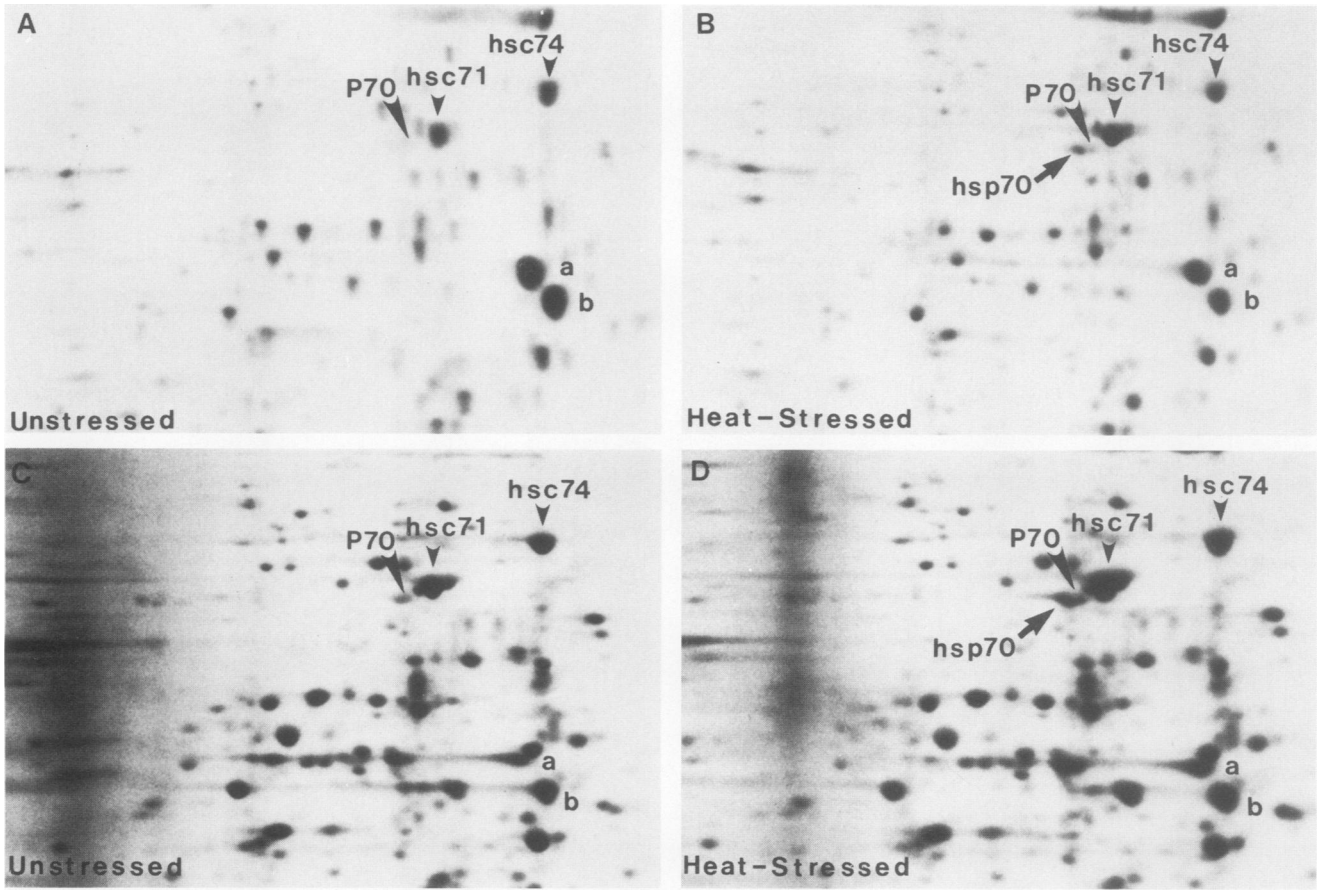

FIG. 2. Effects of heat stress on protein synthesis in isolated populations of preleptotene and leptotene-zygotene spermatocytes. Autoradiograms of two-dimensional gels of proteins from unstressed preleptotene spermatocytes (A) and leptotene-zygotene and spermatocytes (C) and from heat-stressed preleptotene spermatocytes (B) and leptotene-zygotene spermatocytes (D). Germ cells were maintained at 32°C or heat stressed at 42.5°C, allowed to recover for 30 min, and labeled for 5.5 h with L-[^{35}S]methionine. Upward-pointing arrows indicate hsp70 synthesized in germ cells after heat shock. a, α-Tubulin; b, β-tubulin.

44°C for 10 min as previously described (1). Proteins were separated by two-dimensional PAGE and stained with Coomassie blue, and the labeled proteins were visualized by autoradiography. Individual proteins corresponding to the site of hsp70 synthesis were excised carefully from dried gels and rehydrated briefly in distilled water. The proteins were digested with *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc.) and analyzed by the method of Cleveland et al. (11).

RESULTS

Isolation of defined stages of spermatogenic cells. Purities of pachytene spermatocytes isolated from adult mice exceeded 91%, with the remaining cells being multinucleated spermatids and a small number of Sertoli cells. Isolated round spermatids had purities greater than 93% and contained contaminating residual bodies which are derived from haploid cells as they are released from the seminiferous epithelium. Meiotic germ cells isolated from 17-day-old mice had purities greater than 83% for preleptotene spermatocytes, greater than 85% for leptotene-zygotene spermatocytes, and greater than 90% for pachytene spermatocytes. The remaining cells in these preparations were early meiotic germ cells and Sertoli cells.

Identification of hsp70-related proteins in unstressed spermatogenic cells. Three hsp70-related proteins have been identified previously in mixed preparations of unstressed germ cell (1). These include P70, an hsp70-like protein which has been detected only in spermatogenic cells, and the heat shock cognate proteins hsc71 and hsc74. All preparations of isolated germ cells contained relatively large amounts of hsc71 and hsc74 (Fig. 1). Round spermatids and pachytene spermatocytes isolated from adult and 17-day-old mice contained large amounts of P70, whereas only a small amount of P70 was detectable in preleptotene and leptotene-zygotene spermatocytes. There was also a protein between P70 and hsc71 that was consistently observed on two-dimensional gels of pachytene spermatocytes and round spermatids (Fig. 1C and D). This protein reacts with antibodies specific for the hsp70 family and may represent a major charge variant of P70 (unpublished data).

Heat shock response in isolated spermatogenic cells. Isolated populations of unstressed and heat-stressed preleptotene and leptotene-zygotene spermatocytes synthesized relatively large amounts of hsc71 and hsc74 (Fig. 2). Two distinct 70,000-molecular-weight proteins were identified in these cells that corresponded to P70 and heat-inducible hsp70. Biosynthetic labeling of P70 was variable in both

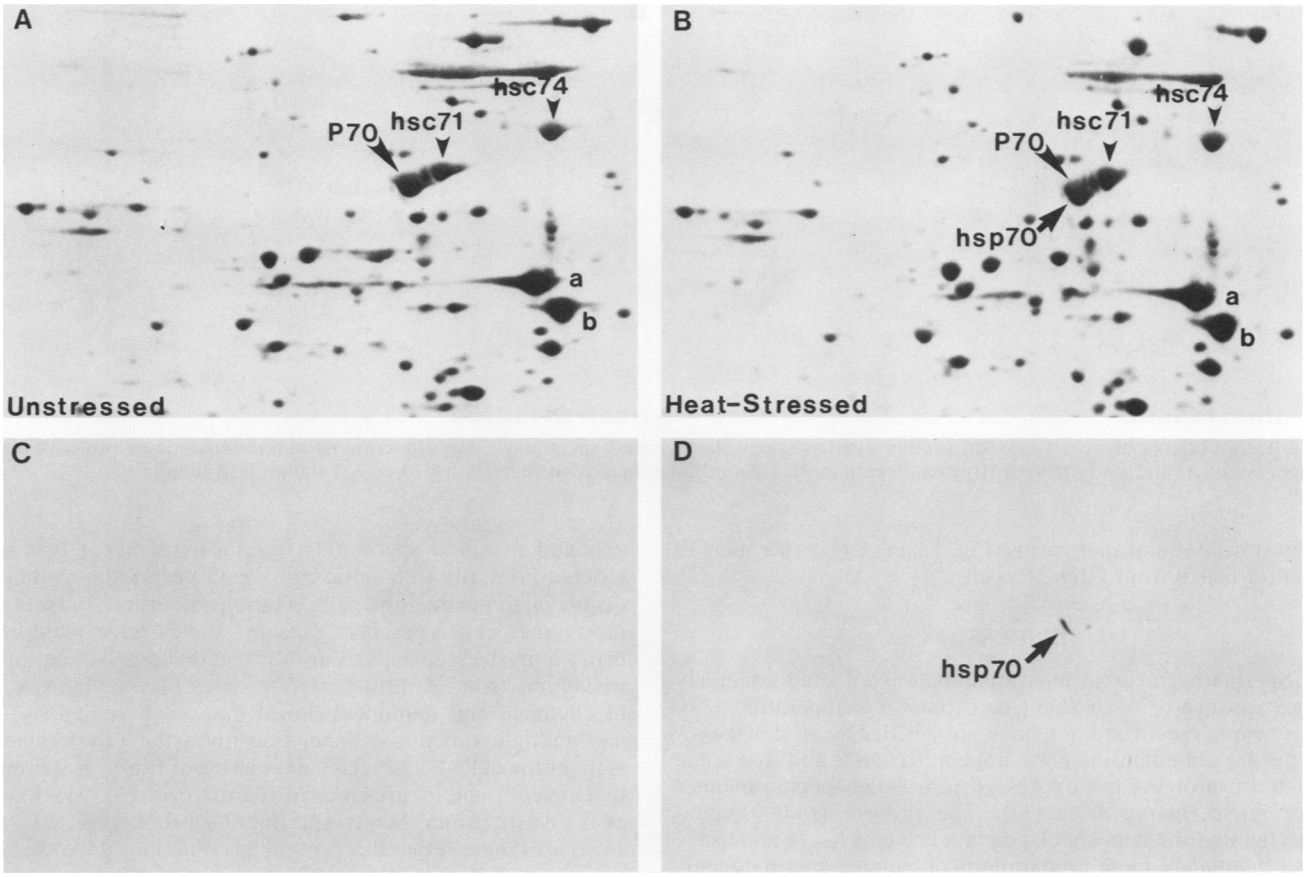

FIG. 3. Effects of heat stress on protein synthesis in pachytene spermatocytes isolated from adult mice. Two-dimensional autoradiograms of proteins from unstressed (A) and heat-stressed (B) pachytene spermatocytes. Corresponding immunoblots from unstressed (C) and heat-stressed (D) pachytene spermatocytes were reacted with a polyclonal antibody specific for hsp70. Upward-pointing arrows indicate hsp70. a, α-Tubulin; b, β-tubulin.

unstressed and heat-stressed preleptotene spermatocytes. Synthesis of P70 was either not detectable in unstressed or heat-stressed cells (Fig. 2A) or detectable at relatively low levels (Fig. 2B). P70 was clearly synthesized by unstressed and heat-stressed leptotene-zygotene spermatocytes although at a much lower level than hsc71 and hsc74 (Fig. 2C and D). Synthesis of hsp70 was not detected in unstressed cells (Fig. 2A and C) but was induced in both preleptotene and leptotene-zygotene stages of spermatogenesis following heat stress (Fig. 2B and D).

P70, hsc71, and hsc74 were synthesized in relatively large amounts in unstressed and heat-stressed pachytene spermatocytes from adult (Fig. 3A and B) and 17-day-old (data not shown) mice. A previous study has shown that both P70 and hsp70 have nearly identical apparent molecular weights and isoelectric points and that biosynthetically labeled hsp70 partially surrounds P70 on two-dimensional gels (1). The presence of labeled P70 made it difficult to visualize induction of hsp70 in heat-stressed pachytene spermatocytes (Fig. 3B). To verify that hsp70 synthesis was induced, the proteins from unstressed and heat-stressed pachytene spermatocytes were separated by two-dimensional PAGE, transferred to nitrocellulose, and immunostained with a polyclonal antibody specific for heat-inducible hsp70 (41). The reaction seen with this antibody indicated that hsp70 was present only in heat-stressed pachytene spermatocytes (Fig. 3C and D).

Coomassie blue-stained gels showed that round spermatids contained P70 and hsc71 (Fig. 1D), whereas autoradiographs of two-dimensional gels indicated that there was relatively little synthesis of P70 and hsc71 in unstressed round spermatids (Fig. 4A). hsp70 was synthesized following heat stress of round spermatids and partially surrounded P70 on two-dimensional gels in a pattern similar to that seen for pachytene spermatocytes (Fig. 4B). Synthesis of hsc71 appeared to be enhanced in heat-stressed round spermatids, whereas synthesis of P70 and hsc74 was not affected by this treatment.

One-dimensional peptide map analysis of hsp70 from heat-stressed mouse germ cells and NIH 3T3 cells. Previous studies showed that the heat-inducible protein partially surrounding P70 on two-dimensional gels of mixed germ cells was quite similar to hsp70 from heat-stressed mouse NIH 3T3 cells by peptide map analysis (1). In the present study, the heat-inducible protein surrounding P70 was examined in pachytene spermatocytes and round spermatids isolated from adult mice. The peptide map for the induced protein in pachytene spermatocytes (Fig. 5, lane A) is similar to that of hsp70 from heat-stressed NIH 3T3 cells (Fig. 5, lane B). An additional band present in the digest of the induced protein from pachytene spermatocytes probably resulted from slight contamination by P70. It corresponds to a major peptide generated by *S. aureus* V8 protease digestion of P70 (1). The

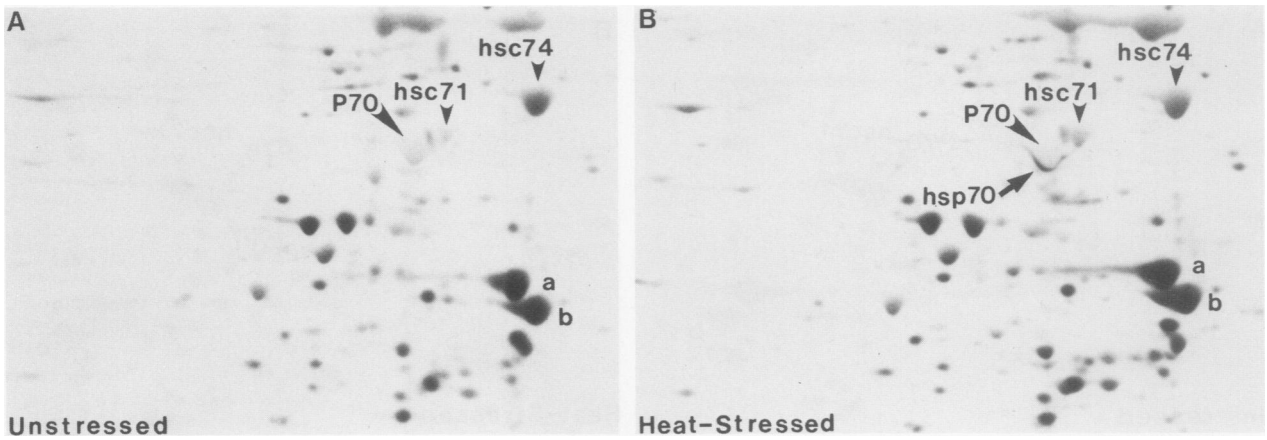

FIG. 4. Effects of heat stress on protein synthesis in isolated round spermatids. Two-dimensional autoradiograms of proteins from unstressed (A) and heat-stressed (B) round spermatids. Upward-pointing arrows indicate hsp70. a, α-Tubulin; b, β-tubulin.

digest from round spermatids (Fig. 5, lane C) was identical to that of hsp70 from NIH 3T3 cells.

DISCUSSION

Spermatogenesis in most mammals is affected adversely by exposure to even slight elevations in temperature (40). However, recent studies have shown that heat shock proteins are abundant in spermatogenic cells (1) and that some of these proteins are expressed in a stage-specific manner during spermatogenesis (31). The present study demonstrated that the heat shock cognate proteins hsc71 and hsc74 were abundant in all preparations of isolated spermatogenic cells. hsc74 was synthesized by germ cells throughout meiosis and in round spermatids. Synthesis of hsc71 was restricted primarily to meiotic cells, with very little synthesis occurring in postmeiotic cells. These proteins are present in most other cell types (34). Recent studies have identified hsc71 in unstressed cells as an ATPase that releases clathrin triskelions from clathrin-coated vesicles (8, 39). However, biochemical and immunochemical data suggest that hsc71 has multiple functions, depending upon the physiological state of the cell (14, 39). The abundance of hsc71 in spermatogenic cells and its presence in spermatozoa (M. Maekewa et al., Abstr. Annu. Meet. Am. Soc. Cell Biol. 1987, 105, p. 167a) also suggest that this protein has functions unrelated to uncoating clathrin-coated vesicles, since clathrin has been detected readily only in the Golgi-acrosome region in spermatocytes and spermatids (15).

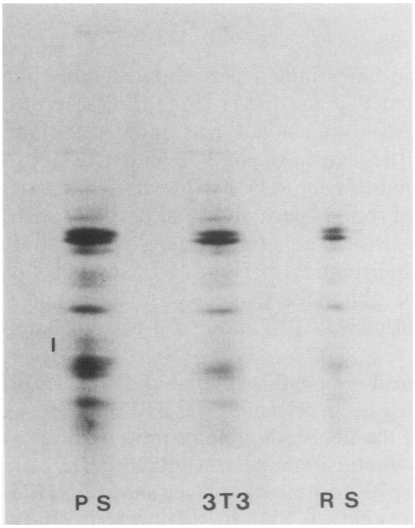

FIG. 5. Comparison of hsp70 proteins from adult pachytene spermatocytes, mouse NIH 3T3 cells, and round spermatids by one-dimensional peptide map analysis. L-[^{35}S]methionine-labeled proteins were separated by two-dimensional PAGE, and spots corresponding to hsp70 were excised and digested with 0.1 μg of *S. aureus* V8 protease. The peptides were resolved on 15% gels and visualized by autoradiography. Lanes: PS, pachytene spermatocytes; 3T3, NIH 3T3 cells; RS, round spermatids. The bar indicates a peptide fragment in pachytene spermatocytes that probably resulted from contamination by P70.

We have shown previously that mouse spermatogenic cells contain an hsp70-like protein (P70) not observed in other cell types (1). Both P70 and hsp70 were found to be antigenically related ATP-binding proteins with nearly identical molecular weights and isoelectric points. However, the peptide map for P70 was different from that of hsp70. This study has shown that P70 is present in relatively large amounts in pachytene spermatocytes and round spermatids. It also confirms that P70 is synthesized predominantly in pachytene spermatocytes, with little biosynthesis detectable in round spermatids or earlier periods of spermatocyte development (31). The stage-specific synthesis of P70 during spermatogenesis suggests that this protein functions in a restricted period of germ cell differentiation.

Some members of the hsp70 family are developmentally regulated during preimplantation mouse embryogenesis (17), *Drosophila* oogenesis, and *S. cerevisiae* sporulation (24). However, hsp70 itself is neither developmentally induced nor inducible by heat during developmental activation of other hsp70 family proteins in these cells. Primary *Drosophila* spermatocytes also fail to synthesize hsp70 in response to elevated temperature (6). Although it had been demonstrated in mice that mixed germ cells synthesize hsp70 following heat stress (1), it remained to be determined whether synthesis of hsp70 occurs in specific populations of spermatogenic cells that are more sensitive to heat than others. Morphological studies have shown that round spermatids and pachytene spermatocytes were more sensitive to heat than germ cells at either earlier or later stages of spermatogenesis (10). It has also been reported that the

inhibitory effects of temperature on total protein synthesis were more pronounced in round spermatids than in pachytene spermatocytes (30).

These studies indicated that induction of hsp70 synthesis occurred in all stages of isolated germ cells examined following exposure to elevated temperature. Induction of hsp70 was difficult to observe clearly on two-dimensional autoradiograms of pachytene spermatocytes because P70 was synthesized in relatively large amounts in these cells and migrated to the same location as hsp70 on two-dimensional gels. However, hsp70 was detected readily on blots of heat-stressed pachytene spermatocytes by using a polyclonal antibody specific for heat-inducible mammalian hsp70 (41, 43). In addition, the peptide map of the heat-inducible protein in pachytene spermatocytes was similar to that of hsp70 in heat-stressed mouse NIH 3T3 cells. Although it was not possible to rule out that some hsp70 was synthesized by contaminating Sertoli cells, it seems unlikely that they were the main source of hsp70 on the basis of the small number of Sertoli cells present in isolated populations of meiotic germ cells. Previous studies also indicated that Sertoli cells did not contribute significantly to synthesis of heat shock proteins in mixed germ cells exposed to elevated temperatures (1).

As in pachytene spermatocytes, heat stress clearly induced hsp70 in round spermatids, the most temperature-sensitive spermatogenic cell type (30). The peptide map of the inducible protein from round spermatids was identical to hsp70 from heat-stressed NIH 3T3 cells. In addition, preparations of isolated round spermatids consisted almost entirely of round spermatids and residual bodies, making it unlikely that contaminating cells were responsible for this hsp70 synthesis.

This study indicates that hsp70 can be induced during stages of spermatogenesis that are sensitive to elevations in temperature and suggests that hsp70 does not protect male germ cells from heat stress (9). These results support the hypothesis that heat shock proteins have a developmental role during spermatogenesis. Furthermore, the presence of P70 in pachytene spermatocytes and round spermatids correlates with an apparent increased sensitivity to heat stress. This hsp70-like protein may even interfere with the function of other members of the hsp70 family, leaving these stages of spermatogenesis particularly sensitive to heat and other environmental stresses (10, 30).

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