Expression of a Testis-specific hsp70 Gene-related RNA in Defined Stages of Rat Seminiferous Epithelium

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Abstract. Changes in the level of a testis-specific hsp70 gene-related transcript (hst70 RNA) and its cellular localization during the cycle of rat seminiferous epithelium have been investigated. Segments of seminiferous tubules at defined stages of the cycle were isolated in living condition by transillumination-assisted microdissection and the exact stages identified by phase-contrast microscopy of live cell squashes. The levels of the hst70 RNA were determined by Northern and slot blotting of whole cell lysates. High levels were found in stages XII-XIV and I to early VII of the cycle, and low levels were found in other stages, i.e., late VII (VIId) through VIII-XI of the cycle. The in situ hybridization revealed that the *hst70* gene was activated in late pachytene primary spermatocytes during stage XII of the cycle, and that mRNA was then present in cells during differentiation through diakinesis, meiotic divisions, and early spermiogenesis (steps 1 through early 7). The activation of the gene coding for hst70 RNA shortly before meiotic divisions may indicate that the gene product is needed either during differentiation of late spermatocytes into spermatids or later during spermiogenesis, and that the mRNA may be stored in early spermatids.

Heat shock proteins are a set of specific proteins synthesized in cells exposed to elevated temperature and a number of other stressing factors (reviewed by Craig, 1985, and Carper et al., 1987). The most prominent and most thoroughly investigated is the heat shock protein of molecular weight of ~70,000. It has been found in cells of all eucaryotes and it also has a counterpart in bacteria (Bardwell and Craig, 1984). It is now well established that in yeast (Ingolia et al., 1982), *Drosophila* (Craig et al., 1983), mouse (Lowe and Moran, 1986), rat (O'Malley et al., 1985; Sorger and Pelham, 1987; Munro and Pelham, 1986), and human (Hunt and Morimoto, 1985; Voellmy et al., 1985; Mues et al., 1986) there are genes that share a high sequence similarity with the *hsp70* gene; these genes, together with *hsp70*, form a group called hsp70 gene family (Pelham, 1986). Besides the *hsp70* genes, which are strictly heat inducible, the family may contain genes that are heat inducible but also become expressed at certain level in the absence of stress (*hsx70* gene), and genes that are constitutively expressed at physiological temperatures and are not heat inducible (*hsc70* gene). In birds and mammals the family may contain a gene which codes for a glucose regulated protein (Lee, 1987). The function of these genes is poorly understood although recent observations suggest that they may be involved in stabilization and assembly of nascent proteins in various cellular compartments, as well as in the recovery of cellular structures from injuries (Pelham, 1986). In addition, some hsp70-related proteins may have a function in cellular growth and differentiation during different phases of the cell cycle (Kao et al., 1985; Milarski and Morimoto, 1986; Kaczmarek et al., 1987). Increased level of the hsp70-related transcript has been also found during S phase of liver regeneration (Carr et al., 1986). A transient expression of *hsc70* gene has been detected in mouse zygote during two-cell stage (Bensaude et al., 1983; Hahnel et al., 1986), but generally it is repressed at early stages of embryogenesis and becomes active when the embryo reaches blastula stage (Heikkila et al., 1986).

Spermatogenesis with its well-defined synchronous stages and spatial arrangement of cells in waves in the seminiferous epithelium provides a good model system for investigations of the regulation and sequential activation of genes during differentiation. The development of spermatozoa includes three main phases: spermatogonial multiplication, meiosis, and spermiogenesis. Cells in these phases are called spermatogonia, spermatocytes, and spermatids, respectively. Different generations of spermatogenic cells form associations with constant composition that give rise to the stages of the cycle of the seminiferous epithelium. They are most commonly defined by morphology of the developing acrosomes and of the nuclei of the young spermatids (Leblond and Clermont, 1952). The stages follow each other along the seminiferous tubules in a wave-like fashion (Perey et al., 1961).

Recently Krawczyk et al. (1987*b*) and Zakeri and Wolgemuth (1987) reported that testes of adult rat and mouse contain abundant levels of the transcript related to the hsp70 gene. This transcript, called hst70 RNA, differs in size from

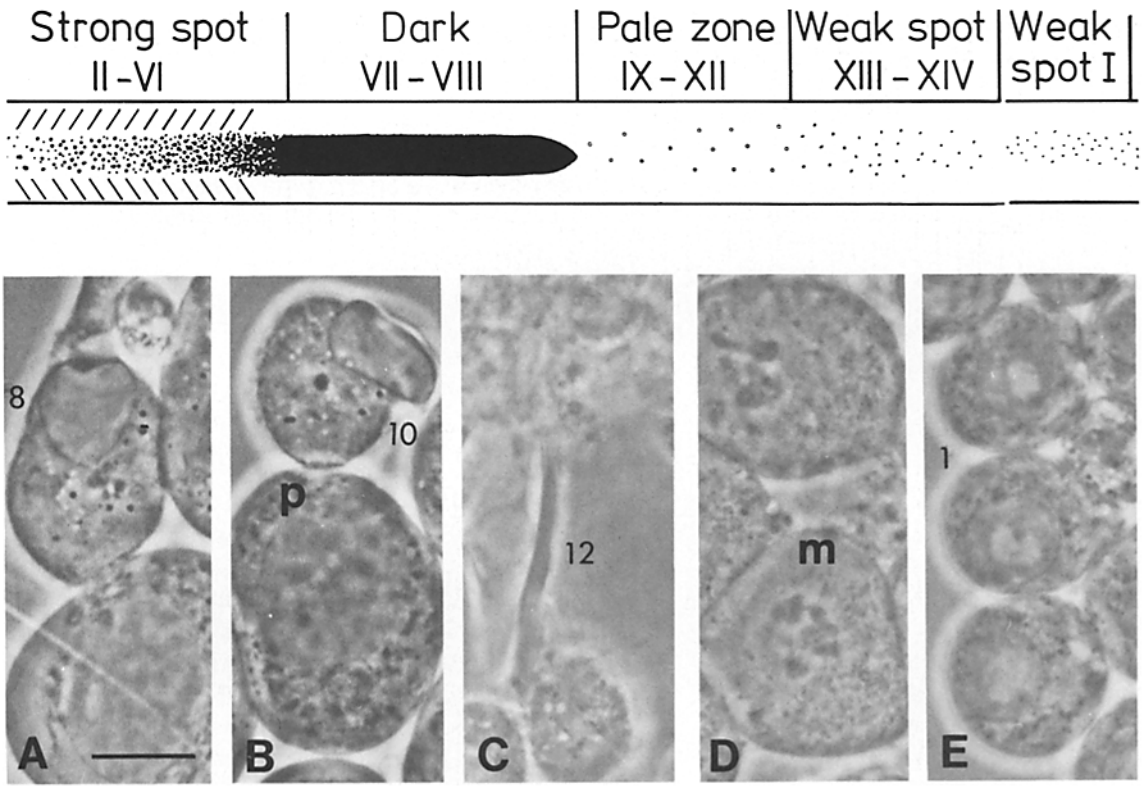

Figure 1. Schematic tracing of a transilluminated rat seminiferous tubulus in living unstained condition. The stages of the cycle can be identified by an increased light absorption induced by late acrosome- and maturation-phase spermatids with condensing nuclei and their characteristic position in the seminiferous epithelium. An abrupt cessation of the dark absorbing zone at spermiation is easily recognized. If any stage should be accurately identified, adjacent microdissected segment may be carefully squashed and the spermatids characteristic for each stage identified under phase contrast optics. In this study, stages VIII (*A*) with polarized spermatids (*8*, step 8 of spermiogenesis), X (*B*) with elongating spermatids (*10*), XII (*C*) with elongated spermatids (*12*), XIV (*D*) with dividing spermatocytes (*m*), and I (*E*) with early spermatids (*1*) were of particular interest. *p*, pachytene spermatocyte. Bar, 10 μm.

other hsp70-related heat shock transcripts and its level is not affected by hyperthermia (Krawczyk et al., 1987*b*; Zakeri and Wolgemuth, 1987; Krawczyk, Z., and J. Wisniewski, manuscript submitted for publication). Its expression is developmentally regulated: it is absent in newborn animals but appears in testes that have initiated spermiogenesis (Krawczyk et al., 1987*a*; Zakeri and Wolgemuth, 1987). In testes depleted of germinal cells after experimental cryptorchidism (Krawczyk et al., 1987*a*), busulphan treatment, increased age (Krawczyk and Szymik, 1988), or certain mutations affecting the reproductive tract (Zakeri and Wolgemuth, 1987), the hst70 RNA is not detectable. In testicular cells separated by gravity sedimentation, the highest level of the transcript was detected in fractions enriched in round and elongated spermatids as well as in residual bodies, but a very low amount was detected in pachytene spermatocytes (Zakeri and Wolgemuth, 1987). Therefore, a major expression of this transcript has been proposed to be during the haploid phase of spermatogenesis.

To gain a more accurate localization of the cellular expression of the hst70 mRNA in the seminiferous epithelium, we have used the possibilities provided by slot-blot hybridization technique in microdissected seminiferous tubules where the exact stage of the epithelial cycle was identified in living condition by transillumination and phase-contrast microscopy, and the results were compared with in situ hybridizations of testis sections.

Materials and Methods

Animals and Seminiferous Tubules Microdissection

Testes of young adult (3 mo) Sprague-Dawley rats were removed and subjected to transillumination-assisted microdissection (Parvinen and Vanha-Perttula, 1972). Segments of seminiferous tubules were pooled from five animals, totaling an amount of 50 cm (ca. 50 mg wet wt) from stages I, II-III, IV-V, VI, VIIab, VIIcd, VIII, IX-XI, XII, and XIII-XIV of the seminiferous epithelial cycle (Parvinen and Ruokonen, 1982). For a more accurate analysis, 2-mm segments of isolated seminiferous tubules from three rats were cut sequentially under a stereomicroscope at 20-fold magnification using a micrometer to assure constant length. A short segment (0.3–0.5 mm) between each 2-mm segment was carefully squashed between coverslip and microscope slide for determination of the accurate stage by phase-contrast microscopy (Söderström and Parvinen, 1976; Toppari et al., 1985; Fig. 1). Before storage at −70°C, the dissection medium was carefully removed from all samples.

Slot-Blot Hybridization

The RNA from 2-mm tubule segment was prepared for slot-blot analysis as described earlier by Krawczyk and Wu (1987) and filtered through nitrocellulose filters (Bio-Rad Laboratories, Richmond, CA) using a Bio-dot apparatus (Bio-Rad Laboratories). For RNA from pooled tubule segments the same procedure was used except that proteinase K treatment was omitted. To detect the transcript, the Sma I–Bam HI (1.6 kb) fragment of the plasmid pMI.8 was used. The plasmid contains a 5′-end fragment of the mouse *hsp70* gene cloned into the pBR322 vector (Morimoto, R., personal communication; for partial restriction map see Krawczyk et al., 1987*b*). The restriction fragment was labeled by random priming either with ^{32}P-dCTP (Amersham International, Amersham, United Kingdom) (for filter

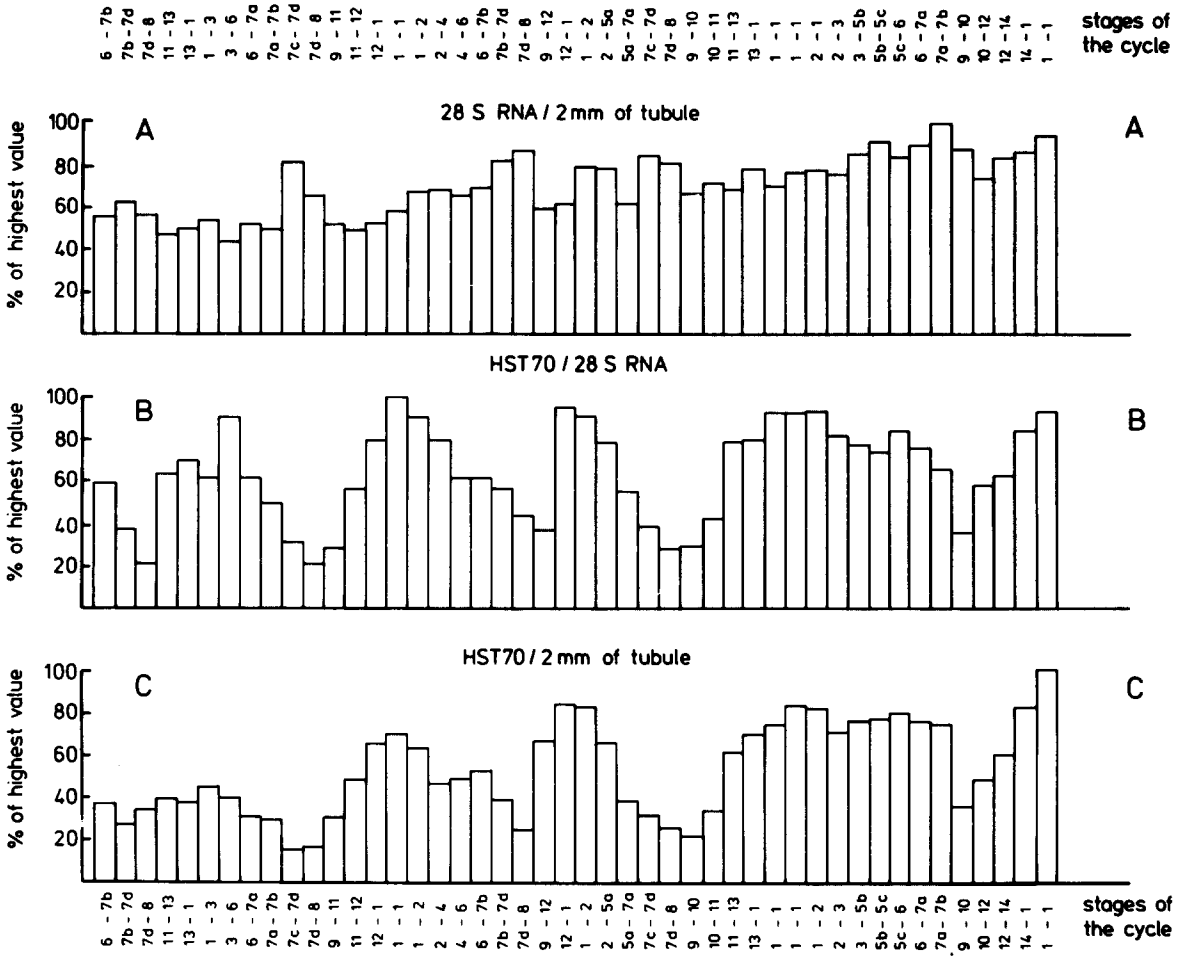

Figure 2. Relative levels of hst70 and 28S RNAs measured at different stages of rat seminiferous epithelium indicated by Arabic numerals at the top and bottom of the figure. The autoradiograms were scanned and related to tubule length (*A* and *C*). In *B*, the level of hst70 RNA was related to the ribosomal RNA in each tubule. The level of hsp70 RNA is repeatedly low in segments containing stages VIId–XI and high in other segments; highest levels were found around stage I of the cycle. The 28S rRNA level shows somewhat higher values in opposite segments containing stages VII and VIII of the cycle.

hybridizations) or with ^{35}S-dATP (for in situ hybridizations) to specific activity of ~10^9 cpm/μg. The prehybridizations, hybridizations with labeled probe, washes, and autoradiography were performed essentially as described earlier (Thomas, 1980; Krawczyk et al., 1987*a*). The autoradiograms were scanned by a Digiscreen (Gelman Sciences, Inc., Ann Arbor, MI) densitometer and the strongest hybridization was expressed as 100%. The densities of other bands were calculated as percentages of this and related either with the length of the tubule or with the levels of ribosomal 28S RNA detected by pI-19 clone (Arnheim, 1979) in the same filter.

In Situ Hybridization

The testes were fixed in 10% buffered formaline for 24 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. 5-μm-thick sections were cut on microscope slides treated with Denhardt's solution (Denhardt, 1966) and acetylated as described by Brahic and Haase (1978). To ensure a firm attachment, the sections were treated with 1% Elmer's glue and heated at 60°C overnight (Sandberg and Vuorio, 1987). Deparaffinization was performed by conventional washes in xylene, absolute ethanol, and 70% ethanol solutions. The prehybridization and hybridization procedures were performed as described earlier (Brahic and Haase, 1978, Syrjänen et al., 1986; for details, see Sandberg and Vuorio, 1987), using 0.1 μg/ml of ^{35}S-labeled heat-denatured probe and a nick-translated Bgl I generated fragments of bacteriophage lambda DNA as control. For autoradiography, the slides were dipped in a Kodak NTB-2 emulsion and exposed for 7–14 d in desiccant-containing boxes at 4°C, developed, and stained with hematoxylin. The exact stages of the cycle were identified from adjacent periodic acid Schiff–hematoxylin-stained sections using the criteria of Leblond and Clermont (1952). The grain densities above different cell types were scored from 1,200× photomicrographs above defined areas (250–4,400 μm^2) that were measured by a morphometer (MOP 3; Reichert-Jung, Austria). For statistical significance, the grain counts per 100 μm^2 (or square root values of grain counts per cell) were assayed by analysis of variance together with Student-Newman-Keuls multiple range test using a BMDP statistical program (Los Angeles, CA).

Results

The Northern blot analysis of total RNA isolated from 10 pools of seminiferous tubule segments showed a single band of hst70 RNA in all stages of the cycle (data not shown). Lower levels of hst70 RNA were found in stages VIII and IX–XI than in other stages both in Northern and slot-blot analyses. Since in the region of stages XII–XIV–I, the change of transillumination pattern is subtle and errors of up to three stages may occur (Parvinen and Ruokonen, 1982), the increase in the hst70 RNA level in pooled stages XII–XIV could result from the contaminating postmeiotic stages I and II. Therefore, the hst70 RNA levels were determined in individual 2-mm segments of the seminiferous tubules containing precise stages identified by squash preparations from adjacent tubule segments. Densitometric analyses of hybrid-

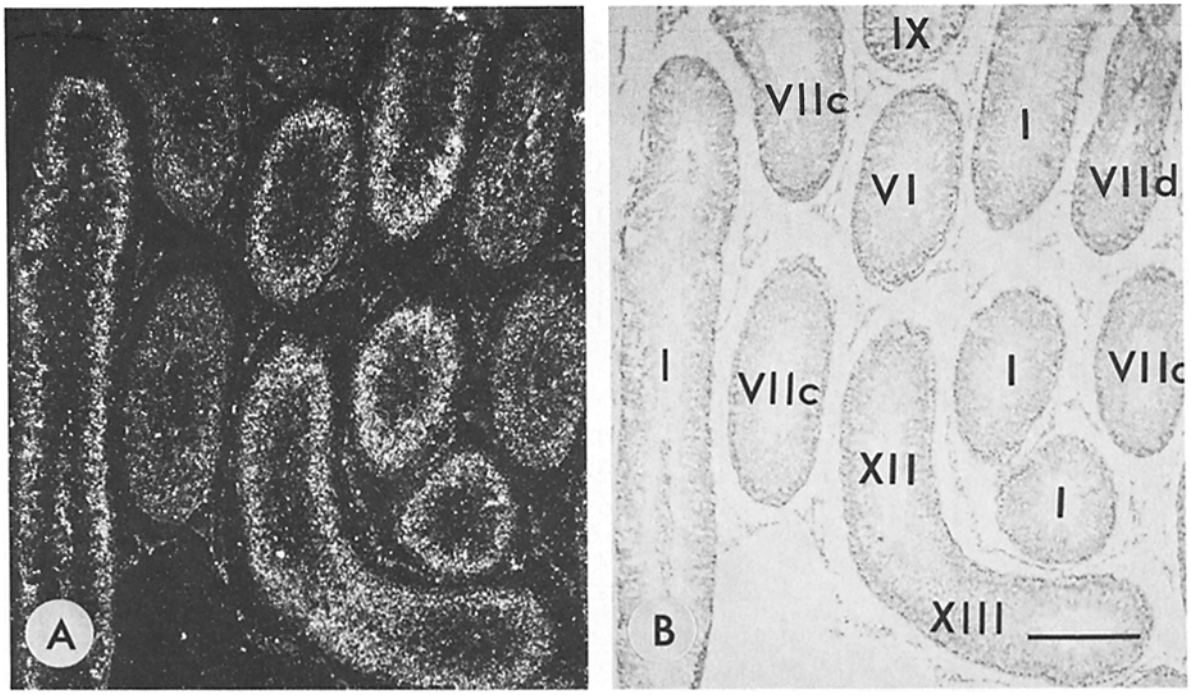

Figure 3. Dark field (*A*) and corresponding normal photomicrograph with epithelial stage identification (*B*) from rat testis sections hybridized in situ with ^{35}S-labeled hst70 cDNA probe. Higher grain densities are found above stages XII, XIII, I, and VI than above stages VIIc, VIId, and IX of the cycle. Bar, 0.5 mm.

izations from 46 consecutive segments covering five complete cycles are shown in Fig. 2. All cycles had a similar pattern of hst70 RNA distribution: the level was highest in stages I–VI, decreased at stages VII and VIII, reached a minimum at stages IX–XI. Then the level of the hst70 RNA raised again and reached a high value at stage XII–XIV, only slightly lower than that observed at stages I–VI. When the same filter was rehybridized with mouse ribosomal 28S RNA, another pattern with a less prominent variation between different stages was found. The maxima were repeatedly found in the region of stages VII and VIII of the cycle (Fig. 2 *A*). The cyclic changes in relative distribution of hst70 RNA between the stages of the seminiferous epithelium were most evident when its levels were related either to arbitrary unit of the rRNA level (Fig. 2 *B*) or to the length of the tubule segment (Fig. 2 *C*), both showing identical patterns. The level of hst70 RNA in 9 out of 10 segments containing stages IX–XI, was lower or the same as in preceding segment containing stages VIId–VIII. In turn the level of the transcript in 9 out of 10 segments containing stages XII–XIV was higher than preceding segment containing stages IX–XI. The relative levels of the transcript at stages XII–XIV and I–II are nearly identical, suggesting that a significant fraction of the total testicular hst70 RNA is synthesized before stage I.

The in situ hybridization analysis showed a variation in the activity between individual seminiferous tubules that was most evident in dark-field photomicrographs taken at low magnification (Fig. 3). Stages I, XII, XIII, and VI displayed higher grain densities over the seminiferous epithelium than stages VIIc, VIId, and IX. A detailed analysis with high magnification revealed that late pachytene primary spermatocytes at stage XII, diakinetic and dividing spermatocytes at stages XIII and XIV, and early round-nucleated spermatids at stages I through early VII of the cycle were significantly more densely labeled than other cell types (Fig. 4). Compared with background counts scored over the lumen of the seminiferous tubules and over the interstitial tissue, some hybridization was found over all cell types analyzed. Significantly higher grain densities were found above meiotic cells from late pachytene (stage XII) to meiotic divisions (stage XIV) and over steps 1–6 spermatids (Fig. 5). Control hybridization with lambda-phage vector cDNA showed evenly distributed low grain densities over all stages of the cycle (Fig. 4 *C*).

Discussion

There are several genes that, as the one coding for the hst70 RNA, are specifically expressed in testis. These include the ones coding for protamines (Hecht et al., 1986; Peschon et al., 1987), transitional proteins (Heidaran and Kistler, 1987), variants of lactate dehydrogenase (Millan et al., 1987), phosphoglycerate kinase (McCarrey and Thomas, 1987), histone (Kim et al., 1987), tubulin (Villasante et al., 1986; Sullivan et al., 1986), and actin (Waters et al., 1985). In addition, transcripts of several genes such as oncogenes *int-1* (Shackleford and Varmus, 1987), c-abl (Ponzetto and Wolgemuth, 1985), *myc* (Stewart et al., 1984) and *c-mos* (Mutter and Wolgemuth, 1987; Goldman et al., 1987) as well as homeobox containing gene (Rubin et al., 1986; Wolgemuth et al., 1987), which are activated at specific stages of embryogenesis have also been found in spermatogenic cells. Interestingly, proopiomelanocortin and proenkephalin genes that originally were thought to be expressed only in pituitary have

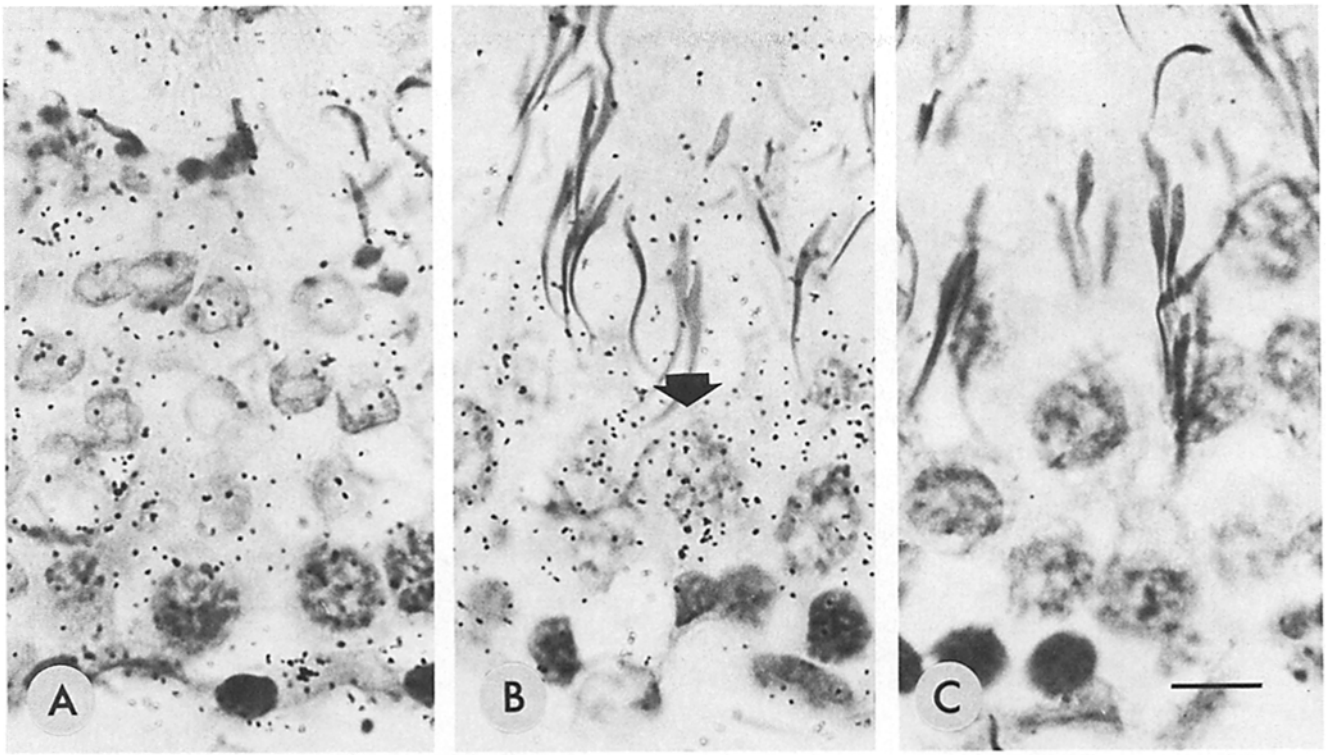

Figure 4. Photomicrographs of the same preparation shown in Fig. 3, from stages VIII (*A*) and XIII (*B*) compared with a control hybridized with labeled vector (*C*, stage XII). Stage VIII shows a low and even grain density above all cell types but the grains above diakinetic primary spermatocytes at stage XIII (*arrow*) suggest a presence of hst70 RNA in these cells. Control hybridization shows a low grain density. Bar, 10 μm.

been found expressed in the spermatogenic cells (Gizang-Ginsberg and Wolgemuth, 1987; Kilpatrick and Millette, 1986).

It is important to determine at what step of spermatogenesis a given gene is activated and transcribed. The most common approach has been the fractionation of cells by sedimentation velocity at unit gravity (Wolgemuth et al., 1985, and references therein). Although this technique is useful in analyses of haploid gene expression (Hecht, 1986), the accurate timing and developmental stages of the onset of transcription of certain genes is not always possible. The cell fractions are usually cross-contaminated. In addition, the lack of distinctions in velocity sedimentation do not permit separation of transcripts in different phases of the meiotic prophase, such as early to mid and late pachytene cells, or in different steps of spermiogenesis. This cell fractionation applied to the determination of the hsp70 gene–related transcript in testis resulted in conclusion that it is primarily expressed postmeiotically (Zakeri and Wolgemuth, 1987).

We present evidence that the gene coding for the hst70 RNA is activated and transcribed before formation of round spermatids. This conclusion was drawn from the analysis of the transcript level in segments of seminiferous tubules containing defined cell associations. Heat shock–related transcript was detected in all stages of the cycle, in agreement with Zakeri and Wolgemuth (1987) who found the transcript in spermatids at all developmental stages and in the residual bodies. However in our study, the pattern of the hst70 RNA

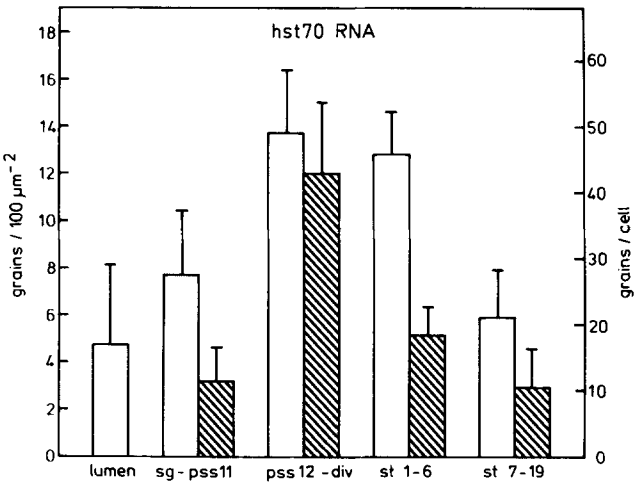

Figure 5. Quantitative grain counts per 100 μm² (*open bars*) and per cell (*hatched bars*) with standard deviation over spermatogonia and spermatocytes up to pachytene at stage XI (sg-pss 11, 60 scored areas), late pachytene, diakinetic, and dividing spermatocytes at stages XII–XIV (*pss 12-div*, 12 scored areas), steps 1–6 round spermatids (*st 1–6*, 12 scored areas) and steps 7–19 spermatids (*st 7–19*, 36 scored areas) compared with background grain densities measured over tubular lumen (*lumen*, 33 scored areas). All cell types show grain densities above background, but late spermatocytes and early spermatids show significantly higher grain densities than other cell types ($P < 0.01$).

expression during the cycle differed from the anticipated one if the hst70 RNA would have been expressed solely in postmeiotic cells.

The level of the transcript was high during stages XII–XIV and I–VI. The level decreased during stage VII showing a minimum at stages IX–X. Since at these stages the only cells which are supposed to express the hst70 RNA are step 10–12 spermatids this also suggest a decrease of the concentration of the transcript in acrosome- and maturation-phase spermatids. The increase of hst70 RNA level at stages XII–XIV is considered to reflect the onset of transcription of the corresponding gene in late pachytene spermatocytes. The transcription in steps 9, 10, and 11 elongating spermatids may be insignificant since late spermatids are transcriptionally inactive (Monesi, 1971), also in agreement with in situ hybridization data.

Our results solve at least partially the controversy that could arise about the origin of the heat shock–related testis-specific RNA. The former results on the transcript level during the maturation of rat testes indicated an expression in late spermatocytes or early spermatids since it first appeared at detectable levels in testes of 3-wk-old rats (Krawczyk et al., 1987*a*). However, reappearance of the transcript during the restoration of spermatogenesis in adult rats treated with busulphan was correlated with the appearance of the spermatids in certain seminiferous tubules (Krawczyk and Szymik, 1988) that is in agreement with the conclusion of Zakeri and Wolgemuth (1987). These data excluded the possibility of the transcription of the hst70 RNA in the leptotene and zygotene spermatocytes and strongly suggested its postmeiotic origin. However they also suggested a possibility of the late meiotic activation of this gene.

Our data, however, does not exclude the possibility that the expression of the hst70 RNA continues in early spermatids being temporarily arrested during metaphase and anaphase I and II. Such a postmeiotic continuation of the transcription started in pachytene spermatocytes has been reported by Tanaka and Fujimoto (1986) for the LDH-X gene. The comparison of the transcript level detected in 2-mm segments of the tubules from stages XI–XIV and I–II with the number of cells which potentially express the hst70 RNA (see morphometric data by Wing and Christensen, 1982) indicate that its concentration in postmeiotic cells is lower than in late meiotic cells. It is possible that the transcription of the gene coding for the hst70 RNA is not continued in postmeiotic cells.

The function of the possible translational product of the hst70 mRNA remains obscure. Recently, Allen et al. (1988) have described a testis-specific hsp70–related protein which is present in spermatocytes as well as in early spermatids. It is not known whether this protein is translated from hst70 mRNA but if this is the case one could speculate its involvement in the differentiation of the spermatocytes into spermatids.

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