A Cytochemical Study of the Transcriptional and Translational Regulation of Nuclear Transition Protein 1 (TP1), a Major Chromosomal Protein of Mammalian Spermatids

Mohammad A. Heidaran,* Richard M. Showman,~ and W. Stephen Kistler*
Department of Chemistry* and the Department of Biology,~ University of South Carolina, Columbia, South Carolina 29208

Abstract. Immunocytochemical localization and in situ hybridization techniques were used to investigate the presence of spermatid nuclear transition protein 1 (TP1) and its mRNA during the various stages of spermatogenesis in the rat. A specific antiserum to TP1 was raised in a rabbit and used to show that TP1 is immunologically crossreactive among many mammals including humans. During spermatogenesis the protein appears in spermatids as they progress from step 12 to step 13, a period in which nuclear condensation is underway. The protein is lost during step 15. An asymmetric RNA probe generated from a TP1 cDNA clone identified TP1 mRNA in late round spermatids beginning in step 7. The message could no longer be detected in spermatids of step 15 or beyond. Thus, TP1 mRNA first appears well after meiosis in haploid cells but is not translated effectively for the several days required for these cells to progress to the stage of chromatin condensation. Message and then protein disappear as the spermatids enter step 15. In agreement with a companion biochemical study (Heidaran, M. A., and W. S. Kistler. J. Biol. Chem. 1987. 262:13309–13315), these results establish that translational control is involved in synthesis of this major spermatid nuclear protein. In addition, they suggest that TP1 plays a role in the completion but not the initiation of chromatin condensation in elongated spermatids.

During spermatogenesis, spermatogonia proliferate mitotically to give rise to primary spermatocytes, which undergo meiosis to yield haploid spermatids, which in turn gradually transform into spermatozoa. About midway through spermatid development in mammals and many other organisms, the nucleus undergoes a rather sudden change in shape, and the chromatin condenses. In mammals this change in the chromatin is accompanied by a transition from histones to a class of novel nuclear “transition proteins” (1, 7, 23, 28, 29, 34), which are later replaced by the characteristic arginine and cysteine-rich mammalian protamines (2, 3, 23, 35). Once the chromatin begins to condense, its nucleosomal structure is lost (22, 30), and available evidence indicates that transcription ceases (10, 22). If this is so, then it follows that any messenger RNAs for proteins that will appear at later stages of spermatogenesis must be laid down before the point of chromatin condensation.

It is well established that the mRNAs for the protamines are transcribed early in spermatogenesis and then regulated at the posttranscriptional level until needed. In trout, protamine messages are synthesized before the end of meiosis and are found in a translationally inert ribonucleoprotein particle throughout the early stages of spermatid development (20, 43). In the mouse, a similar situation occurs though in this case the message first appears in round spermatids rather than in spermatocytes (26). Such translational regulation has not yet been established for other types of mRNAs during spermatogenesis.

We were interested to see if translational control also applies to spermatid nuclear transition protein 1 (TP1),1 one of the first nuclear transition proteins to appear (23, 37). In fact, a biochemical approach to this problem has already shown that TP1 message is detectable during development of young rats several days before synthesis of TP1 can be demonstrated (18). In adult animals, in which TP1 synthesis was occurring, a substantial fraction of its message was associated with polysomes and had variable polyadenylation, as is typical of translationally active mRNAs (II, 41). In contrast, the TP1 mRNA in young animals was found exclusively in a nonpolysomal location and had uniform polyadenylation. Thus, some factor(s) both protects the stored message from nuclease attack and prevents its association with the translational apparatus. While this study provided strong evidence that TP1 message is under translational control, it did not show unequivocally in which testicular cells TP1 message is first made or in which cells TP1 protein first appears. Based on the isolation of specific antisera to TP1, we have now used the techniques of immunocytochemistry and in situ

1. Abbreviation used in this paper: TP1, spermatid nuclear transition protein 1.
hybridization to probe individual spermatogenic cells for each of these molecules.

Materials and Methods

Antibody Preparation

TP1 was purified as described previously (25) incorporating preparative electrophoresis as the final step (24). A female New Zealand White rabbit was immunized by injecting a total of 0.5 mg of TP1 in Freund's complete adjuvant at multiple intradermal sites and by repeating this procedure 10 w later using incomplete adjuvant. Sera were then incubated with TP1 labeled with or TP1 as described by Shima et al. (42) and precipitation of antigen antibody complexes with 4% saturated ammonium sulfate.

Western Blotting

Protein was extracted from fresh or frozen tissues by direct homogenization in cold 4% (wt/vol) trichloroacetic acid, centrifugation (10,000 × g for 10 min) to remove insoluble material, and precipitation of soluble proteins with 20% trichloroacetic acid (23). Duplicate samples of material recovered from ~200 mg of tissue were separated electrophoretically in a 15% polyacryl-

Immunohistochemistry

Immunochemical localization was adapted from Rajaniemi et al. (39). Portions of adult testes were fixed in Carnoy's solution (ethanol:acetic acid:chloroform 6:3:1) for 16 h at 4°C, embedded in paraffin, and cut to give 7-μm sections. After removal of paraffin and step-wise hydration, slides were treated with 3 mM dithiotreitol (DTT) in 50 mM Tris-HCl, pH 8.5, for 1 h at room temp. The slides were then washed in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.5 (PBS), and treated with 0.2% BSA in PBS for 15 min. Next, slides were incubated with a 1:10 dilution of TP1 antiserum in PBS containing 0.3% (vol/vol) Triton X-100 for 1 h at 37°C. They were then washed four times for 5 min in PBS containing 1% gelatin and 1:3,000 dilution of antiserum to TP1. The sheet was then washed twice for 20 min in the incubation buffer lacking gelatin but containing 0.05% (vol/vol) Tween-20, and then incubated for 1 h in buffer containing 1% gelatin and a 1:10 dilution of antiserum to TP1. The sheet was then washed as before followed by one wash without detergent. It was then developed in buffer containing 0.025% (vol/vol) H2O2, 0.025% (wt/vol) 4-chloro-

Results

Antisera to TP1

To investigate the timing of appearance of TP1 mRNA and TP1 protein, we used specific antisera for identification of spermatids that contain the protein and a cDNA-derived RNA probe to identify cells that contain TP1 mRNA. An antisera of suitable specificity was raised in rabbits. As a check of its specificity and also as a demonstration of the conservation of immunological determinants on TP1 found in various species, Western blots of a series of extracts of 4% trichloroacetic acid–soluble testicular proteins were used. After electrophoretic separation in an acetic acid/2.5 M urea gel and transfer to nitrocellulose, a strong reaction was observed for the TP1 band from rat, bull, rabbit, human, boar, and hamster (Fig. 1, lanes b-g). No signal was obtained from a control extract of rat liver (Fig. 1, lane a).

Immunohistochemical Identification of TP1

Using a peroxidase-conjugated second antibody procedure for detection of anti–TP1 immune complexes, we tested a variety of fixation and preparative procedures. Carnoy’s fixative worked well, but fixation in formaldehyde or glutaral-

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spermatids, it was sometimes difficult to see an immunological reaction following hematoxylin counterstaining. Therefore, serial sections of the paraffin block were used with alternate sections stained for immunological identification and for nuclear morphology with hematoxylin.

In the rat, haploid cell (spermatid) development is divided into 19 steps based primarily on acrosome and nuclear morphology (4, 38). Since a new generation of spermatids results from the completion of meiosis every time an existing spermatid cohort completes step 14, only the first 14 steps are necessary to define the 14 stages of the seminiferous epithelium. In each of these stages a characteristic developmental relationship is observed among the several generations of germinal cells present in a tubular cross section. While some of the steps of spermatid development can be identified only based on acrosome morphology, many can be recognized confidently based on the pattern of cells present in the tubule as a whole, that is, by the stage of the tubule. For the problem at hand, it is particularly useful to note that the point at which the spermatid nucleus begins to change shape (step 8) coincides with the release of the most advanced generation of spermatids from the tubule lumen. The period in which the elongating cells progress through steps 9, 10, and 11 is the only one in which no cells are present with the highly elongated and condensed nuclei of advanced spermatids. In step 12, the elongated nucleus begins to condense, a process that is nearly complete by the end of step 13. As the spermatids pass through step 14, the spermatocytes developing behind them in the same tubule divide twice to give rise to a new crop of round spermatids. Thus, beginning at step 15 the condensed spermatids are followed by step 1 spermatids rather than by spermatocytes.

With these reference points in mind, it was possible to fix the point of appearance and disappearance of TP1 immunoreactivity with considerable confidence. The results were a dramatic confirmation of earlier assignments of TP1 to the nuclei of condensing spermatids by other means (15, 23, 37). TP1 was not detectable in any round spermatid, for example in the tubule at Stage VII shown in Fig. 2, a and b. Further, it was not present in early elongated spermatids such as those in the Stage XII tubule of Fig. 2, c and d. Immunochemical reactivity appeared suddenly in tubules containing late condensing (step 13) spermatids (Fig. 2, e and f). In these and other tubules, strong immunological reactivity was seen only when nuclei had been cut in cross section. Immunoreactivity was retained through step 14 (Fig. 2, g and h) and into step 15 (Fig. 2, i and j), but was then lost rapidly and was not seen in step 16–17 cells (Fig. 2, k and l). While loss of immunoreactivity could reflect epitope masking, disappearance of TP1 at this point agrees well with results of a variety of previous studies (14, 32, 33, 37). In summary, TP1 immunoreactivity appeared during the transition of spermatids from step 12 to 13 and was retained until some point in step 15. In one testis cross section, 66:295 (22 %) of tubules were immunologically reactive, which is in good agreement with the expected distribution based on the durations of the various spermatogenic stages (4).

In Situ Hybridization to TP1 mRNA

Localization of the spermatids containing TP1 mRNA was done by the technique of in situ hybridization as used by Cox et al. (6). In adult testis sections, about half the tubules were unlabeled by the tritiated RNA probe (Fig. 3). Of those that did react with the probe, the pattern varied from a light dusting of silver grains scattered about the outer half of the tubule to examples in which a high concentration of silver grains clustered toward the tubular lumen. Closer examination of these tubules indicated that this labeling pattern reflected the progression of round spermatids through the elongating and condensing phases. Fig. 4 highlights examples of these: in 4 A, a tubule with step 7 round spermatids; in 4 B, a tubule
with early condensing spermids of late step 8; in 4 C, a tubule with nearly condensed spermids of about step 13; in 4 D, a tubule with round spermids earlier than step 7 and elongated spermids later than step 14 (e.g., a tubule of stage I to V). These later tubules were uniformly negative. Thus, TP1 message was definitely detectable in step 7 round spermids, several steps younger than the step 13 cells that first displayed the protein. As these cells develop and move toward the lumen of the tubule, TP1 mRNA accumulates, but then disappears as the spermids reach step 15 and begin to associate in clusters around the tubule periphery. In one testis cross section, 160:320 (50%) of tubules were labeled above background by the hybridization probe. This accords well with the expected distribution of tubules having spermids from step 7 through 14 (4).

A summary of the cellular location of TP1 and its mRNA
is diagrammed in Fig. 5, which focuses on just the haploid portion of spermatogenesis. Based on the duration of the various steps of spermatid development (4), we can estimate that TPI mRNA is present for \( \sim 5 \) d before it is translated to a significant extent. The protein is then retained in the nucleus for only \( \sim 2 \) d.

**Discussion**

The results presented here confirm and extend our earlier conclusion (18), based on biochemical techniques, that TPI mRNA accumulates only in haploid cells and that it is subject to translational regulation. In that work we could not identify directly the cells in which either mRNA or protein appeared. Here we have exploited histological techniques and found that TPI mRNA was first detectable in late round spermatids around the beginning of step 7 of development. Immunologically reactive protein was first seen in spermatids that had elongated but which still had incompletely condensed chromatin (late step 12 or early step 13). Both protein and its mRNA were retained during step 14 but were lost rapidly thereafter, though protein was still detected in some step 15 cells.

Certain possible reservations to our conclusions are worth considering. For example, one can imagine certain factors that might mask the presence of a specific RNA in a given cell. One would be the occurrence of an antisense RNA (13) covering the region of homology to the probe. Perhaps other such mechanisms exist as well. We see no reason to suspect such masking of the message in this case as the appearance of TPI mRNA in step 7 spermatids is exactly what was predicted from our developmental analysis, when RNA was extracted and assayed for hybridization to a TPI probe via Northern blotting (18). As with our previous study (18), the technique of in situ hybridization does not address the question of whether transcription is occurring but only whether transcription has led to the accumulation of stable products. Thus it remains a formal possibility that transcription occurs in cells prior to step 7 spermatids but does not lead to accumulation of mRNA. Concerns could also be raised with the possible masking of the antigenicity of TPI. The only suspected modification of TPI is phosphorylation (our unpublished observations), but its extent must be slight since the protein has never been observed to give multiple bands on acetic acid/urea gels under conditions in which rat protamine is readily resolved into several phosphorylated components (31; Kistler, W. S., unpublished observations). Furthermore, newly synthesized TPI has the same electrophoretic mobility as the mature protein (9, 18), which further argues against extensive side chain modifications. Recent analysis of the TPI gene (Heidaran, M., and W. S. Kistler, manuscript submitted for publication) shows that the message can not encode a larger precursor form of the protein. A more difficult issue to address is whether remaining histones might mask TPI immunoreactivity until they were removed or modified. In the absence of any data relative to this last point, we will assume that the cytochemical data are a faithful reflection of the occurrence of both TPI and its mRNA.

TPI was first suggested to be in elongating spermatids based on its appearance during sexual development (24). Later it was localized more accurately by analysis of the nuclear proteins of separated classes of spermatids (14, 37), and...

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**Figure 3.** Cytochemical localization of TPI mRNA by in situ hybridization. The same section is viewed at low magnification by pseudo dark-field optics (top) or by bright field illumination (bottom). The stage of selected tubules is indicated (which corresponds to the developmental step of the least mature spermatid generation). Exposure time was 7 d. Higher power views of portions of tubules designated 7, 8, 13, and 1–5 are shown in Fig. 4. The specificity of the hybridization was shown by the failure of a heterologous probe (to seminal vesicle secretory protein IV) to react with any testis tubules and by the corresponding failure of the TPI probe to react with seminal vesicle epithelial cells (not shown). Bar, 200 \( \mu \)m.
our results agree well with those obtained by Meistrich's laboratory. Similarly, in the mouse (32, 33) tritiated lysine was seen to be incorporated readily into the nuclei of step 12 and 13 nuclei but was retained only through step 14. This label almost certainly reflected the combined presence of transition proteins 1 and 2, which are rich in lysine (5, 14, 15, 23). The low lysine content of protamine 1 (2, 3, 46) was apparently not detected by the conditions of the experiment, and protamine 2, which is prominent in mouse, lacks lysine (46).

A pretreatment with DTT markedly enhanced the immunological detection of TP1 in all classes of positive tubules. This suggests that some type of disulfide barrier blocks access to the protein even when it first appears. TP1 itself lacks cysteine in the rat (25). TP2, which is found in the same isolated cell fractions as TP1 contains substantial cysteine (5, 14, 15). Rat protamine, like other mammalian protamines, is also rich in cysteine (2, 3, 23). Since studies with isolated cell fractions indicated that protamine appears after TP1 in spermatid development (14), TP2 may account for the disulfide barrier.

To the extent that this can be established at the level of the light microscope with the detection reagent we have used, it seems that TP1 is present relatively uniformly throughout the interior of the nuclei of condensing spermatids. It was detected poorly if at all on the nuclear surface and reacted strongly with the antiserum only when a nucleus was cut in cross section. Its presence does not correlate with the early morphological changes that lead to an elongated nucleus or to the earliest steps of chromatin condensation that occur during step 12. Clearly this implies that TP1 does not serve to initiate condensation but rather to play a role during the later phase of this process. TP2 remains a possible candidate for participating in the initiation of condensation.

Translational regulation is now well established for protamine synthesis in both trout (20, 43) and mouse (26). TP1 is the first additional spermatid protein shown to share in this form of control. A clear difference between the regulation of protamine synthesis in trout and in mouse is that the message appears before the meiotic divisions in the former and only postmeiotically in the latter. This difference presumably is dictated by a need to avoid the determination of the spermatid message population by the haploid genome and the attendant dominance of otherwise recessive genetic alleles. In mammals developing spermatogenic cells are joined by cytoplasmic bridges that presumably facilitate the transfer of most proteins and perhaps mRNAs between cells (8) so that each
cell is presumably under control of a polyplody nucleus. The mechanism of translational regulation is not worked out but seems to involve a masking effect by salt-extractable proteins rather than any sort of covalent modification of the message itself (43). The isolation of the gene for rat TPI (Heidaran, M., and W. S. Kistler, manuscript submitted for publication) now makes this system available for the application of modern genetic manipulation as a technique to help define the role of the message sequence in its translational regulation.

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