Development 129, 945-955 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2828

Distinct functions of TBP and TLF/TRF2 during spermatogenesis: requirement of TLF for heterochromatic chromocenter formation in haploid round spermatids

Igor Martianov[1,†], Stefano Brancorsini[1,†], Anne Gansmuller[1], Martti Parvinen[2], Irwin Davidson[1,‡] and Paolo Sassone-Corsi[1,‡]

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire CNRS/INSERM/ULP, B.P. 163, 67404 Illkirch Cédex, C.U. de Strasbourg, France
[2]Department of Anatomy, University of Turku, 20520 Turku, Finland
†These authors contributed equally to this work
‡Authors for correspondence

Accepted 23 November 2001

SUMMARY

TLF (TBP-like factor) is a protein commonly thought to belong to the general transcription initiation complex. TLF is evolutionarily conserved and has been shown to be essential for early development in *C. elegans*, zebrafish and *Xenopus*. In mammals however, TLF has a specialised function, as revealed by targeted mutation of the gene in the mouse germline. The TLF mutation elicits a complete arrest of late spermiogenesis and increased haploid cell apoptosis. We explored in more detail the molecular function that TLF plays in the differentiation program of male germ cells. A comparison of TBP and TLF reveals drastic differences, both in their temporal expression pattern and in their intracellular location. While TBP is ubiquitously expressed, TLF expression is strictly developmentally regulated, being very high in late pachytene spermatocytes, suggesting a function prior to the apoptosis of the haploid cells. A refined study of TLF-deficient mice reveals defective acrosome formation in early stage spermatids. Most importantly, our results uncover an unsuspected function of TLF in chromatin organisation. Indeed, early spermatids in TLF-deficient mice display a fragmentation of the chromocenter, a condensed structure formed by the association of centromeric heterochromatin and containing the HP1 proteins. This defect is likely to be the primary cause of spermatogenic failure in the TLF mutant mice.

Key words: Spermatogenesis, TLF, TBP, Chromatin, Chromocenter, HP1, Mouse. Rat

INTRODUCTION

RNA polymerase II transcription initiation in eukaryotes requires the formation of a multiprotein complex around the mRNA start site. (Hampsey, 1998; Hampsey and Reinberg, 1999; Orphanides et al., 1996). A central factor in this process is transcription factor TFIID comprising the TATA binding protein (TBP) and 14 additional TBP-associated factors ($TAF_{II}s$). TFIID plays an important role in transcriptional regulation through contributing to promoter recognition and acting as a target for transcriptional activators (for reviews, see Albright and Tjian, 2000; Bell and Tora, 1999; Gangloff et al., 2001; Green, 2000). TBP itself is thought to play a role in transcription initiation by all three RNA polymerases through its presence in TFIID for polymerase II, SL1 for polymerase I and TFIIIB for polymerase III (Chiang et al., 1993; Comai et al., 1992; Hernandez, 1993).

TBP has a bipartite structure with a variable N-terminal domain and a highly conserved C-terminal core domain. The core domain of TBP folds into a saddle-like structure (Kim and Burley, 1994; Kim et al., 1993) which is responsible for DNA binding, and interaction with TFIIA, TFIIB and TAFs (Liu et al., 1998; Nikolov et al., 1995; Tan et al., 1996). Two proteins with strong sequence similarity to the core domain of TBP have been described. The first, *Drosophila melanogaster* TRF1, is expressed in a tissue-specific fashion and functions as both a RNA polymerase II and polymerase III transcription factor (Hansen et al., 1997; Holmes and Tjian, 2000; Takada et al., 2000). At present the gene for this has been described only in *Drosophila*.

A second protein with high homology to the TBP core domain has received various names; TLF, TRF2, TLP and TRP (Maldonado, 1999; Moore et al., 1999; Ohbayashi et al., 1999; Rabenstein et al., 1999; Teichmann et al., 1999; Wieczorek et al., 1998). The TLF (TBP-like factor) gene is found in most metazoans (Dantonel et al., 1999) and multiple sequence alignments show that TLFs contain two direct repeats, which are well conserved between all TBP-type factors. Despite this sequence similarity, TLF does not bind classical TATA elements (Dantonel et al., 1999; Moore et al., 1999), but

does interact with TFIIA and TFIIB. Transfection, and overexpression studies in cells and in in vitro experiments, have shown that TLF associates stably with TFIIA and can act as a repressor or activator of RNA polymerase II transcription (Moore et al., 1999; Ohbayashi et al., 2001; Teichmann et al., 1999).

The physiological function of TLF was first addressed using RNAi in *Caenorhabditis elegans* where TLF appears to function as an essential RNA polymerase II transcription factor (Dantonel et al., 2000; Kaltenbach et al., 2000). Injected embryos die at an early stage of embryogenesis before the onset of morphogenesis. Similar results have been obtained using antisense strategies in *Xenopus laevis* (Veenstra et al., 2000) and in the zebrafish *Danio rerio* (Muller et al., 2001). These studies underscored a critical role of TLF in the embryogenesis of these species. It has been suggested that TLF may act as a surrogate for TBP at specific promoters whose expression is required at the onset of zygotic transcription in the developing embryos. However, as it has been demonstrated recently, the situation is radically different in mammals. Indeed, using homologous recombination, it has been shown that mouse TLF is not required for embryogenesis, but that it is essential for spermatogenesis (Martianov et al., 2001; Zhang et al., 2001b).

Spermatogenesis is a cyclic process in which diploid spermatogonia differentiate into mature haploid spermatozoa. Spermatogonia committed for differentiation give rise to spermatocytes which undergo two meiotic divisions, to generate haploid round spermatids. During the process of spermiogenesis the haploid round spermatids undergo an elongation phase in which they are sculptured into the shape of mature spermatozoa. This entails a major biochemical and morphological restructuring of the germ cell in which the majority of the somatic histones are replaced by protamines to pack the DNA into the sperm cell nucleus. In the absence of TLF, round step 7 haploid spermatids die through apoptosis as the transition to elongated spermatids begins. Rare elongating spermatids remain beyond this stage but rapidly degenerate such that no spermatids survive beyond step 13. Hence, TLF is essential for the differentiation of male germ cells. These results highlighted the non-redundant function of TBP and TLF in mammals and solicited further investigations aimed at identifying their distinct roles.

To gain further insight into the molecular function that TLF plays in the differentiation program of male germ cells, we have examined in detail the pattern of TLF expression during spermatogenesis and compared it with that of TBP. Our results show marked differences in temporal regulation of TBP and TLF as well as differences in their intracellular location. In particular, TLF is strongly expressed in late pachytene spermatocytes, suggesting that it has a function prior to the apoptosis of the haploid cells. A detailed analysis of the germ cell phenotype in $TLF^{-/-}$ ($Tlp^{-/-}$) mice shows that is indeed the case since defective acrosome formation is observed in early stage spermatids. Our results also reveal an unexpected role of TLF in chromatin organisation. Strikingly, early stage spermatids of TLF-deficient mice are characterised by fragmentation of the chromocenter, a condensed structure formed by the association of centromeric heterochromatin (Hoyer-Fender et al., 2000; Brinkley et al., 1986; Zalensky et al., 1993). Wild-type spermatids contain a single chromocenter, while in $TLF^{-/-}$ spermatids the single center is fragmented into multiple structures. These results show that TLF plays an essential role in proper chromatin organisation in developing male germ cells. Disorganisation of chromatin domains in the early round spermatids could lead to defects in gene expression and chromosomal packaging in the elongated spermatids and therefore be the primary cause of differentiation arrest and apoptosis of $TLF^{-/-}$ spermatids.

MATERIALS AND METHODS

RNA analysis

Total RNA was extracted from mouse testis and rat spermatogenic cells as previously described (Chomczynski et al., 1986) and analyzed by RNase protection (Foulkes et al., 1991). Rat spermatocyte and spermatid cells were separated by centrifugation as described (Meistrich et al., 1981). The TLF and TBP cDNAs were used to generate a [α-^{32}P]UTP antisense riboprobe using an in vitro transcription kit (Promega). CREM riboprobe was as described previously (Molina et al., 1993). In all RNase protection analyses, transfer RNA was used as a control for nonspecific protection. A mouse β-actin riboprobe was used as internal control to monitor the loading of equal amounts of RNA.

Immunoblots and immunohistochemistry

Rat seminiferous tubule segments at defined stages were isolated using the transillumination-assisted microdissection method (Parvinen and Hecht, 1981) and pooled to obtain 4-5 cm, equivalent to 4-5 mg of wet weight of tissue. Protein extracts were made by shearing the tubule segments in 2× boiling Laemmli buffer containing 10 mM β-mercaptoethanol. The extracts were analyzed by SDS-PAGE and staining with Coomassie Blue stain to normalise each preparation. Immunoblotting and chemiluminescence detection were performed by standard methods. Immunohistochemistry was performed on fixed sectioned seminiferous tubules or from squash preparations of microdissected tubules from wild-type or TLF mutant mice as previously described (Martianov et al., 2001; Nantel et al., 1996). Antibodies against TBP, TLF and CREM are as previously described (Brou et al., 1993; Delmas et al., 1993; Martianov et al., 2001). The anti-HP1α, 2HP1-1H5 and anti-fibrillarin antibodies are also as described previously (Fomproix et al., 1998; Nielsen et al., 2001).

RESULTS

Developmental and stage-specific expression of TLF and TBP

To compare the functions of TBP and TLF in developing male germ cells, we first analysed their respective expression patterns. During the first wave of spermatogenesis, germ cells are synchronised in their development, different cell types appearing at different times. The developmental onset of TBP and TLF expression was examined by RNase protection analysis with 1- to 4-week-old mouse testes.

Low expression of TBP and TLF was observed in 1- to 2-week-old immature testes (Fig. 1A, lanes 2-3). At three weeks, a strong increase in expression of both TBP and TLF was observed, reaching levels similar to those observed at 4 weeks and in the adult (lanes 3-5). The increase in expression correlates with the accumulation of late pachytene spermatocytes and round spermatids, which occurs at this time.

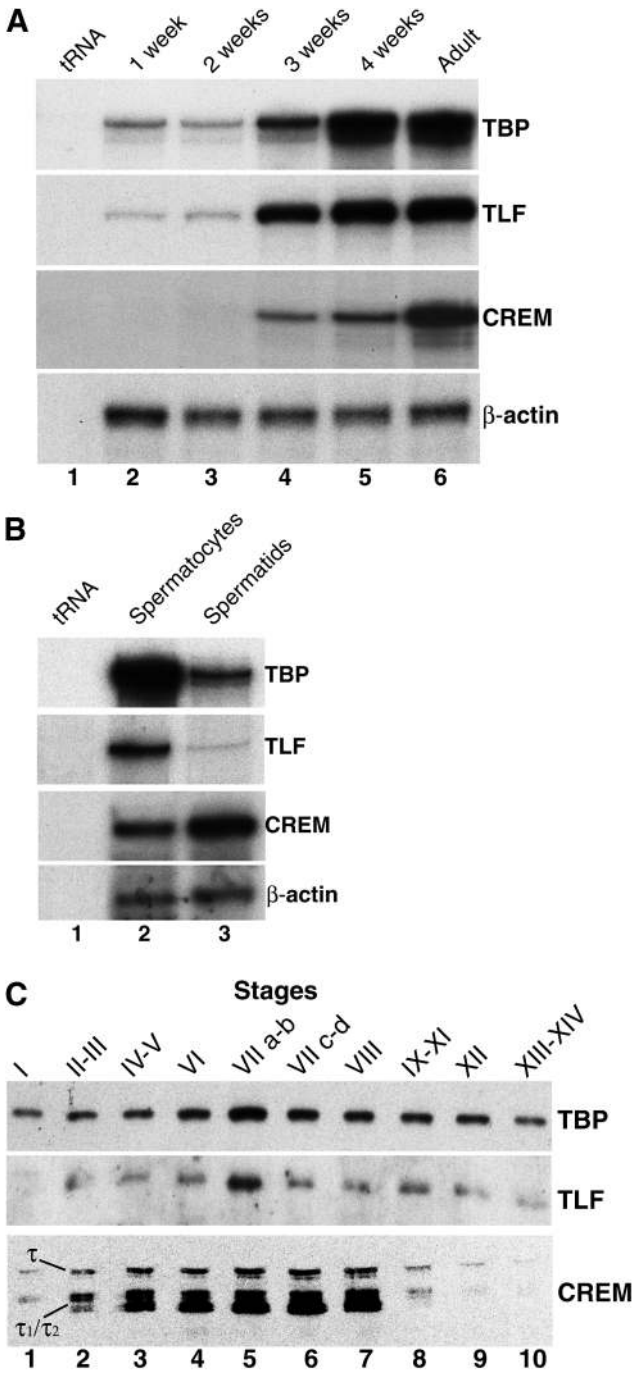

Fig. 1. Developmental regulation of TBP and TLF. (A) RNase protection experiments. The RNA used is indicated above each lane and the detected transcripts identified are listed to the right of each panel. Lane 1 shows a control with only tRNA. (B) The origin of the RNA, pachytene spermatocytes (spermatocytes), and haploid spermatids (spermatids) is shown above each lane and the transcripts are listed to the right of each panel. (C) Immunodetection of TBP, TLF and CREM. The stage-specific protein extracts used are indicated above each lane and the locations of TBP, TLF and the CREM isoforms are indicated to the right of each panel.

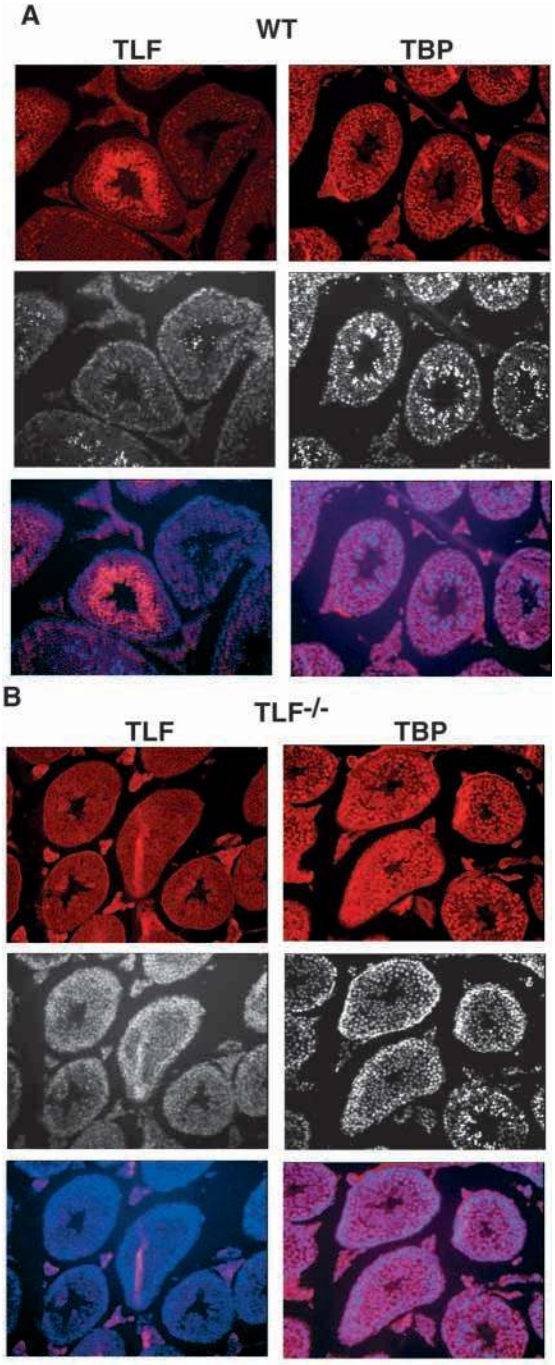

Fig. 2. (A,B) Expression of TLF and TBP in seminiferous tubules of wild-type (A) and TLF null-mice (B). The upper panel in each section shows the immunodetection of TLF and TBP as indicated. The middle and lower panels show the corresponding Hoechst stained DNA and merged images. 40× magnification.

This observation is consistent with previous reports of TBP overexpression in meiotic and post-meiotic cells (Persengiev et al., 1996; Schmidt and Schibler, 1995) with the fact that TBP and TLF overexpression precedes that of the haploid cell-specific CREM activator (Fimia et al., 2001) (Fig. 1A). Hence, TLF is overexpressed in both late meiotic and post-meiotic cells.

In a more refined analysis, TBP and TLF expression were examined in purified adult rat pachytene spermatocytes and haploid spermatids. TBP and TLF mRNAs strongly accumulate in pachytene spermatocytes, while lower levels are

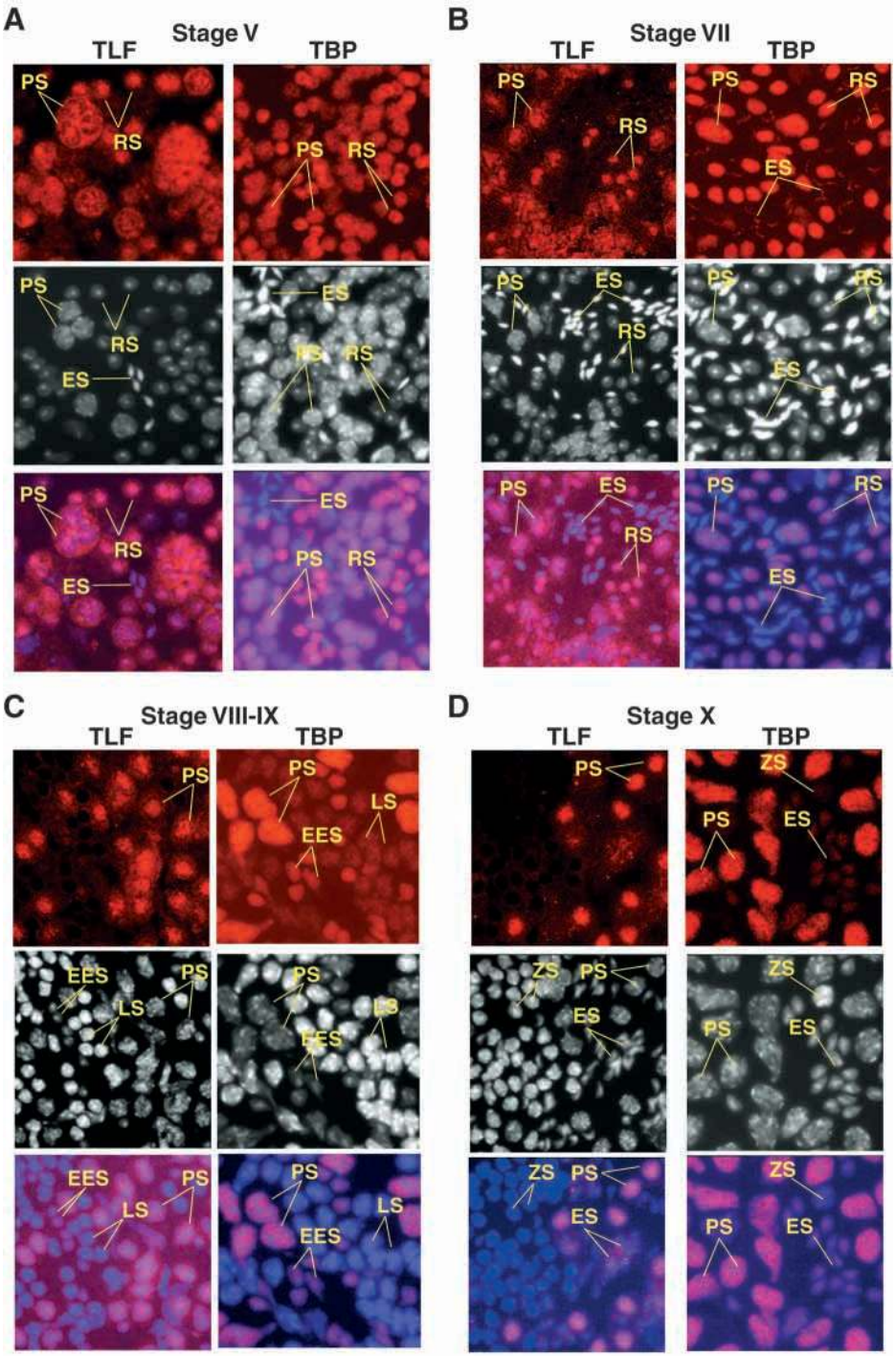

Fig. 3. (A-D) Immunodetection of TBP and TLF in developing male germ cells. TLF and TBP are detected in microdissected segments of seminiferous tubules from various stages as indicated above each panel. Representative examples of cell types are indicated. PS, pachytene spermatocytes; RS, haploid round spermatids; ES, elongating spermatids; EES, early elongating spermatids; LS, leptotene spermatocytes; ZS, zygotene spermatocytes. 40× magnification.

present in haploid spermatids (Fig. 1B, compare lanes 2 and 3). The converse was observed for the CREM activator mRNA (Fig. 1B) (Delmas et al., 1993).

Spermatogenesis occurs in synchronised waves within the seminiferous epithelia. The cycle is divided into 12 stages in mouse and 14 stages in rat, where each stage comprises a mix of cells in given developmental steps. We isolated individual segments from rat seminiferous tubuli corresponding to each developmental stage by transillumination-assisted microdissection (Parvinen and Hecht, 1981) and examined the accumulation of TBP and TLF proteins at each stage by western blotting using specific monoclonal antibodies (Brou et al., 1993; Lescure et al., 1994; Martianov et al., 2001). TBP was detected at each stage with highest expression seen at stage VII (Fig. 1C, lanes 5-6). In contrast, TLF accumulation was weak at stage I, but increased through stages II-VI to peak at stage VII before again decreasing through stages VIII-XIV (Fig. 1C). As a control, strong expression of the CREMτ, τ1 and τ2 isoforms was observed from stages IV-VIII (Fig. 1C, lanes 3-7).

These results show that TBP and TLF have a similar developmental onset of expression, but that they are differentially expressed at different stages of the spermatogenic cycle.

Expression of TLF is dynamic and cell specific, while TBP is ubiquitous

The monoclonal antibodies against TBP and TLF were used to examine the expression and localisation of the corresponding proteins at different stages of differentiation, by immunohistochemistry. We first compared their expression profiles in sectioned seminiferous tubules from wild-type mice. TLF expression is cell-specific and is not visible in any of the randomly sectioned tubuli, indicating that it is dynamically regulated during the cycle (Fig. 2A). In contrast, expression of TBP is much more homogeneous as it is present at all stages in all cell types with the exception of the more mature elongating spermatids (Fig. 2A). We also examined TBP expression in the seminiferous tubules from *TLF*$^{-/-}$ mice to investigate whether its expression was altered in the absence of TLF. As expected, no TLF expression could be seen in the tubuli from the mutant animals. TBP was however expressed in all remaining cell types similar to that observed in wild-type tubuli (Fig. 2B).

To examine the expression of TBP and TLF in more detail, we isolated segments of mouse seminiferous tubuli corresponding to each developmental stage by transillumination-assisted

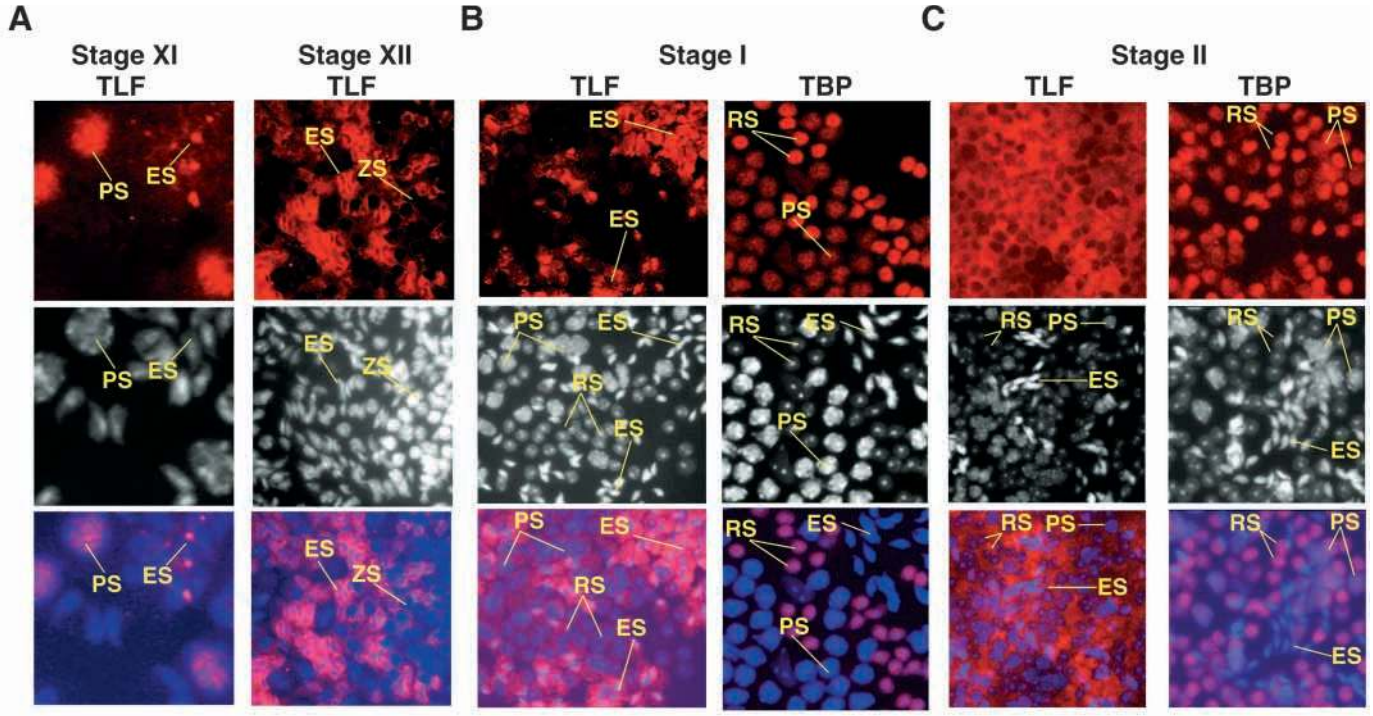

Fig. 4. (A-C) Immunodetection of TBP and TLF in developing male germ cells. The layout and abbreviations are as described in Fig. 3.

microdissection and subjected them to immunostaining with the monoclonal antibodies against TLF and TBP.

At stage V, nuclear and cytoplasmic expression of TLF in pachytene spermatocytes was observed. TLF was also expressed in step 5 haploid round spermatids, predominantly in the nucleus (Fig. 3A). In contrast, no TLF expression was observed in the maturing elongated spermatids at this stage. TBP was strongly expressed in the nucleus of both the pachytene spermatocytes and the round spermatids, but like TLF no expression was seen in the elongated spermatids. At stage VII, a similar, but not identical situation is observed. Expression of TLF is now predominantly, but not exclusively, nuclear in both the pachytene spermatocytes and step 7 round spermatids (Fig. 3B). Strong nuclear expression of TBP is also seen in these cell types at this stage. Interestingly, although no expression of TLF is seen in the now almost mature step 16 spermatozoon, perinuclear expression of TBP can be seen (see also below).

At stage VIII-IX, TLF expression persists in the pachytene spermatocytes, but only very weak expression in the step 9 early elongating spermatids is observed (Fig. 3C). In contrast, no TLF expression is observed in leptotene spermatocytes.

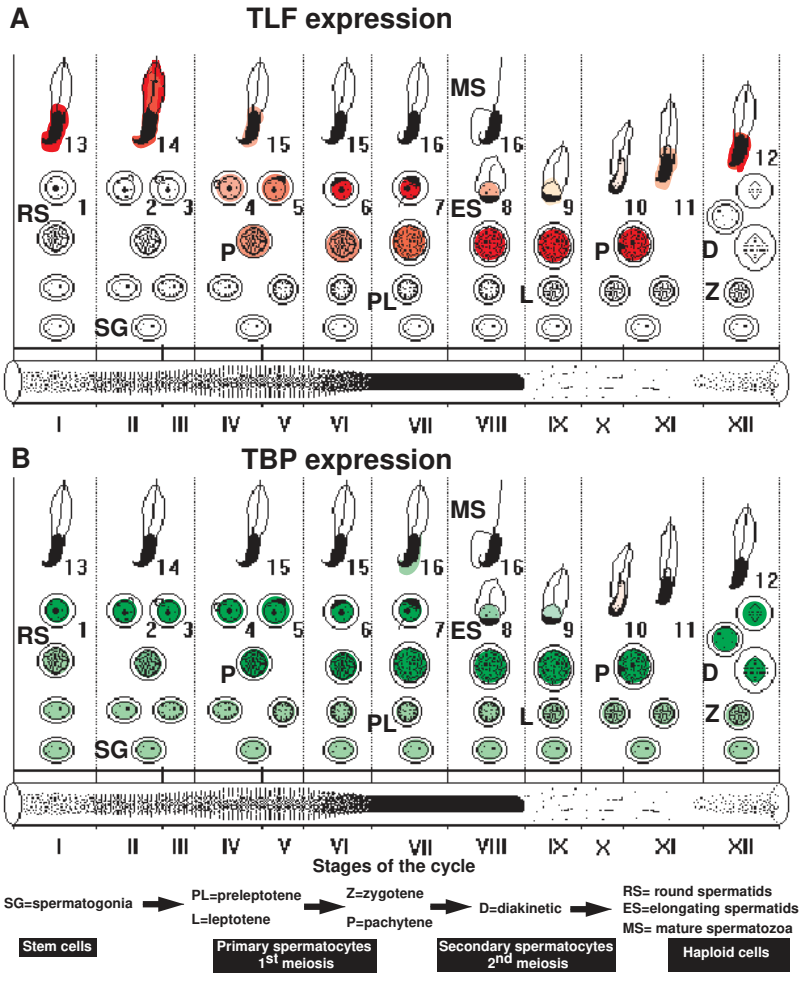

Fig. 5. (A,B) Summary of TLF and TBP expression during spermatogenesis. The spermatogenic wave is schematised and the representative cell types are indicated. Expression of TLF is indicated in A by the red colouring and that of TBP in B by the green colouring.

TBP is expressed in the leptotene spermatocytes, but its expression is much stronger in the pachytene spermatocytes, while it strongly declines in the early elongating spermatids. By stage X expression of TLF in the pachytene spermatocytes has become exclusively nuclear and only very weak expression is observed in the elongating spermatids (Fig. 3D). No TLF expression is seen in the zygotene spermatocytes. TBP expression continues to decline in the elongating spermatids, while it remains strong in the pachytene spermatocytes and weaker in the zygotene spermatocytes.

Strikingly, as the elongating spermatids mature, a strong nuclear/perinuclear expression of TLF is observed beginning at stage XI and becoming stronger at stage XII (Fig. 4A,B). TLF expression persists in step 13 and 14 elongating spermatids and becomes cytoplasmic (Fig. 4C,D). In contrast, no TBP is expressed in the elongating spermatids at this time. TBP is however strongly expressed in the early step 1-2 haploid round spermatids and in the pachytene spermatocytes. Little or no expression of TLF is seen in these cell types at this stage.

The above results indicate that TLF is expressed at three stages in the spermatogenic wave: in stage IV-XI pachytene spermatocyes where its expression is at first predominantly cytoplasmic, but later becomes nuclear, in stage IV-VII haploid spermatids, and in step 11-15 elongating spermatids where its expression is perinuclear and cytoplasmic (summarised in Fig. 5A). In between these stages, TLF expression is weak or undetectable. In contrast, TBP is expressed in all spermatocytes, although its expression strongly increases in late pachytene spermatocytes. TBP is strongly expressed at all stages in round haploid spermatids and its expression declines rapidly at the transition to elongating spermatids. Finally, TBP expression is observed in late step 16 elongating spermatids (summarised in Fig. 5B).

Confocal microscopy was used to compare the intracellular localisation of TLF and TBP. Perinuclear and cytoplasmic TLF expression was observed in elongating spermatids (Fig. 6A, top panel). In contrast, no TBP expression was detected in elongating spermatids (Fig. 6A, middle panel), but perinuclear expression is however seen in late step 16 elongating spermatids as described above (Fig. 6A, lower panel). In round spermatids, TLF was expressed homogeneously in the nucleus, including the chromocenter, whereas most TBP was clearly excluded from the chromocenter (Fig. 6A, compare upper and middle panels). In pachytene spermatocytes, TLF was found in both the cytoplasm and nucleus, whereas TBP was nuclear (Fig. 6B). Within the nucleus, TBP was excluded from the condensed heterochromatin, while TLF was present in both the heterochromatic and euchromatic compartments. Exclusion of TBP from the heterochromatin compartment of pachytene spermatocytes and haploid spermatids is not easily seen in standard immunofluorescence images as presented in Figs. 3 and 4 which do not result from a single focal plane, but rather from the nuclei as whole. The confocal images from a single focal plane give a more accurate picture of TBP distribution. These results reveal significant differences in the intranuclear distribution of TBP and TLF in both pachytene spermatocytes and round spermatids.

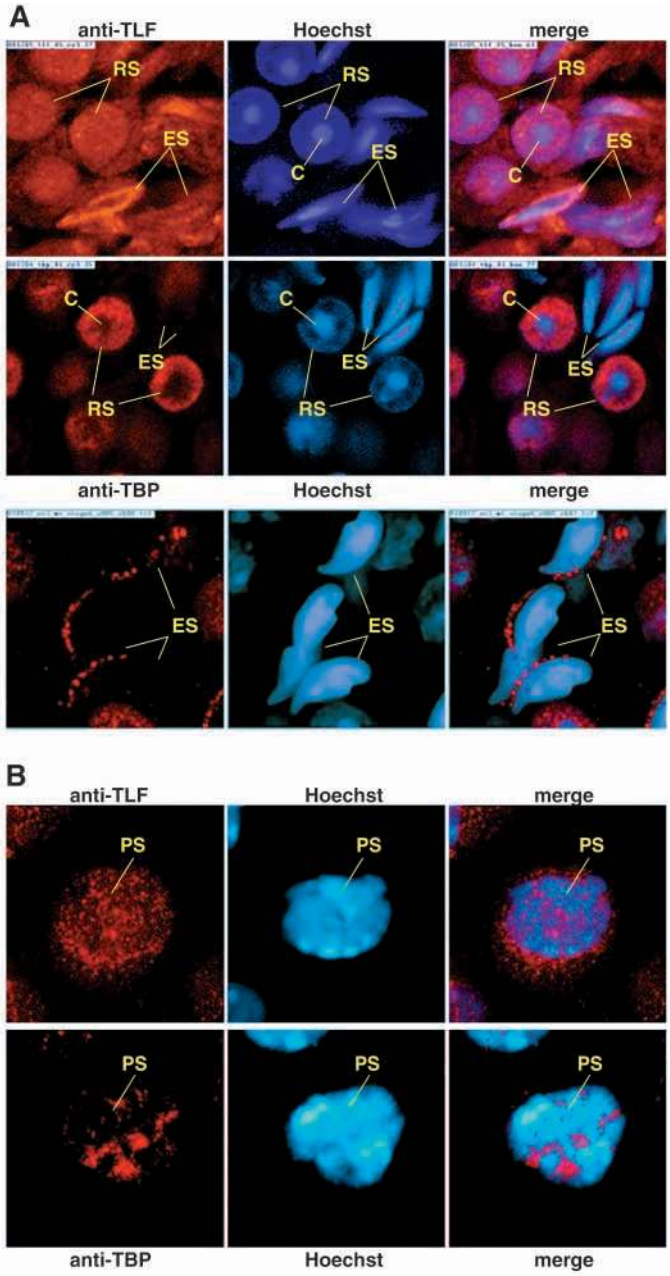

Fig. 6. Different intracellular localisations of TBP and TLF. (A) Confocal images of sectioned seminiferous tubules stained with antibodies against TLF (upper panels) or TBP (lower panels) as indicated. A single representative section is shown. RS, haploid round spermatids; ES, elongating spermatids; C, the chromocenter. The lower panel shows perinuclear TBP detected in late stage elongated spermatids. (B) Confocal sections of pachytene spermatocytes (PS) stained with TLF or TBP as indicated. In all panels the middle and right hand images show the corresponding Hoechst stained DNA and merged images respectively. 400× magnification.

Histological analysis of germ cells in *TLF*$^{-/-}$ seminiferous tubuli

The TLF expression profile suggests that it may have a function in germ cell differentiation before apoptosis is observed in step 7 spermatids. To assess this possibility a comprehensive histological analysis has been performed using transillumination-assisted microdissected segments. In segments from *TLF*$^{-/-}$ mice, no mature spermatozoa were

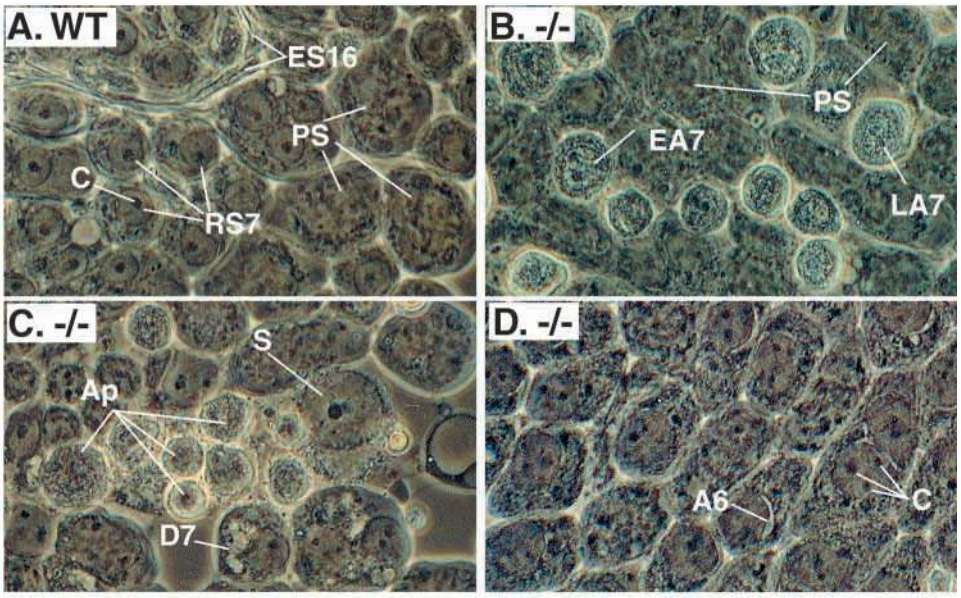

Fig. 7. Live germ cell cytology in microdissected segments of seminiferous tubules from wild-type (WT) and *TLF*$^{-/-}$ (–/–) mice. (A) Stage VII segment from a wild-type mouse. (B,C) Stage VII segments from a *TLF*$^{-/-}$ mouse. (D) Stage VI segment from a *TLF*$^{-/-}$ mouse. EA and LA, early and late apoptotic round spermatids respectively; C, chromocenter; S, Sertoli cell. A, acrosome. Ap, apoptotic round spermatids; D degenerating round spermatids. ES16. Step 16 elongated spermatid. 1000× magnification.

observed, but extensive apoptosis of stage 7 round spermatids was evident. Numerous early and late apoptotic round spermatids are seen in segments from *TLF*$^{-/-}$ mice (Fig. 7B), whereas no such cells are observed in wild-type segments (Fig. 7A). Extensive phagocytosis of apoptotic round spermatids by Sertoli cells was observed (Fig. 7C). In contrast, pachytene spermatocytes appeared normal. No apoptotic round spermatids were observed at earlier stages, but rare degenerating elongating spermatids were observed (Fig. 7D). These observations are consistent with those previously described (Martianov et al., 2001; Zhang et al., 2001b).

Abnormal acrosome formation in *TLF*$^{-/-}$ round spermatids

Acrosome maturation begins in stage I-II round spermatids and can be divided in three stages: Golgi, cap, and acrosomal (for a review, see Abou-Haila and Tulsiani, 2000). During the Golgi-phase, small proacrosomal granules appear from the Golgi apparatus and coalesce to form the acrosomal granule the membrane of which fuses with the outer membrane of the nuclear envelope. Newly synthesised granules and cisternae of the Golgi then form the acrosomal vesicle around the acrosomal granule. The cap-phase corresponds to the expansion of the vesicle over the anterior half of the nucleus. In the acrosomal-phase, the contents of the acrosomal vescicule are gradually distributed throughout the cap to form the final acrosome. Although no increase in apoptosis was observed in early stage round spermatids of the TLF-deficient mice, we have found defects in acrosome formation.

Histological analysis revealed that the acrosome was asymmetric in *TLF*$^{-/-}$ spermatids since the darker acrosomic granule, normally situated in the middle, was found at the edge of the acrosome system (Fig. 7D). Electron microscopy indicated that the acrosomal granules did not coalesce in stage II-III spermatids of *TLF*$^{-/-}$ mice, but were found in the cytoplasm in an asymmetric position, on the outer membrane of the nucleus, or invaginated in the nucleus (Fig. 8B-E compared with wild-type Fig. 8A). In the rare surviving elongating spermatids at stages IX-XI, acrosomes were vacuolated and/or not properly associated with the head (Fig. 8G,I compared with wild type, F,H). Hence, TLF deficiency causes acrosomal abnormalities in round spermatids before the onset of apoptosis.

Absence of chromocenter formation in *TLF*$^{-/-}$ round spermatids

Histological analysis of round spermatids showed that the chromocenter, visible as a single dark body in normal spermatids, was fragmented in TLF mutant spermatids (see C in Fig. 7A,D). To avoid possible confusion between the chromocenter and the nucleolus, we validated the location of the nucleolus by performing immunofluorescence with an anti-fibrillarin antibody, which selectively identifies the nucleolus.

In early stage I-II spermatids, the anti-fibrillarin antibody reveals the presence of 2-3 small nucleoli per nucleus, while staining of DNA with Hoechst revealed the presence of a single dense staining chromocenter in the middle of the nucleus, as previously described (Fig. 9A) (Brinkley et al., 1986; Hoyer-Fender et al., 2000; Zalensky et al., 1993). The merged image substantiates that the nucleoli and the chromocenter are distinct structures in spermatids. While the nucleolar staining in the *TLF*$^{-/-}$ spermatids was comparable to wild type, the Hoechst staining clearly shows that the chromocenter is incompletly condensed and fragmented. Instead of one single center, three or more smaller structures are visible. This result is confirmed by electron microscopic examination. In wild-type spermatids, a single chromatin condensation in the center of the nucleus is observed along with a very small and dense nucleolus as observed in the fibrillarin staining (Fig. 8J). In mutant spermatids, several less dense and more peripheral condensations are observed analogous to that observed with Hoechst staining (Fig. 8K).

It has previously been shown that the heterochromatin protein M31 (HP1β), which is associated with constitutive heterochromatin in somatic cells (Minc et al., 1999), localises to the chromocenter in spermatids (Hoyer-Fender et al., 2000). To determine whether the closely related HP1α protein is also localised to the chromocenter in spermatids and whether

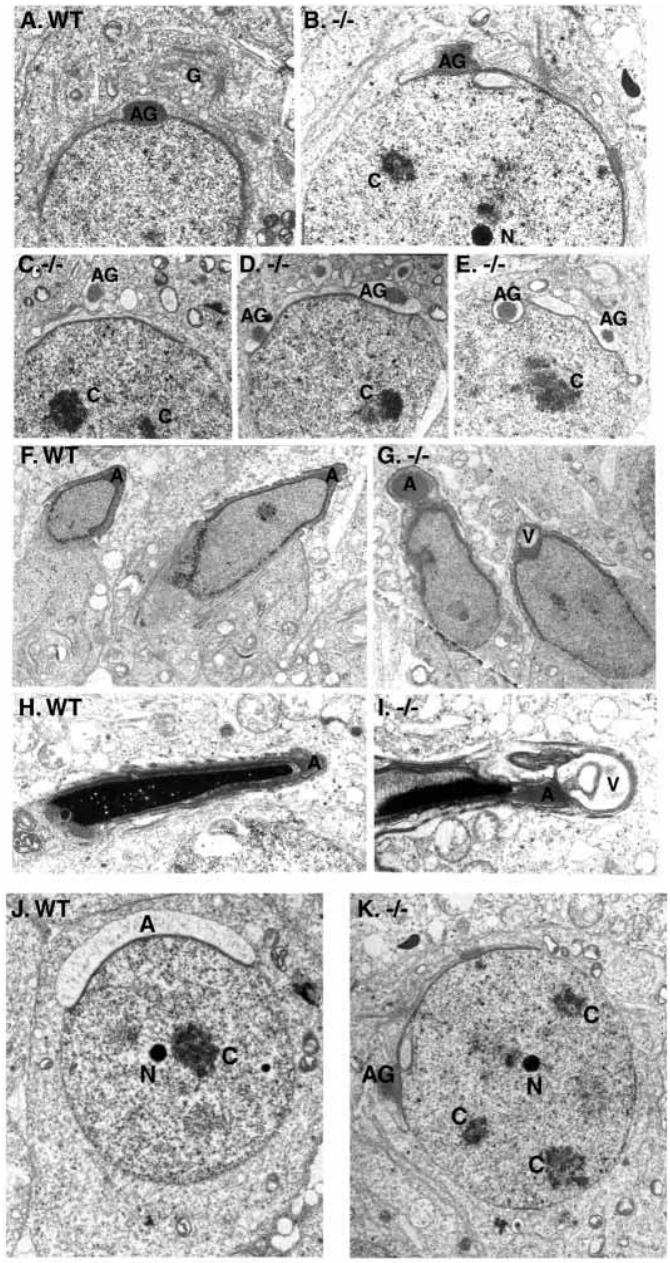

Fig. 8. Electron microscopic sections of wild-type and *TLF*$^{-/-}$ seminiferous tubules. (A-I) High magnification views of defective acrosome formation. (J) Wild-type and (K) mutant cell showing position of organelles. (A) Normal acrosome in wild-type spermatids; (B-E) defective acrosome in round spermatids; (F-G) acrosome in early wild-type or mutant elongating spermatids; (H-I) defective acrosome in late step 13 wild-type or mutant elongating spermatids. AG, acrosomal granule; G, Golgi; N, nucleolus; V, vacuole; C, chromocenter; A, acrosome. B shows a close-up of the acrosome of the cell shown in K. A,C-G: 4000× magnification; B,H-K, 5000× magnification.

fragmentation of the chromocenter observed here in the Hoechst staining is accompanied by a mislocalisation of HP1α, we performed immunofluorescence with an anti-HP1α monoclonal antibody (Nielsen et al., 2001). As described for M31(HP1β) (Hoyer-Fender et al., 2000), HP1α is also uniquely associated with the chromocenter in wild-type spermatids (Fig. 9B). In mutant spermatids, HP1α colocalises with each of the fragmented heterochromatic foci (Fig. 9B). In contrast to spermatids, where abnormal heterochromatin localisation is seen, the distribution of heterochromatic loci as observed by Hoechst staining and HP1α localisation was comparable in both wild-type and mutant pachytene spermatocytes (Fig. 9B). These results indicate that defects in heterochromatin organisation occur only in the round spermatids. Therefore, the organisation of heterochromatin is dramatically altered in *TLF*$^{-/-}$ spermatids, underscoring a critical role for TLF in this process.

DISCUSSION

The identification of TBP-like factors posed a number of questions on their function, their expression patterns, and their molecular mechanisms of action. The finding that mammalian TLF is not essential for embryonic development, as its counterparts in other organisms (Dantonel et al., 2000; Kaltenbach et al., 2000; Muller et al., 2001; Veenstra et al., 2000), but is instead crucial for the differentiation program of spermiogenesis (Martianov et al., 2001; Zhang et al., 2001b), begs the question of what are the distinct functions of TLF and TBP in this system. We have explored this issue by comparing the detailed expression patterns of TBP and TLF in male germ cells. This approach has allowed the uncovering of an unexpected function of TLF in the heterochromatic organisation of the chromocenters of round spermatids.

Distinct expression patterns of TLF and TBP in male germ cells

Expression of TBP and TLF genes is strongly upregulated with the appearance of pachytene spermatocytes and haploid spermatids at post-natal week 3-4, slightly earlier than the upregulation of the transcriptional activator CREM, which is restricted to haploid cells (Foulkes et al., 1992). High expression of TBP and TLF in late pachytene spermatocytes is confimed by RNase protection using purified pacyhtene cells. Our present results are in agreement with those previously obtained showing a developmental upregulation of TBP and TLF expression (Schmidt and Schibler, 1995; Zhang et al., 2001a). However, while it has been shown that TLF mRNA is expressed in pachytene and round spermatids (Martianov et al., 2001; Zhang et al., 2001a), it has previously been suggested that TBP mRNA upregulation and overexpression was specific to round spermatids (Schmidt and Schibler, 1995). The results on mRNA expression and immunofluorescence (see below) shown here indicate that TBP is also strongly overexpressed in late pachytene spermatocytes and is not restricted to haploid cells (see also Persengiev et al., 1996).

The differences in TBP and TLF expression are highlighted when the respective proteins are detected by immunofluorescence. TBP is present in all cell types, although it is expressed at much higher levels in pachytene spermatocytes and haploid round spermatids compared to zygotene and leptotene spermatocytes. This is in agreement with the high mRNA expression seen in the pachytene spermatocytes and confirms that TBP overexpression begins in late meiotic cells. TBP levels decrease as the spermatids

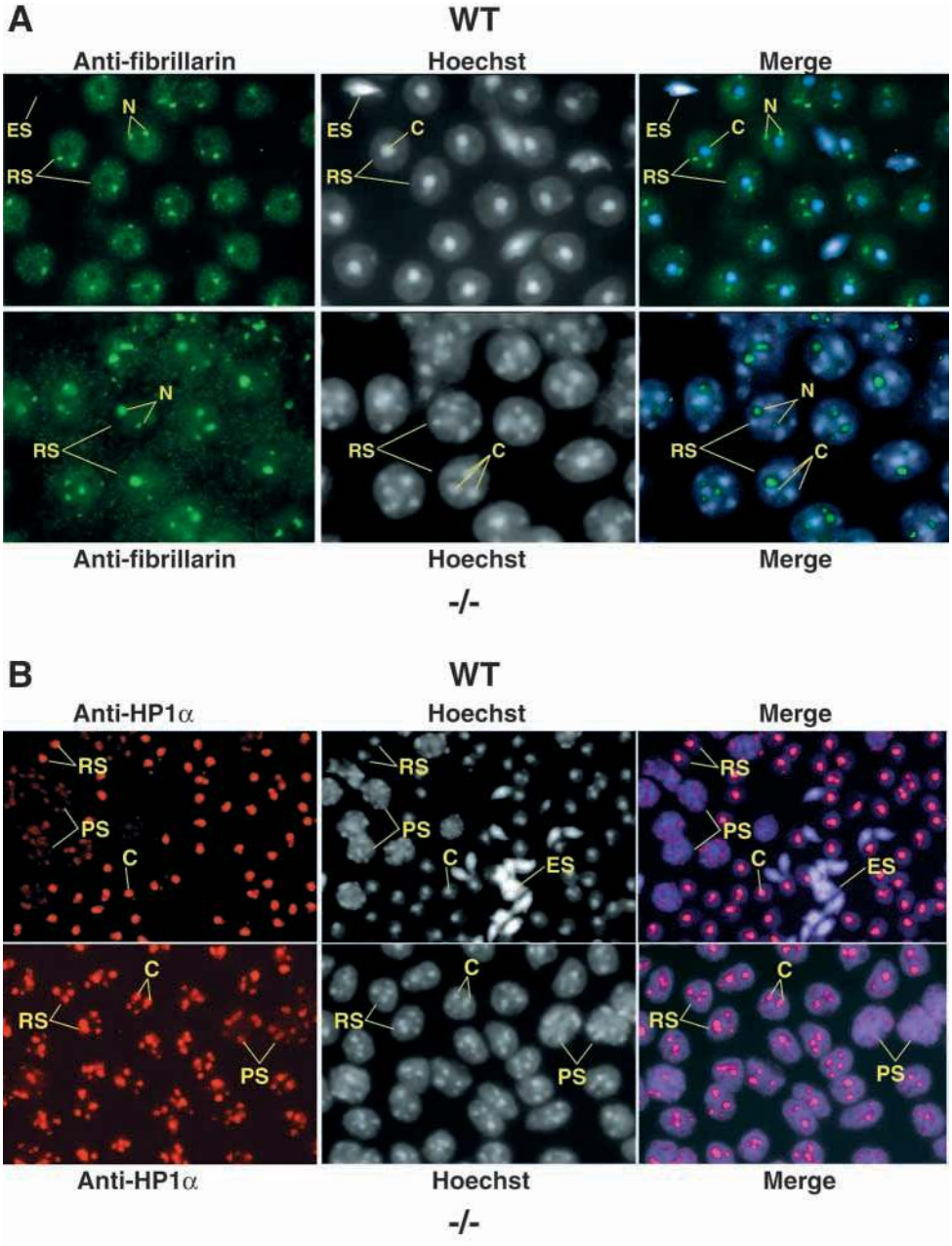

Fig. 9. Immunodetection of fibrillarin and HP1α in wild-type and TLF mutant spermatids. (A) Immunodetection of fibrillarin in wild-type (above) and mutant (below) spermatids. Representative nucleoli (N) and chromocenters (C) are indicated. (B) Immunodetection of HP1α. Nomenclature is as described in A.

elongate, but surprisingly, perinuclear TBP can be detected again in almost mature spermatozoon. This late expression may be due to the translation of stored mRNA, as is often the case for transcripts in germ cells (for a review, see Steger, 1999). Alternatively, it is possible that TBP molecules are present at all stages during the elongation phase, but are shielded from the antibody by the condensed chromatin.

TLF presents a more dynamic pattern of expression. It is first present in stage IV-V pachytene spermatocytes, where it is both cytoplasmic and nuclear, before becoming restricted to the nucleus at later stages. This result differs from that reported by Zhang et al. (Zhang et al., 2001a) where TLF mRNA was first detected only in stage VIII pachytene spermatocytes. The onset of expression is clearly prior to this stage. TLF expression decreases sharply in early round spermatids, but reappears in stage IV spermatids and persists during the early elongation steps. By step 10 little TLF is visible, but it reappears in a perinuclear and eventually cytoplasmic location at steps 12-14.

This expression pattern suggests that TLF functions at multiple steps in the differentiation pathway and not only in step 7 spermatids when apoptosis takes place. The results of the histological analysis presented here provide evidence that this is indeed the case. We observe severe acrosomal defects in early stage spermatids. These defects include aberrant localisation and lack of coalescence of the acrosomal granules and vacuolisation. Despite these abnormalities, we did not observe defects in expression of genes involved in acrosome formation, such as proacrosin binding protein (Martianov et al., 2001), Tpx-1 and Sp56 (data not shown). A more thorough analysis of *TLF*$^{-/-}$ spermatids will be required to identify the changes in gene expression responsible for these defects.

TLF is essential for heterochromatin organisation in haploid cells

In normal mammalian spermatids, the centromeric heterochromatin of each chromosome is condensed into a single chromocenter structure (Brinkley et al., 1986; Zalensky et al., 1993). This structure contains the known heterochromatin associated protein M31 (HP1β) (Hoyer-Fender et al., 2000) and was found to serve as an organiser for the subsequent packaging of DNA in a well defined chromosomal topology in the sperm cell nucleus (Meyer-Ficca et al., 1998; Zalensky et al., 1995). We show here that the structuring of the chromocenter is disrupted in *TLF*$^{-/-}$ spermatids. Instead of a single center, multiple smaller structures are observed. The heterochromatin-associated protein HP1α is localised uniquely at the chromocenter in wild-type spermatids and is found in each of the smaller bodies in mutant spermatids, confirming that they correspond to heterochromatic foci. These results demonstrate that TLF is essential for the formation of the chromocenter and thus for heterochromatin organisation in spermatids.

TLF may regulate heterochromatin topology directly and/or indirectly. TLF is present in late meiotic cells where it may control the expression of genes required for chromocenter formation in early spermatids. The chromocenter is a structure unique to haploid spermatids and at present the proteins responsible for its formation are not known. The finding of differentially expressed genes in wild-type and TLF mutant spermatids may facilitate the identification of proteins involved in this process. In addition, TLF may play a more structural role. This is suggested by the fact that TLF is present in heterochromatic structures in late meiotic cells where it may act to facilitate subsequent condensation. However, TLF is not expressed in very early spermatids when the chromocenter is formed rendering unlikely the possibility that it participates directly in chromocenter formation. However, in later stage spermatids, TLF is localised, although not uniquely, to the chromocenter. TBP, in contrast, is excluded from the transcriptionally silent heterochromatin in both pachytene spermatocytes and haploid spermatids consistent with its role as a transcription factor. The presence of TLF in both the heterochromatic and euchromatic compartments suggests that it may have a dual role, both as a classical transcription factor and as a structural factor as we suggested previously (Martianov et al., 2001).

The fragmentation of the chromocenter and the resulting aberrant heterochromatin organisation may provoke altered gene expression during maturation of round spermatids. It may also interfere with DNA condensation and packaging at the elongation phase of spermiogenesis. Either one of these possibilities or a combination of both may lead to the differentiation arrest and apopotosis at the transition from round to elongating spermatids seen in *TLF*$^{-/-}$ mice. Together our results show that TLF plays an essential role in spermatid heterochromatin organisation and that in the absence of TLF, the disorganisation of heterochromatin is the principle cause of spermiogenesis arrest.

It has been suggested that TLF acts as a surrogate transcription factor for TBP (for a review, see Berk 2000; Verrijzer, 2001). While this may be the case, our findings reveal a novel facet of TLF function. Here we have established a direct link between TLF and chromatin organisation which reasonably explains the drastic spermatogenesis phenotype observed in TLF-deficient mice. It remains to be established whether the important TLF function seen in early embryos in *C. elegans*, zebrafish and *Xenopus* can be ascribed to its ability to act as a modulator of chromatin structuring and/or as a classical transcription factor. Indeed, although this possibility was not investigated, the strong conservation of TLF suggests that its molecular properties have been maintained during evolution.

We thank F. Cammas, R. Losson, L. Tora and D. Hernandez-Verdun for gifts of antibodies and materials. J. L. Vonesch and staff of the confocal microscopy facility. E. Heitz for technical assistance. I. M. was supported by a fellowship from the Human Frontier Science Programme. This work was supported by grants from the CNRS, the INSERM, the Hôpital Universitaire de Strasbourg, the Ministère de la Recherche et de la Technologie, the Association pour la Recherche contre le Cancer, the Ligue Nationale contre le Cancer, Organon (Akzo/Nobel) and the Human Frontier Science Programme research grants RG0196 (to I. D.) and RG0240 (to P. S.-C.).

REFERENCES

Abou-Haila, A. and Tulsiani, D. R. (2000). Mammalian sperm acrosome: formation, contents, and function. *Arch. Biochem. Biophys.* **379**, 173-182.

Albright, S. R. and Tjian, R. (2000). TAFs revisited: more data reveal new twists and confirm old ideas. *Gene* **242**, 1-13.

Bell, B. and Tora, L. (1999). Regulation of gene expression by multiple forms of TFIID and other novel TAF$_{II}$-containing complexes. *Exp. Cell Res.* **246**, 11-19.

Berk, A. J. (2000). TBP-like factors come into focus. *Cell* **103**, 5-8.

Brinkley, B. R., Brenner, S. L., Hall, J. M., Tousson, A., Balczon, R. D. and Valdivia, M. M. (1986). Arrangements of kinetochores in mouse cells during meiosis and spermiogenesis. *Chromosoma* **94**, 309-317.

Brou, C., Chaudhary, S., Davidson, I., Lutz, Y., Wu, J., Egly, J. M., Tora, L. and Chambon, P. (1993). Distinct TFIID complexes mediate the effect of different transcriptional activators. *EMBO J.* **12**, 489-499.

Chiang, C. M., Ge, H., Wang, Z., Hoffmann, A. and Roeder, R. G. (1993). Unique TATA-binding protein-containing complexes and cofactors involved in transcription by RNA polymerases II and III. *EMBO J.* **12**, 2749-2762.

Chomczynski, P., Qasba, P. and Topper, Y. J. (1986). Transcriptional and post-transcriptional roles of glucocorticoid in the expression of the rat 25,000 molecular weight casein gene. *Biochem. Biophys. Res. Commun.* **134**, 812-818.

Comai, L., Tanese, N. and Tjian, R. (1992). The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell* **68**, 965-976.

Dantonel, J. C., Quintin, S., Lakatos, L., Labouesse, M. and Tora, L. (2000). TBP-like factor is required for embryonic RNA polymerase II transcription in C. elegans. *Mol. Cell* **6**, 715-722.

Dantonel, J. C., Wurtz, J. M., Poch, O., Moras, D. and Tora, L. (1999). The TBP-like factor: an alternative transcription factor in metazoa? *Trends Biochem. Sci.* **24**, 335-339.

Delmas, V., van der Hoorn, F., Mellstrom, B., Jegou, B. and Sassone-Corsi, P. (1993). Induction of CREM activator proteins in spermatids: down-stream targets and implications for haploid germ cell differentiation. *Mol. Endocrinol.* **7**, 1502-1514.

Fimia, G. M., Morlon, A., Macho, B., De Cesare, D. and Sassone-Corsi, P. (2001). Transcriptional cascades during spermatogenesis: pivotal role of CREM and ACT. *Mol. Cell Endocrinol.* **179**, 17-23.

Fomproix, N., Gebrane-Younes, J. and Hernandez-Verdun, D. (1998). Effects of anti-fibrillarin antibodies on building of functional nucleoli at the end of mitosis. *J. Cell Sci.* **111**, 359-372.

Foulkes, N. S., Borrelli, E. and Sassone-Corsi, P. (1991). CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**, 739-749.

Foulkes, N. S., Mellstrom, B., Benusiglio, E. and Sassone-Corsi, P. (1992). Developmental switch of CREM function during spermatogenesis: from antagonist to activator. *Nature* **355**, 80-84.

Gangloff, Y., Romier, C., Thuault, S., Werten, S. and Davidson, I. (2001). The histone fold is a key structural motif of transcription factor TFIID. *Trends Biochem. Sci.* **26**, 250-257.

Green, M. R. (2000). TBP-associated factors (TAF$_{II}$s): multiple, selective transcriptional mediators in common complexes. *Trends Biochem Sci* **25**, 59-63.

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. *Microbiol. Mol. Biol. Rev.* **62**, 465-503.

Hampsey, M. and Reinberg, D. (1999). RNA polymerase II as a control panel for multiple coactivator complexes. *Curr. Opin. Genet. Dev.* **9**, 132-139.

Hansen, S. K., Takada, S., Jacobson, R. H., Lis, J. T. and Tjian, R. (1997). Transcription properties of a cell type-specific TATA-binding protein, TRF. *Cell* **91**, 71-83.

Hernandez, N. (1993). TBP, a universal eukaryotic transcription factor? *Genes Dev.* **7**, 1291-1308.

Holmes, M. C. and Tjian, R. (2000). Promoter-selective properties of the TBP-related factor TRF1. *Science* **288**, 867-870.

Hoyer-Fender, S., Singh, P. B. and Motzkus, D. (2000). The murine heterochromatin protein M31 is associated with the chromocenter in round spermatids and is a component of mature spermatozoa. *Exp. Cell Res.* **254**, 72-79.

Kaltenbach, L., Horner, M. A., Rothman, J. H. and Mango, S. E. (2000). The TBP-like factor CeTLF is required to activate RNA polymerase II transcription during C. elegans embryogenesis. *Mol. Cell.* **6**, 705-713.

Kim, J. L. and Burley, S. K. (1994). 1.9 A resolution refined structure of TBP recognizing the minor groove of TATAAAAG. *Nat. Struct. Biol.* **1**, 638-653.

Kim, J. L., Nikolov, D. B. and Burley, S. K. (1993). Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* **365**, 520-527.

Lescure, A., Lutz, Y., Eberhard, D., Jacq, X., Krol, A., Grummt, I., Davidson, I., Chambon, P. and Tora, L. (1994). The N-terminal domain of the human TATA-binding protein plays a role in transcription from TATA-containing RNA polymerase II and III promoters. *EMBO J.* **13**, 1166-1175.

Liu, D., Ishima, R., Tong, K. I., Bagby, S., Kokubo, T., Muhandiram, D. R., Kay, L. E., Nakatani, Y. and Ikura, M. (1998). Solution structure of a TBP-$TAF_{II}230$ complex: protein mimicry of the minor groove surface of the TATA box unwound by TBP. *Cell* **94**, 573-583.

Maldonado, E. (1999). Transcriptional functions of a new mammalian TATA-binding protein- related factor. *J. Biol. Chem.* **274**, 12963-12966.

Martianov, I., Fimia, G. M., Dierich, A., Parvinen, M., Sassone-Corsi, P. and Davidson, I. (2001). Late arrest of spermiogenesis and germ cell apoptosis in mice lacking the TBP-like TLF/TRF2 gene. *Mol. Cell* **7**, 509-515.

Meistrich, M. L., Longtin, J., Brock, W. A., Grimes, S. R., Jr. and Mace, M. L. (1981). Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. *Biol. Reprod.* **25**, 1065-1077.

Meyer-Ficca, M., Muller-Navia, J. and Scherthan, H. (1998). Clustering of pericentromeres initiates in step 9 of spermiogenesis of the rat (*Rattus norvegicus*) and contributes to a well defined genome architecture in the sperm nucleus. *J. Cell Sci.* **111**, 1363-1370.

Minc, E., Allory, Y., Worman, H. J., Courvalin, J. C. and Buendia, B. (1999). Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* **108**, 220-234.

Molina, C. A., Foulkes, N. S., Lalli, E. and Sassone-Corsi, P. (1993). Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell* **75**, 875-886.

Moore, P. A., Ozer, J., Salunek, M., Jan, G., Zerby, D., Campbell, S. and Lieberman, P. M. (1999). A human TATA binding protein-related protein with altered DNA binding specificity inhibits transcription from multiple promoters and activators. *Mol. Cell Biol.* **19**, 7610-7620.

Muller, F., Lakatos, L., Dantonel, J., Strahle, U. and Tora, L. (2001). TBP is not universally required for zygotic RNA polymerase II transcription in zebrafish. *Curr. Biol.* **11**, 282-287.

Nantel, F., Monaco, L., Foulkes, N. S., Masquilier, D., LeMeur, M., Henriksen, K., Dierich, A., Parvinen, M. and Sassone-Corsi, P. (1996). Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature* **380**, 159-162.

Nielsen, A. L., Oulad-Abdelghani, M., Ortiz, J. A., Remboutsika, E., Chambon, P. and Losson, R. (2001). Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol. Cell* **7**, 729-739.

Nikolov, D. B., Chen, H., Halay, E. D., Usheva, A. A., Hisatake, K., Lee, D. K., Roeder, R. G. and Burley, S. K. (1995). Crystal structure of a TFIIB-TBP-TATA-element ternary complex. *Nature* **377**, 119-128.

Ohbayashi, T., Makino, Y. and Tamura, T. A. (1999). Identification of a mouse TBP-like protein (TLP) distantly related to the drosophila TBP-related factor. *Nucleic Acids Res.* **27**, 750-755.

Ohbayashi, T., Shimada, M., Nakadai, T. and Tamura, T. A. (2001). TBP-like protein (TLP/TLF/TRF2) artificially recruited to a promoter stimulates basal transcription in vivo. *Biochem. Biophys. Res. Commun.* **285**, 616-622.

Orphanides, G., Lagrange, T. and Reinberg, D. (1996). The general transcription factors of RNA polymerase II. *Genes Dev.* **10**, 2657-2683.

Parvinen, M. and Hecht, N. B. (1981). Identification of living spermatogenic cells of the mouse by transillumination-phase contrast microscopic technique for 'in situ' analyses of DNA polymerase activities. *Histochemistry* **71**, 567-579.

Persengiev, S. P., Robert, S. and Kilpatrick, D. L. (1996). Transcription of the TATA binding protein gene is highly up-regulated during spermatogenesis. *Mol. Endocrinol.* **10**, 742-747.

Rabenstein, M. D., Zhou, S., Lis, J. T. and Tjian, R. (1999). TATA box-binding protein (TBP)-related factor 2 (TRF2), a third member of the TBP family. *Proc. Natl. Acad. Sci. USA* **96**, 4791-4796.

Schmidt, E. E. and Schibler, U. (1995). High accumulation of components of the RNA polymerase II transcription machinery in rodent spermatids. *Development* **121**, 2373-2383.

Steger, K. (1999). Transcriptional and translational regulation of gene expression in haploid spermatids. *Anat. Embryol.* **199**, 471-487.

Takada, S., Lis, J. T., Zhou, S. and Tjian, R. (2000). A TRF1:BRF complex directs Drosophila RNA polymerase III transcription. *Cell* **101**, 459-469.

Tan, S., Hunziker, Y., Sargent, D. F. and Richmond, T. J. (1996). Crystal structure of a yeast TFIIA/TBP/DNA complex. *Nature* **381**, 127-151.

Teichmann, M., Wang, Z., Martinez, E., Tjernberg, A., Zhang, D., Vollmer, F., Chait, B. T. and Roeder, R. G. (1999). Human TATA-binding protein-related factor-2 (hTRF2) stably associates with hTFIIA in HeLa cells. *Proc. Natl. Acad. Sci. USA* **96**, 13720-13725.

Veenstra, G. J., Weeks, D. L. and Wolffe, A. P. (2000). Distinct roles for TBP and TBP-like factor in early embryonic gene transcription in xenopus. *Science* **290**, 2312-2315.

Verrijzer, C. P. (2001). Transcription factor IID – not so basal after all. *Science* **293**, 2010-2011.

Wieczorek, E., Brand, M., Jacq, X. and Tora, L. (1998). Function of TAF_{II}-containing complex without TBP in transcription by RNA polymerase II. *Nature* **393**, 187-191.

Zalensky, A. O., Allen, M. J., Kobayashi, A., Zalenskaya, I. A., Balhorn, R. and Bradbury, E. M. (1995). Well-defined genome architecture in the human sperm nucleus. *Chromosoma* **103**, 577-590.

Zalensky, A. O., Breneman, J. W., Zalenskaya, I. A., Brinkley, B. R. and Bradbury, E. M. (1993). Organization of centromeres in the decondensed nuclei of mature human sperm. *Chromosoma* **102**, 509-518.

Zhang, D., Penttila, T., Morris, P. L. and Roeder, R. G. (2001a). Cell- and stage-specific high-level expression of TBP-related factor 2 (TRF2) during mouse spermatogenesis. *Mech. Dev.* **106**, 203-205.

Zhang, D., Penttila, T. L., Morris, P. L., Teichmann, M. and Roeder, R. G. (2001b). Spermiogenesis deficiency in mice lacking the Trf2 gene. *Science* **292**, 1153-1155.