# Tetanus toxin light chain expression in Sertoli cells of transgenic mice causes alterations of the actin cytoskeleton and disrupts spermatogenesis

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Communicated by P.Gruss

Tetanus toxin is a powerful neurotoxin known to inhibit neurotransmitter release. The tetanus toxin light chain is a metalloprotease that cleaves some members of the synaptobrevin gene family with high specificity. Here, we report the expression of a synthetic gene encoding the tetanus toxin light chain in the seminiferous epithelium of transgenic mice. Spermatogenesis was severely impaired and mature spermatozoa were completely absent. Late spermatids exhibited pleomorphic shapes and acrosomal distortions. The number of Leydig cells was greatly increased. In situ hybridization analysis revealed that the toxin acts on Sertoli cells. Affected cells exhibited an aberrant distribution of actin filaments and many cells contained large vacuoles. Our results demonstrate that tetanus toxin is active in non-neuronal cells and suggest an important function for members of the synaptobrevin gene family during the late stages of spermatogenesis.
Key words: actin/neurotransmitter release/testis/tetanus toxin/transgenic mice

## Introduction

Bacterial protein toxins are powerful tools in the investigation of cellular functions in transgenic mice. Diphtheria toxin A-chain has been used to ablate acinar pancreatic cells (Palmiter et al., 1987), nuclear fiber cells of the lens (Breitman et al., 1987) and growth hormone-producing cells in the pituitary (Behringer et al., 1988). Pituitary hyperplasia and gigantism have been induced in transgenic mice by cholera toxin (Burton et al., 1991). Tetanus toxin (TeTx) is a highly potent neurotoxin produced by the anaerobic spore-forming bacterium Clostridium tetani (for reviews see Habermann and Dreyer, 1986; Niemann, 1991). It causes convulsions by blocking the release of inhibitory neurotransmitters in the central nervous system. TeTx is composed of two polypeptide chains generated by proteolytic cleavage of a single precursor molecule. The heavy chain (H-chain; mol. wt 98 300) is necessary for internalization in neuronal

cells, retrograde axonal transport and transfer of the light chain from endosomes into the cytoplasm. The light chain (L-chain; mol. wt 52 288) was shown to be sufficient to block $Ca^{2+}$-induced catecholamine release by permeabilized bovine chromaffin cells (Ahnert-Hilger et al., 1989). Injection of in vitro synthesized mRNA encoding the light chain into cholinergic neurons of the Aplysia californica buccal ganglion leads to a blockade of neurotransmitter release within 50 min (Mochida et al., 1990). Recently, it was shown that tetLC is a $Zn^{2+}$-dependent protease and that synaptobrevin-2 and cellubrevin are targets for its proteolytic activity (Link et al., 1992; Schiavo et al., 1992a). Synaptobrevin-2 (VAMP-2) is an integral membrane protein of small synaptic vesicles (Baumert et al., 1989). Cellubrevin is a ubiquitously expressed member of the synaptobrevin family and has been localized in the recycling vesicular compartment (McMahon et al., 1993).

Because of the unusual codon usage of the TeTx gene (Eisel et al., 1986; Fairweather and Lyness, 1986), expression of gene fragments in Escherichia coli and in eukaryotic cells is very inefficient (Makoff et al., 1989; Mochida et al., 1990). To express the TeTx L-chain in mammalian cells, we have therefore synthesized a gene with an altered codon usage (tetLC). We show that tetLC expression in Sertoli cells of transgenic mice induces an alteration in the F-actin distribution and results in the disruption of spermatogenesis.

## Results

### Synthesis and expression of a gene encoding the tetanus toxin light chain

We synthesized a 1496 bp gene encoding for tetLC by using 50 oligonucleotides (Figure 1). The codon usage was adapted to fit the translational requirements of mammalian cells by raising the [G+C] content. This was achieved mainly with changes in the second and third codon position. In addition, we eliminated putative splice and polyadenylation signals from the coding sequence.

In vitro transcription/translation followed by SDS–PAGE shows that the synthetic gene can be efficiently translated in rabbit reticulocyte lysates and yields the expected mol. wt 52 000 protein. The tetLC gene could also be expressed in CV-1 cells (Figure 2).

### Tetanus toxin light chain constructs

Three different constructs were generated for tetLC expression in transgenic mouse lines (Figure 3). SV40tetLC (Figure 3A) was expressed at low levels in several tissues in earlier experiments (U.Eisel and A.Zimmer, unpublished results). POMCtetLC contains the rat pro-opiomelanocortin promoter (Figure 3B). This promoter has been used to direct the expression of the bacterial enzyme β-galactosidase to the anterior lobe of the mouse pituitary (Hammer et al., 1990).
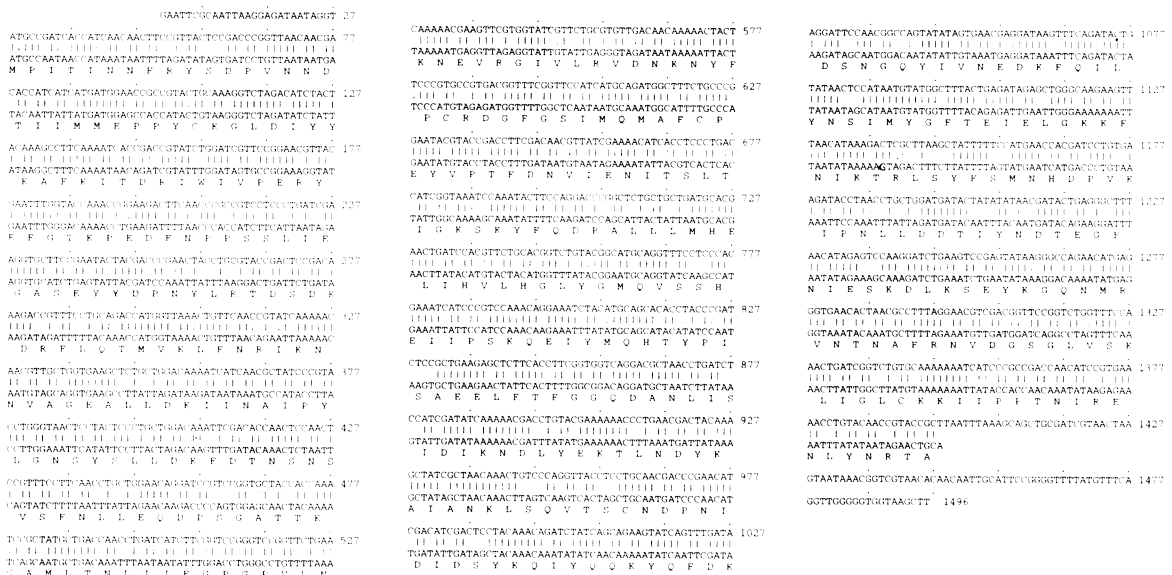
**Fig. 1.** Sequence of the synthetic tetLC gene. The alignment of the synthetic tetLC gene (upper sequence) with the coding sequence of the original gene from *C.tetani* (lower sequence; Eisel *et al.*, 1986) demonstrates the multiple changes that were introduced to improve expression. The amino acid sequence is identical in both the synthetic and the original gene. EMBL/GenBank accession number L19522.

The third construct, L7tetLC, was generated by introducing the tetLC cassette upstream of the initiation codon in the second exon of the L7 gene (Figure 3C). An L7−LacZ fusion construct has been shown to be expressed in Purkinje cells of the cerebellum and in bipolar cells of the retina of transgenic mice (Oberdick *et al.*, 1990). In Northern blot analysis, L7 transcripts were not detected in the testis (Oberdick *et al.*, 1988), while the endogenous POMC gene is expressed there (Gizang-Ginsberg and Wohlgemuth, 1987). Surprisingly, all three constructs expressed in the same pattern in seminiferous tissue. We believe that a cryptic Sertoli cell-specific promoter element may be present in the synthetic tetLC gene, although we cannot exclude the possibility that all three promoters are coincidentally active in Sertoli cells. Such a cryptic promoter element would not be unusual. The Herpes Simplex Virus (HSV) type 1 thymidine kinase (tk) gene, for example, contains a promoter that directs HSV-1 tk expression to haploid germ cells (Al-Shawi *et al.*, 1991).

## Male transgenic mice expressing tetanus toxin light chain in the seminiferous tissue are sterile

We obtained several transgenic founder mice from each construct (Table I). None of the animals were obviously impaired in their development or behavior. We could establish only one transgenic mouse line derived from a male founder (POMCtet26). All other male founder animals were either sterile or did not transmit the transgene to their offspring. A total of five transgenic lines were derived from female founder animals. All male transgenic offspring from these lines were sterile.

Semi-thin sections of transgenic testes revealed that spermatogenesis in these animals was severely impaired (Figure 4). Late stages of spermatids were much less frequent than in non-transgenic animals. Mature sperm cells corresponding to stage VIII (step 16 spermatids; Oakberg, 1956) were never seen in transgenic testes. The number of residual bodies was increased in all atrophic tubules. In some of these
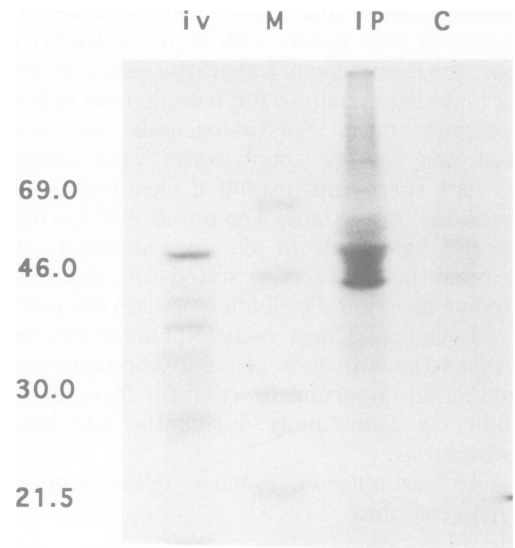


**Fig. 2.** Expression of tetLC *in vitro* and in CV-1 cells. Autoradiograph of a SDS−polyacrylamide gel shows that tetLC product after *in vitro* translation (iv) and immunoprecipitation of transiently transfected CV-1 cells (IP). The immunoprecipitated product also shows some smaller bands, possibly degradation products. CV-1 cells transfected only with pSV-SPORT1 showed no such products after immunoprecipitation (C). The size of mol. wt markers (M) is indicated at the left.

residual bodies we found structures resembling remnants of later spermatids. We could also detect a large number of vacuoles in the transgenic seminiferous epithelium. However, the average number of spermatogonia per tubule, and the number and morphology of round spermatids, seemed to be unaffected. These findings indicate that germ cells may disintegrate at later stages of development.

The heads of later spermatids from transgenic mice were pleomorphic (Figure 4E and F). Some nuclei contained invaginations or intranuclear vacuoles and some of the intranuclear vacuoles showed inclusions (Figure 4E). The

**A**

SV40    tetLC    tl pA

500bp

**B**

rPOMC    tetLC    tl pA

500bp
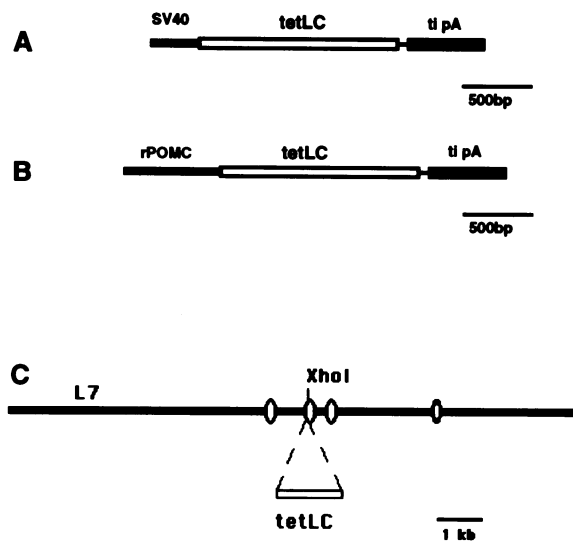
**C**    L7    XhoI

tetLC    1 kb

Fig. 3. Schematic representation of three constructs used for tetLC expression in transgenic mice. (A) pSVtetLC contains the SV40 early promoter, the tetLC fragment and the small t-intron and polyadenylation signal. This construct was also used for transient tetLC expression in tissue culture cells. (B) In pPT1, the SV40 promoter was exchanged for a 770 bp fragment containing the rat POMC promoter. (C) pL7tetLC contains a genomic clone with the L7 promoter, the four exons of the L7 structural gene and 3'-non-coding sequences. The tetLC fragment was introduced into a XhoI site 12 bp upstream of the initiator ATG in the second exon of the L7 gene.

**Table I.** Transgenic mouse lines

| Founder | Sex | Offspring | Male sterility[a] | Expression in testis |
|---------|-----|-----------|-------------------|----------------------|
| L7tetLC/C1 | M | 0 | + | + |
| L7tetLC/C4 | M | 57 | − | − |
| L7tetLC/E2 | F | 12 | + | + |
| SVtetLC/B7 | F | 20 | nd | nd |
| SVtetLC/B8 | F | 37 | + | + |
| SVtetLC/D5 | M | 0 | + | nd |
| SVtetLC/D6 | M | 45 | nd | nd |
| POMCtet8 | M | 0 | + | + |
| POMCtet9 | M | 0 | + | nd |
| POMCtet11 | M | 30 | − | − |
| POMCtet15 | F | 14 | + | + |
| POMCtet18 | F | 21 | + | nd |
| POMCtet26 | M | 73 | + | + |
| POMCtet32 | M | 0 | + | nd |
| POMCtet57 | F | 20 | nd | nd |

M = male founder; F = female founder; nd = not determined.
[a]Assessed in founder and/or filial generation.

acrosome of later spermatids from transgenic testes was very often irregularly shaped and in some cases hyperplastic. We also found clusters of spermatids that appeared to be at different stages of spermatogenesis (Figure 5F). This observation is surprising because germ cell development in normal testes is highly synchronized (Figure 4A) (Willison and Ashworth, 1987). The number of Leydig cells in the interstitium of transgenic testis was also increased (Figure 4B).

To analyze the tetLC expression pattern, we performed *in situ* hybridization studies. We found expression in the seminiferous epithelium of 15- and 22-day-old mice, and in adult animals. No expression was seen in the interstitium

of testes or in brain, pituitary, heart, muscle, kidney, liver lung, intestine, spleen and ovary (data not shown).

We could not conclude from the *in situ* hybridization data whether expression occurs in one or both cell types of the seminiferous epithelium as Sertoli cells form a close meshwork in which germ cells are embedded.

POMCtet26 was the only male founder from which we could establish a transgenic line. This founder was probably highly mosaic, as he had 73 offspring and only one of these was a transgenic female (Figure 5). This hypothesis was supported by *in situ* hybridization analysis of testis sections from transgenic F2 males and from the founder animal (Figure 6). Transgene expression in F2 animals could be seen in all of the seminiferous tissues (Figure 6C). In contrast, expression in the founder testis was patchy; only ~30% of the tubules showed strong expression (Figure 6A). tetLC-expressing tubules in POMCtet26 were abnormal, whereas the other tubules produced spermatozoa (Figure 6B). This indicates that transgenic spermatozoa are viable and expression of tetLC in Sertoli cells caused sterility.

### Expression of tetLC in Sertoli cells alters the actin pattern

To determine whether tetLC expression affects cytoskeletal components of the Sertoli cells, we used rhodamine-coupled phalloidin to stain actin filaments in cryosections. In the testes of non-transgenic animals, we detected the typical stage-specific actin filament pattern (Vogl, 1989). A strong linear actin staining at the tunica propria shows actin filaments in myoid cells. Finger-like structures in the Sertoli cell cytoplasm and cap-like structures around the heads of late spermatids represent ectoplasmic specializations. Dense staining near the concave surface of the late spermatids comes from F-actin associated with tubulobulbar complexes (Figure 7C and E).

The actin filament pattern in tetLC-expressing tissue was changed dramatically (see Figure 7D and F). The amount of actin filament was increased and it was distributed all over the Sertoli cell cytoplasm. Double staining with DAPI and phalloidin showed that most actin filaments were not associated with spermatids in transgenic testes. No alterations of the actin distribution in myoid cells or in interstitial cells were observed. It was important to determine whether the disturbed actin pattern in transgenics preceded the pathological effects or was caused by the failure to complete spermatogenesis. Therefore, we stained immature testis from day 22 non-transgenic and transgenic mice. At this stage, we did not find any obvious morphological abnormalities in transgenics. However, as shown in Figure 7A and B, the F-actin distribution in Sertoli cells of transgenic mice was already altered at this age. In non-transgenic testes, a network of actin was found throughout the tubule. Actin in the Sertoli cells of transgenic testes exhibited dense staining in the epithelium, often accumulating in parallel fibers or in patches, instead of forming a network-like structure. Thus, changes in actin distribution preceded other morphological effects.

### Discussion

We have generated transgenic mice expressing a synthetic tetLC gene under the control of either the POMC, the L7 or the SV40 promoter. All three constructs expressed at high
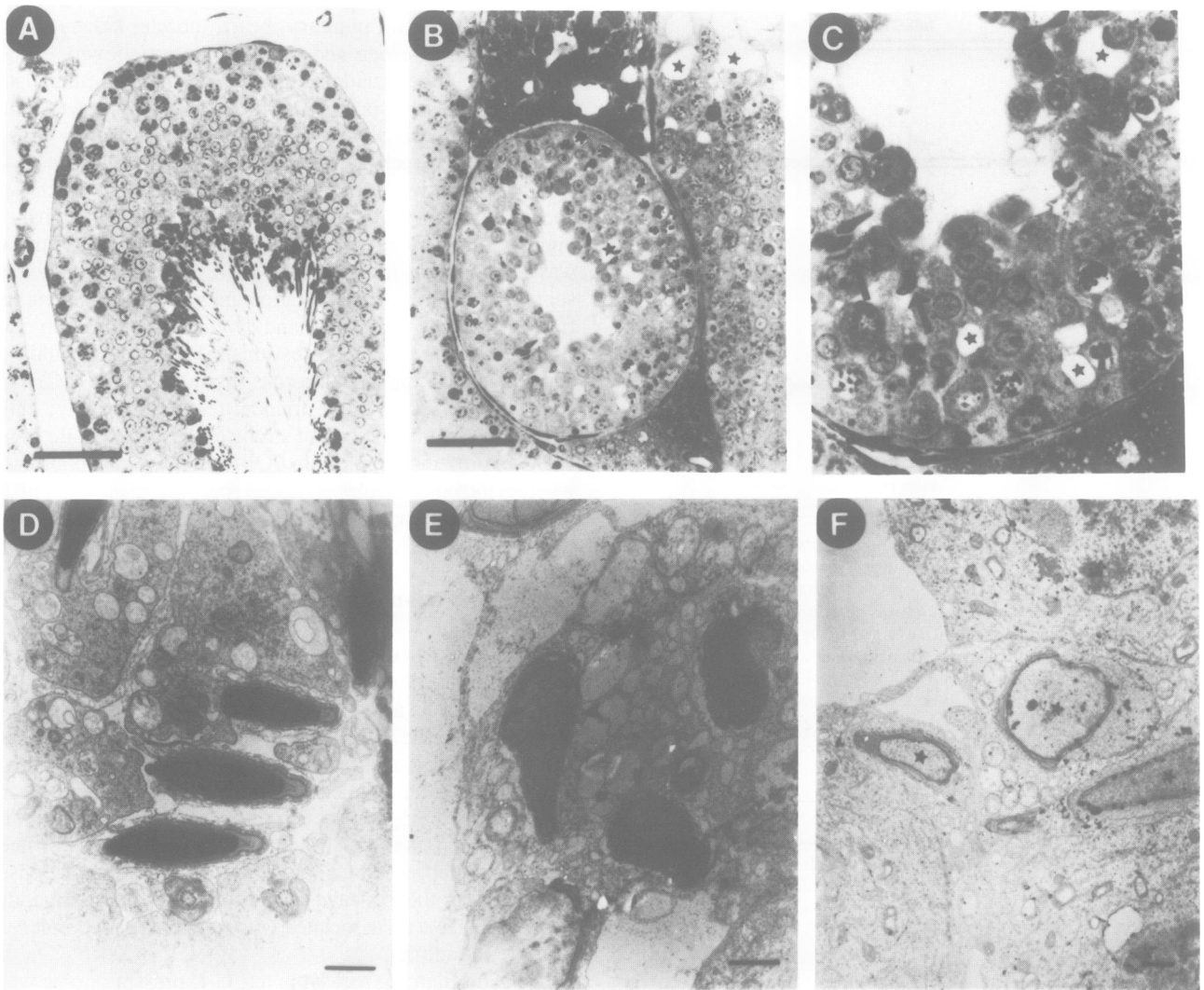
**Fig. 4.** Histological analyses of testes from transgenic and non-transgenic animals. (A) Semi-thin section of a stage VIII seminiferous tubule of a non-transgenic animal. Ony a few Leydig cells can be seen in the interstitium. Round spermatids, beginning to form acrosomes, and step 16 spermatids prior to release can be seen in the adluminal part of the seminiferous epithelium. (B) Overview of a seminiferous tubule, interstitium and neighbouring tubules of a transgenic animal. Note that the tubule is atrophic and the interstitium is almost completely filled with Leydig cells. (C) At higher magnification, a large number of round cell bodies can be seen close to the lumen of the tubule. Arrows point to abnormally shaped late spermatids. Stars indicate vacuoles in the seminiferous epithelium. Bars represent 50 $\mu$m in (A) and (B), and 10 $\mu$m in (C). (D) Electron micrograph of spermheads from non-transgenic seminiferous epithelium. Note that germ cell development is highly synchronized. (E) Electron micrographs of transgenic spermheads exhibiting intranuclear inclusions (arrowhead) or acrosomal hyperplasia and deformation (arrow). Spermheads often exhibit bizarre shapes and the surrounding tissue shows many vacuoles. No organized structure is detectable. (F) Clusters of spermheads in transgenic animals at different stages of nuclear condensation (stars). Bars represent 1 $\mu$m in (D)−(F).

levels in the seminiferous epithelium, which resulted in failure to complete spermatogenesis. While early germ cell differentiation appeared normal, spermatids (step 11−13) failed to elongate and were clearly defective. Late spermatids were reduced in their number and exhibited distorted spermheads, acrosomal defects and intranuclear vacuoles. Similar observations can be made in a number of human andrological disorders characterized by oligo-, terato- or azoospermia (Holstein, 1975), as well as in infertile patients with inflammatory bowel disease (Hrudka and Singh, 1984). Several lines of evidence suggest that Sertoli cells are primarily affected by tetLC expression: (i) transgenic spermatozoa appear to be viable in non-transgenic seminiferous epithelium of a mosaic founder; (ii) the tetLC expression pattern is compatible with expression in Sertoli cells, but not with expression in late spermatids; (iii) vacuoles found in the seminiferous epithelium are typically associated
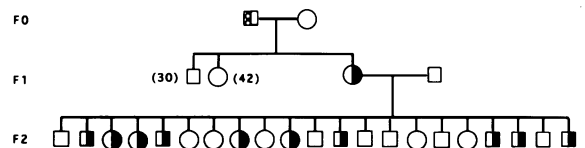


**Fig. 5.** Pedigree of a mouse line derived from POMCtet26. The mosaic founder (F0) was fertile and had 73 offspring. The only transgenic animal of the F1 generation was a female. Male transgenic animals of the F2 generation were sterile. Squares represent male and circles female mice. Blank squares or circles indicate non-transgenic animals, half-dotted indicates mosaic, half-black represents hemizygous animals.

with Sertoli cell damage (Russell *et al.*, 1990); (iv) characteristic alterations in the Sertoli cell cytoskeleton are observed in juvenile mice prior to the accumulation of germ
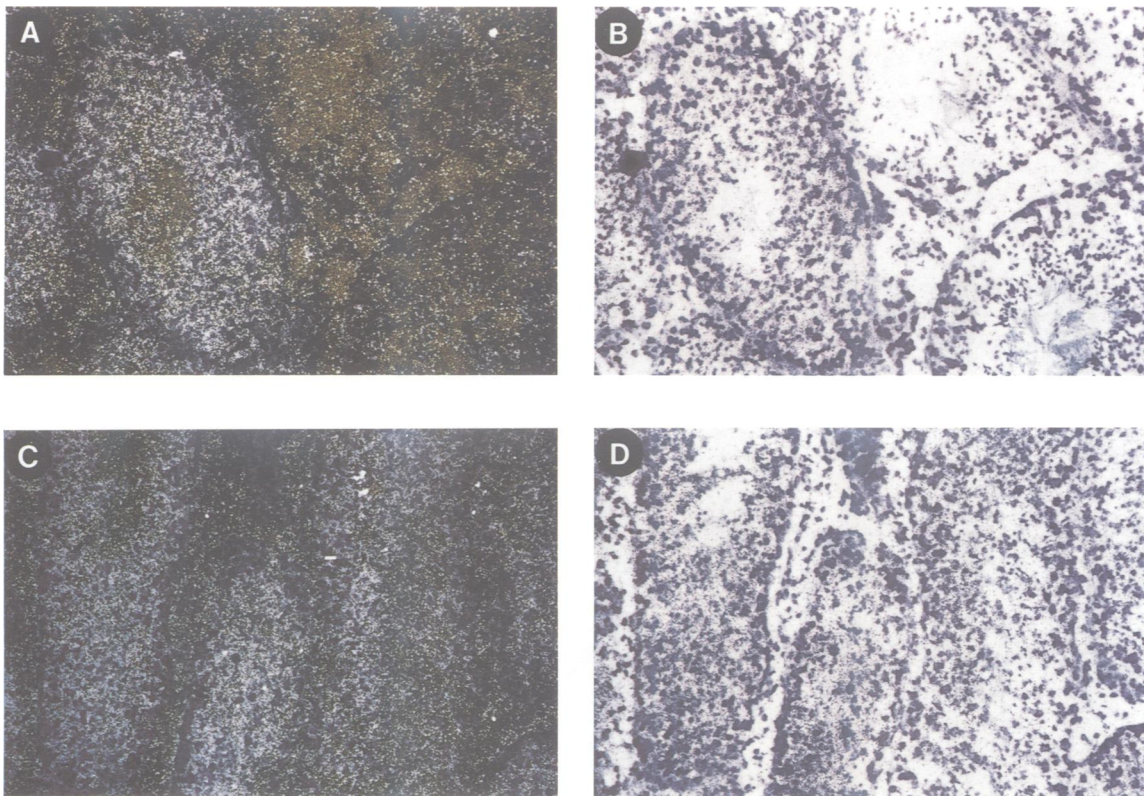
**Fig. 6.** Transgene expression in mosaic and hemizygous POMCtet26 mice. (**A**) Darkfield photograph of testis cryosections from the mosaic founder hybridized with an anti-sense riboprobe against the synthetic tetLC gene. Strong expression can be observed in a tubule in the left part of the photograph. Tubules on the right show no expression. Expression was always observed in large areas of the tubule, indicating a clonal origin of Sertoli cells. (**B**) Brightfield photograph of (A). Expressing tubules exhibited the same phenotype as in sterile mice, while normal spermatogenesis was observed in non-expressing tubules. (**C**) Darkfield photograph of testis from a sterile F2 mouse shows expression throughout the whole testis. (**D**) Brightfield photograph reveals that all tubules are atrophic.

cell defects. Defects observed in germ cells are, therefore, most likely caused by Sertoli cell damage.

Interestingly, the number of Leydig cells in the interstitium is also increased in transgenic testes. An increase in the steroid-producing Leydig cells in the interstitium can also be seen in the testes of infertile patients suffering from Klinefelter syndrome or cryptorchidism (Wong *et al.*, 1973; Lipschultz, 1976). Because tetLC expression is restricted to the seminiferous epithelium, the increased number of Leydig cells in the interstitium could be the result of a paracrine disturbance. Paracrine factors such as angiotensin II, epidermal growth factor, macrophage-derived growth factor, interleukin I, insulin-like growth factor and a luteinizing hormone-releasing hormone (LHRH)-like factor have been reported to be involved in Leydig cell regulation (Chubb and Ewing, 1991). The loss of later spermatids or impaired Sertoli cells could result in the absence of controlling factors for Leydig cell growth.

TeTx L-chain inhibits neurotransmitter release from most, if not all, neuronal cells. To our knowledge, there has been no previous observation of TeTx L-chain effects on non-neuronal cells. This may be due to an intrinsic cell type specificity, e.g. inefficient transportation of TeTx L-chain to its molecular site of action in non-neuronal cells. Despite the fact that TeTx has been known for several decades to interfere with synaptic transmission, the molecular mechanisms of the toxin action remained elusive. One report indicated that TeTx and botulinum toxin type A (BoNTA) block the $Ca^{2+}$-induced reorganization of F-actin in bovine

chromaffin cells during exocytosis (Marxen and Bigalke, 1991). It is thought that the nerve terminal actin cytoskeleton plays an important role in the regulation of vesicle traffic and neurotransmitter release (Walker and Agoston, 1987; Trimble *et al.*, 1991). Actin assembly/disassembly during the exo-endocytotic cycle seems to be calcium regulated (Bernstein and Bamburg, 1989). Other reports suggested that TeTx stimulates a $Ca^{2+}$-dependent, GTP-modulated tissue transglutaminase (Fachiano and Luini, 1992), and indirectly affects the phosphorylation and translocation of synapsin I (Presek *et al.*, 1992). Synapsin I is involved in the regulation of the actin rearrangement during neurotransmitter release and is also associated with synaptic vesicles (Trimble *et al.*, 1991).

It has recently been shown that tetanus and botulinum toxins are metalloproteases that cleave synaptobrevin-2 (Link *et al.*, 1992; Schiavo *et al.*, 1992a), an 18 kDa protein of small synaptic vesicles (Baumert *et al.*, 1989). The C-terminal part forms a hydrophobic domain that is inserted into the vesicle membrane, while the N-terminal region of synaptobrevin-2 is exposed to the cytoplasm. Synapto-brevin-2 can function as a receptor for soluble *N*-ethylmale-imide-sensitive fusion protein (NSF) attachment proteins. NSF is an essential component of the constitutive fusion machinery and is required for transport vesicle fusion. The yeast gene SEC18, which is homologous to synaptobrevins, is required for transport from the endoplasmic reticulum to the Golgi. The fact that components involved in constitutive fusion events and induced exocytosis interact has been
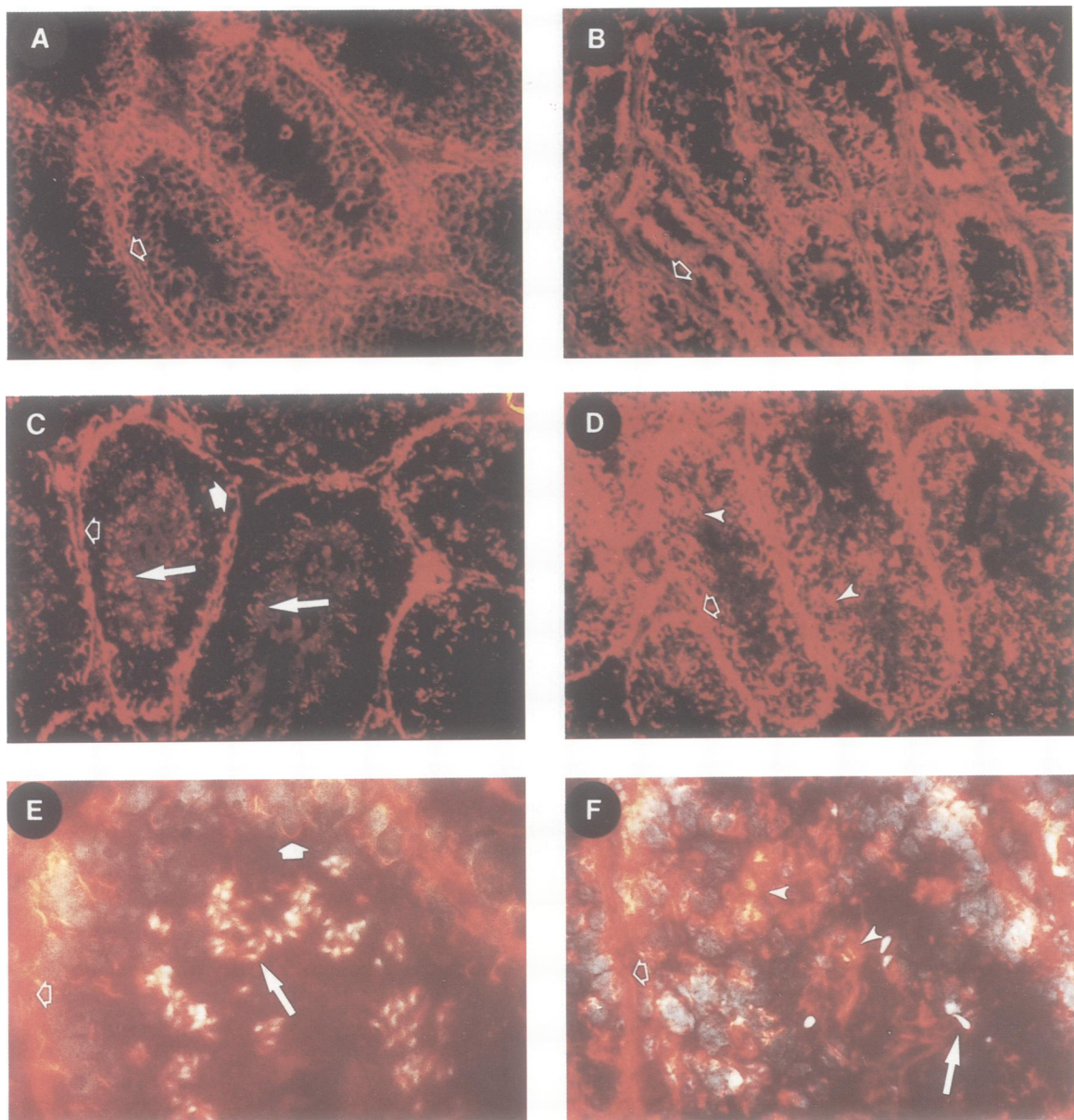
**Fig. 7.** Distribution of F-actin in seminiferous tubules of transgenic and non-transgenic mice. (**A**) F-actin staining of day 22 non-transgenic mice shows a regular network-like pattern. At this age spermiogenesis has not yet started. (**B**) Transgenic tissue of the same age shows a patchy actin distribution. Actin filaments are also arranged in parallel fibers throughout the seminiferous tubules. (**C**) Adult non-transgenic testis shows the typical stage-dependent distribution of F-actin. F-actin is associated mainly with myoid cells (small open arrow) at the basement membrane, Sertoli–Sertoli cell ectoplasmic specializations (small arrow) and Sertoli–germ cell ectoplasmic specializations surrounding the heads of late spermatids (long arrow). (**D**) Transgenic seminiferous epithelium of adult animals shows irregular accumulations of actin filaments in Sertoli cell cytoplasm (arrowheads). The F-actin distribution in myoid cells is not affected (small open arrows). Double staining with DAPI reveals at higher magnification that intense staining is associated with late spermatids in (**E**) non-transgenic tissue, but not in (**F**) transgenic tissue.

interpreted as an indication that the same molecular machinery may underlie both processes (Söllner *et al.*, 1993).

Our results indicate that a substrate for the TeTx L-chain proteolytic activity must also be present in Sertoli cells. We were unable to detect any synaptobrevin-2 expression in Sertoli cells (data not shown). Recently, a new member of the synaptobrevin family, termed cellubrevin, was identified (McMahon *et al.*, 1993). Cellubrevin is expressed in all cell types analyzed and also at high levels in Sertoli cells. The subcellular localization of cellubrevin suggests that it may be involved in the constitutive recycling vesicular pathway. Like synaptobrevin-2, cellubrevin is cleaved by the TeTx

L-chain. This suggests that tetLC acts in Sertoli cells by cleaving cellubrevin, which may interfere with constitutive and/or regulated vesicular fusion processes.

Sertoli cells produce and secrete a large number of nutritional and regulatory products. If these excretory Sertoli cell functions are affected by tetLC expression, this would account for germ cell defects. In this case, alterations in the actin cytoskeleton may be secondary, caused by altered paracrine and/or mechanical influences of Leydig and/or germ cells. An alternative, but not mutually exclusive, hypothesis takes into account that synaptobrevin/cellubrevin may be involved in intracellular vesicle fusion (Söllner *et al.*, 1993). Thus, tetLC expression could interfere with

intracellular membrane traffic and perhaps with the formation of complex membrane structures such as ectoplasmic specialization and tubulobulbar complexes (Vogl, 1989). This would also explain the changes observed in the actin cytoskeleton. Ectoplasmic specializations, tubulobulbar complexes and the accompanying actin cytoskeleton undergo characteristic cycles (Vogl, 1989). This process is hormonally regulated and probably $Ca^{2+}$ dependent. Follicle-stimulating hormone (FSH) acts stage-specifically on Sertoli cells and is modulated by other factors such as testosterone (Kerr et al., 1992). It is important to note that the accumulation of defects in germ cells coincides precisely with the formation of ectoplasmic specializations.

## Materials and methods

### Synthesis of the tetLC gene and plasmid construction

Design of the synthetic tetLC gene was aided by the GCG program (Devereux et al., 1984). Standard procedures were performed as described previously (Sambrook et al., 1989). Synthetic oligonucleotides (30–80mers) were purified, phosphorylated and annealed. Linker molecules (3 or 4 linkers/reaction) were ligated for 3–8 h at room temperature using T4-DNA-ligase (Boehringer/Mannheim) in buffer supplied by the manufacturer. DNA fragments (200–400 bp) were separated from smaller molecules by agarose gel electrophoresis. Bands of the appropriate size were excised and DNA was extracted using the Geneclean or Mermaid Kit (Bio101) according to the manufacturer's instructions. Overhanging ends were designed to fit into unique sites of the recipient vector pSP73 (Promega). 5'-Ends of the flanking oligonucleotides were not phosphorylated to avoid multiple insertion. A total of six different subfragments were cloned and finally sequenced with Sequenase Kit (United States Biochemical) using [$\alpha$-$^{35}$S]dCTP (New England Nuclear) and primers specific for the SP6 and T7 promoter sequences. Subfragments were isolated and finally assembled to one synthetic gene fragment designated tetLC. The sequence of the final construct pLC2 was verified.

pSVtetLC was generated by insertion of the EcoRI−HindIII tetLC fragment into the polylinker of the expression vector pSV-SPORT 1 (Bethesda Research Laboratories). To create a construct containing the rat POMC promoter, pSVtetLC was cut with AseI and overhangs were filled with Klenow polymerase. After subsequent EcoRI digestion, a 2 kb fragment was isolated and cloned into a Bluescript II SK$^+$ vector cut with EcoRI and EcoRV. The resulting clone was designated pbluetetLC. A 770 bp blunt-ended HindIII−XhoI rat POMC promoter fragment (kindly provided by J.L.Roberts) was inserted into the SamI site of pbluetetLC. The final construct was named pPT1. The L7 gene was inserted into pGEM 3 vector and contained a linker-created XhoI site in the second exon 12 bp upstream of the initiator ATG (a generous gift from J.I.Morgan). The L7 fragment (8 kb) was excised with SalI and cloned into the XhoI site of Bluescript II SK$^+$. The now singular XhoI site was cut and filled using Klenow polymerase (Boehringer/Mannheim). The EcoRI−HindIII overhangs of the tetLC fragment were made blunt ended and the fragment inserted into the pre-treated L7 construct, resulting in the plasmid pblueL7tetLC.

### In vitro transcription/translation and expression in tissue culture cells

The pLC2 construct was linearized with HindIII for in vitro transcription. SP6 polymerase (Boehringer/Mannheim) was used to synthesize in vitro mRNA in the presence of 5 mM 5'-GpppG-3' as described previously (Mayer et al., 1988). In vitro translation using rabbit reticulocyte lysate was performed as recommended by the suppliers (Promega). A typical assay was performed using 2 $\mu$g mRNA in 50 $\mu$l total volume for 1 h at 37°C in the presence of 50 $\mu$Ci [$^{35}$S]L-methionine (New England Nuclear).

The transfection method and tissue culture conditions for CV-1 cells were described previously (Chen and Okayama, 1988). pSVtetLC DNA (25 $\mu$g) was used for transfection of 10$^6$ cells on a 10 cm plate. In vivo labeling of protein was performed 12 h after transfection by growing the cells in the presence of DMEM (Gibco) containing 10% fetal bovine serum (Gibco) and 50 $\mu$Ci [$^{35}$S]L-methionine (New England Nuclear) per ml for 24 h at 37°C. Both pSVtetLC-transfected and pSV-SPORT1-transfected cells were harvested and washed three times in phosphate-buffered saline (PBS). Preparation of cell extracts and immunoprecipitation using a human hyperimmunoglobulin antiserum against tetanus toxoid (a generous gift from Miles Inc.) and protein A−Sepharose (Pharmacia) were performed as described previously (Niemann et al., 1984).

### Pronuclear injection and identification of transgenic mice

For microinjection into mouse oocytes, DNA was excised from the plasmids and purified from vector fragments by sucrose gradient centrifugation. DNA fragments were dialyzed and the DNA concentration was adjusted to a final concentration of 2 $\mu$g/ml in injection buffer [10 mM Tris (pH 7.5), 0.25 mM EDTA]. Transgenic mice were generated as described previously (Hogan et al., 1986). Superovulated FVB/N female mice were used as embryo donors and B$_6$D$_2$F$_1$ females as fosters. Transgenic animals were analyzed by polymerase chain reaction as previously described (Zimmer and Gruss, 1991) using DNA extracted from tail tips.

### Histology, actin staining and in situ hybridization

Testis fixation was performed by perfusion through the heart as described by Russell et al. (1986). Testes were excised, diced into 1 mm cubes and immersed in 5% glutaraldehyde/PBS at 4°C overnight. They were subsequently washed in PBS and post-fixed in 1% osmium tetroxide/1.25% ferrocyanide. After dehydration, tissue samples were embedded in Epon. Semi-thin sections (1 $\mu$m) were cut using an LKB 2088 ultrotome V and stained with toluidine blue for light microscopic examination. Sections for electron microscopy were stained using Ultrastain I and II (Reichert−Jung) and analyzed in a Zeiss EM 109.

For staining of F-actin, testes were quickly frozen, 12 $\mu$m sections were cut in a cryostat (Reichert-Frigocut 2800) and subsequently fixed in acetone at −20°C for 20 min. Sections were washed three times in PBS/1% fetal calf serum and stained for 45 min in 2×10$^{-6}$ M rhodamine-coupled phalloidin (Sigma) at room temperature, and incubated for 10 min at room temperature with 1 $\mu$g/ml DAPI (Sigma) in PBS. After washing three times for 10 min in PBS, the slides were mounted and analyzed in the fluorescence microscope.

For in situ hybridization, an anti-sense riboprobe corresponding to a region between nucleotide 1000 and 1300 of the tetLC fragment was prepared. A sense probe against the same region was used as a negative control. Tissue and probe preparation and in situ hybridization were performed as described previously (Kurtz et al., 1990).

## Acknowledgements

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