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Tctex2: A Sperm Tail Surface Protein Mapping to the *t*-Complex

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Transmission ratio distortion (TRD) in mouse *t*-haplotypes remains the most significant example of meiotic drive in vertebrates. While the underlying mechanism that fuels it is still mysterious, TRD is clearly a complex multigene phenomenon. The characterization of *Tctex2* (*t*-complex testis expressed 2) shows it to be one of several candidates for involvement in TRD. *Tctex2* maps to the *t*-complex and encodes a membrane-associated protein found exclusively on the sperm tail. The *t*-haplotype form of *Tctex2* is aberrant in both the level of its expression and its primary amino acid sequence, but is nonetheless translated and transported to its normal location. The multiple amino acid changes in the *t*-form make it extremely unlikely that it can function normally and, since it is found on sperm tails, suggest that it may actively interfere with the development of normal gamete function in males. The possible role of *Tctex2* in *t*-complex transmission ratio distortion and sterility is discussed.

INTRODUCTION

Meiotic drive, found in several organisms from yeast to mammals, is defined as the non-Mendelian transmission of one allele or a chromosomal segment to more than 50% of the gametes or offspring of a heterozygote. The most dramatic example of meiotic drive is transmission ratio distortion in *t*-bearing male mice. In TRD[3] the transmission of a variant form of mouse chromosome 17, known as a *t*-haplotype, is promoted from heterozygous (+/*t*) males to up to 99% of the offspring. Intensive study of *t*-haplotypes has revealed that they can be found in 25% of wild mouse populations and contain a structurally rearranged region, known as the *t*-complex. The *t*-complex spans the proximal one-third of mouse chromosome 17 and consists of at least four inversions (Hammer *et al.*, 1989). Complete *t*-haplotypes contain all four inversions and usually carry recessive mutations that cause embryonic lethality, while partial *t*-haplotypes, derived in the laboratory from rare recombinants between *t*-chromosomes and wild-type homologs, contain only a portion of the *t*-complex and usually do not carry lethal mutations. Mice homozygous for two complementing *t*-haplotypes (t^x/t^y) are viable, but the males are sterile.

Superficially both +/*t* and *t*/*t* mice produce morphologically normal sperm (Nadijcka and Hillman, 1980). However, accumulated evidence indicates that TRD in +/*t* males is due to dysfunction of the +-sperm, although both types are found in expected numbers in testes and in the uterus after mating (Silver and Olds-Clarke, 1984; Olds-Clarke and Peitz, 1985; Seitz and Bennett, 1985). For sterile t^x/t^y males, their sperm do not reach the oviduct, but are also incapable of fertilization *in vitro*, even when the cumulus cells and zona pellucida are removed from the oocyte (McGrath and Hillman, 1980).

Genetic analysis of partial *t*-haplotypes allowed Lyon to propose a model attributing the *t*-related TRD and sterility to the action of at least four distorters (*Tcd1* to *Tcd4*) on a common target, the responder (*Tcr*). In +/*t* males, the *t*-alleles of the distorters (Tcd^t) "poison" their meiotic partners carrying the wild-type form of the responder (Tcr^+), leading to the transmission in favor of *t*-sperm (Lyon, 1984, 1986; Silver and Remis, 1987). Since the effect of *Tcd*s on *Tcr* can be observed in the *cis*- or *trans*-configuration, it is thought that the products of *Tcd*s must be expressed prior to meiosis and must freely diffuse through the cytoplasmic bridges that link the developing gametes. Furthermore, since the distorters act in an additive way to control the extent of distortion, they must have similar functions or act in the same pathway. In contrast, the Tcr always behaves in a cell autonomous manner, so its product must be retained in the cells in which it is produced (Schimenti *et al.*, 1988). The sterility of *t*-homozygous males is thought to be caused by the same set of genes effecting TRD, but this is still an open issue.

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[3] TRD, transmission ratio distortion; *Tctex2*, *t*-complex testis expressed 2; GST, glutathione *S*-transferase; ER, endoplasmic reticulum; SRP, signal recognition particle; IL-1β, interleukin-1β.

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Attempts to identify genes responsible for *t*-related TRD or sterility have produced several candidate genes. One of these genes, *Tctex2*, was cloned in a subtractive cDNA library screening (Lader *et al.*, 1989). It is expressed exclusively in male germ cells. Compared to wild type, its message is sixfold underexpressed in sterile *t/t* mice (Ha *et al.*, 1991). Moreover, *Tctex2* maps to the third inversion in *t*-complex, the putative location of the *Tcd3* distorter gene (Rappold *et al.*, 1987; Willison *et al.*, 1990).

The map position of *Tctex2* and its aberrant expression in *t*-haplotypes made it a good candidate for a distorter or a sterility factor. The aim of this study was to characterize the biological function of *Tctex2* during spermatogenesis and investigate the mechanism causing its underexpression in *t*-haplotypes. We demonstrate that the aberrant level of message is probably due to a gene copy number difference between wild type and *t*-haplotypes and leads to lower levels of the protein synthesized. We also show that Tctex2 is a sperm tail surface protein stored in cytoplasmic granules during spermatogenesis. The coding sequence of the *t*-form revealed several deletions and base pair transitions compared to wild type. Among them, a proline substitution in the *t*-form alters the secondary structure of the putative protein and could render the protein nonfunctional. Nevertheless, it was determined that the altered protein does not seem to be affected in its localization and although *Tctex2* is underexpressed in *t*-haplotypes, it is equally distributed in all +/*t* spermatids.

MATERIALS AND METHODS

Nomenclature

Tctex2 and *Tcte3* (formerly named *117c3*) were cloned independently in our (Lader *et al.*, 1989) and Lehrach's (Rappold *et al.*, 1987) laboratories. *Tctex2* maps to the same position as *Tcte3* does. A sequence of several independent clones indicated that the first 54 bp of the published *117c3* sequence is a cloning artifact; otherwise, *Tctex2* and *117c3* are virtually identical. Thus, *Tctex2* and *Tcte3* are probably the same gene. However, a missing "G" in the *Tcte3* cDNA shifts the open reading frame 3′ from amino acid 125 and results in a protein that is shorter by 21 amino acids. Since we have sequenced several wild-type and *t*-type cDNAs, as well as genomic DNAs, we consider the sequence reported here to be more reliable. For simplicity's sake, we will refer to this gene by our original name, *Tctex2*.

Mice

All mice used in this study were bred in the *t*-complex colony at The University of Texas at Austin. t^{w5}, t^{w5g}, and t^{w12} are complete *t*-haplotypes. For all quantitative experiments, wild-type and *t*-bearing male mice are congenic on the C3H/DiSn/Art background. When *t/t* mice are indicated, t^{w5g}/t^{w5g} or t^{w5}/t^{w12} mice were used and considered equivalent.

Southern Blot Analysis

Eight micrograms of mouse liver DNA was digested with various restriction enzymes at a concentration of 3 U/μg DNA under standard conditions and loaded onto 0.7% TPE (Tris–phosphate buffer) agarose gel. After electrophoresis, DNA were denatured, neutralized, and transferred to a nylon membrane, Hybond-N (Amersham). The blots were then hybridized with probes which were radiolabeled by a standard random hexamer priming reaction. Exon 2 probe was made by RT-PCR. Total testis RNA from +/+ mice was reverse-transcribed with oligo(dT) primers and amplified with $oligo_{212-232}$ (nucleotides 212–232 of wild-type *Tctex2* transcript) and $oligo_{443-420}$ (complement of nucleotides 420–443) (see Fig. 3B).

RNA Purification and Northern Blot Analysis

Total RNA isolation from mouse testis was prepared with the LiCl/urea method (Ha *et al.*, 1991). Ten micrograms of RNA samples was size-fractionated on 1.1% formaldehyde gels and blotted onto Hybond-N nylon membranes according to the manufacturer's instructions (Amersham). Northern blots were hybridized with random-primed DNA probes, according to standard techniques. The intensity of signals on X-ray films was measured by an ISCO scanner (Model 1312) and an ISCO absorbance/fluorescence detector (UA-5).

Nuclear Runoff

Single-cell suspension of mouse testis was accomplished by the collagenase/trypsin method (Ha *et al.*, 1991) with several modifications. After digestion with trypsin/collagenase, cells were centrifuged through a 5% BSA cushion and washed twice with 10 ml enriched Krebs–Ringer bicarbonate medium plus 10% fetal bovine serum. Nuclei preparation and nuclear runoff assay were performed as described (Linial *et al.*, 1985). Twenty micrograms of each plasmid DNA was slot-blotted and hybridized with [^{32}P]UTP-labeled runoff transcripts at a concentration of 10^7 cpm/ml.

RT-PCR

First-strand cDNA was synthesized with AMV (avian myeloblastosis virus) reverse transcriptase (BRL, Life Technologies, Inc.) from 1 μg total testis RNA in a 20-μl reaction volume. Reverse transcriptase was then heat-inactivated at 95°C for 5 min and 500 μl of 10 m*M* Tris–HCl, 1 m*M* EDTA was added to the reaction. Two microliters of the cDNA pool was used for PCR reactions with

forward primer $oligo_{67-84}$ (nucleotides 67–84) and reverse primer $oligo_{750-733}$ (complement of nucleotides 733–750) for 20 cycles. PCR products were then mixed with nine volumes of 80% formamide loading buffer to ensure complete denaturation and run on 5% urea–polyacrylamide gels. After electrophoresis, DNA was blotted to a nylon membrane by semi-dry transfer blotter (Bio-Rad) and hybridized to the internal primer, $oligo_{212-232}$.

Primer Extension

Primer extension was performed as described (Triezenberg, 1992). Sequences of the primer ($oligo_{204-175}$) used are complementary to nucleotides 175–204 of the wild-type *Tctex2* transcript. Ten micrograms total testis RNA and 10^5 cpm end-labeled primers were used. The RNA–oligomer mixture was heated for 5 min at 85°C and then gradually cooled to 45°C overnight to anneal the oligomers to the RNA.

Screening of Testis cDNA Library and Mouse Genomic Library

A mixed mouse *t/t* testis library (kindly provided by Dr. J. Schimenti, The Jackson Laboratory), made from testis RNAs from t^{Lub1}, t^{Tuw24}, t^{Lub8}, and t^{w2} mice, and a wild-type testis cDNA library (Clontech, U.S.A.) were screened as described (Lader *et al.*, 1989). Sequences of double-stranded DNA were obtained by dideoxy sequencing using Sequenase (USB). A mouse cosmid library from BALB/c and t^{w5g}/t^{w5g} mice were screened with *Tctex2* cDNA.

Antibody Production and Affinity Purification

A GST (glutathione *S*-transferase) expression vector, pGEX 3X, was used to express +-form and *t*-form of *Tctex2* (amino acid 38 to C-terminus) in *Escherichia coli.* The fusion protein was solubilized by adding Sarkosyl to a final concentration of 1.5% and affinity-purified by glutathione beads (Hay and Hull, 1992; Smith and Johnson, 1988). Two rabbits each were immunized with +- and *t*-form of Tctex2 fusion protein prepared by affinity chromatography against the GST resin or by minced polyacrylamide gel slices containing the denatured chimeric protein. Four weeks after the first complete adjuvant subcutaneous injection of 500 μg GST/Tctex2 fusion protein, rabbits were boosted with the same amount of the fusion protein (in incomplete adjuvant) every 2 weeks. Rabbit sera were collected at Day 10 following each boost and immunoaffinity-purified against the GST/Tctex2 fusion protein by MAC active capsules (Amicon). Preabsorption of affinity-purified antisera with acetone powder of bacteria transformed with GST or GST/Tctex2 plasmid was carried out as described (Harlow and Lane, 1988).

Western Blot Analysis

Testis lysate was made by heating two testes in 1.5 ml 1× sample buffer [62.5 m*M* Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol] for 5 min, followed by brief sonication (to shear DNAs) and centrifugation at 12,000*g* for 20 min at 4°C to pellet debris (Harlow and Lane, 1988). Twenty microliters of the supernatant was loaded onto a 15% SDS-PAGE gel. After electrophoresis, proteins were transferred to PVDF membrane (Millipore) by a semi-dry blotting system (Bio-Rad). Sperm proteins were also prepared in 1× sample buffer at a concentration of 1.5×10^7 sperm/ml. Protein concentration was determined by Bio-Rad protein assay kit. Western blots were stained with anti-Tctex2 rabbit sera (1/1000 dilution for crude antisera and 1/200 dilution for affinity-purified antisera) and alkaline phosphatase-conjugated secondary antibody (Boehringer Mannheim) at 1/1000 dilution.

Immunocytochemistry

Frozen, unfixed testes were cryostat-sectioned at 5 μm thickness and mounted on gelatin-coated slides (Beltz and Burd, 1988). Spermatozoa collected from caudae epididymis or vas deferens were air-dried on poly-L-lysine-coated slides. Spermatogenic cells, prepared either by the trypsin/collagenase method or by mechanical disruption (Pratt and Shur, 1993), were also air-dried onto poly-L-lysine-coated slides. In some cases, the slides of cryosectioned testis or spermatogenic cells were fixed with 4% paraformaldehyde plus 0.1% Triton X-100 for 3 min at room temperature, followed by two washes of TBS [20 m*M* Tris-HCl (pH 7.4), 0.9% NaCl]. Immunostaining was accomplished using the Zymed Histostain-SP kit, which utilizes a biotinylated second antibody, a horseradish peroxidase–streptavidin, and a substrate–chromogen (aminoethyl carbozole) mixture to produce a red color at antigen-containing sites. Primary and secondary antibodies were incubated overnight at 4°C and 1 hr at room temperature, respectively. Only immunoaffinity-purified anti-Tctex2 rabbit sera were used for immunocytochemistry.

Immunoprecipitation

Spermatogenic cells were metabolically labeled with [^{35}S]methionine based on the method of Willison *et al.* (1989). The Tctex2 immunocomplex was eluted by boiling in an equal volume of 2× sample buffer. After SDS-PAGE electrophoresis, gels were dried and fluorographed.

Salt Extraction and Trypsin Treatment of Sperm Cells

Freshly isolated spermatozoa (2×10^6/ml) in phosphate-buffered saline (PBS) were washed once at 1000*g*

for 5 min and resuspended in the following solutions containing protease inhibitors (0.5 m*M* PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin, 4 μg/ml bestatin): 3 *M* KCl solution made in PBS buffer with 3 *M* KCl and 1 m*M* EDTA; 100 m*M* Na_2CO_3 solution (pH 11.5); 3 *M* KCl extraction was done at room temperature for 30 min; Na_2CO_3 extraction was performed on ice for 30 min. Sperm were then pelleted and washed once with PBS. The supernatants were concentrated in Centricon 10 (Amicon).

The sperm for trypsin treatment were collected in a modified Hepes buffer [140 m*M* NaCl, 4 m*M* KCl, 4 m*M* Hepes (pH 7.4), 10 m*M* glucose]. After washing once, $MgCl_2$ was added to 5 m*M*. The cells were digested with various concentrations of trypsin for 5 min at room temperature; the reaction was terminated by adding soybean trypsin inhibitor to 100 μg/ml. The spermatozoa were washed with PBS three times, boiled in sample buffer, and loaded onto 15% SDS gels.

In Vitro Translation

Tctex2 cDNAs were generated by RT-PCR from total testis RNA with primers 67 bp 5′ to the first methionine ($Oligo_{68-84}$) and 45 bp 3′ to the last residue of Tctex2 protein ($Oligo_{750-733}$) and were subsequently subcloned into a TA cloning vector (Invitrogen). cDNA clones corresponding to the larger and smaller transcripts were isolated and sequenced. Capped sense RNAs were made by *in vitro* transcription with T7 or Sp6 RNA polymerase in the presence of all four ribonucleotides and 7-methylguanosine (m^7G (5′)ppp(5′)G). The ratio of 7-methylguanosine and guanosine is 3:1. Capped RNAs were then translated in wheat germ cell lysates (Ambion) according to the manufacturer's instructions.

RESULTS

Wild-Type and t-Haplotypes Have Different Copy Numbers of Tctex2

Previous experiments have demonstrated that *Tctex2* is expressed exclusively in male germ cells and that *t*-haplotypes have about sixfold less transcripts than those in wild type (Ha *et al.*, 1991; Willison *et al.*, 1990). To investigate whether the reduced level of *Tctex2* message in *t*-haplotypes is due to divergence of the gene copy numbers, a genomic Southern blot of DNAs from congenic +/+ and *t/t* mice digested with various restriction enzymes was probed with the full-length *Tctex2* cDNA. As shown in Fig. 1A, the band intensity was about six times greater in +/+ when several different restriction enzymes were used. The apparent multiple copies of *Tctex2* in wild type must be very similar to one another because no polymorphism was detected between them when nine enzymes were used. Three different exons

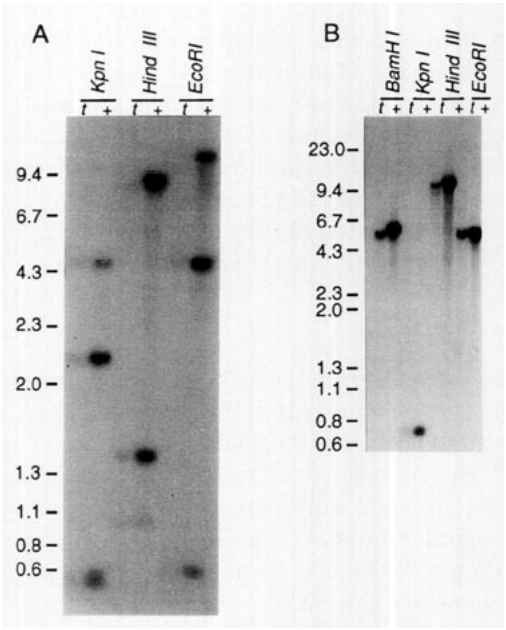

FIG. 1. Southern analyses of *Tctex2* copy number and nonpolymorphism in +-type and *t*-haplotypes. (A) Genomic DNA from *t/t* and +/+ digested with indicated restriction enzymes were probed with full-length *Tctex2* cDNA. (B) Representative autoradiogram of a Southern blot hybridized with exon 2 of *Tctex2*. Molecular size markers are indicated at left in kb.

(first, second, and last) were used as probes on genomic DNA restricted with several enzymes that did not cut in the probes. The result was always one band in both genotypes (Fig. 1B). Furthermore, a pulsed-field gel analysis showed that the multiple copies of *Tctex2* in wild type are clustered in a 480-kb *Not*I fragment, suggesting that they are probably present in a tandem array.

Less Tctex2 Message Is Synthesized in t/t Testis Nuclei

Given the multiple copies present in wild type, it was not clear whether all of the copies were transcribed. It is formally possible that only one of the copies of *Tctex2* in wild type is transcribed at the same rate as the one in *t*-haplotype, but that its transcripts are more stable or are more efficiently transported out of the nuclei. This could lead to the higher message level in wild-type mice. To test this possibility, a nuclear runoff assay was performed on isolated nuclei from +/+ and *t/t* testis. Since the reinitiation of RNA polymerases, as well as the rates of RNA degradation, might vary between samples, experiments were performed with or without heparin, which prevents new initiation of RNA polymerases and acts as an inhibitor of ribonuclease (Groudine *et al.*, 1981). Under both conditions, less *Tctex2* is transcribed in *t/t* nuclei than in +/+ nuclei by a factor of about 6 (Fig. 2A) (data with heparin are not shown). This indicates that the observed relative levels of steady-state

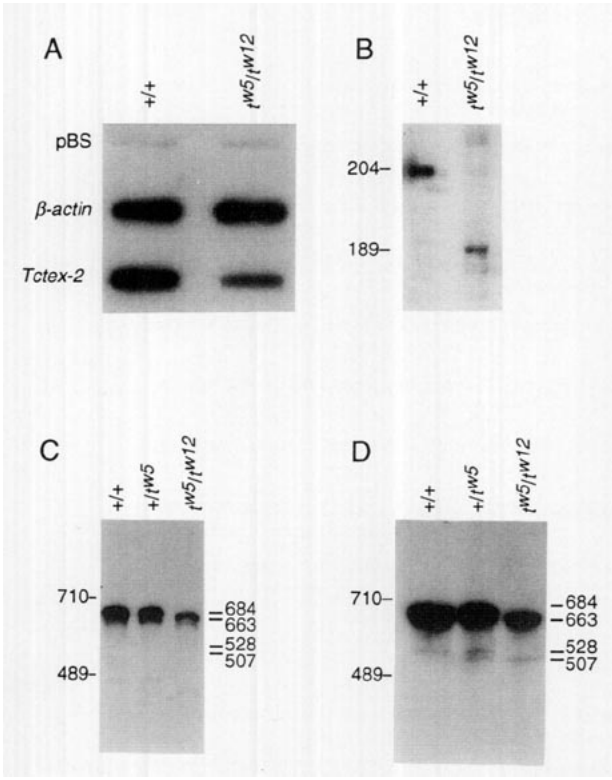

FIG. 2. Nuclear runoff, primer extension, and alternative splicing analyses of *Tctex2* transcripts. (A) [^{32}P]UTP-labeled runoff transcripts from +/+ and *t/t* testis nuclei were used to hybridize blots containing the indicated plasmids. Plasmid DNAs used were pBS, pBluescript (KS) (Stratagene); *Tctex2*, *Tctex2* cDNA in pBluescript vector, and actin, hamster β-actin in pGEM3 vector. (B) Primer extension of total testis RNAs from +/+ and *t/t* mice with oligo$_{204-175}$. The sizes of the extended products are indicated in nucleotides. (C and D) Total testis RNAs from +/+, +/*t*, and *t/t* mice were reverse-transcribed using oligo$_{750-733}$ and amplified with oligo$_{67-84}$ and oligo$_{750-733}$. Amplified products were hybridized to oligo$_{212-232}$. The filter was exposed for 30 min (C) or 5 hr (D). *Hpa*II fragments of pBluescript were end-labeled and used as size markers, indicated at left. The size of each amplified product is indicated in nucleotides at right.

message of *Tctex2* in the two genotypes is not due to post-transcriptional regulation. This experiment does not rule out the possibility that only one of the wild-type copies is transcribed six times more efficiently than the *t*-copy. However, taken together, the results of the nuclear runoff experiment and the homogeneity of the wild-type copies argue that all of the copies are equally transcribed and that the copy number difference most probably accounts for the underexpression of *Tctex2* message in *t*-bearing mice.

The Sequences of the cDNAs and Promoter Regions Are Divergent

The above explanation of the relative differences in amount of *Tctex2* notwithstanding, its male germ-cell-specific expression and its genetic location still qualify it as a candidate gene involved in TRD and/or sterility of the *t*-haplotypes. Thus, we embarked on a comparison of the sequences of cDNA clones and promoter regions from +/+ and *t/t* mice. To define the transcription initiation sites, primer extension and RNase protection assays were carried out and compared (Goldsborough, 1991). The position of the transcription start site defined by primer extension was 3 nucleotides upstream of that located by RNase protection assay (Fig. 2B). Since RNA migrates 5–10% slower than DNA of the same length in a sequencing gel, the 3-nucleotide error was acceptable and the start site defined by primer extension was accepted. Note that the extension product from +/+ RNA was 15 nucleotides longer than that from *t/t* testis (Fig. 2B). This is due to 15 nucleotides deleted in the 5′ untranslated region of the *t*-type of *Tctex2* (see below).

The complete wild-type *Tctex2* cDNA sequence is 0.76 kb in length and revealed an open reading frame encoding 191 amino acids with an inferred molecular mass of 22 kDa and a p*I* of 8.8. When + and *t* copies of the full-length cDNA sequences were compared, several nucleotide substitutions and deletions were noted (Fig. 3B). One of them is a 15-nucleotide deletion revealed by the primer extension. It comprised a 14-nucleotide and one other single-nucleotide deletion in the 5′ untranslated region. Interestingly, two of the three GC consensus core sequences (GGGCGG) for Sp1 binding were in the 14-nucleotide deletion (Fig. 3A). The implication of the deletion in *t*-haplotypes is unclear. However, an electrophoretic mobility shift assay showed that the binding pattern of both the +-probe and the *t*-probe for this region was not affected when tested with various tissue extracts, notably when testis extract was included (data not shown).

The most dramatic change in the open reading frame between +- and *t*-forms of *Tctex2* is that eight nucleotides, GAAAGACT, in the wild-type form are replaced by two nucleotides, CC, in the *t*-form. This results in a six-nucleotide deletion and the conversion of three amino acids, Glu, Arg, and Leu, to one proline in *t*-haplotypes. Data from the predicted secondary structure of the protein indicate that the substitution of proline, a known α-helix breaker, changes the surrounding region from α-helix to β-sheet. The repercussions of this structural change are discussed below.

The sequences of 1.3 kb upstream of the start of transcription in +/+ and *t/t* genomic DNAs are shown in Fig. 3A. Several features within the first 1 kb of the putative promoter were noted. First, three distinct 50-bp repeats, starting at positions −106, −63, and −11 upstream of the transcription start site, were found in a region rich in CpG dinucleotides (Fig. 3C). Second, there are several other consensus sequences for DNA-binding proteins, including TIE (TGF-β inhibitory element) and SRE (serum response element). Third, no TATA box was found even though *Tctex2* has only one transcription

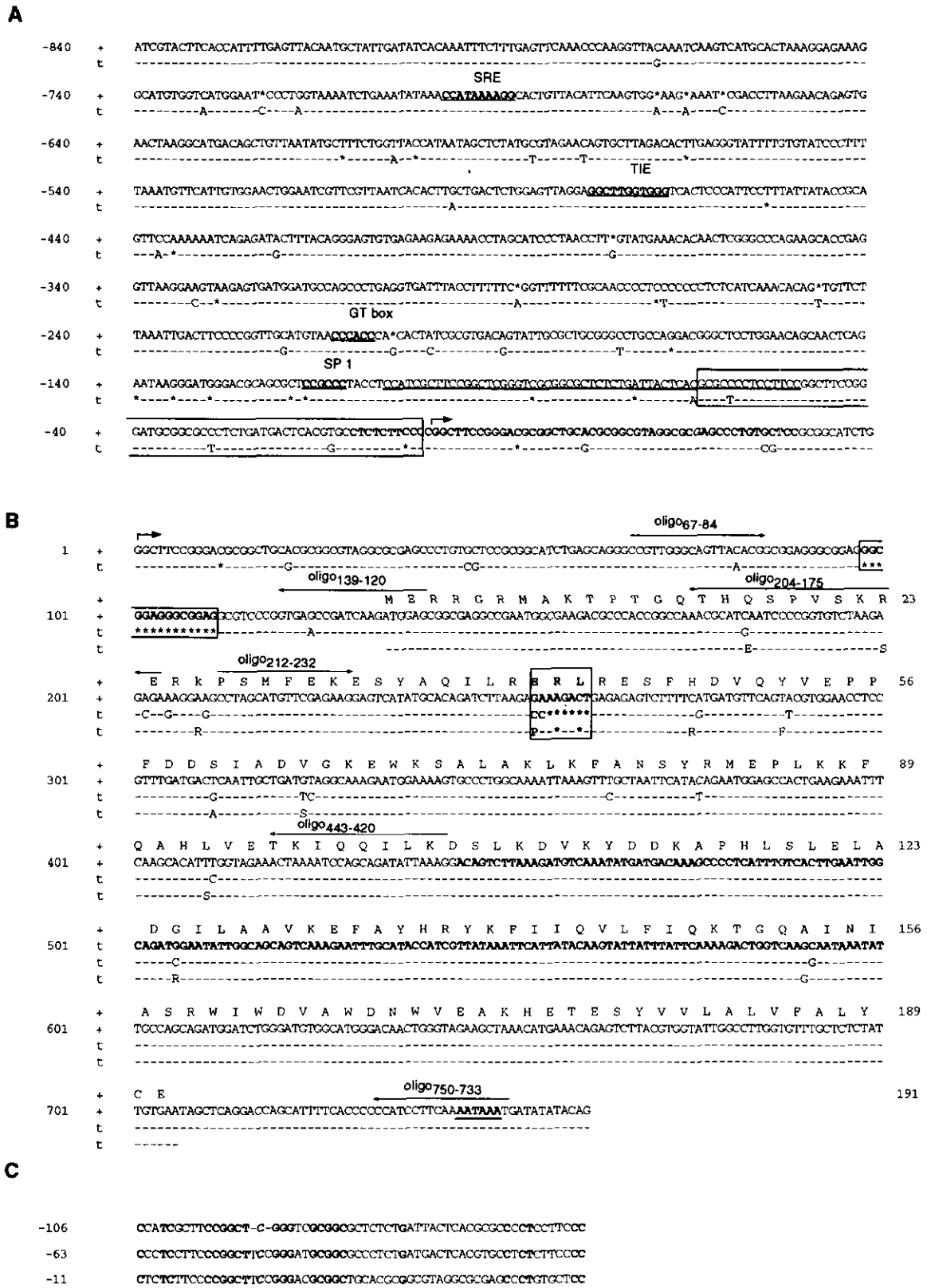

FIG. 3. Sequence of coding and promoter regions of *Tctex2* in wild type and *t*-haplotypes. (A) Promoter sequences of *Tctex2* between wild type and *t*-haplotypes. Only the 840-bp upstream sequence is shown here. Asterisks and dashes denote deletions and identities as in (B). The putative transcription factor binding sites are labeled above the representative sequences, which are boldfaced and underlined. The three overlapping repeats are shown underlined, in a box, and boldfaced, respectively. An arrow indicates the transcription start site. (B) Nucleotide and amino acid sequence of wild type and *t*-type of *Tctex2*. Identities are marked by dashes. Asterisks represent deletions. The amino acid sequence of the

start site. There may be some factors other than the TATA-binding protein that form complexes with RNA polymerase II to define the transcription start site of *Tctex2* and initiate its transcription (Pugh and Tjian, 1990). Finally, a GT box (GGGTGGG) was present in the promoter. This is characteristic of three other testis-specific genes: *mouse protaminel, proacrosine,* and *human phosphoglycerate kinase2* (Goldsborough, 1991). To date it is not known whether the GT box binds to any testis-specific protein and whether it has a role in transcription regulation. Moreover, a number of nucleotide changes were observed between +- and *t*-forms of *Tctex2,* but no major deletions or insertions were found.

There Are Two Tctex2 Transcripts

The sequence data obtained from cDNA clones showed two different splicing products of *Tctex2;* one contains an additional exon, 156 nucleotides in length (Fig. 3B). To further analyze the splicing patterns which might be affected by the sequence divergence between +- and *t*-copies of *Tctex2,* RNA from +/+, +/*t*, and *t*/*t* mice was reverse-transcribed and amplified by PCR with two primers located at the first and last common exons of these two kinds of cDNA clones. All three genotypes have these two transcripts. In addition, the longer one is more abundant than the other by a factor of more than 10 (Fig. 2D). The restriction fragment length polymorphism between +/+ and *t*/*t* testis RNA is due to the deletions (21 nucleotides total) in the *t*-copy. This result demonstrates that the splicing pattern of *Tctex2* and the relative amount of each splicing product are the same in different genotypes.

Underexpression of Tctex2 in t-Haplotypes Is Also Evident at the Protein Level

Translational control, both temporal and quantitative, of several testis-specific genes has been documented. For example, *mouse protamine1,* which replaces histones in sperm nuclei, is not translated until 7 days after its message is synthesized (Kleene *et al.*, 1984). Another example of translational control is *Tctex1,* which was identified in the same differential screening experiment that yielded *Tctex2.* In contrast to *Tctex2,* the *Tctex1* transcript is eightfold overexpressed in *t*/*t* testes, but surprisingly, equal amounts of Tctex1 protein are seen in + and *t* mice (O'Neill and Artzt, 1995). Because of the findings for *Tctex1* and since *Tctex2* message is underexpressed in *t*-haplotypes, it was important to examine the abundance of Tctex2 protein in + and *t* testes. To do so, the *Tctex2* cDNA was subcloned into a GST expression vector to produce a large quantity of GST/Tctex2 fusion protein which was used to immunize rabbits. A protein of about 22 kDa was detected in Western blots of testis by immune sera, but not by preimmune sera (data not shown). This size agreed with the conceptual molecular mass of the polypeptide encoded by the larger transcript which is 22 kDa. The predicted size of the protein encoded by the smaller *Tctex2* message is about 15 kDa. However, since the amount of small transcript is far less than that of the larger one, this may be the reason why the 15-kDa Tctex2 protein was not detected in Western blots.

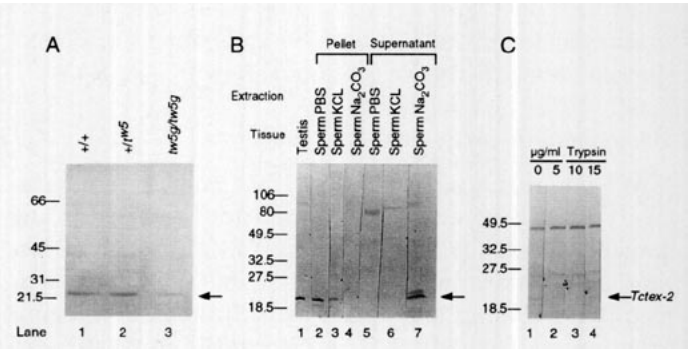

FIG. 4. Quantitative Western blot analysis of Tctex2 in testis and extraction of Tctex2 protein on sperm. (A) Forty micrograms of testis proteins from +/+, +/*t*, and *t*/*t* was stained with crude anti-Tctex2 antisera and alkaline phosphatase-conjugated secondary antibody. Molecular weight markers are shown in kDa. (B) Sperm from the caudae epididymis of wild-type mice were extracted with phosphate-buffered saline (lanes 2, 5), 3 *M* KCl (lanes 3, 6), and 100 m*M* Na_2CO_3 (lanes 4, 7) and immunostained as in (A); lanes 2, 3, and 4 are sperm pellets after treatments; lanes 5, 6, and 7 are supernatants. Lane 1 is total testis protein as positive control. (C) Intact sperm digested with various concentrations of trypsin. Bands of higher molecular masses are due to the background binding of crude rabbit sera to sperm proteins.

To quantify and compare the abundance of Tctex2 protein among different genotypes, equal amounts of testis homogenates from +/+, +/*t*, and *t*/*t* were immunostained with the antisera (Fig. 4A). In contrast to what was observed for *Tctex1,* the underexpression in the message level of *Tctex2* was also reflected at the protein level. It is unlikely that the intensity difference observed between wild type and *t*-haplotypes results from the differential binding affinity of the antibodies to the two allelic forms of the protein, because antisera raised

+-copy of *Tctex2* is indicated above the cDNA sequence; *t*-type is shown below the nucleotide sequence. Nucleotides are numbered on the left and amino acids on the right. The transcription start site is marked with an arrow and numbered 1. The 14-bp deletion is boxed and boldfaced, as is the proline substitution. The polyadenylation site is underlined and in boldface. Sequences of the alternative exon are displayed in boldface. Primers used for RT-PCR and primer extension are indicated by arrows above the relevant sequences. (C) An alignment of the three direct repeats. Nucleotides in common are in boldface.

against fusion protein of both +- and *t*-forms of Tctex2 showed identical results (data not shown).

Tctex2 Is Localized on the Sperm Tail

A comparative search of the DNA and protein database with *Tctex2* revealed no significant homology to any known genes. Thus, to gain further insight into its function, its temporal and spatial distribution were examined in developing male germ cells. Immunocytochemical staining using affinity-purified anti-Tctex2 antisera was performed on testis sections, single-cell preparations from testes, and spermatozoa from epididymi and vas deferens, as described under Materials and Methods. These experiments showed that Tctex2 is distributed along the entire length of the sperm tail, as well as in numerous small granule-like structures in the cytoplasm of spermatogenic cells (Figs. 5A–5F). These granules become denser and more prominent as spermatogenesis proceeds and then decrease in number to three to five granules per spermatid in the late stages of spermiogenesis. The staining for germ cells was specific because Sertoli cells and Leydig cells were negative. Moreover, the positive staining disappeared when the antiserum was preabsorbed with GST/Tctex2 fusion protein, but not with GST protein alone.

It has been shown that some proteins on the surface of sperm are added, modified, or redistributed during epididymal passage or after acrosomal reaction in the oviduct. For example, the distribution of β-1,4-galactosyltransferase in sperm changes dramatically during epididymal passage (Myles, 1993). To investigate whether Tctex2 undergoes any change in distribution, sperm collected from different parts of epididymis, vas deferens, and acrosome-reacted sperm were analyzed on Western blots and by immunocytochemistry. In brief, the data showed that there was no extensive glycosylation or proteolytic cleavage of the protein observed as the sperm move through the genital tract, nor was there any redistribution of the protein on sperm surface during its journey to oocyte (data not shown).

Tctex2 Is a Surface Protein

In an early attempt to localize Tctex2, sperm heads and tails were separated by sonication and separately subjected to Western blot analysis. Surprisingly, Tctex2 was detected in intact sperm, but not in either the head or the tail fractions. This indicated that it was localized to a sonication labile region, either the acrosome or the plasma membrane (Calvin, 1976). Since staining was seen only on the sperm tail, it is likely that the protein is associated with the plasma membrane of the tail. Two lines of evidence further support its cell surface localization. First, Tctex2 protein was completely extracted by 100 m*M* sodium carbonate at pH 11.5. This procedure disassociates peripheral membrane protein from the cell membrane by flattening the membrane bilayer, while leaving the integral membrane proteins still associated with phospholipids (Fujiki *et al.*, 1982) (Fig. 4B). Second, and more importantly, Tctex2 was removed by a mild trypsin treatment of intact sperm (Fig. 4C). Trypsin is known to digest only proteins located on the outside of the membrane. The immunocytochemical and biochemical studies together argue that Tctex2 is a sperm tail surface protein.

Tctex2 Associates with a 14-kDa Protein

As mentioned above, the *t*-form of Tctex2 differs from its wild-type counterpart in 13 of 191 amino acids (6.8%). These differences included several conserved and nonconserved amino acid substitutions and a noteworthy deletion, which alters the predicted secondary structure of the protein. To address the significance of these changes, immunoprecipitation was used to see if any other proteins interact with Tctex2, and if so, whether the interaction is abolished by the changes in the protein. Affinity-purified antibodies were used to immunoprecipitate Tctex2 from total [^{35}S]methionine-labeled testicular proteins from +- and *t*-mice. As shown in Fig. 6A, two bands of molecular weights 22 and 14 kDa were resolved in SDS–PAGE gels. These two proteins were not covalently linked by disulfide bonds, since the immunoprecipitation pattern was not affected by the addition of the reducing agents DTT and β-mercaptoethanol. The 22-kDa protein is recognized by anti-Tctex2 antisera by Western blot analysis; thus, it represents intact Tctex2 protein. The proteins with molecular masses between 22 and 14 kDa in lanes 1, 3, and 5 of Fig. 6A are probably degradation products of Tctex2 proteins because they are recognized by anti-Tctex2 antisera on immunoblot analysis and because they did not appear in fresh testis lysates. In contrast, the 14-kDa protein failed to be detected by anti-Tctex2 antisera. This coprecipitated protein is not a degradation product of Tctex2 since exogenously added Tctex2 translated and radiolabeled by an *in vitro* wheat germ translation system was stable under these conditions of immunoprecipitation (Fig. 6B). Furthermore, the 15-kDa protein, encoded by the smaller *Tctex2* message, is distinguishable from the coprecipitated 14-kDa protein by its lower mobility on the protein gels. Nevertheless, it is still possible that the 14-kDa protein could be generated by cleavage of Tctex2 *in vivo* and thus represents a processed form of Tctex2 which is not recognized by anti-Tctex2 antisera in Western blots. Exclusion of this possibility requires sequencing of this protein. Last, it is worth noting that the 22-kDa *in vitro*-translated Tctex2 (lanes 2 and 4) migrates to the same position as the endogenous 22-kDa Tctex2 in testis (lane 1). This further supports the idea that

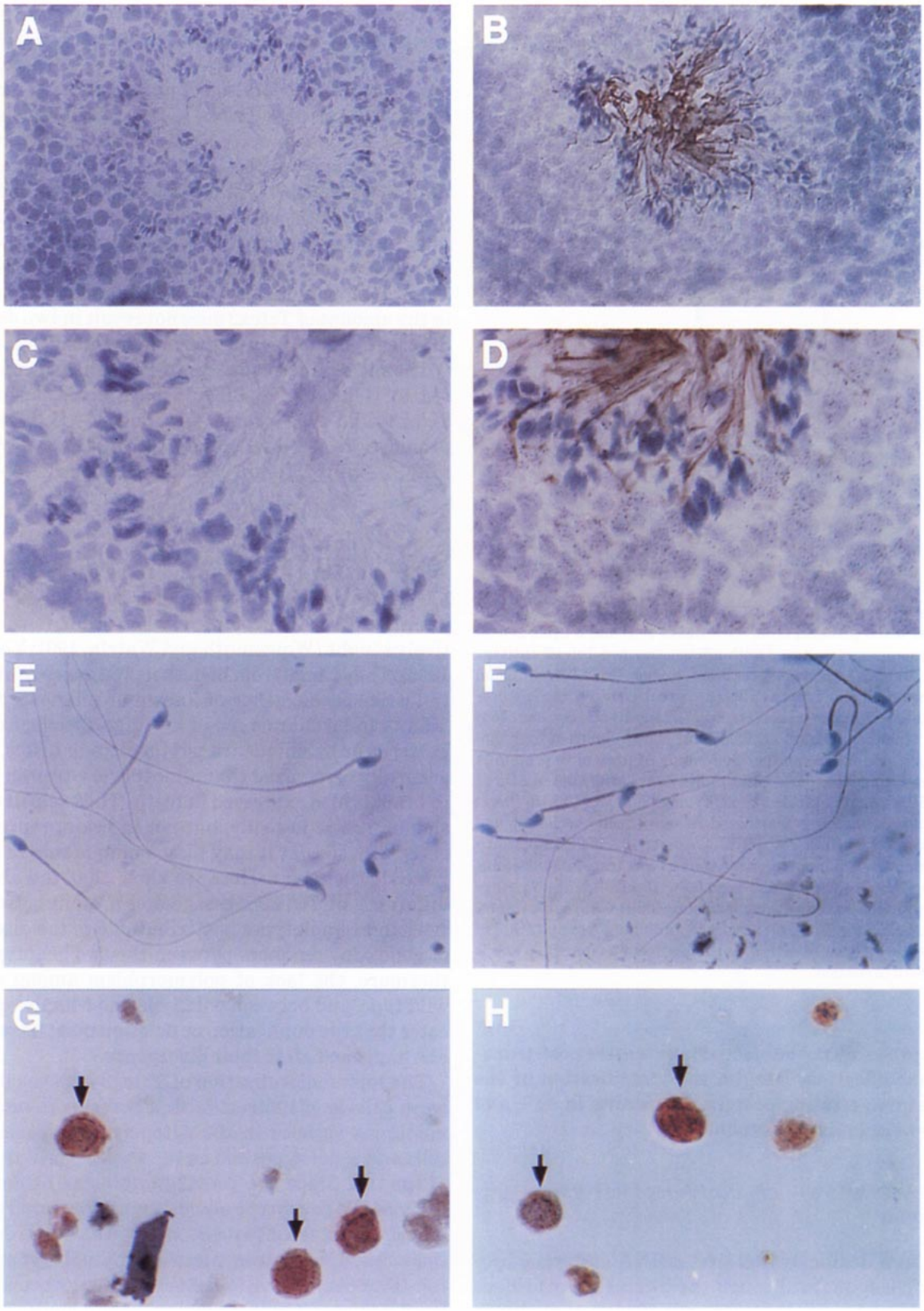

FIG. 5. Localization of Tctex2 protein in testis and on sperm. Testis sections (A, B, C, and D) and sperm (E and F) from wild-type mice were stained with affinity-purified Tctex2 antisera blocked with GST/Tctex2 bacterial fusion protein (A, C, and E) or preabsorbed with GST protein only (B, D, and F). Positive staining is evident as red-brown deposits. Nuclei were counterstained with hematoxylin. (G) A typical microscopic field of testicular cells from +/*t* mice stained with affinity-purified Tctex2 antisera. (H) A mixture of +/+ and *t*/*t* testicular cells. Arrows indicate spermatids. Samples are photographed at 400× (A and B) or at 1000× (C–H).

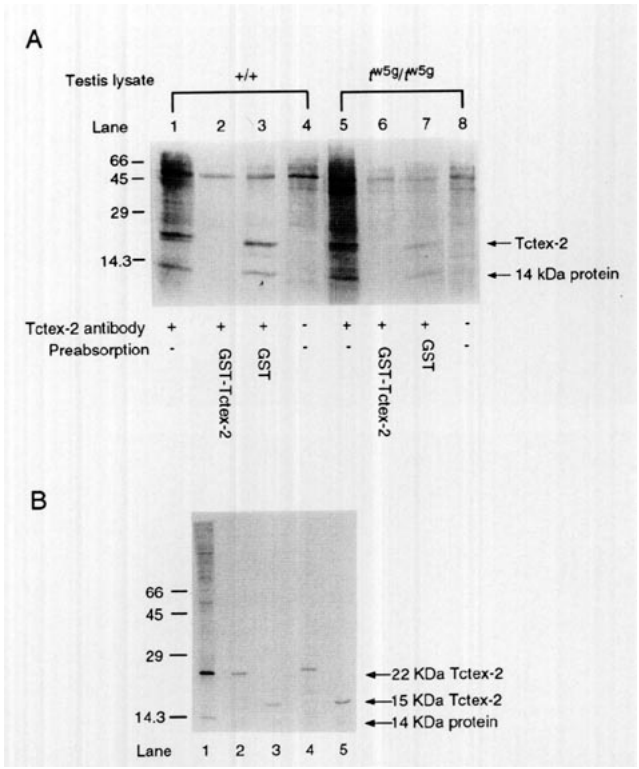

FIG. 6. Immunoprecipitation of Tctex2 protein. (A) [^{35}S]Methionine-labeled total testis proteins from +/+ (lanes 1-4) and *t/t* (lanes 5-8) mice were immunoprecipitated with affinity-purified Tctex2 antisera (lanes 1, 5), Tctex2 antibodies preabsorbed with GST/Tctex2 fusion protein (lanes 2, 6), and Tctex2 antibodies preabsorbed with GST protein (glutathione *S*-transferase) (lanes 3, 7). Lanes 4 and 8 are reagent controls to which no primary antibody was added. Bands of high molecular weight are due to nonspecific binding of protein to protein-A beads (lanes 4, 8). Molecular weight markers are indicated in kDa at left. (B) [^{35}S]Methionine-labeled 22-kDa (lanes 2, 4) and 15-kDa (lanes 3, 5) Tctex2 proteins were translated *in vitro* from the larger and smaller *Tctex2* transcripts, respectively. Affinity-purified Tctex2 antisera were used to immunoprecipitate the *in vitro*-translated proteins alone (lane 4 for 22-kDa protein; lane 5 for 15 kDa) or the *in vitro*-translated proteins mixed with nonradiolabeled testis lysates (lane 2 for 22 kDa; lane 3 for 15 kDa). Lane 1 is an immunoprecipitation of radiolabeled testis lysates with the same antisera as lanes 2-5 and is used as a positive control.

Tctex2 is probably not subjected to extensive post-translational modification. Despite the identification of the coimmunoprecipitating protein, the results in +/+ and *t/t* testes were indistinguishable.

Tctex2 Is Not Differentially Partitioned in + and t Spermatids

It has been demonstrated that mRNA and some proteins can freely move through the intercellular bridges between developing male germ cells (Braun *et al.*, 1989). We thought that we could take advantage of the sixfold lower level of Tctex2 to ask whether +- and *t*-populations of gametes could be differentiated. An artificial mixture of +/+ and *t/t* testicular cells was compared to a population derived from heterozygotes (+/*t*) for their staining intensity with the anti-Tctex2 antiserum. In order to easily compare the staining intensity, cells were permeabilized with Triton X-100, a nonionic detergent, so that the whole cytoplasm, including the prominent Tctex2 storage vesicles, were stained. The results were that all of the spermatids from +/*t* testes had similar levels of Tctex2 protein, whereas in the artificial mixture, two discrete populations of spermatids were observed, one more strongly stained than the other (Figs. 5G-5H). This indicates that the quantitative difference in the amount of Tctex2 does not result in two detectably different populations of sperm in heterozygous mice.

The nucleotide sequences of Fig. 3 have been submitted to GenBank/EMBL and have accession numbers: *Tctex2* wild-type promoter sequence—U21671; *Tctex2* promoter from *t*-haplotype—U21672; *Tctex2* wild type, mRNA—U21673; and *Tctex2 t*-haploid, mRNA—U21674.

DISCUSSION

The number of genes found to be expressed in testis have increased dramatically over the last few years, especially in the *T/t* complex, where they have been actively sought (Wolgemuth and Watrin, 1991; Yeom *et al.*, 1992). They range from housekeeping genes to oncogenes to testis-specific genes of known or unknown function. Many can be characterized by stage-specific expression patterns or by unique transcript sizes in testes, and it is generally recognized that almost every transcript made in brain is also expressed in testis. The exclusive expression of *Tctex2* in testis, but not in brain, represents an exception, so that it may play a unique role in male gamete development. Here we show that the abundance difference of *Tctex2* message and protein between wild type and *t*-haplotypes is attributable to the discrepancy of gene copy numbers between the two genotypes. Furthermore, the lack of polymorphism among copies in wild type, and between wild type and *t*-haplotypes, indicates that the duplication or deletion must be recent and has happened after their divergence.

The immunolocalization of Tctex2 in developing male germ cells is of interest in that it seems to be stored in numerous vesicles in the cytoplasm of spermatogenic cells and is not deposited on the surface until the genesis of the tail. Since the Tctex2 protein can be detected as early as the pachytene stage, it must be stored cytoplasmically for at least 15 days. There are several dense particles found in electron microscopic studies of male germ cells that could serve as a storage site for Tctex2: granulated bodies, reticulated bodies, radial bodies, and chromatoid bodies (Desjardins and Ewing, 1993). However, at present it is not clear which, if any, of these anatomically correspond to the Tctex2 storage vesicles.

The majority of presecretory proteins are exported via the classical endoplasmic reticulum (ER)–Golgi pathway by a mechanism involving a signal peptide, signal recognition particle (SRP), receptor of SRP, and translocating proteins (Rapoport, 1992). The association of Tctex2 with the sperm tail cytoplasmic membrane presents an enigma, because Tctex2 protein lacks a typical signal sequence. This feature of Tctex2 is unusual, but is not unique. Several secretory proteins, such as interleukin-1β (IL-1β) (Rubartelli *et al.*, 1990), basic fibroblast growth factor (Abraham *et al.*, 1986), blood coagulation factor XIII (Grundmann *et al.*, 1986), ATL-derived factor (Tagaya *et al.*, 1989), platelet-derived endothelial cell growth factor (Ishikawa *et al.*, 1989), and yeast mating factor-a (Kuchler *et al.*, 1989), do not contain signal peptides, yet they are all secreted extracellularly. Although the mechanism for their transport is still mysterious, studies done on interleukin-1β are relevant. It is a cytokine released by activated monocytes. Pro-IL-1β is synthesized in the cytosol as a 33-kDa polypeptide and then processed into a mature 17-kDa protein in intracellular vesicles, where it is protected from proteolysis. These vesicles are not derived from ER or Golgi, as judged by immunoelectron microscopy and biochemical studies. The mechanism for secretion of IL-1β is distinct from the classic secretory pathway in that drugs that block vesicular transport of ER or Golgi do not inhibit its processing or secretion (Rubartelli *et al.*, 1990). Tctex2 protein is similar to IL-1β in that it lacks a signal sequence and can be found in the cytosol in vesicles. Thus, the mechanism for translocating IL-1β and Tctex2 into vesicles may be very similar. A gene family encoding ATP-binding transporter proteins may in part involve their transport. Members of this family include prokaryotic permeases, multiple drug-resistance transporters, and *TAP* genes. They are transmembrane proteins which facilitate the transport of a variety of substrates across the cell membrane (Kuchler *et al.*, 1989; Doige and Ames, 1993).

Although *t*-bearing sperm are functionally unique, there is no distinct morphological abnormality observed in distorting sperm from +/*t* males or sterile *t*/*t* mice (Nadijcka and Hillman, 1980). As mentioned above, physiological studies of sperm from +/+, +/*t*, and *t*/*t* congenic mice have suggested that there are several *t*-related alterations of sperm motility (Olds-Clarke and Johnson, 1993). All these may contribute to both TRD and/or sterility or may simply be the effects of some undetected dysfunction. The localization of Tctex2 to sperm tails and the amino acid changes seen in *t*-haplotypes described here suggest that *Tctex2* cannot be ruled out as a candidate for involvement in these phenomena.

To date, five of eight *t*-complex testis-expressed genes characterized in our laboratory show marked abundance and sequence differences between wild type and *t*-haplotypes (Ha *et al.*, 1991). In addition, two of these genes sequenced subsequently, *Tctex4* and *5*, show a 5% difference at the amino acid level (H. Uehara, and K. Artzt, unpublished information). Also, nine testicular proteins identified in two-dimensional gel analysis also have *t*-specific polymorphisms in their isoelectric points (Silver *et al.*, 1983). However, it is worth noting that changes in any one gene do not have to be causal in *t*-related male germ cell phenomena. Virtually all *t*-haplotypes exist in mouse populations in a heterozygous state, since they are meiotically driven to high frequency by ratio distortion and usually do not exist as homozygotes due to the embryo lethals they carry. Consequently, their genes involved in spermatogenesis can wander into deleterious states because homozygosity for more than one recessive sterility gene carries no further detriment to the individual or to the population as a whole. If so, such parasitic sterility mutations could explain why many testis genes in *t*-haplotypes differ from their wild-type counterparts. This would also make it difficult to decide which few or all of them contribute to the prototypical TRD and sterility.

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