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Expression of three *t*-complex genes, *Tcp-1*, *D17Leh117c3*, and *D17Leh66*, in purified murine spermatogenic cell populations.

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Summary

Transmission ratio distortion (TRD) is a property of the complete *t*-haplotype which results in the preferential transmission of the *t*-haplotype chromosome from heterozygous *t*/+ males to the majority of the progeny. Available data suggest that in *t*/+ males, a dysfunction of the wild-type sperm within the female reproductive tract is responsible for the observed deviation from Mendelian segregation ratios. Genetically, Lyon has shown that multiple loci within the *t*-complex are required for maximum levels of TRD. These loci include multiple *t*-complex distorters (*Tcd*s) which act upon a single *t*-complex responder (*Tcr*). Testis-expressed genes have been cloned which map to the same subregions of the *t*-complex as the *Tcd*s and *Tcr* and are thus considered candidate genes for the products of these loci. To begin to understand how the products of these loci biochemically control TRD, the expression of three TRD-candidate genes (*Tcp-1*, *D17Leh117c3*, and *D17Leh66*) has been determined in populations of spermatocytes and differentiated spermatids purified to near homogeneity by unit gravity sedimentation. Fractions covering the entire gradient were analysed resulting in a more accurate picture of the precise timing of expression than previously reported. Transcription of all three genes was up-regulated in pachytene primary spermatocytes and persisted at stable levels through the haploid spermatid stages. Significantly, only levels of mRNA encoded by *D17Leh66*, the candidate gene for *Tcr*, increased from early round to elongating-stage spermatids. If this pattern of expression does, in fact, represent *Tcr*, these data provide the first direct evidence that wild-type and *t*-haplotype *Tcr* elements could be differentially expressed in haploid spermatids.

1. Introduction

The *t*-complex is a naturally occurring variation of murine chromosome 17 which is found in approximately 40% of wild mouse populations (for review see Silver, 1985). Two features of this gene complex are responsible for its persistence within the mouse gene pool. The first is the presence of four inversions relative to wild-type chromosome 17 which result in the suppression of recombination between chromosomes over approximately 40 cM (Hammer *et al.* 1989). The second, transmission ratio distortion (TRD), is the phenomenon whereby the *t*-carrying sperm from heterozygous *t*/+ males preferentially fertilize 95% or more of the eggs. Multiple genetic loci are involved in TRD and map to at least four distinct regions of the *t*-complex (Lyon, 1984; Lyon, 1986; Silver & Remis, 1987; Silver, 1989). Interestingly, it appears that homozygosity at any of these individual loci results in varying degrees of sterility, while complete sterility is observed only when all the loci are homozygous (Lyon, 1986). Lyon's current model of the mechanism of TRD postulates the existence of a major cis-acting genetic element, the *t*-complex responder (*Tcr*), which is acted upon in *cis* or in *trans*, by the products of at least three to four other loci called *t*-complex distorters (*Tcd*).

Analysis of total sperm DNA by restriction fragment length polymorphisms (RFLPs) to distinguish wild-type and *t*-haplotype chromosome contribution suggests that approximately equal numbers of wild-type and *t*-haplotype sperm are present in the female reproductive tract up to 90 minutes after fertilization (Silver & Olds-Clarke, 1984). Physiological manipulations which vary the time of fertilization relative to the age of the unfertilized egg can alter the degree of TRD in certain haplotypes (Braden, 1958) as can *in vitro* fertilization (McGrath & Hillman, 1980). These results

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suggest that the sperm carrying the wild-type chromosome is neither disadvantaged in numbers available at the site of fertilization nor irrevocably damaged. Finally, mixed insemination experiments using genetically marked epididymal sperm from TRD males (*t*-haplotype) and normal males (wild type) demonstrate that when differentiation occurs in the same seminiferous epithelium, it is the wild-type sperm which are impaired in their ability to fertilize, rather than the *t*-haplotype sperm being at an advantage (Olds-Clarke & Peitz, 1985). Available data therefore suggest that a delicate balance between the products of the *t*-haplotype and the wild-type loci is required to achieve high levels of TRD, and that various other factors including differences in *Tcr* and *Tcd* alleles, time of fertilization and genetic background (Olds-Clarke & McCabe, 1982; Gummere *et al.* 1983, Lyon, 1990) can all influence the final degree of distortion.

Although the genetic and physiological bases of TRD are becoming clearer, the biochemical mechanisms responsible for the described effects remain obscure. The isolation of candidate genes for three of the TRD-defined loci, *Tcr* (*D17Leh66*, Schimenti *et al.* 1988), *Tcd-3* (*D17Leh117c3*, Rappold *et al.* 1987 and *Tcd-4* (*Tcp-1*, Willison *et al.* 1986) has allowed the analysis to proceed from the genetic to the molecular level. We stress that these are candidate genes only; they show no homology to one another and to date have no known function; however, the characterization of the patterns of expression of these genes during spermatogenic differentiation may provide further evidence for their association with the TRD loci. In the research presented here, mRNA expression of the *Tcp-1*, *D17Leh66*, and *D17Leh117c3* genes has been analysed during spermatogenesis. In the case of TCP-1, protein synthesis and steady state levels were also determined using rat anti-TCP-1 monoclonal antibodies (Willison *et al.* 1989). The implications for the genetic control of mammalian spermatogenesis, the nature of gene activity in haploid spermatids, and the role of *D17Leh117c3*, *D17Leh66*, and *Tcp-1* in TRD are discussed.

2. Materials and methods

(i) *Cell purification*

The testes from four mature MF1 mice were dissected and used to prepare a suspension of cells from the seminiferous epithelium by the collagenase/trypsin method of Romrell *et al.* (1976). The normal Ringer's buffer (EKRB) was supplemented by pyruvate (1 mM) and lactate (6 mM) in all steps. The yield from eight testes was reproducibly between 2–4 x 10^8 cells. The cells were resuspended in 100 mls of EKRB plus 0·5% bovine serum albumin (BSA) and loaded onto the top of a 900 ml 2–4% BSA gradient prepared in a CELSEP chamber (Dupont) with a 50 ml 10% BSA cushion at the bottom. Gradient formation, cell loading and cell sedimentation (unit gravity, 90 min) were essentially as described by Wolgemuth *et al.* (1985) except that all solutions and cell suspensions were kept at room temperature. Sixteen fractions of 50 mls each were collected from the bottom (high density) to the top (low density) of the gradient after discarding the 50 ml cushion of 10% BSA. Cell yield was determined using a Coulter counter. Representative populations of live cells from each fraction were examined by phase-contrast and Nomarski differential interference microscopy using a laser-sharp confocal microscope (Bio-rad). Recovered cells were pelleted (300 *g*, 10 min, 4 °C) and used for further analysis.

(ii) *RNA purification*

The procedure of Chomczynski & Sacchi (1987) was followed exactly to isolate total RNA from cells recovered in each CELSEP gradient fraction. The cell pellet from four-fifths of each fraction was initially solubilized in 400 μl of denaturing solution (4 M-guanidinium thiocyanate [Fluka], 25 mM-Na citrate pH 7·0, 0·5% Sarcosyl, 0·1 M-2-mercaptoethanol). Northern analysis was carried out by electrophoresis of equivalent volumes of RNA in formaldehyde agarose gels (Thomas, 1980) and transfer to Genescreen membrane (Dupont) which allowed multiple reprobings of the same filter. The filter was stripped between sequential hybridizations by three washes of 0·1% SDS at 100 °C. Hybridizations signals were quantitated by densitometric scanning (Joyce-Loebel, Chromoscan 3) of autoradiograms (Kodak, XAR5 film) obtained from multiple exposures of the filter. Data were plotted with corrections for equivalent amounts of total RNA in μg per fraction.

(iii) *DNA probes*

Tcp-1 was detected using the plasmid pB1·4 (Willison *et al.* 1986). *D17Leh117c3* was detected using the full length cDNA described by Rappold *et al.* (1987) and kindly provided by Hans Lehrach. *D17Leh66* was detected using a full length cDNA (66·5·1) isolated in our laboratory from a *t6/tw1* testis cDNA library. The 66·5·1 cDNA is 98% homologous to the *D17Leh66* cDNAs described in Schimenti *et al.* (1988). Mouse protamine-1(PRM-1) was detected using a 500 bp *NcoI/BglII* fragment from pmP1–P3·2 (Peschon *et al.* 1987), kindly provided by Richard Palmiter, covering most of the gene from +95 to just distal of the polyA addition site. All DNA probes were made according to the oligo-labelling method of Feinberg & Vogelstein (1984) using an oligo-labelling kit (Amersham).

(iv) *TCP-1 protein analysis*

The cell pellet from one-tenth of each fraction was resuspended into 500 μl of EKRB/0·5% BSA con-

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taining 30 μCi of [^{35}S]methionine (Amersham, 800 Ci/mmole) and distributed into one well of a 24-well Linbro plate. Cells were radiolabelled for 90 min at 33 °C. Lysis was accomplished by the addition of 500 μl of 2 × RIPA buffer (1 × = 150 mM-NaCl, 10 mM-Tris pH 7·2, 1 % deoxycholate, 0·1 % SDS, 1 % Triton® X-100[Pierce, Surfact-Amps]). Lysates were cleared by centrifugation for 30 min (4 °C, 2500 g). TCP-1 was quantitatively immunoprecipitated from 200 μl of precleared cell lysate/fraction using rat anti-TCP-1 monoclonal antibody (23c), directly coupled and crosslinked to protein A-Sepharose beads (Pharmacia) according to the method of Simanis & Lane (1985). SDS-polyacrylamide gel electrophoresis (PAGE) analysis of immunoprecipitates and Western blotting were performed as previously described (Willison *et al.* 1989). Autoradiograms of immunoprecipitated, ^{35}S-methionine-labelled TCP-1 and calf alkaline phosphatase reaction product signals were quantitated by desitometric scanning as for Northern analysis. All reagents were from Sigma unless otherwise indicated.

3. Results

(i) *Assessment of recovered spermatogenic cell populations*

The analysis of cells from CELSEP gradient fractions was performed on individual fractions across the entire gradient. This had the advantage of allowing the identification of, and obtaining results for, the purest peak fractions of each cell type. Fractions were analysed for cell numbers recovered, homogeneity of cell type with regards to stage of spermatogenic differentiation, and for yields of isolated RNA.

Morphological analysis of the cells in each fraction was by light microscopy using Nomarski differential interference (Fig. 1 *a*). Since separation relies primarily on differences in cell volume, the largest cells sedimented in the high density fractions (fraction 1, bottom of gradient) and the smallest cells (mature sperm) were recovered along with small cellular debris in the lowest density fractions (fraction 16, top of gradient). As previously described by Wolgemuth

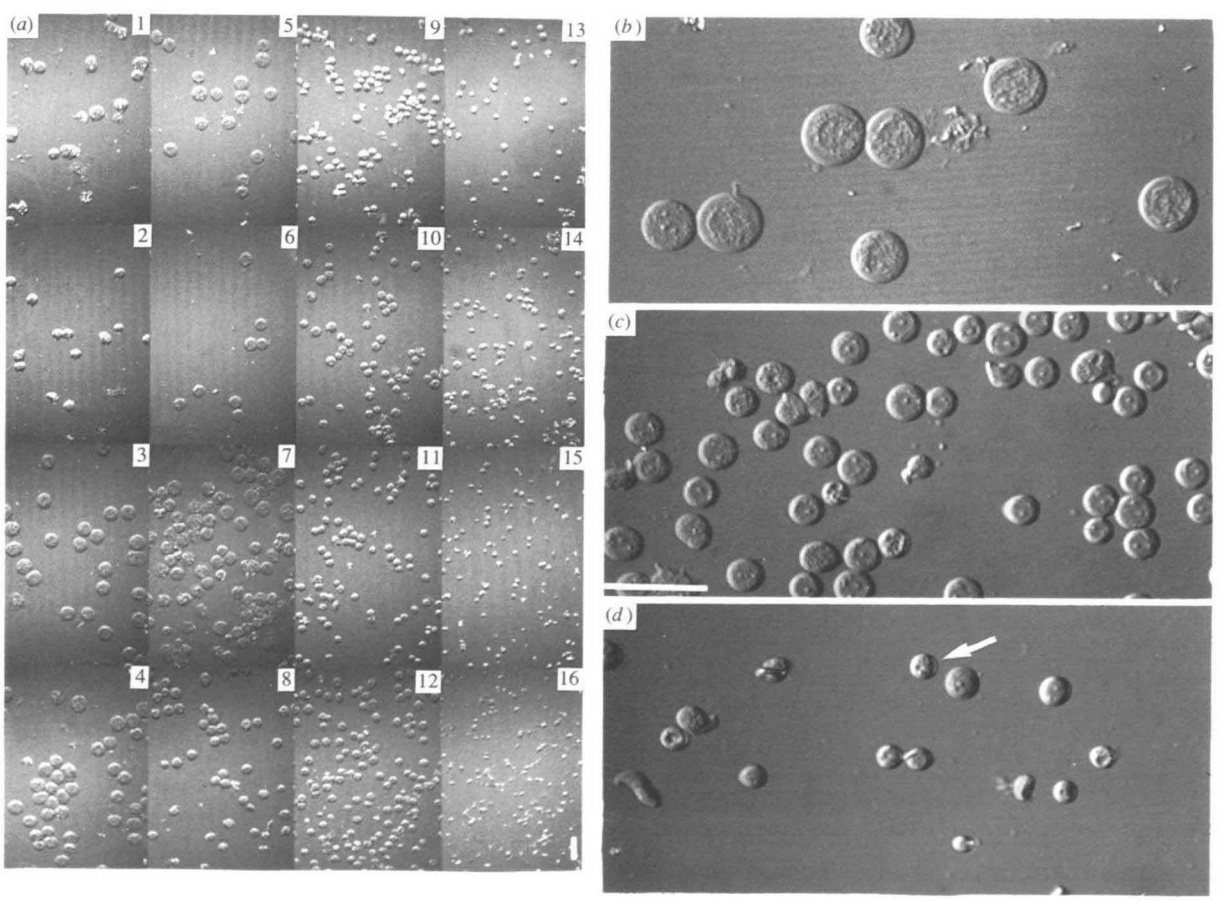

Fig. 1. Nomarski differential interference photomicrographs of live murine spermatogenic germ cells as recovered from individual CELSEP fractions. Separation of specific cell types is based primarily on cell volume. Fraction no. 1 corresponds to the bottom, or heaviest, and fraction no. 16 the top, or lightest part of the gradient. A profile of representative cells recovered in individual fractions no. 1–16 is compiled in panel A. Higher magnification of populations obtained in fractions no. 5 (B), no. 12 (C) and no. 13 (D) represent highly enriched primary spermatocytes, early spermatids, and later elongating spermatids, respectively. Arrow in D indicates the elongating cap structure on a single representative late stage spermatid. Scalebar equals 50 μm.

et al. (1985), the most homogeneous cell populations recovered were the primary spermatocytes (Fig. 1*b*, peak fraction no. 5), the early stage round spermatids (Fig. 1*c*, peak fraction no. 12) and the later, elongating spermatids (Fig. 1*d*, peak fraction no. 13). The yield of cells from each fraction and corresponding amount of total RNA isolated in μg is plotted in Fig. 3*a*. We estimate the amount of total (i.e. ribosomal) RNA in a primary spermatocyte and an early spermatid to be 15·1 and 4·1 pg per cell, respectively. Our yields are slightly higher than the previously reported estimates of 4–11 pg (spermatocyte) and 1–2·5 pg (spermatid) (Kleene *et al.* 1983), however, this is probably due to the higher efficiency of recovery of RNA when using the guanidinium thiocyanate method of extraction (Chomczynski & Sacchi, 1987). The approximate four-fold difference in recovery from spermatocytes versus spermatids is consistent with the generation of 4 spermatids from each spermatocyte and the fact that ribosomal RNA synthesis is shut-down in spermatids (Hecht, 1986).

(ii) *Distribution of TCP-1 protein expression in fractionated spermatogenic cells*

The distribution of TCP-1 protein expression across the CELSEP gradient fractions was analysed using monoclonal anti-TCP-1 antibodies (Willison *et al.* 1989). Protein analysis was limited to TCP-1 since, to date, no antibodies specific for the protein products of *D17Leh117c3*, *D17Leh66* or *Prm-1*, have been generated. Protein distribution was assessed using two methods: (1) immunoprecipitation from ^{35}S-methionine-labelled cell lysates; and (2) Western blot analysis of cell lysates subjected to SDS-PAGE and transferred to nitrocellulose. Since radiolabelling was limited to 90 min, analysis by SDS-PAGE of immunoprecipitated TCP-1 (Fig. 2*a*) represented the contribution from newly synthesized material. The

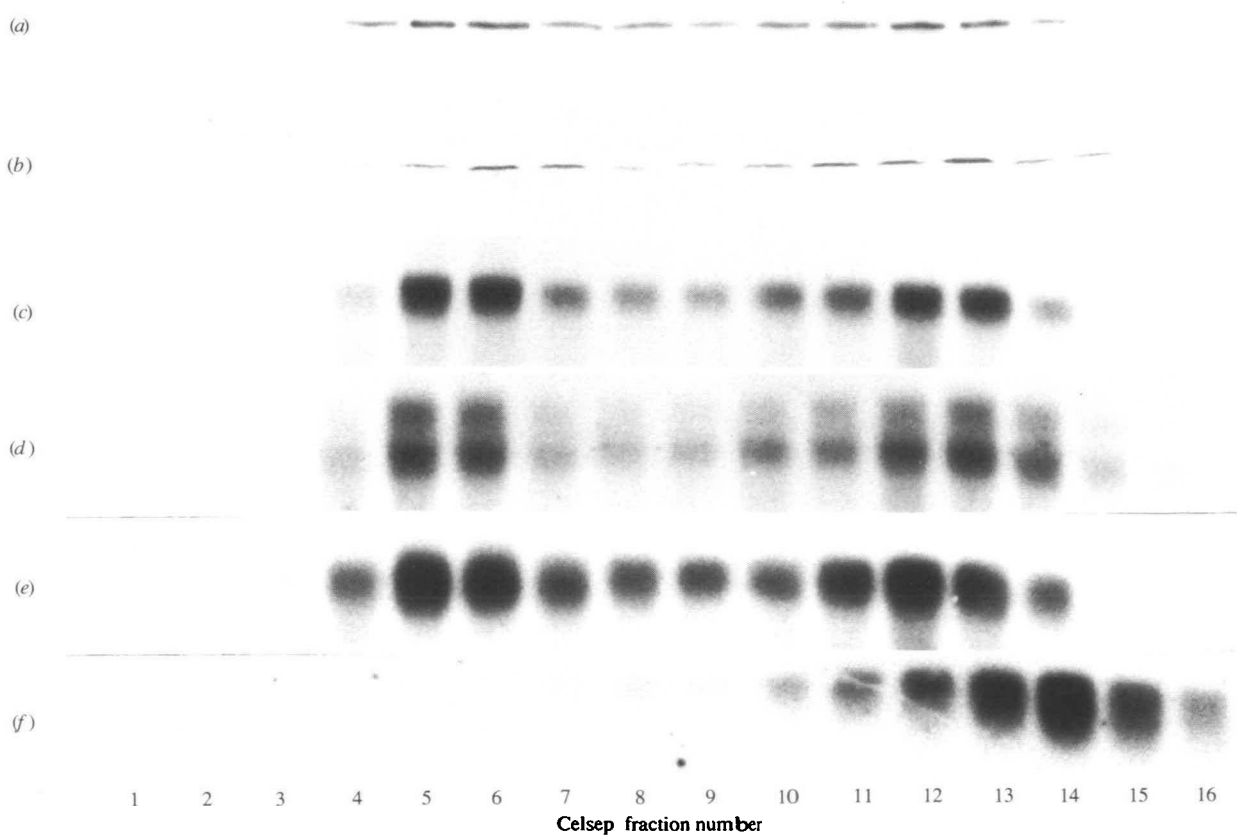

Fig. 2. Analysis of the distribution of TCP-1 protein (a and b) and various mRNA transcripts (c–f) expressed in spermatogenic populations from individual CELSEP fractions (fractions no. 1–16, left to right). The distribution of TCP-1 was determined by SDS-PAGE and autoradiography of quantitative immunoprecipitates from ^{35}S-radiolabelled cells (a). Cells, lysed in RIPA were immunoprecipitated with rat anti-TCP-1 monoclonal antibody (23c) directly coupled to Protein A-Sepharose beads. TCP-1 distribution was confirmed by Western blot analysis (B) of cold lysates separated by SDS-PAGE, electroblotted to nitrocellulose and probed with a cocktail of 3 rat anti-TCP-1 monoclonal antibodies (23c, 84a, 91a) followed by goat anti-rat Ig coupled to calf alkaline phosphatase. For Northern blot analysis of the distribution of mRNA transcripts, equivalent volumes of total RNA recovered per fraction were loaded in each lane. Samples were separated by electrophoresis through a formaldehyde agarose gel and transferred to a single sheet of Genescreen. This single Genescreen blot was probed sequentially using the DNA probes for (C) *Tcp-1*; (D) *D17Leh66*; (E) *D17Leh117c3*; and (F) mouse protamine-1 *Prm-1*).

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peak of TCP-1 synthetic activity was observed in fractions containing spermatocytes (4, 5, 6) and spermatids (10, 11, 12), which agrees with previous results obtained by quantitative 2-D gel electrophoresis of total, *in vivo*-labelled, spermatid and spermatocyte cell extracts (Silver *et al.* 1987). Although the Western blot analysis profile (Fig. 2*b*) is similar to that obtained by immunoprecipitation, one significant difference is the persistence of high levels of protein through the elongating spermatid stage (fraction 13 and 14) despite new synthesis of the protein having substantially declined. Since the analysis by Western blot detects steady-state levels of TCP-1, rather than just newly synthesized material, this suggests that TCP-1 protein turnover is relatively slow in spermatogenic cells, a result which is consistent with that

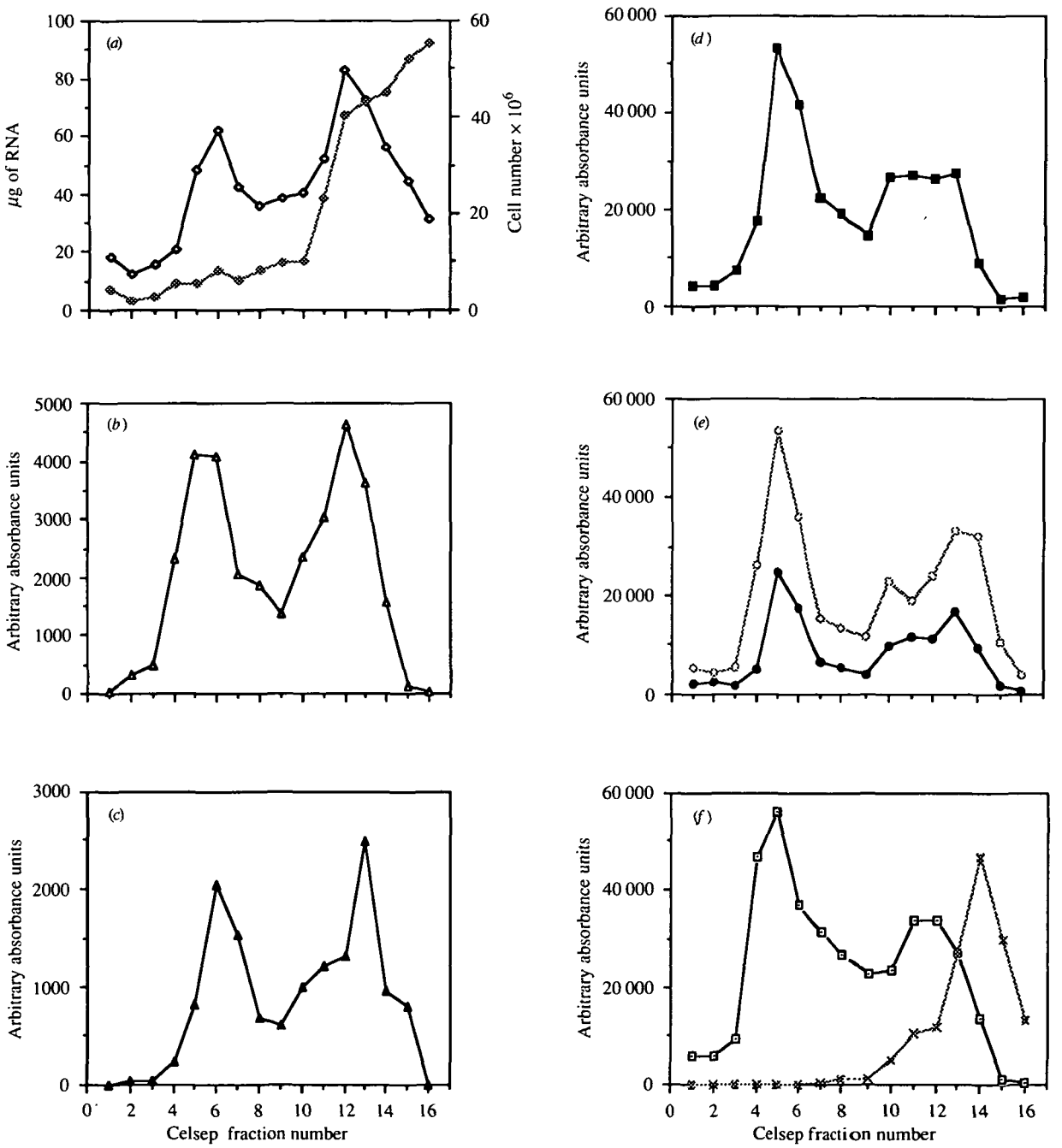

Fig. 3. Quantitative analysis of CELSEP gradient fractions. Panel (*a*) represents the recovery of cells (—●—) and the total RNA yields in μg per fraction (—◇—). Panels *b–f* indicate the quantitation by densitometetric scanning of autoradiograms from Fig. 2. TCP-1 protein recovered by immunoprecipitation (—△—) and Western blot analysis (—▲—) is indicated in panels *b* and *c*, respectively. Panels *d–f* correspond to hybridization of mRNA transcripts to DNA probes for *Tcp-1* (D, —■—), *D17Leh66* (E, —●— 2·2 kb transcript and —○— 2·7 kb transcript), *D17Leh117c3* (F, —⊡—), and *Prm-1* (F, —×—). Quantitative results as plotted in panels D–F were corrected for equivalent amounts of total RNA in μg per fraction and therefore are not directly comparable with the autoradiograms as shown in Fig. 2.

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observed in somatic cells by pulse-chase analysis (V. A. Lewis & K. R. Willison, unpublished data). Quantitation by densitometric scanning of the results of immunoprecipitation and Western blotting experiments is shown in Fig. 3, panels *b* and *c*, respectively.

(iii) *Distribution of mRNA expression in fractionated spermatogenic cells*

The expression of mRNA transcripts corresponding to three *t*-complex genes: *Tcp-1*, *D17Leh117c3*, *D17Leh66*, and the control *Prm-1* gene was determined by Northern blot analysis of total RNAs isolated from individual CELSEP gradient fractions. Labelled probes were sequentially hybridized to one Genescreen membrane filter with 0·1 % SDS (100 °C) stripping in between. The autoradiographic signals from each hybridization (Fig. 2*c–f*) were quantitated by densitometric scanning and corrected for RNA loading. The graphs of these quantitations (Fig. 3*d–f*) therefore represent the signal obtained for an equal mass of total RNA in μg from each fraction.

The control protamine-1 gene is expressed only in spermatids and accumulates to high levels in late elongated spermatids, at which stage it is translated (Peschon *et al.* 1987). Northern filter hybridization of the protamine-1 cDNA confirmed this pattern of expression Fig. 3*f*), and the signal peak in fraction 14 was therefore used as a reference point for the other genes analysed.

All three *t*-complex genes showed high levels of transcript accumulation by the primary spermatocyte stage, with hybridization signal peaks in fraction no. 5 (Fig. 2*c–e* and 3*d–f*). The later stage transcription of *Tcp-1* mRNA remained relatively constant from fractions 10–13 (Fig. 2*c* and 3*d*) corresponding to early round spermatids through to the elongating stage spermatids. The transcription of *D17Leh117c3* mRNA (Fig. 2*e* and 3*f*) was at peak levels in fractions 11 and 12, representing the round spermatids, but declined significantly in subsequent fractions.

Two distinct transcripts (2·2 and 2·7 kb) were detected by filter hybridization with the 66·5·1 cDNA (Fig. 2*d*). These two mRNAs represent differential polyadenylation events rather than the alternatively spliced forms of *D17Leh66* (Schimenti *et al.* 1988; P. Davies & K. R. Willison, unpublished data). The transcript sizes reported here are slightly larger than those previously reported by Schimenti *et al.* (1988) (1·8 and 2·2, respectively). However, the higher values were determined using the 2·0 kb *Tcp-1* transcript as a known size standard and agree with our cDNA cloning results (P. Davies & K. R. Willison, unpublished data). The cell-stage distributions of the two *D17Leh66* transcripts were similar to each other, with peak accumulation in fractions 13 and 14, representing the late haploid stages. In contrast to the *Tcp-1* and *D17Leh117c3* patterns of expression, levels increased significantly from fraction 12 to fraction 13, and persisted into fractions 14 and 15, indicating *de novo* synthesis of *D17Leh66* mRNA during the haploid stages of spermatogenesis.

4. Discussion

(i) *The staging of gene expression during spermatogenesis*

Detailed and elegant morphological analysis has resulted in the extensive characterization of spermatogenic development into fourteen discernible stages (Perey *et al.* 1961, Clermont, 1972; Fawcett, 1975). Unfortunately, the resolution of the temporal expression of genes during these developmental stages has been limited by the indirect methods used to define the stage-specific cell types. Mutant mice, arrested in different stages of development (Dudley *et al.* 1984), have been used to represent expression in defined cell types. Likewise, the first wave of spermatogenesis in prepuberal mice has been used in an attempt to catch the first cohort of individual cell types: cells recovered at time points of 1, 2, 3 and 4 weeks after birth are generally taken to screen the whole process of spermatogenic differentiation which in mature mice lasts approximately 31 days (Willison & Ashworth, 1987). Both these methods suffer from contamination of total testis extracts analysed with non-germinal (Sertoli, Leydig, interstitial fibroblast, myoid) cells as well as the need to extrapolate data obtained from cells which are morphologically, but possibly not biochemically, similar to normal mature spermatogenic cells (for review see Hecht, 1986).

The use of *in situ* hybridization to RNA in tissue sections has been the method of choice for localization of transcription in many tissues; however, there are substantial drawbacks when this technique is applied to the testis (Gizan-Ginsberg & Wolgemuth, 1985). The distinctions between cells within the fourteen stages of differentiation are based on their cellular associations within the seminiferous epithelium and adjacent sectors along the tubules. Although this complex arrangement can be defined in well-preserved, conventional epon-embedded sections, the same is not true of poor quality frozen sections processed for *in situ* hybridization. The limitations in interpreting *in situ* hybridization signals in testis sections are particularly significant for sequences which are expressed for extended periods during spermatogenesis (Haffner & Willison, 1987).

Finally, gene expression has been analysed using individual populations of enriched spermatocytes and spermatids prepared by sedimentation (Shackelford & Varmus, 1987; Rappold *et al.* 1987) or centrifugal elutriation. The nature of the spermatogenic differentiation process, whereby cell volume is progressively decreased as differentiation proceeds, fortuitously facilitates the use of unit gravity sedimentation to separate successive developmental stages. We believe

that the isolation of mRNA and corresponding protein products from stage-specific cell populations purified to near homogeneity represents the most accurate and unequivocal method for analysis of differential gene expression. Therefore, we have refined the procedure of unit gravity sedimentation to allow the rapid separation of preparative numbers of spermatogenic cells on a CELSEP gradient apparatus. Data thus obtained allowed the quantitative, as well as qualitative, comparison of expression during spermatogenesis.

(ii) *Behaviour of spermatogenic cells in CELSEP sedimentation gradients*

The CELSEP® apparatus has been used for the isolation of may cell types and has recently been applied to the purification of testicular cells (Wolgemuth *et al.* 1986). We have combined the use of the CELSEP separation chamber with the collagenase/trypsin method of preparing a spermatogenic single-cell suspension. The input cells displayed minimal cell damage or contamination with multinucleated cells or aggregates. The gradient formed in the CELSEP apparatus is shallow, maximizing the surface area over which sedimentation occurs, and allowed the rapid and efficient separation of over 4×10^8 cells in a single run.

Over thirty gradients were run and analysed for cell-type recovery in each of sixteen 50 ml fractions. The rate of sedimentation and yields of cells were entirely reproducible in our hands, and agreed well with those reported by Wolgemuth *et al.* (1986). Under the sedimentation conditions used, the peak of spermatocytes with respect to purity and yield was fraction 5 (fraction 1 = highest density) and consisted of $5{\cdot}3 \times 10^6$ cells or 1·6% of the input cell number. The secondary spermatocytes, being of intermediate cell volume, were recovered in fractions between the primary spermatocytes and the spermatids. However, since they are such a short-lived cell population (approximately 24 h) when compared with pachytene spermatocytes (9–10 days) and spermatids (10–11 days), they represented less than 10% of the cells recovered from either peak and therefore the total RNA recovery from this fraction was relatively low.

The greatest analytical use of the gradient comes from the resolution obtained over the top portion of the gradient where the differentiating spermatids are separated. The peak of round, early spermatids was found at fraction 12 (4×10^7 cells recovered = 11·9% of input), and progressively more elongating and elongated haploid spermatids were recovered in fractions 13, 14 and 15, respectively. An accurate profile of the developmental distribution during spermiogenesis of any chosen species of molecule can thus be derived by the analysis of individual fractions across this region. Spermatogenic cells, prepared by the methods described, provided sufficient total RNA from individual stage-specific cells for Northern blot analysis.

(iii) *Developmental profiles of* t-*complex cDNA probes*

Of the three genes analysed, the first, *Tcp-1*, is ubiquitously expressed in all cells examined, with the exception of the two-cell embryo and epididymal sperm (Silver *et al.* 1979; Sanchez & Erickson, 1985; Willison *et al.* 1989). Although it is not testis-specific, it is more than 20× more abundant in testis than in other somatic tissues and is up-regulated during spermatogenesis, between day 14 and day 21 in prepuberal mice (Dudley *et al.* 1984; Willison *et al.* 1986). The protein product, TCP-1, exists in two polymorphic forms distinguished by unique isoelectric points, with the more acidic form, TCP-1A, representing the *t*-specific form (Silver *et al.* 1979). TCP-1 has been shown to be abundant in pachytene spermatocytes, and to increase in abundance relative to other proteins such as actin in haploid spermatids (Silver *et al.* 1987). Our results show that *Tcp-1* mRNA and protein expression parallel one another closely through the fifteen-day period during which it is highly expressed. Up-regulation begins early, since high levels of both protein and mRNA were observed as early as the pachytene spermatocyte stage; however, levels remain high and stable through the round to elongating spermatid stages. Taken together with our observation that TCP-1 protein has a relatively slow rate of turn-over, these results suggest an important role for the presence of TCP-1 throughout all stages of differentiation.

The *D17Leh117c3* gene was originally isolated on the basis of its localization to the *t*-complex and its proximity to a CpG-rich island (Rappold *et al.* 1987). It displays highly abundant, testis-specific transcription, and maps to the ~ 1 cM region of the *t*-complex which contains *Tcd-3*. The transcriptional distribution of this gene in our experiments was similar to *Tcp-1*, although protein production was not analysed. Accumulation of transcripts was observed in spermatocytes with high levels detected at the round spermatid stage. Significantly, a marked decline in hybridizing mRNA was detected one fraction earlier than observed with *Tcp-1* or *D17Leh66*. Therefore, the profile of developmental expression observed in these studies confirms data presented by Rappold *et al.* (1987) in which RNA levels in various gradient-purified cell fractions were analysed.

Like *D17Leh117c3*, the expression of *D17Leh66*, is also testis-specific, but has been shown to be limited to primary spermatocytes, as demonstrated in studies with prepuberal mice and *in situ* hybridization (Schimenti *et al.* 1988). Our data confirm that this is the onset of mRNA accumulation, but also provide evidence that transcription levels are equivalent in the spermatid peak fractions. Significantly, abundance of

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mRNA increased from the round to the elongating spermatid stage, and high levels persisted into the late haploid stages. Therefore, we suggest that some or all of the transcription units must remain active throughout the final stages of spermiogenesis. Although our studies were limited to non-*t*-haplotype germ cells, Schimenti *et al.* (1988) reported no differences in the RNA profiles between various inbred and *t*-haplotype stains.

(iv) *Implications for understanding spermatogenic gene expression*

A whole class of genes have now been identified which either produce novel testis-specific transcripts or whose levels of transcription are up-regulated in the testis relative to other somatic tissues. (for review see Willison & Ashworth, 1987). The patterns of expression of these genes during spermatogenic differentiation fall roughly into two categories. In the first, the accumulation of transcripts begins early in pachytene primary spermatocytes, while the up-regulation of the second class doesn't occur until later in haploid spermatids. The latter class appears to be more unusual, and consists primarily of those genes encoding gene products which act late in spermatogenesis such as the protamines, including PRM-1 which was used as a control in the experiments reported here (Peschon *et al.* 1987). Certain genes of unknown function, such as the proto-oncogenes *int-1* (Shackelford & Varmus, 1987) and *c-abl* (Meijer *et al.* 1987), also fall into the spermatid-specific pattern of expression.

The class of genes which are up-regulated before the spermatid stage is much more common, and includes the *t*-complex genes characterised in this report. Although transcription of these genes begins in spermatocytes, high levels of mRNA persist through to various points during spermatid differentiation. The parallel analysis of *Tcp-1* mRNA levels and protein products using the *in vitro* metabolic labelling of purified cell populations has allowed us to show conclusively that the high levels of *Tcp-1* mRNA are efficiently translated late into spermiogenesis. The biosynthetic capacity of late-stage spermatids is important for the *de novo* synthesis of mRNAs differentially transcribed during the haploid spermatid stages.

It would be useful to know if all genes involved in a particular process, for example the construction of the sperm acrosome or flagellum, were exactly co-regulated. Recently, taking advantage of the protocols described here to analyse protein and mRNA from individual gradient fractions, we have found that the mRNA encoding acrosin was first detectable in early spermatids which implies that haploid stage translational regulation is involved in acrosin biosynthesis (S. Keime & K. R. Willison, unpublished data).

(v) *Implications for potential mechanisms of TRD*

Tcp-1, *D17Leh117c3*, and *D17Leh66* all map within the *t*-complex and have been defined as candidate genes for the control of TRD. *D17Leh117c3* has been identified as *Tcd-3* and *Tcp-1* has been proposed to be *Tcd-4* (Silver & Remis, 1987; Silver, 1989), although evidence contradicting the *Tcp-1*/*Tcd-4* identity has recently been published (Lyon, 1990). Our results show that two candidate genes for *Tcd*s were expressed during spermiogenesis and that, at least for *Tcp-1*, protein products were translated during the final elongation processes. Therefore, as would be required by the postulated action of the *Tcd*s, products of these loci may be functionally active at all stages of spermiogenesis. *D17Leh66* is an attractive candidate for *Tcr* since in addition to its mapping to the same genetic locus, it is testis-specific and displays strain and *t*-haplotype specific differences in copy number and polymorphisms in sequence. (Schimenti *et al.* 1988; P. Davies & K. R. Willison, unpublished data). The data presented here provide the first molecular evidence that *D17Leh66* gene transcripts are not only present in high levels late into the haploid stages of spermiogenesis, but that levels increase from the round to the elongating stages, indicating that the genes must be transcribed in haploid cells. The pattern of expression for the *D17Leh66* gene family provides essential support for TRD models which invoke differential gene activity of the wild-type and *t*-haplotype copies of these transcription units. Further DNA sequence analysis and characterization of the genes in conjunction with the protocols described here will be required to identify which transcription units most closely fulfill the requirements for the *Tcr* candidate gene.

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