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Quantitation of two-dimensional gel proteins reveals unequal amounts of *Tcp*-1 gene products during mouse spermatogenesis but no correlation with transmission ratio distortion

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SUMMARY

The discovery of a protein, p63/6.9, that is synthesized by both somatic and germ cells and coded for by a gene, *Tcp*-1, within the *t* complex provides a molecular probe for examining transmission ratio distortion in *t* mice. Two electromorphs of this protein exist. The acidic protein (a) is encoded by *t*-haplotype chromosomes, while the basic protein (b) is encoded by wild-type 17th chromosomes. We have measured the relative amounts of p63/6.9a and p63/6.9b for various *t*-complex bearing males and for several stages of spermatogenesis. There was no correlation between the ratio of p63/6.9a to b and the magnitude of transmission ratio distortion but the relative amounts of these proteins present in testicular cells can vary depending on the method of labelling. *In vivo* labelling results in the detection of two-fold greater amounts of p63/6.9a while *in vitro* labelling produces equimolar amounts of these two proteins. These data suggest that unequal synthesis or degradation of the p63/6.9 proteins occurs during spermatogenesis. It is proposed that increased synthesis of p63/6.9a *in vivo* is an intrinsic property of *t*-haplotypes.

INTRODUCTION

Sperm development and function in the mouse are affected by the interaction of genes within the *t* complex (Lyon & Mason, 1977; Hammerberg, 1981; Lyon, 1984). Most *t* haplotypes are transmitted by the male in abnormal numbers, either higher or lower than the expected Mendelian ratios. Transmission ratio distortion by the *t* complex has been demonstrated to be a postmeiotic event by using a chromosomal marker (Hammerberg & Klein, 1975) and because it can be altered

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by changing the time between insemination and fertilization (Braden, 1958; Erickson, 1978). However, until the discovery of p63/6.9 on two-dimensional, protein electrophoresis gels, no identifiable protein coded for by the *t* complex had been available (Silver, Artzt & Bennett, 1979). More recently, several other *t*-chromosome-specific proteins have been identified by two-dimensional gel techniques (Silver, Uman, Danska & Garrels, 1983). These proteins may now be studied as potential molecular markers for the mechanisms of tranmission ratio distortion. If the cellular event responsible for transmission ratio distortion occurs during spermiogenesis, such an event could be reflected in a change in the synthesis rate of p63/6.9a, the *t* variant of p63/6.9, as compared to the synthesis rate of p63/6.9b, the wild-type protein, in postmeiotic spermatids. Such changes in rates of synthesis could correlate with the degree of transmission ratio distortion of the male.

We have measured the relative ratios of p63/6.9a to p63/6.9b in testicular cells and other spermatogenic stages of males exhibiting varying degrees of transmission ratio distortion. No simple correlation between p63/6.9a or b synthesis and transmission ratio distortion could be established. However, equimolar amounts of p63/6.9a *vs.* b were not discovered under *in vivo* conditions. In contrast, equimolar amounts of a and b were observed when labelling was performed *in vitro*. We found that a high rate of synthesis of the *t*-specific *Tcp*-1 gene is an intrinsic property of all the *t*-haplotypes studied and that control of differential synthesis is dependent on interactions within the intact testes.

MATERIALS AND METHODS

The *t*-haplotypes t^{w100} *tf* were obtained from Dr D. Bennett, T^{Or1} from Dr J.-L. Guénet, t^6 and T^{hp} from Dr M. F. Lyon; t^0 and t^{12} were originally from Dr S. Waelsch but have been maintained in our mouse colony for more than 15 generations. The t^{hes}*tf* chromosome is a spontaneous recombinant of the t^6 haplotype originating in our colony. It contains the tail-interaction (t^T) and abnormal transmission ratio (A) regions, but has lost the lethal (t^1) region. It also contains the *Tcp*-1^a allele. The transmission ratios of $+/t^0$ and $+/t^{12}$ males were determined by mating them to $T/+$ females and comparing the number of tailless (T/t) offspring to tailless (T/t) and short-tail ($T/+$) offspring. The transmission ratio for T/t^{w100} was obtained from matings to random-bred CD-1 females.

Spermatids that were used for measuring rates of synthesis of p63/6.9 from males with known transmission ratios were obtained by the following method: males were given 300 rads on Day 0 and 100 rads on Days 6 and 13 from a cobalt -60 source. This irradiation protocol results in a germ-cell population consisting of spermatids (Geremia, D'Agostine & Monesi, 1978). The testes from irradiated males were labelled *in vivo* by intratesticular injection with [^{35}S]methionine (Amersham, 20 μl, 100 μCi/testes) on Day 13. Fourteen to sixteen hours later the mouse was sacrificed and the seminiferous tubules freed by digestion with 0·1 % collagenase. The tubules were then gently pipetted to release a single cell suspension while tubule basement membranes and attached Sertoli cells were allowed to settle out. Cells were washed and lysed at a concentration of 160×10^6 cells ml^{-1} of DPBS (Dulbecco's phosphate-buffered saline) containing 0·5 % NP-40. Lysates were centrifuged at 100 000 *g* for 60 min and stored at −70 °C. Two-dimensional gels (Silver *et al.* 1979) were run on the NP-40 lysate and after fluorography the p63/6.9a and b spots plus a blank portion of the gel above the p63/6.9a spot (background value) were cut out, rehydrated in 100 μl H_2O, digested overnight in 1·0 ml NCS at 46 °C, then 10 ml of PPO-POPOP was added and the samples were counted for 50 min in a Packard liquid scintillation counter.

Testicular cells that were used to measure the ratio of a to b in a comparison of *in vivo versus in vitro* labelling were obtained as follows: testes were teased apart, treated with 0·1 % collagenase at 32 °C for 20 min; cells were freed from the seminiferous tubules by pipetting and filtering. This suspension was then overlaid on an 8 % albumin cushion and spun at 600 *g* and cells were washed and counted. *In vivo* labelled cells (100 μCi/testes of [^{35}S]methionine 14 h before sacrifice) were placed in lysis buffer (O'Farrell, 1975) at a concentration of 10×10^6 cells ml^{-1}. Cells destined for *in vitro* labelling were starved in methionine-free DMEM at 32 °C for 15–30 min followed by addition of 100–200 μCi [^{35}S]methionine 2×10^{-6} cells ml^{-1} for 4–5 h in DMEM with 1 % FCS. Cells were washed in complete DMEM without FCS and taken up in lysis buffer at 10×10^6 cells ml^{-1}. To test for the possible effect of collagenase on the ratio of p63/6.9a to b, *in vitro* labelled cells were also subjected to an additional incubation in 0·1 % collagenase at 32 °C for 20 min.

Two-dimensional gel fluorograms of the above samples were performed according to the method of O'Farrell (1975), except plates were pre-exposed in order to obtain a linear response between protein concentration and spot density (Laskey & Mills, 1975) and gels were impregnated with 'Enhance' scintillation compound (New England Nuclear). All film used was Kodak X-Omat XR-5 and all exposures took place at −70 °C.

The relative amounts of the p63/6.9a, b and z proteins seen in the fluorograms were measured using a Joyce Loebl microdensitometer. Absorption peaks of these spots were made in the isoelectric focusing dimension with the beam width adjusted to include the entire area of the spot measured. The area under the curves in mm^2 were determined with a Leitz Digitizing Analyzer.

Fractions of cells from several stages of spermatogenesis were obtained by centrifugal elutriation (Grabske, Lake, Gledhill & Meistrich, 1975) from unirradiated males which had been labelled *in vivo* with [^{35}S]methionine. Each fraction containing spermatocytes, spermatids or sperm was lysed at a concentration of 40×10^6 cells per ml of 0·5 % NP-40 in DPBS and prepared for two-dimensional gels as described above. Autoradiograms were made from these gels and microdensitometric tracings were performed.

RESULTS

Two-dimensional fluorographs of spermatid-specific proteins are shown in Fig. 1. These patterns clearly demonstrate that new synthesis of p63/6.9 occurs at this stage of spermiogenesis, thereby indicating postmeiotic synthesis of this chromosome 17 marker. Spermatids obtained by elutriation (Fig. 1A) and by irradiation (Fig. 1B) both show synthesis of p63/6.9, suggesting that the respective contaminating cells of each purification protocol are not likely to be the only source of *Tcp*-1 expression. The elutriation gel corresponds to the late spermatid stage (stages IX–XVI).

The foregoing results have allowed us to study the postmeiotic effects of *t*-haplotypes and the *Tcp*-1 locus on transmission ratio distortion by measuring the relative amounts of *in vivo* labelled p63/6.9a and b detectable during the various stages of spermatogenesis (Table 1). The results demonstrate that both pre- and postmeiotic stages produced an excess of p63/6.9a as compared with b. However, there did not appear to be much variation between stages in the ratios observed. Therefore, postmeiotic (spermatid) type cells were chosen as the source of p63/6.9 in males of known *t*-determined transmission ratio distortion.

Since the irradiation procedure of Geremia *et al.* (1978) yielded a spermatid preparation which was 90 % or more pure, this method was used in examining the synthesis rate of p63/6.9 in males with predetermined transmission ratios. The ratio of the rate of synthesis of *in vivo* labelled p63/6.9a (*t* variant) to p63/6.9b

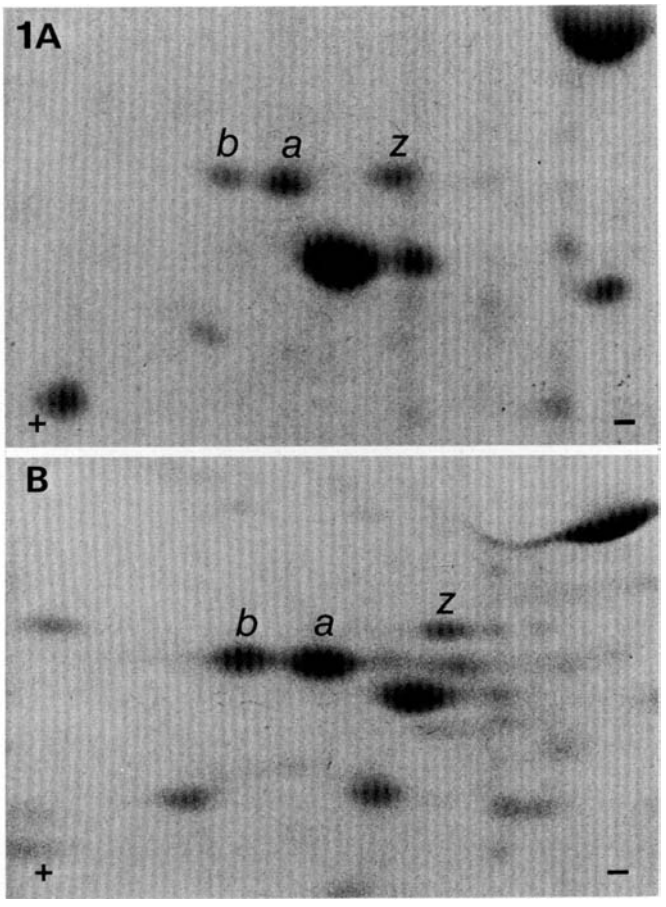

Fig. 1. Partial 2-D gel fluorographs of NP-40 soluble proteins of spermatids obtained by elutriation (A) and irradiation (B) procedures. The p63/6.9 proteins are indicated a, b and z. Basic end (+) on left, acidic end (−) on right. Relative molecular masses decrease from top to bottom. (A) 46 000 c.p.m. loaded. T/t^0 male. (B) 147 000 c.p.m. loaded. $+/t^{12}$ male.

(wild-type) of individual *t*-bearing mice was compared to their transmission ratio. As shown in Table 2, no correlation between the ratio of the rate of synthesis with transmission ratio distortion was found (r = 0·08) which could suggest a simple role for p63/6.9 in transmission ratio distortion at the level of spermiogenesis.

However, *t* mice, either low or high transmitters, consistently produced more p63/6.9a than b. These results were unexpected since subjective estimates of the amounts of p63/6.9a and b from the autoradiograms of Silver *et al.* (1979, 1980) do not demonstrate differential quantities of these two proteins. We attribute this discrepancy to the methods of labelling employed. Table 3 shows the relative amounts of the p63/6.9a, b and z proteins produced for *in vitro versus in vivo* labelling with [^{35}S]methionine. The T^{Or1} chromosome has previously been shown

to have both a *Tcp*-1^a and *Tcp*-1^b allele (Silver, 1981), presumably due to an unequal crossing-over event. The T^{hp} chromosome contains a deletion that includes the *Tcp*-1 locus. The t^{hes} chromosome, as previously mentioned, is type *Tcp*-1^a. T/t^0 or $+/t^0$ males (*Tcp*-1^a/*Tcp*-1^b) showed an a/b ratio of 1.31. $T^{Or1}/+$ males (*Tcp*-1^{ab}/*Tcp*-1^b) demonstrated an a/b ratio of 0.66. $T^{hp}/+$ males (*Tcp*-1^{null}/*Tcp*-1^b) showed a b/z ratio of 0.92, while +/+ males (*Tcp*-1^b/*Tcp*-1^b) showed a b/z ratio of 1.98. t^{hes}/t^{hes} males (*Tcp*-1^a/*Tcp*-1^a) exhibited an a/z ratio of 2.09. For each genotype measured, *in vitro* conditions yielded amounts and ratios of the p63/6.9 proteins consistent with non-differential rates of synthesis.

Table 1. *Ratio of p63/6.9a and b rates of synthesis for various stages of spermatogenesis (*in vivo *labelling, quantitation by microdensitometry, one gel per genotype)*

Male genotype	Fraction	Areas (mm²) a	b	a/b
T/t^0	Sperm	309·7	260·2	1·19
T/t^0	Late spermatids	46·5	28·4	1·64
T/t^0	Round spermatids	2194·4	1084·5	2·02
T/t^0	2^0 Spermatocytes and binucleate spermatids	798·4	492·0	1·62
T/t^0	Mid-pachytene spermatocytes	1160·8	574·8	2·02
$+/t^0$	Spermatids	1241·0	909·6	1·36
$+/t^{12}$	Spermatids	1109·2	396·4	2·80

Table 2. *Ratio of p63/6.9a and b rates of synthesis in spermatids from males with varying transmission ratio distortion (*in vivo *labelling, quantitation by scintillation counting)*

t-Haplotype	Transmission ratio*	p63/6.9a† / p63/6.9b
$T/t^{w100}tf$	4 % (2/57)	2·61 ± 0·4
$+/t^0$	55 % (77/141)	4·75 ± 1·51
$+/t^0$	64 % (68/106)	1·93 ± 0·47
$+/t^{12}$	65 % (34/52)	2·12 ± 0·22 (duplicate)
$+/t^0$	66 % (25/38)	1·63 ± 0·15
$+/t^0$	76 % (13/17)	2·15 ± 0·3
$+/t^{12}$	88 % (70/79)	2·28 ± 0·45
$+/t^{12}$	96 % (62/64)	5·45 ± 2·26 (duplicate)
$+/t^{12}$	96 % (46/48)	1·81 ± 0·06

* Percent transmission of the *t* chromosome by the male. Number *t* offspring divided by total number of informative progeny in parentheses.

† $\frac{\text{p63/6.9a - background}}{\text{p63/6.9b - background}}$, done in triplicates (except where noted, mean ± standard error).

In contrast, *in vivo* labelling resulted in an apparent increase in the amounts of p63/6.9a relative to b for each genotype studied. The magnitude of the increase is approximately two-fold since the $T^{Or1}/+$ genotype resulted in an a/b ratio of 1.09, which is in agreement with the gene dosage expectation. $+/t^0$ or T/t^0 males yielded an a/b ratio of 1.99. Additionally, the overall amounts of p63/6.9a and b proteins produced appear to be decreased for *in vivo* as opposed to *in vitro* conditions. This can be seen when the (a+b)/z ratios are compared (1.06 *versus* 2.60 for $+/t^0$; 1.56 *versus* 4.02 for $T^{Or1}/+$) and when the b/z ratios are compared 0.60 *versus* 0.92 for $T^{hp}/+$: 1.14 *versus* 1.98 for +/+).

In order to determine if the difference in a/b ratios obtained between *in vivo* and *in vitro* labelling methods was due to a postlabelling collagenase treatment (*in vivo*) as opposed to a prelabelling collagenase incubation (*in vitro*) an additional collagenase incubation was performed on *in vitro* labelled cells. The results of Table 3 demonstrate no effect of collagenase on the a/b ratio (1.31 *versus* 1.35).

DISCUSSION

Although the *Tcp*-1 locus resides within the *T/t* complex of the mouse and has an allele (*Tcp*-1^a) which is specific to all complete *t*-haplotypes, its role in *t*-complex genetics (if any) remains to be discovered. Evidence that the *Tcp*-1^a allele correlates with a proximal *t*-haplotype factor affecting transmission ratio distortion has been reported (Silver, 1981). Our observation that the p63/6.9 proteins are synthesized in postmeiotic cells raised the possibility for this protein product to be involved in *t*-allele-mediated transmission ratio distortion. We have measured the amounts of p63/6.9a and b proteins produced during spermatogenesis in an attempt to determine if any correlation exists between the expression of this allele

Table 3. *Quantitation of* Tcp-*1 proteins during* in vivo *and* in vitro *labelling, of testicular cells*

Labelling method	Genotype	Number of gels	p63/6.9a,b,z ratios*			
			a/b	a/z	b/z	(a+b)/z
In vivo	T/t^0 or $+/t^0$	3	1.99	0.71	0.36	1.06
	$T^{Or1}/+$	1	1.09	0.81	0.74	1.56
	$T^{hp}/+$	1			0.60	
	+/+	1				1.14
In vitro	T/t^0 or $+/t^0$	3	1.31	1.49	1.12	2.60
	$T^{Or1}/+$	1	0.66	1.59	2.43	4.02
	$T^{hp}/+$	1			0.92	
	+/+	1			1.98	
	t^{hes}/t^{hes}	1		2.09		
In vitro with collagenase	T/t^0 or $+/t^0$	2	5	1.69	1.25	2.94

* Values for duplicate or triplicate measurements are reported as means.

and transmission ratio distortion. It is apparent from our results that no direct relationship between *Tcp*-1 and *t*-complex effects on segregation ratios exists.

Although the techniques here employed cannot discriminate between rates of synthesis or rates of degradation, it is obvious that one or the other of these processes can be equalized under *in vitro* conditions and that control of the relative rates of p63/6.9a to b is due to interactions in the physiological environment of the testes. It has been reported that p63/6.9 is a major constituent of the extracellular matrix of testicular cells (Silver & White, 1982). It is possible that the unequal amounts of the p63/6.9 proteins seen *in vivo* are due to unequal loss or secretion of p63/6.9b to the extracellular environment. Presumably, *in vitro* conditions would block this event and cause equalization of the a/b ratio. The observation that testicular cells incubated *in vivo* produce less overall p63/6.9 protein ((a+b)/z = 1.06) than *in vitro* cells ((a+b)/z = 2.60) supports this idea.

One- and two-dimensional gel electrophoresis is increasingly being used to characterize spermatogenesis. Selective solubilization and fractionation into heads and tails has revealed a considerable array of proteins in spermatozoa (Mujica, Alonso & Hernandez-Montes, 1978; O'Brien & Bellvé, 1980). These methods have also been used to detect unique sperm mitochondrial (Hecht & Bradley, 1981) and tail proteins (Bradley, Meth & Bellvé, 1981). We have previously used two-dimensional gel electrophoresis to analyse stage-specific protein synthesis during mouse spermatogenesis, finding 52 proteins which were detectable only in haploid sperm cells (Kramer & Erickson, 1982). The present work extends such analyses to genetic variants detected in spermatogenic cells.

It has previously been argued that transmission ratio distortion is most simply explained by either: 1) differences in numbers of the two kinds of spermatozoa or 2) differences in functional properties of spermatozoa present in equal numbers (Erickson, 1978). Meiotic studies of chromosome 17 segregation (marked by a Robertsonian translocation) in *t*-heterozygous males showed that there were equal numbers of the two kinds of early spermatids (Hammerberg & Klein, 1975). Since we find postmeiotic synthesis of p63/6.9a and p63/6.9b, a loss of the wild-type protein synthesis in spermatids is not apparent in *t*-heterozygous males. Thus, if the *Tcp*-1 gene product is not equilibrated by the intercellular bridges connecting spermatids (Erickson, Lewis & Butley, 1981), qualitatively different sperm could result. Evidence for two functionally distinguishable classes of spermatozoa in *t*-heterozygous males has been presented using antigenic properties (Yanagisawa *et al.* 1974) and sperm motility (Katz, Erickson & Nathanson, 1979). Demonstrations that mRNAs for sperm proteins increase in quantity in postmeiotic cells (Erickson, Kramer, Rittenhouse & Salkeld, 1980; Fujimoto & Erickson, 1982; Distel, Kleen & Hecht, 1984; Dudley, Potter, Lyon & Willison, 1984) also argues for the possibility of haploid gene expression which could result in functionally distinct populations of *t* and normal sperm. Although the products of the *Tcp*-1 locus do not quantitatively correlate with *t*-complex transmission ratio distortion, their possible roles in spermatogenesis or fertilization events, independent of *t*-complex phenomena, remain to be investigated.

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