

**Table 2** Recovery of spermatozoa from females mated with  $t^x t^y$  and control males

$t^x t^y$ sterile males			Control males		
Male	No. of matings	Sperm count log mean $\pm$ s.d.* (linear value)	Male	No. of matings	Sperm count log mean $\pm$ s.d.* (linear value $\times 10^6$ )
$t^6 t^{w5}$	4	4.30 $\pm$ 0.25 (22,500 $\pm$ 12,000)	$Tt^{w32\ddagger}$	1	6.96 $\pm$ 0.04 (9.13 $\pm$ 0.9)
$t^6 t^{w5}$	4	5.10 $\pm$ 0.53 (209,000 $\pm$ 244,000)	$Tt^{w32\ddagger}$	1	6.77 $\pm$ 0.09 (5.95 $\pm$ 1.1)
$t^6 t^{w5}$	3	<3.00 (<1,000) $\dagger$	$Tt^{w5\ddagger}$	2	6.85 $\pm$ 0.35 (8.35 $\pm$ 6.0)
$t^6 t^{w5}$	4	<3.00 (<1,000) $\dagger$	$Tt^{w5\ddagger}$	2	7.11 $\pm$ 0.08 (13.00 $\pm$ 2.5)
$t^6 t^{w5}$	2	<3.00 (<1,000) $\dagger$	$T+$	1	7.30 $\pm$ 0.05 (19.82 $\pm$ 1.9)
$t^6 t^{w32}$	5	4.99 $\pm$ 0.30 (113,000 $\pm$ 54,000)	$T+$	1	6.44 $\pm$ 0.13 (2.82 $\pm$ 0.8)
$t^6 t^{w32}$	1	3.79 (7,000) $\dagger$	$+ + C57BL$	1	7.14 $\pm$ 0.06 (13.87 $\pm$ 1.8)
$t^{w5} t^{w32}$	2	4.88 $\pm$ 0.51 (100,000 $\pm$ 98,000)	$+ + C57BL$	2	6.86 $\pm$ 0.04 (7.17 $\pm$ 0.6)
			$+ t^{w5}$	1	7.23 $\pm$ 0.06 (16.77 $\pm$ 2.5)

Female mice were either induced to ovulate (5 IU follicle stimulating hormone followed 48 h later by 5IU human chorionic gonadotropin; ovulation assumed to occur 13 h later<sup>13</sup>), or used when naturally ovulating as assessed by vaginal smear test.  $T+$  and  $+t$  mice were obtained as  $F_1$  from  $C57BL+ \times Tt$  crosses. The  $t^x t^y$  males were considered sterile if they were run with five known-fertile females for 4 weeks or more without pregnancies (five 'mating units'<sup>14</sup>).

\* Standard deviations of pooled counts per mating where the s.d. of each count <20% of mean; except where only one count is given, then the s.d. of that count is shown.

$\dagger$  These counts were based on very few (<20) spermatozoa per spot.

$\ddagger$  Known fertile control males.

male. However, they are at least two orders of magnitude lower than the figures from the control males. In the human just one order of magnitude difference seems to confer a state of clinical sterility, so this hundredfold difference would adequately explain the sterility of these  $t^x t^y$  males.

In addition to the other suggested causes of the sterility<sup>5-7</sup>, the extreme  $t^x t^y$  oligozoospermia reported here presents a convincing explanation; it is tempting to consider this as the chief cause, with reduced motility and lack of fertilizability being secondary phenotypic expressions arising from the infertile state<sup>15</sup>. In this simple solution to the  $t^x t^y$  sterility it is not necessary to invoke altered sperm surface antigens interacting within the female<sup>16</sup>; the answers appear to lie in the course of spermatogenesis, which is in accordance with and extends the suggestion<sup>17</sup> that events before mating affect  $t^x t^y$  fertility. Preliminary studies so far indicate fewer spermatozoa present in the epididymides and vasa deferentia of  $t^x t^y$  males compared with controls<sup>18</sup>. It is to be hoped that further work will elucidate the actual processes resulting in this severe oligozoospermia found in these heterozygous  $t^x t^y$  male mice.

I thank Dr Jack Cohen for helpful criticism. This research was supported by a studentship from the SRC.

Received 1 August; accepted 19 September, 1980.

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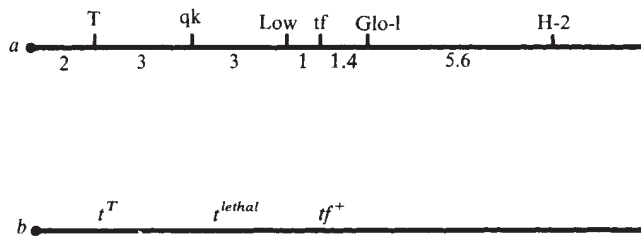
## Molecular analysis of the genetic relationship of *trans* interacting factors at the T/t complex

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The T/t complex is an extensive genetic region proximal to the H-2 complex on mouse chromosome 17, with multiple effects on embryonic development, spermatogenesis and recombination<sup>1-3</sup>. Recently, two-dimensional gel analysis of testicular cell proteins identified a gene within the T/t complex that codes for a major cell surface-associated protein, p63/6.9 (ref. 4). The wild-type gene, *Tcp-1*<sup>b</sup>, codes for a 63,000-molecular weight protein (p63/6.9b), whereas a mutant allele, *Tcp-1*<sup>a</sup>, which occurs in all intact *t* haplotypes, codes for a more acidic form of the protein (p63/6.9a). Analysis of partial *t* haplotypes obtained from rare recombination events showed that *Tcp-1*<sup>a</sup> correlated completely with the tail interaction factor  $t^T$ , which is thought to be a genetic allele of *T*, thus raising the possibility that the locus of *T* codes for the p63/6.9 protein. We report here that the p63/6.9 proteins produced by seven chromosomes carrying independently derived dominant mutations at the locus of *T* are all indistinguishable from the wild-type form; thus, the cumulative data indicate that the *Tcp-1* gene is most probably not at the locus of *T*.

The T/t complex was originally defined by the dominant mutation *Brachyury* (*T*), which shortens the tails of heterozygotes and is lethal at 10.5 days of gestation when homozygous. Recessive lethal *t* haplotypes are found at high frequency in wild mice and are identified by their interaction with *T* to produce a tailless phenotype. Recombination data demonstrate that naturally occurring *t* haplotypes are separable into at least two well defined genetic factors (see Fig. 1): a proximal factor,  $t^T$ , is allelic to and interacts in *trans* with *T* to produce taillessness; a distal factor,  $t^{lethal}$ , results in embryonic death when it is homozygous. Other data indicate that the *t* haplotype may be



**Fig. 1** a, Mouse chromosome 17 and relevant markers. Genetic distance between markers is given in centimorgans. b, *t* Haplotype showing the separation of *t* factors.

separated into several other factors including those factors responsible for male sterility and distorted segregation ratios<sup>5</sup>.

Partial *t* haplotypes, recovered from recombination events, were used to map the *Tcp-1* gene: *Tcp-1*<sup>a</sup> was not associated with the one available partial haplotype (*t*<sup>h17</sup>) carrying only the *t*<sup>lethal</sup> factor, but correlated completely with the presence of the tail interaction factor, *t*<sup>T</sup>, in a variety of partial haplotypes examined. As *t*<sup>T</sup> and *T* behave as genetic alleles, the simplest explanation for the data was that the p63/6.9 protein was the direct gene product of the locus of *T*. However, the form of p63/6.9 produced by the one *T* mutation examined (*Brachyury*) was indistinguishable by two-dimensional gel analysis from wild type.<sup>4</sup> Nevertheless, as two-dimensional gel analysis is capable of defining only about one-third of all point mutations, the possibility remained that the locus of *T* codes for the p63/6.9 protein.

Seven independently derived dominant tail mutations at the locus of *T* have been analysed here (Table 1). These include two radiation-induced mutations and five spontaneous mutations which occurred in laboratory stocks. Of the five spontaneous mutations at *T*, two seem to be deletions of a portion of chromosome 17 including the *T* locus and another locus, *quaking* (*qk*). All *T*-locus mutations meet the following criteria: (1)

they are dominant mutations mapped to the *T/t*-complex region of chromosome 17, and are lethal when homozygous; (2) they interact with *t* to produce a tailless phenotype in *T/t* individuals; (3) they fail to complement one another and hence *T*<sup>+</sup>/*T*<sup>+</sup> individuals die during embryonic development.

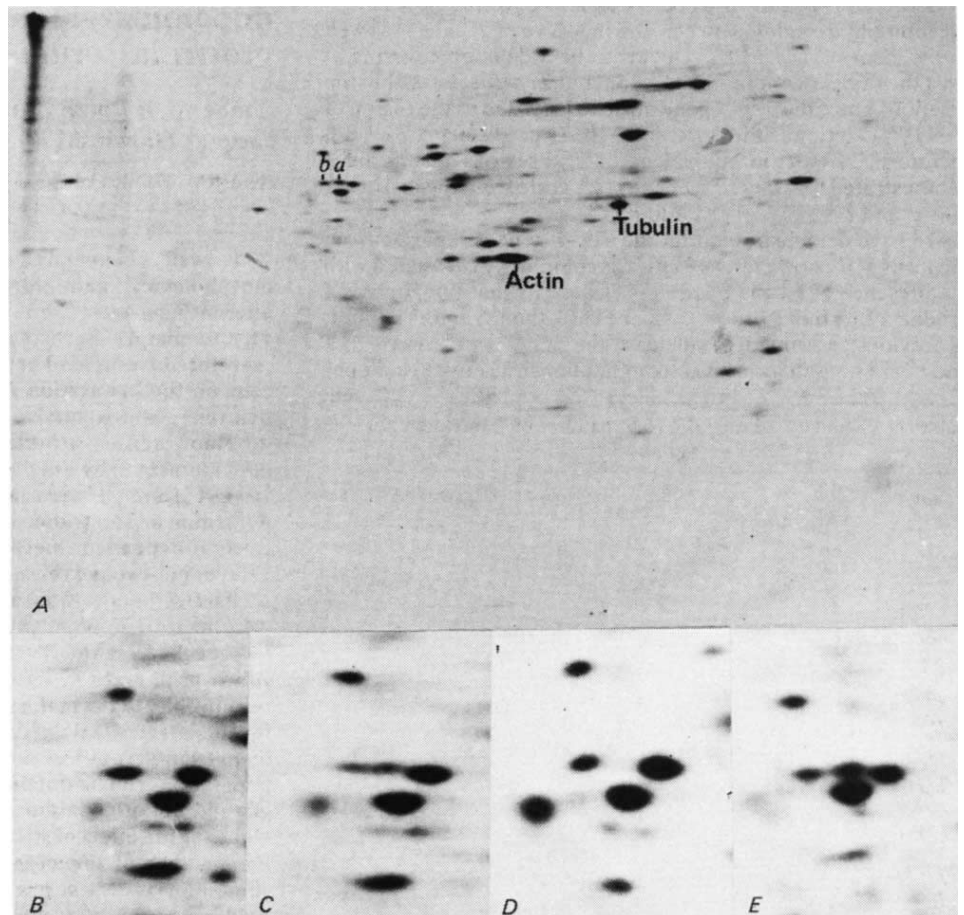
The expression of the p63/6.9 protein in heterozygotes for seven independent *T*-locus mutations was analysed by two-dimensional gel electrophoresis of radio-labelled testicular cell proteins (Fig. 2, Table 2). Animals which carried *T*, *T*<sup>hp</sup>, *T*<sup>Wis</sup>, *T*<sup>J</sup>, *T*<sup>c</sup> and *T*<sup>OR4</sup> opposite a chromosome with a wild-type *Tcp-1*<sup>b</sup> allele expressed a distinct p63/6.9b protein spot, but showed no additional spots (relative to known *Tcp-1*<sup>b</sup> homozygotes) in the p63/6.9 region of two-dimensional gels. In each case, this result indicates that the chromosome opposite *Tcp-1*<sup>b</sup> either expresses p63/6.9b or does not express any form of the p63/6.9 protein.

To distinguish between these two possibilities, we analysed the protein patterns of mice carrying each of these mutations opposite a chromosome carrying a *Tcp-1*<sup>a</sup> allele. Animals which carried *T*, *T*<sup>Wis</sup>, *T*<sup>J</sup>, *T*<sup>c</sup> and *T*<sup>OR4</sup> and a *Tcp-1*<sup>a</sup> allele all expressed both p63/6.9b and p63/6.9a. Therefore, each of these *T*-locus mutations expresses the wild-type form of the p63/6.9 protein. As described previously, mice with a *T*<sup>hp</sup>/*Tcp-1*<sup>a</sup> genotype express only p63/6.9a (ref. 4). Therefore, chromosomes with the *T*<sup>hp</sup> deletion do not have a functional *Tcp-1*<sup>a</sup> gene.

The apparent deletion *T*<sup>Or1</sup> was analysed for *Tcp-1* gene expression. *T*<sup>Or1</sup>/*Tcp-1*<sup>a</sup> mice express both p63/6.9b and p63/6.9a. This indicates that the *T*<sup>Or1</sup> deletion does not cover the *Tcp-1* gene. However, *T*<sup>Or1</sup>/*Tcp-1*<sup>b</sup> mice also express both p63/6.9b and p63/6.9a. These data imply that the *T*<sup>Or1</sup> chromosome, far from containing a deletion of *Tcp-1*, actually possesses both the *Tcp-1*<sup>b</sup> and *Tcp-1*<sup>a</sup> alleles. Further analysis of this chromosome is in progress.

The *Tcp-1* gene codes for a major cell surface-associated protein, p63/6.9. Two structural alleles of this gene have been

**Fig. 2** A, Complete two-dimensional fluorograph of Nonidet P40-soluble proteins from *T*<sup>c</sup>/*t*<sup>w73</sup> testicular cells. b, p63/6.9b; a, p63/6.9a. The basic side of the gel is on the left. B-E, The p63/6.9 regions of fluorographs are shown for the following genotypes: B, *T*<sup>Wis</sup>/+; C, *T*<sup>Wis</sup>/*t*<sup>w73</sup>; D, *T*<sup>OR4</sup>/+; E, *T*<sup>OR4</sup>/*t*<sup>w18</sup>. Testicular cells were isolated essentially as described by Romrell *et al.*<sup>12</sup> as modified by Silver *et al.*<sup>4</sup>. Cells were labelled in medium containing 200–250 μCi ml<sup>-1</sup> <sup>35</sup>S-methionine, for 4–5 h at 34 °C. Cells were washed and lysed at a concentration of 10<sup>7</sup> cells ml<sup>-1</sup> with phosphate-buffered saline containing 1% Nonidet P40, 2 mM methionine and 1 mM phenylmethylsulphonyl-fluoride. Nonidet P40-soluble proteins were obtained after centrifugation at 15,000g at 4 °C for 60 min and were stored at -80 °C. Proteins were separated according to the procedure described by O'Farrell and O'Farrell<sup>13</sup> as modified by Silver *et al.*<sup>4</sup>.



**Table 1** Origin of the various *T* mutations

<i>T</i> Mutation	Origin	Ref.
<i>T</i> <i>Brachyury</i>	Spontaneous in laboratory stock	7
<i>T<sup>hp</sup></i> <i>T<sup>hairpin</sup></i>	Spontaneous in an AKR mouse	8
<i>T<sup>Or1</sup></i> <i>T<sup>Orleans</sup></i>	Spontaneous in a Swiss/Orleans mouse	9
<i>T<sup>Wis</sup></i> <i>T<sup>Wisconsin</sup></i>	Spontaneous in laboratory stock	Moutier (personal communication) D.B. (unpublished data)
<i>T<sup>J</sup></i> <i>T<sup>Jackson</sup></i>	Spontaneous in a BALB/CHu mouse	Hummel (personal communication)
<i>T<sup>c</sup></i> <i>T<sup>curtailed</sup></i>	Radiation induced	10
<i>T<sup>OR4</sup></i> <i>T<sup>Oak Ridge4</sup></i>	Radiation induced	11

identified. All wild-type chromosomes carry *Tcp-1<sup>b</sup>* whereas all naturally occurring *t* haplotypes carry *Tcp-1<sup>a</sup>*. As *Tcp-1<sup>a</sup>* and the *t<sup>T</sup>* allele of the *T* locus seemed to be completely correlated<sup>4</sup>, we sought to determine whether the *Tcp-1* gene and the locus of *T* were identical. Of seven independently derived *T*-locus mutations examined, six allowed expression of a form of p63/6.9 which seems to be identical to the wild-type p63/6.9. The seventh, *T<sup>hp</sup>*, acted as a null allele of *Tcp-1* (*Tcp-1<sup>a</sup>*).

At least 33% of all single base pair substitutions will result in an alteration in protein charge that can be readily detected by standard isoelectric focusing techniques<sup>6</sup>. If we take a conservative estimate, 66% of all single base pair substitutions go undetected (that is, those resulting in a neutral-to-neutral amino acid change). If we assume that the *Tcp-1* gene is at the locus of *T*, the probability that in six independent mutational events at *T* such a shift has occurred and that it remains undetected is (0.66)<sup>6</sup>. The probability of detecting at least one such shift is 1 - (0.66)<sup>6</sup> or 92%. Because, in fact, no shifts were detected, we can say with 92% probability that the *Tcp-1* gene is separate from the locus of *T*. This view is compatible with other genetic evidence. *T<sup>hp</sup>* is known to be a deletion covering at least the 3-centimorgan region between and inclusive of *T* and *qk* as well as the locus of *Tcp-1*. *T<sup>Or1</sup>* seems to be a deletion covering at least the same 3-centimorgan region, but *Tcp-1* is not within the deletion. Thus, the *Tcp-1* gene must be localized to the region of the *T<sup>hp</sup>* deletion either proximal to *T* or distal to *qk* and, therefore, cannot be equivalent to *T*. Previous results have demonstrated that the *Tcp-1* gene is separate from the loci of *qk*, *low*, *tf* and *t<sup>lethal</sup>* (see Fig. 1 and ref. 4).

We have demonstrated that the *Tcp-1* gene is separate from the locus of *T*; however, as *Tcp-1<sup>a</sup>* is completely correlated with the presence of *t<sup>T</sup>*, the possibility still exists that p63/6.9a is a product of *t<sup>T</sup>*. If this proves to be the case, then *T* and *t<sup>T</sup>* cannot, as previously assumed, be alleles at the same locus. Although *T* and *t<sup>T</sup>* are not separable by recombination, one must remember

**Table 2** Summary of the p63/6.9 pattern of expression for the various *T<sup>x</sup>*

Genotype	p63/6.9b	p63/6.9a
<i>T</i> /+	+	-
<i>T</i> / <i>t</i>	+	+
<i>T<sup>hp</sup></i> /+	+	-
<i>T<sup>hp</sup></i> / <i>t</i>	-	+
<i>T<sup>Wis</sup></i> /+	+	-
<i>T<sup>Wis</sup></i> / <i>t</i>	+	+
<i>T<sup>J</sup></i> /+	+	-
<i>T<sup>J</sup></i> / <i>t</i>	+	+
<i>T<sup>c</sup></i> /+	+	-
<i>T<sup>c</sup></i> / <i>t</i>	+	+
<i>T<sup>OR4</sup></i> /+	+	-
<i>T<sup>OR4</sup></i> / <i>t</i>	+	+
<i>T<sup>Or1</sup></i> /+	+	+
<i>T<sup>Or1</sup></i> / <i>t</i>	+	+

that one of the effects of *t* haplotypes is the suppression of recombination along chromosome 17. *T* and *t<sup>T</sup>* interact in *trans* to produce taillessness, but it is possible that *trans* interactions can occur even between two interacting factors at separate loci. Until the precise relationship between *t<sup>T</sup>* and *Tcp-1* can be determined, one cannot comment on the validity of the assumption that *T* and *t<sup>T</sup>* are alleles at the same locus.

Although it is possible that the p63/6.9 protein may be associated with some other property of the *T*/*t* complex, such as segregation distortion or sterility, for which others had suggested factors existed<sup>5</sup>, our data do not fit the distribution of any known *T*/*t*-complex factor other than *t<sup>T</sup>*.

This work was supported in part by NCI core grant CA-08748, grants CA 21651 and HD 10668, and research contract EV04159. A.K.A. is a pre-doctoral fellow at the Sloan-Kettering Division of the Cornell Graduate School of Medical Sciences and received support from the American Cancer Society institutional grant IN-114. We thank Dr T. Alton for his help with the figures, and Drs H. Axelrod, P. McCormick and T. Alton for their critical reading of the manuscript.

Received 19 June; accepted 25 September 1980.

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## Effect of striatal cells on *in vitro* maturation of mesencephalic dopaminergic neurones grown in serum-free conditions

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It is well documented that target cells can regulate the morphological and biochemical development of peripheral afferent neurones<sup>1,2</sup>, but little is known about the existence of such regulatory mechanisms in the central nervous system. We therefore investigated previously the influence of striatal target cells on the maturation *in vitro* of nigrostriatal dopaminergic neurones, which survive in culture for more than 5 weeks, develop dense arborizations and both take up <sup>3</sup>H-dopamine (DA) by a high-affinity specific process and synthesize <sup>3</sup>H-DA from <sup>3</sup>H-tyrosine<sup>3</sup>. Furthermore, depolarization by potassium or veratridine stimulates the release of DA through a calcium-dependent mechanism and tetrodotoxin prevents the veratridine-evoked release of the transmitter<sup>4</sup>. Both the number of <sup>3</sup>H-DA uptake sites and the capacity for <sup>3</sup>H-DA synthesis were at least doubled when the neurones were cultured with target cells from the striatum<sup>3</sup>. To determine whether glial cells which proliferate in serum-complemented medium are partly responsible for the maturation of dopaminergic neurones and/or for the effect of striatal cells, we have now repeated the experiment using serum-free medium in which virtually pure neuronal populations can be obtained<sup>5-7</sup>. The reduction in the number of glia did not affect either the maturation of dopaminergic cells alone, or the effect of striatal cells. Autoradiographic analysis of the number of dopaminergic cells strongly suggests that the stimulatory effect is related to increased capacities of <sup>3</sup>H-DA uptake and synthesis per dopaminergic neurone.