Molecular Cloning and Genetic Mapping of the *t complex responder* Candidate Gene Family

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ABSTRACT

Male transmission ratio distortion (TRD) is a property of mouse *t* haplotypes requiring the *t complex responder* locus (*Tcr*). *Tcr* maps to the central region of *t* haplotypes, and is embedded within a series of large duplicated tracts of DNA known as "T66 elements." In previous work, a family of genes (the "T66" genes) was identified within this region that encodes male germ cell-specific transcripts. Genetic and molecular data indicate that one of these genes represents *Tcr*. Here, we describe the molecular cloning of the four members of the T66 gene family, the genetic mapping of these genes to three adjacent *t* haplotype loci, and comparative restriction enzyme analysis of the genes. The results indicate that these genes are highly similar to one another, and were created by recent, complex duplication events. This suggests that a minor alteration(s) could have been responsible for conferring "mutant" responder activity upon *Tcr*, while the other homologs retained "wild-type" biochemical function. In addition, we have identified and mapped three T66 genes in wild-type *t* complexes. They reside in two separate loci at the opposite ends of the proximal *t* complex inversion, and are separated by at least 3 cM.

t haplotypes are variant forms of the *t* complex, a 15-cM stretch of DNA located in the proximal third of mouse chromosome *17* (for a review, see SILVER 1985). This represents approximately 0.5–1% of the mouse genome. Although most *t* haplotypes contain at least one recessive developmental lethal mutation, and males heterozygous for two complementing *t* haplotypes are sterile, these variant chromosomes propagate to high frequencies in wild mouse populations due to male transmission ratio distortion (TRD). This causes male mice heterozygous for a *t* haplotype and a wild-type form of the *t* complex (+/*t*) to transmit the *t* chromosome to nearly all of their offspring.

TRD is believed to occur through the action of at least four *trans*-acting *t* complex distorter (*Tcd*) loci upon a *t* complex responder (*Tcr*) locus (LYON 1984; SILVER and REMIS 1987; see Figure 1). If a male carries all the *Tcd* loci and is heterozygous for *Tcr*, the *Tcr*-containing chromosome *17* homolog can be transmitted to progeny at frequencies greater than 95%. However, if a male carries the *Tcr* locus in the absence of *Tcd* loci, the frequency is reversed, and the *Tcr*-containing homolog is transmitted to less than 20% of the offspring (LYON and MASON 1964; DUNN and BENNETT 1968). The distorters act in an additive fashion: as doses are removed, *Tcr* transmission declines (LYON 1984). Finally, if the *Tcr* locus is absent from both chromosomes, each is transmitted at 50%, irrespective of the presence or absence of distorters.

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The presence of at least four rearrangements of genetic material in *t* relative to wild-type chromosomes suppresses recombination in +/*t* heterozygotes throughout the *t* complex region (ARTZT, SHIN and BENNETT 1982; PLA and CONDAMINE 1984; HERRMANN *et al.* 1986; SARVETNICK *et al.* 1986; HAMMER, SCHIMENTI and SILVER 1989). This region of recombination suppression formally defines the *t* complex (SILVER 1985), and allows *t* haplotypes to propagate as single genetic units in mouse populations.

Tcr has been mapped to a small region in the center of *t* haplotypes called the *D17Leh66b* locus (abbreviated throughout the text as *T66B*) by molecular and genetic analyses of recombinant chromosomes known as partial *t* haplotypes (Figure 1). Partial *t* haplotypes are the products of rare recombination events between a *t* haplotype and a wild-type form of the *t* complex. These recombinants contain only a portion of *t* haplotype DNA. By using DNA probes which detect restriction fragment length polymorphisms (RFLPs) between wild-type and *t* haplotype loci, the recombination breakpoints of partial *t* haplotypes have been mapped relative to one another. Combined with the genetic testing of these partial *t* haplotypes for responder activity, *Tcr* was localized to *T66B* (LYON and MASON 1964; LYON 1984; FOX *et al.* 1985; HERRMANN, BARLOW and LEHRACH 1987; LYON and ZENTHON 1987).

The *T66B* locus is part of a family (the T66 family) of large, duplicated blocks of DNA sequences called T66 elements. Individual members of the T66 family can be up to 110 kb in length, and are not found

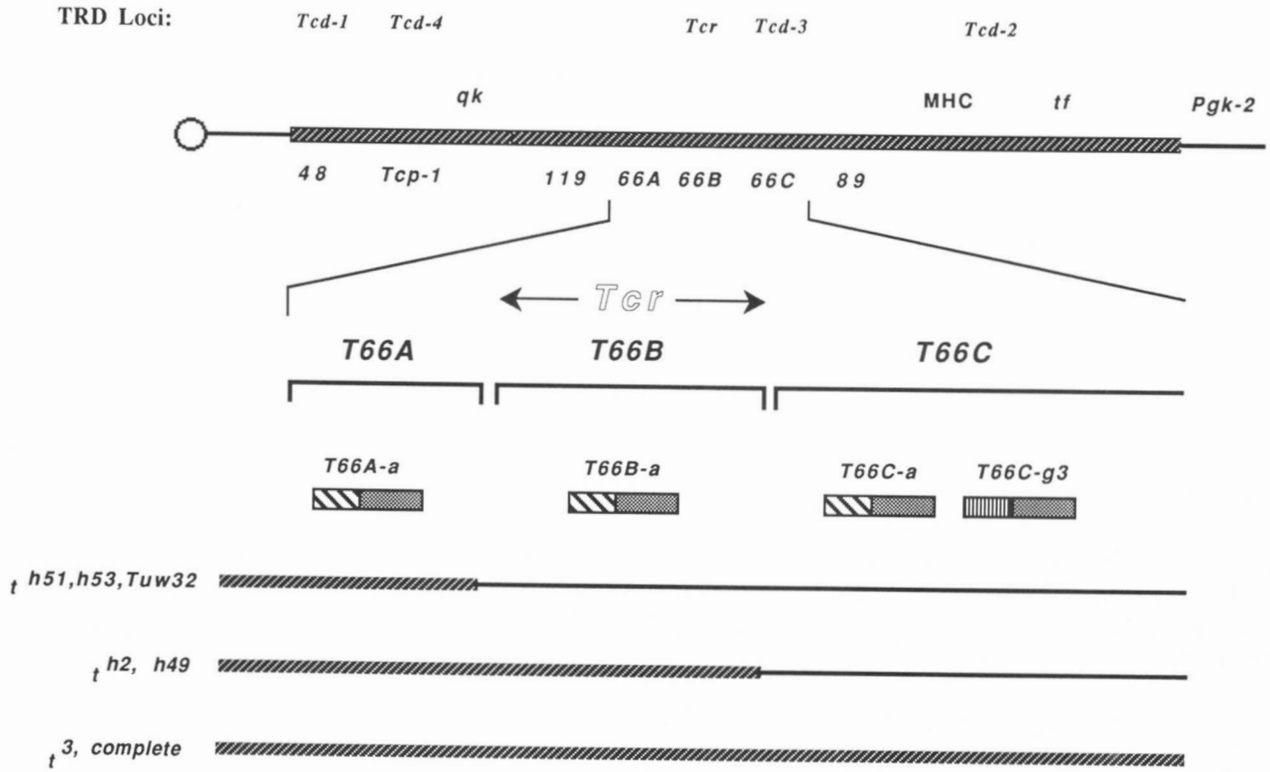

FIGURE 1.—Breakpoint chromosomes in the *Tcr* region. A genetic map of the *t* haplotype form of the *t* complex is at the top of the diagram. The *t* complex is flanked by the centromere on the left (empty circle) and *Pgk-2* (*phosphoglycerate kinase-2*) distally. The genetic loci *qk* (*quaking*), major histocompatibility complex (MHC), and *tf*(*tufted*) are shown above the chromosome, while the following DNA microclone markers are shown below: *48*(*D17Leh48*; FOX *et al.* 1985), *119* (*D17Leh119*; HERRMANN *et al.* 1986), *89* (*D17Leh89*, SILVER and REMIS 1987), and *66A*/*66B*/*66C* (*D17Leh66A*/*B*/*C*; FOX *et al.* 1985). Also shown are loci involved in transmission ratio distortion: the *t* complex distorters *Tcd-1*–*Tcd-4*, and the *t complex responder*, *Tcr* (LYON 1984; SILVER and REMIS 1987). *Tcd-2* is located somewhere between *T66C* and the distal end of the *t* complex. The central region of the *t* haplotype is expanded in the middle of the figure to emphasize the three loci *T66A*, *T66B* and *T66C*, first characterized by FOX *et al.* 1985. *Tcr* is localized to the *T66B* region as indicated. Various partial *t* haplotypes having breakpoints which have been used to define these loci are indicated on the left: t^{h51}, t^{h53}, t^{h2}, t^{h49}, t^{3} (FOX *et al.* 1985); t^{Tuw32} (SILVER *et al.* 1987; SCHIMENTI *et al.* 1987). The locations of the T66 genes discussed in this report are shown at the bottom. The hatched region at the right side of each box represents T66 gene coding and about 15 kb of 5′ flanking sequences (see text; SCHIMENTI *et al.* 1987; L. SNYDER, J. SCHIMENTI, and L. SILVER, unpublished observations). The diagonally striped portions in *T66A-a*, *T66B-a* and *T66C-a* represent homologous "α-like" sequences upstream of the T66 genes. The corresponding portion of the T66C-g3 gene is vertically striped to indicate its dissimilar "γ-like" sequences when compared to the other three genes (SCHIMENTI *et al.* 1987). The order (relative to the centromere) of the two genes in the T66C region are unknown and are shown arbitrarily. Sizes of the genes are greatly exaggerated with respect to the actual regions in which they reside.

elsewhere in the genome (SCHIMENTI *et al.* 1987; J. SCHIMENTI, unpublished observations). The *T66B* locus is flanked on the centromeric and distal sides by the *T66A* and *T66C* loci, respectively, which also contain T66 elements (see Figures 1 and 4). The contiguous *T66A*, *T66B*, and *T66C* loci contain one, two, and eight T66 elements, respectively (see Figure 4). A detailed analysis of these elements through molecular cloning and genomic blotting grouped them into three subclasses–α, β and γ–based on relative similarity to one another (SCHIMENTI *et al.* 1987). These elements arose by a complex series of large scale duplications. The divergence between the α, β, and γ subclasses suggest that considerable periods of time passed between early duplications, followed by more recent events that created multiple subclass members.

Some T66 elements contain sequences (genes) which hybridize to a species of RNA transcripts found specifically in male germ cells (SCHIMENTI *et al.* 1988). Several cDNA clones representing these transcripts were isolated, sequenced, and compared to portions of two cloned genomic genes or pseudogenes (SCHIMENTI *et al.* 1988). One of these genes, called the *T66B-a* gene, maps to *T66B*. Molecular cloning of the *T66B* locus has revealed that it is approximately 220 kb in length (J. SCHIMENTI, unpublished observations). The *T66B-a* gene is a strong candidate for *Tcr* since it maps to the responder locus, is expressed exclusively in male germ cells, and is the only detectable gene within the 220 kb *T66B* locus.

In this report, we present the genomic identification, molecular cloning, genetic mapping, and comparative restriction enzyme analysis of the 4 structural members of the T66 gene family. These results indicate a strong sequence similarity among the genes, which appear to have been created as parts of larger

TABLE 1

Restriction fragments of the T66 genes

Probe	Enzyme	Gene			
		T66A-a	*T66B-a*	*T66C-a*	*T66C-g3*
Tcr16 R/H.2	*Ban*II	**4.2 + 1.5**	1.8 + 0.4	**1.75** + 0.4	**2.1** + 1.8
	*Pst*I	6.25	6.3	7.1	6.3 + **0.6**
	*Bam*HI	**3.4**	6.0	6.0	6.0 + **2.0**
Cg3-79	*Ban*II	**1.5**	1.3	1.3	**1.2**
	*Pst*I	1.6	**1.0**	1.6	1.5

Restriction fragment sizes are in kilobases. Boldfaced numbers denote that the fragment is *t*-specific, *i.e.*, it was not detected in the wild-type chromosomes used in this study.

duplication events. If indeed only one of these genes represents the responder, the molecular change(s) which have conferred this biochemical property must be quite minor, and may be revealed by further detailed comparative analyses. This report is a step toward determining the nature of such mutations.

MATERIALS AND METHODS

Southern blotting: Mouse genomic DNA (5 μg) was digested with the appropriate restriction enzyme, electrophoresed on 0.8% agarose gels, soaked 30–60 min in 1.5 M NaCl, 0.5 M NaOH and transferred to Genescreen membranes (New England Nuclear) overnight in the same solution. Following a 1 min neutralization in 1.5 M NaCl, 0.5 M (Tris pH 7.6), membranes were baked at 80° for 2 hr, then UV cross-linked 15 sec at a distance of 15 cm by 2–254 nm bulbs. The blots were prehybridized at least 5 min in "Church" buffer (CHURCH and GILBERT 1984) at 65°, and hybridized overnight at 65° to random primed probes (FEINBERG and VOGELSTEIN 1984). The blots were washed four times for 5 min in 2× SSC, 0.1% SDS at room temperature, followed by 2–30 min washes in 0.1× SSC, 0.1% SDS at 65°.

Cosmid cloning: Two mouse cosmid libraries containing different *t* haplotypes (genotypes: t^{w2}/t^{w2} and t^{lub1}/t^{w5}) were constructed in the vector pWE15 (EVANS and WAHL 1987). Spleen DNAs were prepared as described (BLIN and STAFFORD 1976). Partial *Sau*3A digests were performed on these DNAs, followed by size fractionation on 0.4% low gelling temperature agarose gels. DNA in the 30–50-kb size range was purified and ligated to *Bam*HI-cut, phosphatased pWE15 cosmid vector DNA and packaged *in vitro* using Gigapack Gold extracts (Stratagene Cloning Systems). Packaged cosmids were infected into either *Escherichia coli* 490A or NM554 (a gift from Heidi Short, Stratagene cloning systems), and plated onto nonsterilized, Millipore HATF 137 mm nitrocellulose membranes placed on top of LB agar plates containing 25 μg/ml kanamycin. The filters were briefly rinsed in sterile water prior to plating. Colonies were grown for 12 hr, then two replicas were made onto nonsterile but rinsed nylon membranes (Nytran, Schleicher & Schuell). The masters were regrown for 2–3 hr at 37°, sandwiched against a second membrane soaked in glycerol, placed between two moist filters (Whatman 3MM), and frozen at −70°. Colonies on the replica filters were lysed according to the instructions outlined by the manufacturer (Schleicher & Schuell), but the DNA was fixed by UV cross-linking as described above. All clones prefixed by "CW" in this report were isolated from the t^{lub1}/t^{w5} library. Clones prefixed by "CD" were isolated from the t^{w2}/t^{w2} library.

Restriction mapping: Cosmids were restriction mapped for the enzymes *Eco*RI, *Bam*HI and *Hin*dIII by an indirect end labeling/partial digestion method (SCHIMENTI *et al.* 1987). These results were compared to those obtained from complete digests of the clones on ethidium bromide stained agarose gels.

DNA Probes: Three probes were used in this study. Tcr16R/H.2 is a 426-bp *Hin*dIII fragment extending from bases 510–936 of a cDNA clone called Tcr16. The sequence of this clone has been published (SCHIMENTI *et al.* 1988). This fragment spans 4.5 kb of genomic DNA (see Figure 3). Cg3-79 is a 400 bp subclone derived from the 5′ end of the *D17Leh66c-g3* gene (SCHIMENTI *et al.* 1987; Figure 3). Cg3-100 is a 534-bp *Eco*RI/*Bam*HI fragment from the 3′ end of the *D17Leh66c-g3* gene (Figure 3). It corresponds to bases 1621–2155 of the cDNA sequences in Figure 4 of SCHIMENTI *et al.* (1988).

Nomenclature: The five genetic subregions discussed in this report are formally designated *D17Leh66a*, *D17Leh66b*, *D17Leh66c*, *D17Leh66d* and *D17Leh66e*. In this text, however, and in earlier publications, they are abbreviated as *T66A*, *T66B*, *T66C*, *T66D* and *T66E*, respectively. The genes described in this text are informally named with regard to the DNA element of which they are a part. These elements, designated *D17Leh66aa*, *D17Leh66ba*, *D17Leh66ca* and *D17Leh66cg-3* are abbreviated in this report as *T66A-a*, *T66B-a*, *T66C-a* and *T66C-g3*. Since it has been shown that at least one of the members of this gene family is translated, this family of genes has been designated *t complex protein-10* (*Tcp-10*; SCHIMENTI *et al.* 1988). The different family members are named *Tcp-10a*, *Tcp-10b*, *Tcp-10c* and *Tcp-10d* in order from the centromere. However, since the order of the *Tcp-10c*/*Tcp-10d* (*T66C-a*/*T66C-g3*, or vice versa) genes is not yet known, we will at present refrain from using this terminology.

RESULTS

Isolation of T66 gene family genomic clones: Prior to this work, a cosmid clone had been isolated which contained the entire genomic coding region of a T66 gene, *T66C-g3* (map location 78-101 in Figure 2 of SCHIMENTI *et al.* 1987). Fragments from this clone were used to identify the T66 family of RNA transcripts (SCHIMENTI *et al.* 1988). Comparison of cDNA sequences to those from the genomic clone allowed an approximate determination of the 5′ and 3′ termini of this gene (SCHIMENTI *et al.* 1988; J. SCHIMENTI and L. SILVER, unpublished observations).

To isolate the remaining members of the family, a cDNA probe corresponding to the central portion of the gene (Tcr16R/H.2; Figure 3) was used to screen genomic cosmid libraries constructed from t^{lub1}/t^{w5} and t^{w2}/t^{w2} mice. Positive clones were tested for hybridization to the probes Cg3-79 and Cg3-100 (see Figure 3), which contain sequences corresponding to the 5′ and 3′ ends, respectively, of the presumably canonical *T66C-g3* gene. Clones hybridizing to both sequences would therefore contain an entire T66 gene.

Mapping of genes and cosmids to *t* haplotype subregions: To map the genetic location of the cosmid clones, we used a strategy which is routinely employed

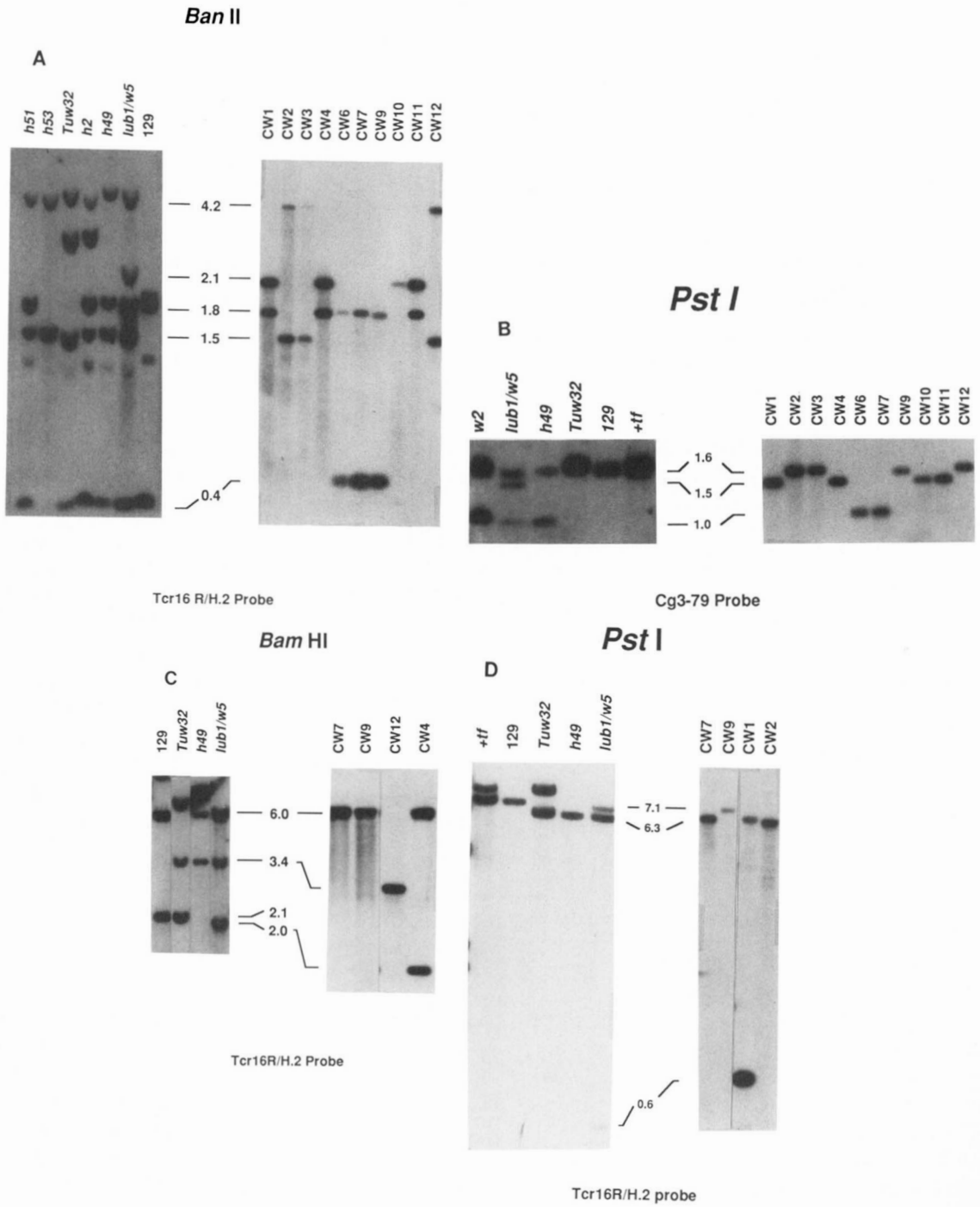

FIGURE 2.—Mapping of T66 genes and cosmid clones by Southern blotting. Genomic DNAs from various *t* complexes, indicated at the top of the lanes, have been abbreviated as follows: *h51* (t^{h51}/+); *h53* (t^{h53}/t^{h53}); *Tuw32* (t^{Tuw32}/t^{Tuw32}); *h2* (t^{h2}/t^{h2}); *h49* (t^{h49}/t^{h49}); *t3* (t^{3}); *lub1/w5* (t^{lub1}/t^{w5}); *w2* (t^{w2}); 129 (*129/Sv*); *+tf* (*+tf/+tf*–a noninbred sample of wild-type chromosome *17* kept in the colony). Cosmid clone DNAs are prefixed by "CW." The sizes of bands are shown in kilobases. A, *Ban*II digests probed with Tcr16R/H.2, B, *Pst*I digests probed with C-g3-79. C, *Bam*HI digests probed with Tcr16R/H.2, D, *Pst*I digests probed with Tcr16R/H.2.

for the mapping of *t* complex DNA probes (FOX *et al.* 1985; HERRMANN *et al.* 1986; SCHIMENTI *et al.* 1987). First, probes for the T66 genes were identified which detect RFLPs between *t* haplotypes and wild type forms of the *t* complex. Second, an RFLP specific for each *t* haplotype gene was identified, in part by probing restriction enzyme digests of cosmid clones. The identification of gene-specific RFLPs was complicated by the presence of numerous genes (at least two or three exist in various inbred mouse strains, and three to four in *t* haplotypes–data presented in this manuscript) and the high similarity between them. Third, these probes were hybridized to panels of DNAs from various partial *t* haplotypes (rare recombinant chromosomes with only a portion of *t* haplotype chromatin) known to have breakpoints within the T66 loci *T66A*, *T66B* and *T66C*. The haplotypes used and their breakpoints within this region are diagrammed in Figures

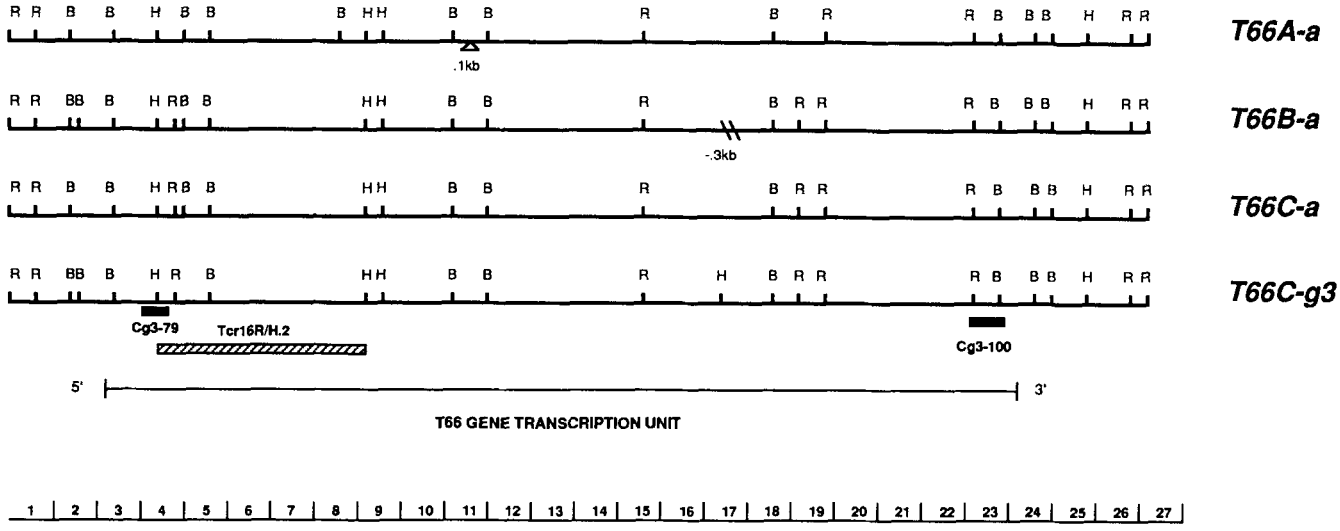

FIGURE 3.—Restriction enzyme map of the T66 genes. The restriction maps of the four T66 genes and a few kilobases of flanking sequences are aligned. The location and orientation of the transcription unit is indicated, and the size in kilobases is shown on the bottom of the diagram. A relative insertion of 0.1 kb is present in *T66A-a* as indicated by a triangle below the map. A 0.3-kb deletion is indicated in the *T66B-a* gene. The maps of the genes were derived from the following cosmid clones: *T66A-a*-CW2, CW12; *T66B-a*-CW6, CW7, and cosu, a cosmid clone isolated from a different t^{lub1}/t^{w5} cosmid library which has been described (SCHIMENTI *et al.* 1987); *T66C-a*-CW9, CD10 (derived from the t^{w2} library); *T66C-g3*-CW1, CW4, CW11. The locations of probes Cg-79 and Cg-100 are indicated by solid black rectangles below the map. The genomic sequences spanned by the cDNA probe Tcr16R/H.2 are shown as a hatched rectangle.

1 and 4, and fall into three classes: those containing just *T66A*, those containing *T66A* and *T66B* but not *T66C*, and those containing all three loci (including complete *t* haplotypes). These haplotypes are scored for the presence or absence of gene-specific RFLPs. It is then possible to localize a RFLP. For example, if an RFLP is present in a partial *t* haplotype which spans both the *T66A* and *T66B* loci, but is absent in a partial *t* haplotype which contains only *T66A*, then it must map to *T66B*. Finally, the cosmid clones can be mapped by scoring for the corresponding RFLPs. It should be noted that the partial *t* haplotypes used in this study contain wild-type T66 loci, and will therefore contain fragments representative of wild-type genes (presented below; Figure 6).

Two probes were informative for these analyses: Cg3-79, which represents the 5′ region of T66 genes, and Tcr16R/H.2, representing the central portion (see MATERIALS AND METHODS; Figure 3). Eleven restriction enzymes were tested for the identification of RFLPs: *Bam*HI, *Ban*II, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II, *Sca*I and *Taq*I. Using RFLPs identified by some of these restriction enzymes, we were able to detect and map four T66 genes in some *t* haplotypes, and three in others. One gene maps to the *T66A* subregion, one to *T66B*, and one or two (depending on the *t* haplotype) within *T66C*.

The gene in the *T66A* region, *T66A-a*, is characterized by *t*-specific 4.2-kb and 1.5-kb *Ban*II fragments detected by the Tcr16R/H.2 probe (Figure 2A; Table 1). These bands on Southern blots are present in all partial *t* chromosomes that extend into *T66A* but not *T66B*, such as t^{h53} and t^{Tuw32} (Figures 1 and 2A). These RFLPs are displayed by the cosmid clones CW2, CW3 and CW12 (Figure 2A). This probe similarly hybridizes to a *T66A*-specific 3.4-kb *Bam*HI band in the same cosmid clones (Figure 2C; Table 1). In addition, *T66A-a* can be mapped by a 1.5-kb *Ban*II RFLP detected by the probe Cg3-79 (Table 1). This RFLP also correlates with the CW2, CW3 and CW12 cosmid clones (not shown).

The *T66B-a* gene was mapped by a 1.0-kb *Pst*I RFLP detected by the Cg3-79 probe. This *t*-specific band is not present in t^{Tuw32}, a partial *t* haplotype which has a breakpoint between the *T66A* and *T66B* regions, but is contained in those with breakpoints between *T66B* and *T66C*, such as t^{h2} and t^{h49} (Figure 2B; Figure 1; Table 1; t^{h2} is not shown). This fragment is contained in cosmids CW6 and CW7 (Figure 2B).

The cumulative data indicate the presence of two genes in the *T66C* interval of the t^{lub1} and t^{w5} haplotypes: *T66C-g3* and *T66C-a*. The *T66C-g3* gene is characterized by four *t*-specific RFLPs indicated in Table 1: 0.6-kb *Pst*I, 2.1-kb *Ban*II and 2.0-kb *Bam*HI fragments with the Tcr16R/H.2 probe (see Figure 2, A and C for the *Ban*II and *Bam*HI RFLP data, respectively, and Table 1 for *Pst*I) and a 1.2-kb *Ban*II fragment with the Cg3-79 probe (Table 1). These RFLPs are present in DNA from the compound heterozygote t^{lub1}/t^{w5}, but not in partial *t* haplotypes which have a breakpoint between *T66B* and *T66C* (Figures 1 and 2, A and C). They are contained in the cosmid clones CW1, CW4, CW10, CW11 and other previously isolated cosmids corresponding to the *T66C-g3* element (Figures 2, A and C; SCHIMENTI *et al.* 1987, 1988).

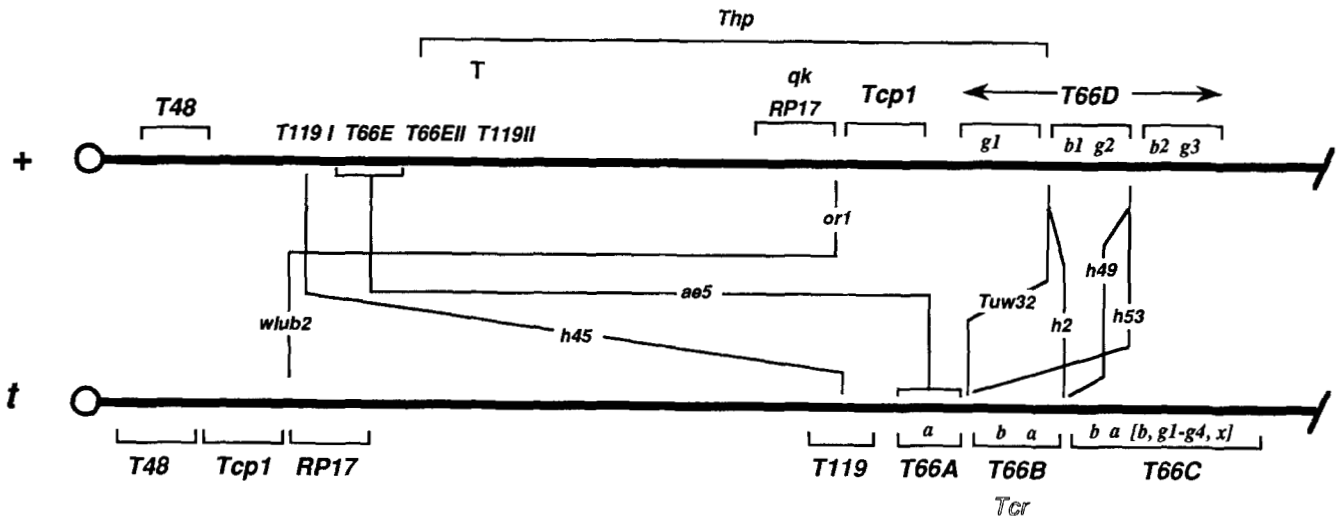

FIGURE 4.—Recombination breakpoints of partial *t* haplotypes. Maps of the proximal portion of wild-type and *t* haplotype forms of the *t* complex are aligned. The relative locations of genetic intervals which have been defined by recombination data are indicated by brackets. Relevant molecular markers for each of these intervals are indicated. The sizes of genetic intervals are not to scale. The classical distance between Brachyury (*T*) and quaking (*qk*) is about 3 cM. The physical size of the *T119-T66E-T66EII-T119II* segment is approximately 1300 kb (HERRMANN, BARLOW and LEHRACH 1987). Individual "T66 elements" in the *T66A, B, C* and *D* loci are listed within brackets. The relative order of T66 elements in the *T66C* locus is not known, but the order in the *T66B* locus is as indicated (J. SCHIMENTI, unpublished observations). The relative order of neither the *T66D-g2/T66D-b1* nor *T66D-g3/T66D-b2* pairs are known. *a, b* and *g* stand for α, β, and γ, respectively, as originally designated by SCHIMENTI *et al.* (1987). Formal nomenclature designations and references on the mapping of the loci *T48* and *T119* are given in the Figure 1 legend. Other markers are: *RP17* (*D17Rp17*; MANN, SILVER and ELLIOTT 1986); and *Tcp-1* (SILVER 1981). An example of formal nomenclature for the various T66 element loci is: *D17Leh66Aa* for the "α" element in *T66A*. The vertical lines connecting the chromosomes represent the breakpoints of various partial *t* haplotypes. The abbreviated names (they should be preceded by a "*t*") of these haplotypes are shown. t^{Tuw32}, t^{h2}, t^{h45}, t^{h49}, t^{h53}, Tt^{or1} and t^{ae5} contain *t* haplotype-derived centromeric ends, while the centromeric end of t^{wlub2} is wild type. The region of DNA deleted by T^{hp} is bracketed. Published reports which have characterized the breakpoints of these recombinant chromosomes are as follows: t^{h51}, t^{h53}, t^{h2}, t^{h49}, t^{3} (FOX *et al.* 1985); t^{Tuw32} (SILVER *et al.* 1987; SCHIMENTI *et al.* 1987); Tt^{or1} and t^{wlub2} (SARVETNICK *et al.* 1986); t^{h45} and t^{ae5} (HERRMANN, BARLOW and LEHRACH 1987).

In contrast, we have not obtained evidence for the presence of the *T66C-g3* gene in the t^{w2} haplotype. This is in agreement with earlier work which reported a deletion of the *T66C-g3* DNA element in this haplotype (SCHIMENTI *et al.* 1987). This apparent deletion was identified by analysis with the probes Cg3-38, Bb-40 and Tu66; these probes are situated approximately 40, 38 and 25 kb upstream, respectively, of the *T66C-g3* gene (SCHIMENTI *et al.* 1987; this report). t^{w2} lacks the 1.5-kb *Pst*I fragment detected by the Cg3-79 probe, which is contained in t^{lub1}/t^{w5} mice and the *T66C-g3* gene cosmids CW1, CW4, CW10 and CW11 (Figure 2B). Similar results have been obtained for t^{3}, a partial *t* chromosome which has a breakpoint distal to the T66 family of DNA sequences (FOX *et al.* 1985). We have probed Southern blots of t^{w2} homozygous DNA cut with 14 restriction enzymes using the Tcr16R/H.2 probe. Seven of these enzymes (*Bgl*II, *Eco*RI, *Hin*dIII, *Nco*I, *Pst*I, *Taq*I and *Xba*I) generated three bands of apparently equal intensities, while none yielded four bands (data not shown). The transmission frequency of t^{w2} in heterozygous males is 95% (DUNN and SUCKLING 1956), indicating that this gene (which is believed to be a pseudogene–see DISCUSSION) does not participate in TRD.

A fourth gene, *T66C-a*, was identified and mapped indirectly. Because no partial *t* haplotypes have been described with breakpoints in *T66C* (to a resolution determined by the current availability of DNA probes), demonstration of multiple genes in this locus depended on a compilation of the following pieces of data: (1) identification of nonallelic RFLPs mapping to this region, (2) restriction patterns of cosmid clones, and (3) physical linkage to diagnostic RFLPs outside the T66 gene sequences.

The presence of the *T66C-a* gene was predicted from previous studies of the T66 family of DNA elements and T66 gene sequences. The *T66C-a* element contains sequences homologous to the 5′ end of T66 gene transcripts (SCHIMENTI *et al.* 1988). Cloned DNA upstream of these sequences were clearly distinct from those flanking the *T66C-g3* gene (see DISCUSSION pertaining to alpha *vs* gamma T66 elements). One cosmid clone isolated from the t^{lub1}/t^{w5} library, CW9, has a set of restriction fragments which differ from the *T66A-a*, *T66B-a* and *T66C-g3* genes (Table 1). CW9 has a unique 7.1-kb *Pst*I fragment which hybridizes to the Tcr16R/H.2 probe (Table 1; Figure 2D). While this fragment is present in DNA from both wild type and t^{lub1}/t^{w5} mice, it is not contained in the partial *t* haplotypes t^{Tuw32}, t^{h49} or t^{h2} (Figure 2D; t^{h2} is not shown). This indicates that the fragment does not map to the *T66A* or *T66B* loci (Figure 1). We conclude that this fragment (and therefore the CW-9 cosmid)

maps to *T66C*, and is representative of the *T66C-a* gene.

Because the cosmid library and genomic blots were made with compound heterozygote t^{lub1}/t^{w5} DNA, it remains possible that the *T66C-a* and *T66C-g3* genes are alleles. However, several pieces of data indicate that these two genes are nonallelic. First, for all five combinations of probes and restriction enzymes listed in Table 1, the two cloned genes have different patterns. Second, these two genes are linked to vastly different upstream sequences–*T66C-a* to "α"-like and *T66C-g3* to "γ"-like DNA (SCHIMENTI *et al.* 1987; see DISCUSSION). Thirdly, it has been shown that the t^{lub1} and t^{w5} haplotypes contain sequences representative of both the *T66C-a* and *T66C-g3* elements (SCHIMENTI *et al.* 1987).

Comparative restriction enzyme mapping and gene boundaries: Our cosmid clones were mapped with three enzymes, *Eco*RI, *Bam*HI and *Hin*dIII. The analysis is shown in Figure 3. It is evident that all four genes are highly similar in DNA sequence. If the genes are superimposed, a total of 26 different restriction enzyme sites are present in the region examined. Twenty of these are shared by each of the four genes, while five represent a presence or absence of a site specific to only one of the genes. In only one case (a *Bam*HI site at position 1.5) is a site shared by two genes and absent in the others. We detected one relative insertion in the *T66A-a* gene and one relative deletion in the *T66B-a* gene after comparison to the others. The nature of these size differences is unknown.

T66 genes in wild-type *t* complexes: Although we have not cloned wild-type T66 gene homologs, they can be identified and genetically mapped by Southern blot analysis of recombinant chromosomes. An informative probe for these analyses is Cg-100, which was isolated from the 3′ end of the *T66C-g3* gene (Figure 3). When hybridized to Southern blots of *Taq*I digested t^{lub1}/t^{w5} DNA, a single fragment of 9.6 kb is detected (see Figure 5). This demonstrates that all T66 genes in these *t* haplotypes possess a fragment of this size. 129/Sv mice display three bands of 9.4, 7.2 and 5.8 kb, whereas C3H/He mice have 9.6- and 7.2-kb bands (Figure 5).

The wild-type-specific 7.2-kb *Taq*I band, which is shared by 129/Sv and C3H/He, is present only in those recombinant chromosomes which contain wild-type DNA proximal to the RP17 locus. These includes t^{ae5}, t^{h45}, t^{wlub2}, and T^{hp} (see Figures 4 and 5). It is absent from partial *t* haplotypes which contain all (Tt^{or1}) or part (t^{h53}, t^{Tuw32}, t^{h49}) of the *T66D* locus, but not *T66E* (Figures 4 and 5). These data allow the localization of this fragment between the wild-type breakpoints of T^{hp} and t^{ae5} (Figure 4), a segment of DNA approximately 500 kb in length (HERRMANN, BARLOW and LEHRACH 1987). This genomic region

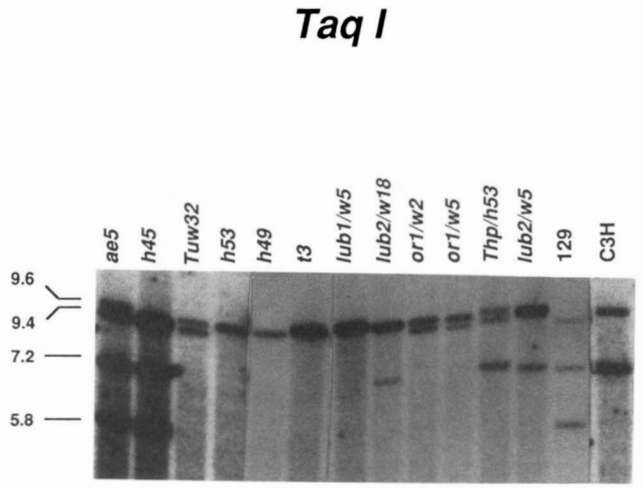

FIGURE 5.—Mapping of wild-type T66 genes by Southern blotting. All of the DNAs shown have been digested with *Taq*I and probed with Cg-100. The molecular weight of the four bands are shown in kilobases. The abbreviated names of various *t* haplotypes (they should be preceded by a "*t*") are shown. 129 stands for 129/Sv, and C3H for C3H/He. The t^{w18} chromosome contains only the *t* forms of the T66 loci (BUCAN *et al.* 1987).

contains a large inverted duplication of DNA, part of which contains T66 element sequences (HERRMANN, BARLOW and LEHRACH 1987; SCHIMENTI *et al.* 1987). The order of loci in this region was determined to be: centromere-*T119I-T66E-T66EII-T119II* (Figure 4; HERRMANN, BARLOW and LEHRACH 1987). The T66 gene represented by the 7.2-kb *Taq*I fragment must map to either *T66E* or *T66EII*.

The 5.8- and 9.4-kb bands present in 129/Sv must be interpreted with caution since they are not present in C3H/He. This discrepancy is indicative of polymorphism in wild-type *t* complexes. One of these two bands may be allelic to the 9.6 kb band in C3H/He (Figure 5), and the other may represent a gene absent in this strain. Support for this interpretation comes from the observation that C3H/He mice are deleted for the *T66D-b1* and *T66D-g2* loci (SCHIMENTI *et al.* 1987). However, it must be considered that one of the C3H/He bands actually represents a doublet.

The interpretation most consistent with our data maps the 5.8 kb fragment to the *T66D-g1* locus. All of the proximal partial *t* haplotypes tested which contain wild-type chromatin distal, but not proximal, to *T66D-g1* do not contain this band (Figures 4 and 5). It is present in the t^{ae5} and t^{h45} haplotypes, which possess the entire *T66D* locus (Figures 4 and 5). It is absent in t^{wlub2}, which contains wild-type chromatin proximal to the *Tcp-1* locus. These data localize the 5.8 kb band distal to *Tcp-1* and proximal to *T66D-b1/T66D-g2*. *T66D-g1* represents the only characterized locus containing a T66 element in this region, and is the likely location of the T66 gene homolog containing the 5.8-kb fragment. Interestingly, this band is absent from the deletion chromosome T^{hp} (Figure 5), which is known to be deleted for Tcp-1 (SILVER,

WHITE and ARTZT 1980), but not sequences in *T66D* detected by a probe called p66M-RT (HERRMANN, BARLOW and LEHRACH 1987). This probe hybridizes to a 10-kb wild-type specific *Bam*HI fragment in t^{h2} but not t^{h49} DNA (HERRMANN *et al.* 1986). This would appear to localize this 10-kb *Bam*HI fragment to the *T66D-b1/T66D-g2* locus (Figure 4). We conclude that the distal T^{hp} deletion breakpoint lies between *T66D-g1* and *T66D-b1/T66D-g2*, thereby deleting the *T66D-g1* locus and a resident T66 gene homolog represented by the 5.8-kb *Taq*I restriction fragment.

The partial *t* haplotype Tt^{or1} seems to contradict the mapping of a T66 gene to *T66D-g1*. This chromosome does not have the *T66E* loci, but does contain the entire *T66D* locus (Figure 4). It does not exhibit the 5.8-kb *Taq*I fragment (see the Tt^{or1}/t^{w2} and Tt^{or1}/t^{w5} samples in Figure 5). In isolation, this might suggest that the 5.8-kb band maps to T66E or T66EII. However, neither t^{wlub2} nor t^{h45}, both of which contain *T66E* and *T66EII*, have this band. It is likely that polymorphism in the wild-type T66 gene loci, such as the variation seen between 129/Sv and C3H/He, is responsible for this discrepancy. Specifically, allelism between the 9.6 kb C3H/He and 5.8 kb 129/Sv *Taq*I fragments could explain the disconcordance (Figure 5).

The remaining 129/Sv 9.4-kb *Taq*I band appears to map to the *T66D-g2/T66D-b1* subregion. It is present in the recombinant chromosomes which contain the entire *T66D* locus (t^{ae5}, t^{h45} and Tt^{or1}), and t^{Tuw32}, which has a breakpoint between *T66D-g1* and the distal *T66D* subregions (Figures 4 and 5). It is absent in the partial *t* haplotypes t^{h49} and t^{h53}, which recombined between *T66D-b1/T66D-g2* and *T66-b2/T66-g3*, as well as t^{wlub2}, which has none of the *T66D* loci. Finally, it has been reported that the C3H/He chromosome is deleted for the *T66D-b1* and *T66D-g2* elements (SCHIMENTI *et al.* 1987), which could account for the absence of this band/gene.

In summary, we have found evidence for the existence of three T66 genes in wild-type forms of the *t* complex. One maps to *T66E/T66EII* (the *T66E* gene), a second to *T66D-g1* (the *T66D-g1* gene), and a third to *T66D-b1/T66D-g2* (the *T66D-b1g2* gene). Contrary to a report which cited preliminary evidence for the presence of a T66 gene associated with the *T66D-g3* element (SCHIMENTI *et al.* 1988), we have not found any evidence for such a gene in this study.

DISCUSSION

Evolution of the T66 genes: The genomic analyses and cloning data presented here demonstrate the existence of three to four T66 gene homologs in those *t* haplotypes examined. The comparative restriction mapping shows that these genes are highly related, and suggest that this gene family was either recently created, or has been subject to concerted evolution. Rat and *Mus spretus* appear to have only one T66 gene copy as assayed by Southern blot analysis (J. SCHIMENTI, unpublished observations). While this suggests a recent expansion in *t* haplotype gene copy number, the possibility that these species have deleted genes subsequent to diverging cannot be ruled out. As we have found in this study, different inbred strains appear to have different numbers of T66 genes. The variety in copy number suggests that unequal recombination has been a mechanism for expansion and/or contraction of this gene family (SCHIMENTI *et al.* 1987).

The restriction site analyses of the genes presented here are insufficient to allow a determination of their evolutionary relatedness. Closer molecular examination of flanking and intron regions, coupled with a complete linkage map of the various elements, may elucidate the events leading to the creation of this multigene family. Similar work on the wild-type loci must be performed in order to reconstruct events which have occurred in the T66 family since the divergence of + and *t* chromosomes. These questions are also significant in regard to the T66 family's ostensible involvement in the "proximal" *t* complex inversion event (SCHIMENTI and SILVER 1986; SCHIMENTI *et al.* 1987). Recent work with interspecific crosses has supported the hypothesis that the proximal inversion actually occurred in the wild-type lineage, with a breakpoint in the T66 family that resulted in the transposition of two T66 DNA elements several centiMorgans towards the centromere (HAMMER, SCHIMENTI and SILVER 1989). It appears that the *T66E* gene was contained in this inversion, resulting in its separation from the other T66 genes by at least 3 cM.

Relationship of T66 genes to T66 elements: The initial studies of the T66 region of *t* haplotypes resulted in the identification of a family of large, duplicated tracts of DNA called "T66 elements" (SCHIMENTI *et al.* 1987). These elements were classified into three subfamilies (α, β and γ) based on differential hybridization to various probes. The size of individual elements range up to 110 kb (J. SCHIMENTI, unpublished observations), and in some cases include T66 genes. It is likely that the T66 genes expanded in copy number by inclusion in larger scale T66 element duplications. Three of the T66 genes described here, *T66A-a*, *T66B-a* and *T66C-a*, comprise portions of the α-class elements. In contrast, the *T66C-g3* gene adjoins distinct γ-type sequences; these sequences map about 20 kb 5′ (at map position 60 in SCHIMENTI *et al.* 1987) to the transcription initiation site (L. SNYDER, J. SCHIMENTI and L. M. SILVER, unpublished results). The α and γ sequences have diverged to a degree where they do not cross-hybridize over large stretches (SCHIMENTI *et al.* 1987). The *T66C-g3* gene and its adjacent sequences may have arisen by either of two means: (1)

a partial duplication event not involving an entire α element, or (2) a deletion which removed the upstream 5′ α sequences, resulting in juxtaposition to γ-type DNA.

Two of the wild-type T66 gene homologs, *T66D-g1* and *T66D-b1/g2*, colocalize with γ elements. The *T66E* gene resides within a region which contains both an α and β element (SCHIMENTI *et al.* 1987). Although the wild-type genes and their flanking sequences have not been cloned, we can speculate that the *T66D-g1* and *T66D-b1/g2* genes are parts of γ elements (as the *t* haplotype *T66C-g3* gene), and the *T66E* gene is part of an α element (as the *T66A-a*, *T66B-a* and *T66C-a* genes). That *t* haplotypes have three α-associated (and sometimes one γ-associated) T66 genes while wild type appears to have two γs and one α indicates that a dramatic series of evolutionary changes occurred in this gene family since the divergence of *t* and + forms of the *t* complex. A clear understanding of the exact structural relationships between the two *t* complex forms will require a higher resolution analysis of the wild-type T66 loci.

Function of the T66 gene family members: The apparent absence of the *T66C-g3* gene from some *t* haplotypes suggests that its function is either redundant, unnecessary, or nonexistent. Since the t^{w2} haplotype (which is deleted for *T66C-g3*) has a 96% distortion ratio in males, this gene does not seem to play a role in TRD. The predicted amino acid coding sequence of a portion of this gene is divergent compared to sequenced cDNAs (SCHIMENTI *et al.* 1988), and the sequences of several PCR-amplified T66 transcripts do not match that of *T66C-g3* (L. SILVER personal communication). These early indications raise the possibility that *T66C-g3* is a pseudogene.

If indeed the *T66B-a* gene represents the responder as postulated, what is the function of the other T66 genes? There are three likely possibilities: (1) They are pseudogenes; (2) They also possess "mutant" responder-like activity; and (3) They play a "normal" or wild-type functional role in spermatogenesis. Although the *T66C-g3* gene may be a pseudogene, both the *T66A-a* and *T66C-a* genes are transcribed (SCHIMENTI *et al.* 1988; J. THOMAS and L. SILVER, personal communication). However, it is unknown whether the messages they encode are translated.

The cumulative genetic evidence argues against the possibility that the *T66A-a* and *T66C-a* genes have responder activity. The *T66A* locus, in which the *T66A-a* gene maps, does not appear to play a role in TRD (SILVER and REMIS 1987). Although the distorter gene *Tcd-3* maps to *T66C*, it has been impossible to assay potential responder activity associated with this locus due to the non-existence of recombinant haplotypes which contain *T66C* but not *T66B*. Nevertheless, several studies have indicated that the *T66B* locus possesses full ability to mediate TRD (FOX *et al.* 1984; LYON 1986; SILVER and REMIS 1987).

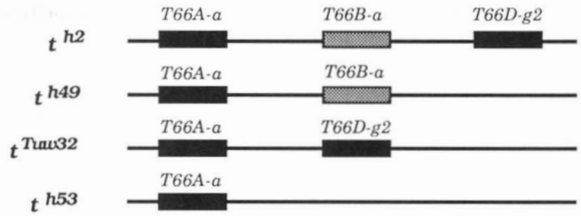

FIGURE 6.—Arrays of T66 genes in partial *t* haplotypes. Individual T66 genes which have been positively identified in this study are depicted as boxes. The candidate *Tcr* gene (*T66B-a*) is represented by a shaded box. Names of the individual genes are shown. For additional evidence that t^{h53} contains no wild-type T66 genes, note the absence of wild-type fragments in Figure 2A.

We have found evidence in this study which supports the possibility that the *T66A-a* and *T66C-a* genes have wild-type function. The proximal partial haplotype t^{h53} appears to contain only one T66 gene, the *T66A-a* gene (Figure 6). This haplotype has a normal transmission ratio (FOX *et al.* 1984), indicating that sperm can function normally without other T66 genes. We postulate that the *T66A-a* gene is capable of performing a "normal" biochemical function, and can substitute for wild-type T66 genes during spermatogenesis.

A model for TRD: If the *T66A-a* and *T66C-a* genes are essentially wild type, this raises the possibility that *t* sperm require "wild-type" responder function in *cis* for normal development or viability. We can build upon earlier hypotheses concerning the mechanism of TRD and propose the following scenario: (1) At least one "wild-type" T66 gene is required for the production of viable sperm, and the *T66A-a* and *T66C-a* genes are equivalent to wild-type. (2) *Tcr* encodes a nonfunctional (in the context of wild type responder function) or aberrant polypeptide which is localized inside the sperm in which it is produced (the *t* haplotype sperm). (3) The *Tcr* protein has a high affinity for interacting with the various putative distorter proteins. (4) According to the model of LYON (1986), the distorters act deleteriously upon the wild-type forms of the responder. (5) The avid interaction between *Tcr* and distorters decreases the concentration of free distorters inside sperm with *Tcr*, allowing the products of the biochemically "normal" *T66A-a* and *T66C-a* genes to form functional protein complexes with the trans-active wild-type distorters.

This model is consistent with the observation that no partial *t* haplotypes have yet been identified which contain the *T66B-a* gene in isolation (Figure 6). Although the t^{low} series of recombinants contain the *T66B* locus, but not *T66A* or *T66C*, and t^{low} homozygotes are fertile (DUNN and BENNETT 1971), it is unknown whether they possess any wild-type T66 genes as a consequence of the recombination events which created them. We are currently investigating this question.

Due to the unique structure and properties of *t* haplotypes, it has been impossible to generate ideal recombinant chromosomes which would allow us to address the functional roles of the individual T66 genes. It is clear that more modern approaches, such as transgenesis and gene disruption by homologous recombination, will be required to thoroughly understand the T66 gene family. Such experiments are under way.

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