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Genetics

Testis-/embryo-expressed genes are clustered in the mouse H-2K region

(*t* complex/endogenous retrovirus)

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ABSTRACT **The major histocompatibility complex (MHC) of the mouse is located on chromosome 17 in the distal inversion of the *t* complex. In addition to genes playing major roles in the immune response, it contains a diversity of genes. In humans, numerous diseases are known to be associated with the MHC loci. Moreover, at least three recessive embryonic *t*-lethal mutations have been mapped to the MHC. Here a molecular genetic approach was used to study the detailed genomic structure of 240 kilobases (kb) surrounding the *H-2K* gene and 150 kb of a partly homologous region located in the distal inversion of the *t* complex. Combined with previous findings, the H-2K region was found to contain an impressively high density of genes—12 transcription units in 240 kb. Surprisingly, virtually all of these genes are expressed in testis and/or embryos. The genomic organization of this region is contrasted with the 150 kb of the homologous area where only three genes and an endogenous retrovirus reside.**

The major histocompatibility complex (MHC) is located on the short arm of human chromosome 6 and the proximal third of mouse chromosome 17. This 3500-kilobase (kb) chromosome segment is one of the best studied areas of the mammalian genome. It is chiefly known for its content of genes important in the immune system: The highly polymorphic class I and class II genes, the class III components of the complement cascade, and a peptide transporter gene that has recently been found in the human class II region (1–3). The MHC also encodes a number of molecules of known function not obviously related to the immune system. This list now includes genes as diverse as tumor necrosis factors, steroid 21-hydroxylase, neuraminidase 1, heat shock proteins, *Oct-3/4* transcription factor, and *int-3* oncogene (4).

The MHC has been molecularly characterized so well that gene searching has become feasible and a striking number of transcription units of unknown function have been revealed. In the human class III region, a 435-kb genomic segment was found to contain 9 additional genes (*BAT1–9*) in one study (5) and, in another, 10 transcripts (*G1–G10*) (6). In the human and mouse class II regions, 4 (7) and 6 (8) genes were identified, respectively, and in the mouse H-2K region, the tally to date is 5 genes (*KE1–5*) (9).

There are at least three compelling reasons for scrutinizing this complex and diverse chromosomal region. First, some MHC haplotypes are known to be involved in susceptibility to diseases (10). In several cases, a direct association between disease susceptibility and a particular class I or II molecule has been demonstrated (11, 12); in other cases, the genetic predisposition maps to a gene of nonimmunological or unknown function residing in the MHC (13, 14). However, in many MHC-associated diseases, it is not conclusively established whether the genetic predisposition is directly associated with a particular class I or II molecule or is due to an as yet unidentified gene closely linked to the class I or II loci.

Second, in the mouse, the MHC is embedded in the middle of the *t* complex in the distal inversion (reviewed in ref. 15). In mutant *t* haplotypes, this particular segment of chromosome contains at least three recessive early embryonic lethal genes in or very near the MHC. One of them, *tcl-w5*, is genetically inseparable from *H-2K* (16). In addition, in mutant haplotypes, the distal inversion contains several genes involved in male transmission ratio distortion and sterility. The molecular identification of these *t*-mutant genes is expected to help understand key developmental pathways controlling the early stages of mammalian embryogenesis and sperm maturation and function. Lastly, knowledge of the detailed genomic organization of the MHC should allow an in-depth understanding of the evolutionary history of a sizable segment of mammalian chromosome.

Here we describe an extended chromosomal walk in the H-2K region, which led to the identification of seven additional genes. Five of them are located proximal to *H-2K* in wild type. This brings the regional total to at least 12 transcribed sequences or 1 every 20 kb.

MATERIALS AND METHODS

Mice. Except for t^{w12}/t^{Lub1}, mice bearing *t* haplotypes are congenic on C3H/DiSn (C3H) and are bred in our mouse colony. C3H.t^{w5}/t^{12} compound mice were obtained as normal-tailed offspring from crosses of tailless C3H.T/t^{w5} × C3H.T/t^{12}. t^{w5g} is a viable revertant of the t^{w5} haplotype, isolated as a normal-tailed progeny from the balanced lethal cross of C3H.T/t^{w5}; the t^{w5g} chromosome appears to be identical to t^{w5} except for the loss of the embryonic lethality (unpublished data). CF1 is a random bred, wild-type stock. W/W^v lacks germ cells and was purchased from The Jackson Laboratory.

Cell Lines. C44 (17), t^{w5}/t^{w5} cells (18), and nulli (19) are embryonal carcinoma (EC) cell lines. MAO-A 1A9 (B-cell line), BW5147 (T-cell line), and NS1 (a myeloma line) were from P. D. Gottlieb (University of Texas at Austin). E36 is a hamster cell line, and R4.4-1 is a mouse–hamster hybrid cell line that contains mouse chromosomes 17 and 18 fused as a single metacentric chromosome (20).

Cosmid and cDNA Libraries. The cosmid libraries from C3H.t^{w5g}/t^{w5g} or t^{w5}/t^{w5} EC cells were made in pWE15 and screened as described (21). The clones were restriction mapped by the terminase oligomer method (22). cDNA libraries: the 5.5-day and 10.5-day embryo libraries are described by Abe *et al.* (9); the wild-type testis library was purchased from Clontech; the *t*-haplotype testis library was

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Abbreviation: MHC, major histocompatibility complex.

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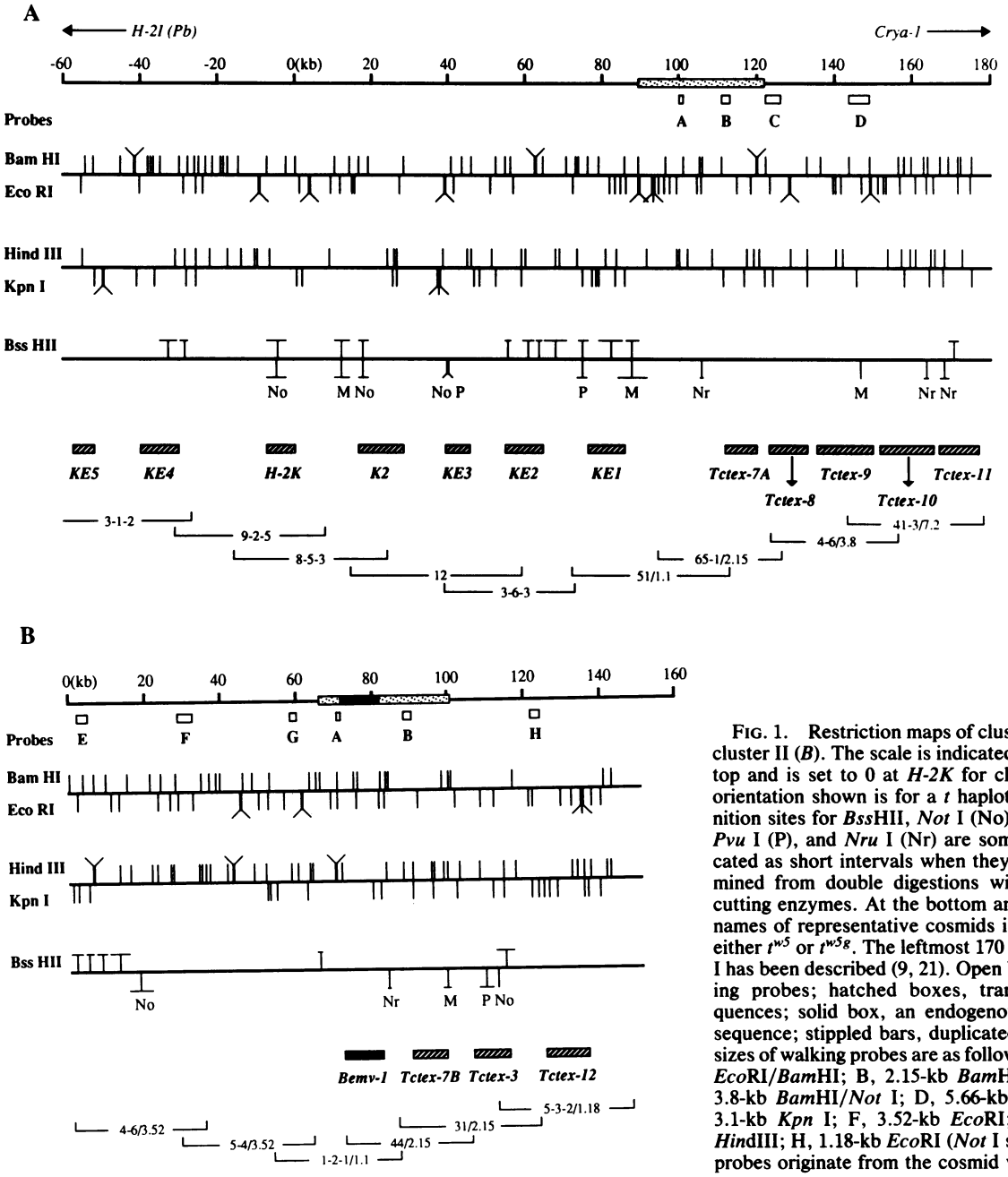

FIG. 1. Restriction maps of cluster I (*A*) and cluster II (*B*). The scale is indicated in kb at the top and is set to 0 at *H-2K* for cluster I. The orientation shown is for a *t* haplotype. Recognition sites for *Bss*HII, *Not* I (No), *Mlu* I (M), *Pvu* I (P), and *Nru* I (Nr) are sometimes indicated as short intervals when they were determined from double digestions with frequent-cutting enzymes. At the bottom are shown the names of representative cosmids isolated from either t^{w5} or t^{w5g}. The leftmost 170 kb of cluster I has been described (9, 21). Open boxes, walking probes; hatched boxes, transcribed sequences; solid box, an endogenous retroviral sequence; stippled bars, duplicated areas. The sizes of walking probes are as follows: A, 1.1-kb *Eco*RI/*Bam*HI; B, 2.15-kb *Bam*HI/*Not* I; C, 3.8-kb *Bam*HI/*Not* I; D, 5.66-kb *Bam*HI; E, 3.1-kb *Kpn* I; F, 3.52-kb *Eco*RI; G, 1.45-kb *Hin*dIII; H, 1.18-kb *Eco*RI (*Not* I sites of these probes originate from the cosmid vector).

a gift from J. Schimenti (Case Western Reserve University). These libraries were screened as described (9).

Southern and Northern Blot Analyses. Restriction-digested DNA (10 μg) was size-fractionated on a 0.7% agarose gel in Tris/phosphate buffer and transferred to a nylon membrane (Hybond N; Amersham). Blots were hybridized at 42°C in a mixture containing 50% formamide, 50 mM sodium phosphate (pH 6.5), 5× standard saline citrate (SSC), 5× Denhardt's solution, 0.1% SDS, 1 mM EDTA, 7.5% dextran sulfate, and heat-denatured salmon sperm DNA (100 μg/ml). Radiolabeled genomic probes were treated before hybridization with 1.5×10^4 times excess (wt/wt) amount of unlabeled mouse DNA at 42°C to block highly repetitive sequences. Blots were washed in 2× SSC/0.1% SDS for 50 min at room temperature and then in 0.1× SSC/0.1% SDS for 40 min at 65°C. In zoo blots, the washing stringency was reduced to 2× SSC/0.1% SDS at room temperature (twice for 25 min), followed by 0.5–2× SSC/0.1% SDS at 50°C–65°C (10–20 min). Only 1 μg of DNA was used for *Drosophila*. For Northern blots, total RNA (10 μg per lane) was size-fractionated on a 1% agarose gel containing 6.7% formaldehyde. Northern blots were hybridized and washed as described above.

Pulsed-Field Gel Analysis. DNA plugs were prepared (23) from spleen cells aiming to have 5 μg of DNA in a 70-μl block of 0.5% low-melting-point agarose. The plug was digested overnight using 3 units of enzyme per μg of DNA and run in 1.2% agarose gels in 0.25× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) using a hexagonal gel box (24). Running conditions were a 45-sec pulse time at 3 V/cm for 16 hr, followed by a 35-sec pulse time at 5 V/cm for 43 hr at 4°C–6°C. Gels were UV irradiated 2 min per side, denatured, and Southern-transferred to membrane by vacuum blotting for 5 hr at a pressure of 50–60 cm_{H_2O}. The blot was hybridized as described above and washed in 2× SSC/0.1% SDS for 50 min at room temperature with one change.

RESULTS AND DISCUSSION

The Extended Walk Tripped over a 35-kb Duplicated Region. We previously described five embryo-expressed genes

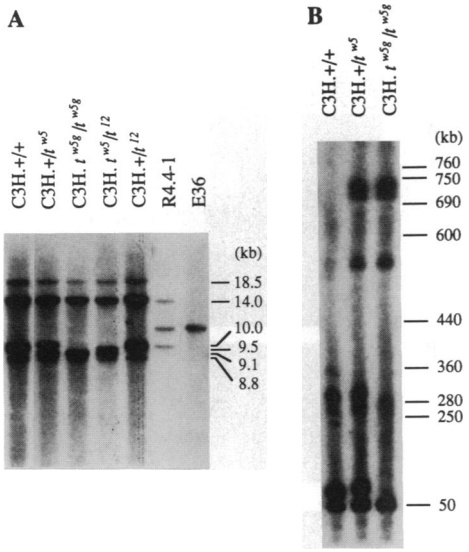

FIG. 2. Mapping of the duplication shared by clusters I and II. A 9.1-kb *Bam*HI fragment derived from the duplicated area of cluster I (see Fig. 1, positions 111–120 kb) was used as probe. (*A*) Hybridization to a *Bam*HI-digested genomic Southern blot. The presence of two C3H-type bands (10.0 and 14.0 kb) in the R4.4-1 lane indicates that they are probably located on chromosome 17. (*B*) Hybridization to a pulsed-field gel blot of DNAs digested with *Mlu* I. The smallest fragment is ≈50 kb and represents the cluster I copy because it corresponds to the size of the t^{w5}-derived *Mlu* I fragment contained in cluster I. The cluster II copy shows a polymorphism between C3H and the t^{w5} haplotype; it is ≈75 kb in C3H but at least 520 kb in the *t* haplotypes. The 280-kb band present in all lanes may correspond to one of the non-*t*-complex copies. The polymorphic bands at 300 kb (+/+ and $+/t^{w5}$) and 720 and 745 kb ($+/t^{w5}$ and t^{w5g}/t^{w5g}) could be due to differential methylation of *Mlu* I sites flanking cluster II in spleen cell DNA.

found in 170 kb around *H-2K* (9). The cloned DNA was further extended in the present study by a chromosomal walk from the end of *H-2K* toward α-crystallin (*Crya-1*). To this end, a total of 240 kb of DNA was cloned from the H-2K region of the t^{w5} haplotype. Fig. 1*A* shows the restriction map for the newly cloned 70-kb DNA and for completeness includes the previously described area. The entire region is designated cluster I. The walk in the present study was initiated by using probe A (Fig. 1*A*); this probe recognizes two groups of cosmids, whose restriction maps do not overlap. It became evident that ≈35 kb in cluster I (marked with a stippled bar in Fig. 1*A*) encompassing probes A and B exists in duplicated copies. Probe B identifies four copies of homologous sequences in a genomic Southern blot analysis with *Bam*HI; two of them map to the *t* complex (Fig. 2*A*). One of the *t*-complex copies is contained in cluster I, as judged by overlapping restriction maps and shows a restriction fragment length polymorphism among C3H and *t* haplotypes (Fig. 2*A*); it is 10.0 kb in C3H, 9.5 kb in t^{12}, and 9.1 kb in t^{w5} and t^{w5g}. The other copy (the 14.0-kb *Bam*HI band in Fig. 2*A*) was not polymorphic between *t* and + in Southern blots but was mapped to the *t* complex when a *t*-specific polymorphism was revealed in a pulsed-field gel using *Mlu* I (Fig. 2*B*). This copy served as the starting point for a bidirectional chromosomal walk in which 150 kb of DNA, designated cluster II, was cloned (Fig. 1*B*).

Once we walked out of the duplicated region, unique sequences made it possible to map cluster II more precisely. For this purpose, DNA from a series of partial haplotypes that contain different portions of *t* chromosome was analyzed in Southern blots using probe E (Fig. 3). It detects 3.5- and 2.9-kb *Bam*HI fragments unique to *t* haplotypes in contrast to a 3.8-kb fragment in C3H (Fig. 3). The results show that *t*-specific bands are present only in distal partial haplotypes such as t^6 and Tt^{s6}. t^{w18}, which retains *t* chromatin from the centromeric inversion up to the beginning of the distal inversion, and the short proximal partial t^{w111} do not show *t*-type bands. Therefore, cluster II maps within the distal inversion of the *t* complex.

The Search for Transcribed Sequences. The cosmid DNAs were restriction digested to derive a collection of genomic probes. Individual probes or pools of probes were hybridized to Northern blots to search for transcribed sequences. Genomic probes that detected message were then used to screen cDNA libraries. Two embryo libraries (5.5- and 10.5-day) (9) and two testis libraries (a wild-type and a t^x/t^y-mixed *t* haplotype) were used. These experiments enabled us to identify eight additional genes; all of them are expressed in the testis and, hence, are generally named "*Tctex* genes" (*t*-complex testis-expressed genes; see below).

Fig. 1 shows the map position of the *Tctex* genes along with *H-2K* and *KE* genes. There are at least 12 transcribed sequences mapping in the 240 kb of cluster I, whereas only three or four genes are found in the 150 kb of cluster II. The number of genes in the H-2K region is unusually high considering the predicted gene density for mammals of 1 in 35–60 kb (25). A comparable but slightly lower gene density has been reported for the human MHC class III region (5, 6).

Newly Discovered Genes of Cluster I. The genes were analyzed for their expression pattern, copy number, and conservation in other species. Table 1 summarizes these results for all of the genes discussed below (see also Fig. 4 for the Northern blot data).

Tctex-7 is expressed predominantly in spleen, EC cells, and testis. In addition to the list in Table 1, oocytes, embryos older than 7 days p.c., bone marrow, and thymus are also positive (26); heart and macrophages are negative. *Tctex-7*

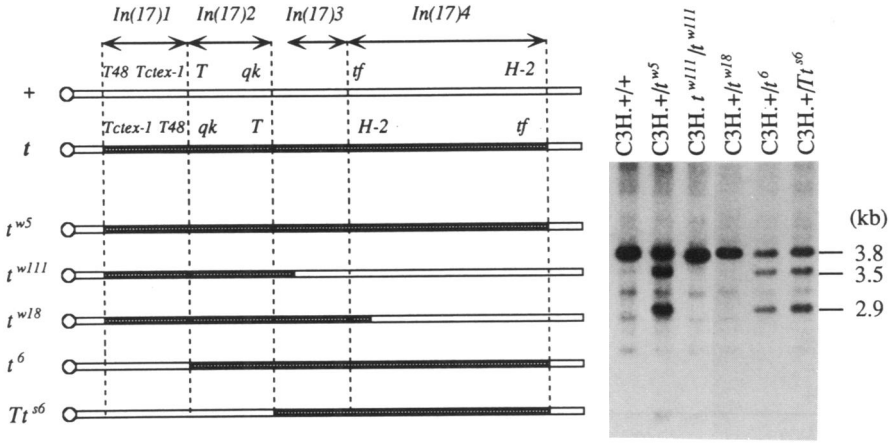

FIG. 3. Mapping cluster II in the *t* complex. A 3.1-kb *Kpn* I fragment at positions 1.8–4.9 kb in cluster II (see Fig. 1*B*, probe E) was hybridized to a genomic Southern blot of *Bam*HI-digested partial *t* haplotypes. C3H shows a unique 3.8-kb band whereas t^{w5}, a complete *t* haplotype, shows two *t*-specific bands of 3.5 and 2.9 kb. A diagram showing the extent of *t* chromatin in each haplotype is presented along with the positions of the four inversions and relevant markers. Open bar, wild-type chromatin; shaded bar, *t* chromatin.

forms a small multigene family with four copies in the mouse genome; two of them map to the *t* complex in the duplicated area shared by clusters I and II. We have named these two *Tctex-7A* and *-7B*, respectively. In Southern blots with relevant genomic probes from cluster I or II, the two *t*-complex copies give much stronger signals than the non-*t*-complex copies, indicating the existence of significant sequence divergence among the four members (see Fig. 2*A*). It is not clear how many of the four copies actually encode the *Tctex-7* message detected in testis. However, at least one of the *t*-complex copies is actively transcribed, since an 840-base-pair (bp) cDNA probe cloned from a wild-type testis library gives the same pattern of band intensity in Southern blot as was seen with the genomic fragments (data not shown).

Tctex-8 detects two transcripts (Fig. 4*B*): One of 4.1 kb is expressed in testis, thymus, and spleen; while another 2.85-kb transcript is seen only in testis. Neither message is expressed in myeloma cells, a B-cell line, a T-cell line or macrophages, as well as the negative tissues listed in Table 1. The expression level of the 4.1-kb message is genetic background dependent. CF1, a random-bred stock, and C3H/DiSn have an almost undetectable level of message when compared to noninbred t^x/t^y testis (Fig. 4*B*). However, when congenic C3H.+/+ and +/*t* are compared, the message levels are approximately equal (data not shown). The abundance of the 2.85-kb message is invariant regardless of the genetic background (Fig. 4*B*). *Tctex-8* must exist in at least two copies in the genome since the genomic fragment detects several extra bands in a Southern blot that are not present in the cluster I restriction map. These are apparently not located on chromosome 17 because they are not detected in the R4.4-1 mouse–hamster hybrid cell line containing mouse chromosomes 17 and 18 (data not shown). It appears that each of the 4.1- and 2.85-kb messages is encoded by a different genomic copy because the genomic probe hybridizes more strongly to the 4.1-kb message, whereas cDNA probes isolated from testis libraries show two kinds of hybridization pattern on Northern blots. One group of cDNAs yields the same pattern of band intensity as the genomic probe, while the other group hybridizes more strongly with the 2.85-kb message (data not shown). These results also indicate that the 4.1-kb transcript is the product of the *Tctex-8* sequence in cluster I.

Tctex-9 is predominantly expressed in testis (Fig. 4*A*). It appears to be single copy since with three restriction enzymes the relevant probes only detect bands that are present in the cluster I map. Two independent cDNA clones (570 and 680 bp) were isolated from a *t*-haplotype testis library.

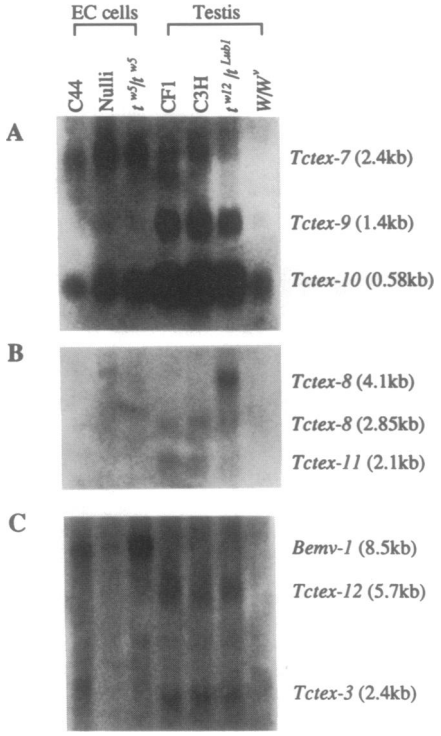

FIG. 4. Northern blot analyses of the newly identified genes in embryonic cells and testis. cDNA fragments were used as probes except for *Tctex-3* and *Tctex-11*.

Tctex-10 is expressed in all tissues tested. Its ubiquitous expression implies that it is a housekeeping molecule. This gene must have at least one cross-hybridizing sequence, since Southern blots with either genomic or cDNA probes always reveal extra restriction fragments not found in the map of cluster I (data not shown). Although the message is small, it has a relatively large transcription unit (≈14 kb) because a 460-bp cDNA probe cloned from a wild-type testis library hybridizes with several noncontiguous restriction fragments.

Tctex-11 is exclusively expressed in germ cells of the testis since its message is not present in the testis of W/W^v, a mutant lacking germ cells (Fig. 4*B*). Like *Tctex-8*, the expres-

Table 1. Summary of newly identified genes in clusters I and II

	Gene (copy number; message size, kb)								
	Tctex-3 (1; 2.4)	*Tctex-7* (4; 2.4)	*Tctex-8* (2–3; 4.1)	*Tctex-8* (2–3; 2.85)	*Tctex-9* (1; 1.4)	*Tctex-10* (2; 0.58)	*Tctex-11* (1; 2.1)	*Tctex-12* (1; 5.7)	*Bemv-1* (40–50; 8.5)
Tissue expression									
Brain	−	−	−	−	±	+	−	+	−
Kidney	−	−	−	−	±	+	−	−	−
Liver	−	−	−	−	−	+	−	−	−
Muscle	−	−	−	−	−	+	−	−	−
Spleen	−	+	+	−	−	+	−	−	+
EC cells	−	+	−	−	±	+	−	−	+
Testis	+	+	+	+	+	+	+	+	−
W/W^v testis	−	−	ND	−	−	+	−	−	−
Conservation									
Rat	+	+	+		+	+	+	+	±
Mink	+	+	+		−	+	+	+	±
Cat	+	+	±		−	+	+	+	±
Human	+	+	±		−	+	+	+	±
Chicken	ND	+	−		−	+	−	−	±
Drosophila	ND	+	+		−	−	−	−	−

ND, not determined.

sion of *Tctex-11* varies depending on the genetic background (Fig. 4*B*). *Tctex-11* appears to be single copy because only the band sizes present in the cluster I map are detected in Southern blots. No cDNA clones were isolated after screening 7×10^5 plaques from the two testis libraries.

Genes of Cluster II. *Tctex-3* is expressed only in testis (Fig. 4*C*): Negative tissues not listed in Table 1 are ovary, oocytes (26), thymus, muscle, and heart. It appears to be a single-copy gene.

Tctex-12 detects a broad signal of ≈5.7 kb on a Northern blot (see Fig. 4*C*). It is expressed in brain and testis only. Genomic Southern blots with several restriction enzymes suggest that *Tctex-12* is a single-copy gene. Four cDNA clones (380, 630, 1130, and 1730 bp) were isolated from a testis library.

There is one more sequence worth describing here. When the structure of the duplication common to clusters I and II was compared in detail, the cluster II copy was shown to have an insertion of 8–10 kb. Probes from this insertion detect 40–50 bands in Southern blots from both wild-type and *t* haplotypes (data not shown). In a Northern analysis, a 6.04-kb *Bam*HI genomic fragment from this region detects multiple messages of 8.5, 6.8, 4.5, 2.05, and 1.55 kb (data not shown). A cDNA clone with a 970-bp insert was isolated from a 10.5-day embryo library. The sequence of 271 bp revealed that it is 97% homologous to the envelope region of B-26, an endogenous murine retrovirus (27). We named this copy *Bemv-1* for B-26 envelope mouse retrovirus. In Northern blots the cDNA probe only detects the 8.5-kb message (Fig. 4*C*). It is present in EC cells, spleen, myeloma cells, a B-cell line, and lymph node, but not in macrophages and a T-cell line. The transcripts smaller than 8.5 kb are presumed to be subgenomic messages of the retrovirus. Unlike the 8.5-kb transcript, the smaller messages are expressed in all tissues tested; the 6.8-kb message is especially predominant in brain and kidney while the 1.55-kb transcript is most expressed in lymph node and spleen (data not shown). It is not known whether the *Bemv-1* copy is expressed.

Overall Expression Pattern of Cluster I and II Genes in Testis and Embryos. Except for the *H-2K* gene, none of the genes in clusters I and II have known functions. The distal inversion of the *t* complex contains a number of genes that are important in spermatogenesis and sperm function and in embryonic development (for review, see ref. 15). It is noteworthy that all of the newly identified genes are expressed in either testis or embryonic cells or both. Moreover, the expression patterns of *KE3, Tctex-3, -7, -8, -11*, and *-12* are tissue specific, making it likely that some of them will play a significant role in spermatogenesis or embryogenesis.

We examined whether the expression of the *Tctex* genes in clusters I and II is specific to germ cells using RNA from W/W^v testis. With the exception of *Tctex-10*, none of the other *Tctex* messages are detected in this mutant, and thus they are germ-cell specific.

Except for *KE1* and *Tctex-9*, all the genes in cluster I are conserved in humans. Since *KE3, KE4*, and *KE5* were demonstrated to be syntenic with their human homologs near *HLA-DP* (28), the other cluster I genes are also expected to be syntenic in the region proximal to *HLA-DP*. Thus, it will be interesting to investigate whether any of the cluster I genes are responsible for the disease susceptibility associated with the human class II loci.

D.B. participated actively in the work reported in this paper, but because of her death in August 1990, we missed the many improvements to the manuscript she would have made. This work was supported by grants from the National Institutes of Health (HD10668 and CA21651).

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