Identification and characterization of the t(w73) candidate gene Ortc3
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Chapter 3

Genetic analysis of the organic cation transporter genes Orct2/Slc22a2 and Orct3/Slc22a3 reduces the critical region for the t haplotype mutant $t^{w73}$ to 200 kb

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Genetic analysis of the organic cation transporter genes *Orct2/Slc22a2* and *Orct3/Slc22a3* reduces the critical region for the *t* haplotype mutant *t*<sup>W73</sup> to 200 kb

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Here we report an analysis of two candidate genes for the *t*<sup>W73</sup> implantation mutation. The *t*<sup>W73</sup> gene maps to a 20 cM region of mouse chromosome 17 known as the *t* complex, that exists in a wildtype and *t* haplotype form in present day mice. The *t* haplotype variants contain several mutant alleles affecting male fertility and embryonic viability and offer the opportunity to identify genes critical for these processes. *t*<sup>W73</sup> homozygous embryos are defective in trophoblast production and fail to implant adequately, with death occurring at approximately 7.5 days post coitum. Two recently described organic cation transporter genes, *Slc22a2 (Orct2)* and *Slc22a3 (Orct3)*, fulfill criteria predicted for *t*<sup>W73</sup> candidate genes, since both map to the previously defined 500 kb *t*<sup>W73</sup> minimal region and both are also expressed in 7.5 days pc post-implantation embryos. The genomic locus of the *Orct2* gene appears similar in wildtype and *t*<sup>W73</sup> chromosomes. In contrast, the genomic locus of *Orct3* is amplified and displays an altered expression profile in all *t* haplotype variant chromosomes tested. In addition, *Orct3* shows a *t*<sup>W73</sup>-specific polymorphism. To test if either *Orct2* or *Orct3* are involvement in the *t*<sup>W73</sup> phenotype, we have performed a genetic rescue experiment using YAC transgenes overexpressing *Orct2*, and genetic complementation with an allele in which the *Orct3* gene was inactivated by homologous recombination. The results eliminate both *Orct2* and *Orct3* as candidates, and further reduce the critical region containing the *t*<sup>W73</sup> mutant from 500 kb to 200 kb.

Keywords: *t* complex, *t*<sup>W73</sup>, *Orct3/Slc22a3/EMT, Orct2/Slc22a2*, organic cation transporter
Chapter 3

Introduction

The proximal 40 Mbp of mouse chromosome 17 is found in a naturally variant form in t haplotype mice, who comprise 10-20% of the mouse populations in the wild in Europe, America, and Asia (1). The t haplotype form of mouse chromosome 17 consists of four neighboring inversions that severely suppress recombination with the wildtype homologue, and which also link together a set of mutant genes that promote a form of male-specific meiotic drive, also known as ‘transmission ratio distortion’ (TRD) (2-4). Although several gene candidates for involvement in TRD have been proposed (5-8), the first molecular component in TRD has only recently been identified. The t complex responder gene (Tcr) is a fusion of the ribosomal S6 kinase gene and a newly identified sperm motility kinase (Smok). The resultant protein shows impaired kinase activity and may be involved in a signal cascade that controls sperm motility (9-11). TRD has been a major contributor to the continual maintenance of t haplotype alleles in wild mouse populations despite the accumulation of more than 30 recessive embryonic lethal mutations in the region spanned by the inversions (12-14). These embryonic lethal genes affect many stages of pre-implantation and early post-implantation development and have the potential to be informative about genetic programs regulating these events. However, none have yet been cloned.

The mutant t haplotype allele t*73 carries a mutation resulting in defective development of the trophoderm lineage. t*73 homozygous embryos die early in embryonic development, around 7.5 days post coitum (dpc), most likely due to an impairment of invasive trophoblast giant cell formation (15,16). Mice heterozygous for the t*73 allele do not show any abnormalities, whereas mice that are heterozygous for both the t*73 allele and the T° allele (which contains a large 3 cM deletion on the proximal part of wildtype chromosome 17) are indistinguishable in their phenotype from t*73 homozygotes (17). This argues that the t*73 allele carries a recessive lethal mutation, and that the t°73 mutation is present within the T° deletion. More precise mapping was performed by genetic complementation experiments with the partial t haplotype alleles t°Lab2 and T°Or1 (18). Partial t haplotype alleles are the products of rare recombination events between wildtype and t haplotype chromosomes. Both the t°Lab2 and T°Or1 alleles probably arose through reciprocal recombination events at the Plasminogen (Plg) gene locus. Plg is present as a single copy on wildtype chromosomes, but is contained in a multicopy repeat on all t haplotype variants (19). The t°Lab2 chromosome carries a deletion of a DNA segment of approximately 500 kb that is duplicated on T°Or1. In addition, it also contains a duplication of the region that includes the T, qk, and D17Rp17 loci, which is deleted in T°Or1 (20). The t°Lab2 allele failed to complement the t*73 mutation, whereas T°Or1/t°73 double heterozygotes are viable. These data define the 500 kb t°Lab2 deletion as the t°73 critical region (17,20).

A schematic representation of this region and the known genes it contains is shown in Figure 5. The centromeric end of the t°Lab2 deletion was mapped 40 kb downstream of Plg, whereas the distal end mapped approximately 10 kb downstream of Sod2 (19,21). In addition to t*73, the t°Lab2 deletion includes the Tme mutation, the imprinted Igf2r and Air genes, a cluster of the organic cation transporter genes Orct1/Slc22a1, Orct2/Slc22a2, and Orct3/Slc22a3, the proto-oncogene Mas, the Tcp-1/x and Acat-1/2 gene duplications, and the superoxide dismutase 2 (Sod2) gene. The mapping of these genes within the critical region presented them as potential t°73 candidate genes. However, candidate genes should also be expressed in early post-implantation embryos when the phenotype becomes apparent. This excludes the Mas
Reduction of the \( t \) haplotype mutant \( t^{w73} \) critical region

proto-oncogene, as well as the \( Orct1/Slc22a1 \) gene (22,23). Since the \( t^{w73} \) phenotype is not inherited in a parental-specific fashion this also excludes an involvement of imprinted genes present within the region, including the \( Igf2r \) gene that is most likely the \( Tme \) gene ((24), A. Wutz et al., submitted), and the recently identified paternally expressed \( Air \) RNA that overlaps the \( Igf2r \) gene (25). Mice deficient for \( Mas, Sod2, Igf2r, \) and \( Air \) have been generated by homologous recombination and do not recapitulate the \( t^{w73} \) embryonic lethal phenotype, thus excluding those as candidate genes ((22,26,27), A. Wutz et al., submitted).

Recently, we reported the isolation of a new gene from the \( t^{w73} \) critical region, called \( Slc22a3 \) or \( Orct3 \) (28). \( Orct3 \) is a new member of the solute carrier family of organic cation transporters (Orct), which are thought to function in the clearance of endobiotic and xenobiotic organic cation compounds. \( In vitro \) transport studies with the rat and human \( Orct3 \) genes have shown that the substrate specificity, transport kinetics, and sensitivity to various antagonists correspond closely with the parameters described for the extraneuronal monoamine transporter (EMT) system, or uptake, (29,30). Using a BAC contig that spans the mouse \( Orct3 \) gene, a close physical linkage was observed with two additional Orct family members: the previously identified \( Orct1 (Slc22a1) \) gene and \( Orct2 (Slc22a2) \), a clustering that is conserved in humans (28). Northern blot analyses showed that in embryonic development in mice, \( Orct3 \) is absent from embryonic tissue and highly expressed in placenta, a tissue derived from the trophoderm cells that are affected in the \( t^{w73} \) homozygous mutant embryos (28). The expression profile of \( Orct2 \) in embryonic development is not known, however, the gene was first isolated from an enhancer-trap transgene integration locus, that caused an embryonic recessive lethal mutation, although it is not known when the lethality occurs (31). Thus, the expression profile of \( Orct3 \), and the enhancer trap mutation of \( Orct2 \), suggest them as possible candidates for the \( t^{w73} \) phenotype.

In this report we have further investigated the possible involvement of the \( Orct2 \) and \( Orct3 \) genes in the \( t^{w73} \) phenotype. Both genes are expressed in early post-implantation embryos. \( Orct3 \), but not \( Orct2 \), is contained within a large multicycopy repeat array that is present in all \( t \) haplotype alleles that were examined and also includes the \( Pfg \) locus ((19) and Fig. 5). Expression analysis in 12.5 dpc \( t^{w73} \) heterozygous placentas compared to wildtype litter mates, revealed a nearly two-fold reduction in \( Orct3 \) expression. However, a similar reduction was observed from \( t^e \) and \( t^{w12} \) \( t \) haplotype alleles, suggesting that \( Orct3 \) expression was deregulated in the multicycopy repeat array in all \( t \) haplotypes. To directly test the role of \( Orct3 \), an \( Orct3 \) knockout allele was used in a genetic complementation test. Mice doubly heterozygous for a \( t^{w73} \) allele and an \( Orct3 \) null allele were born according to the expected ratios and are viable, excluding \( Orct3 \) as a candidate gene for the \( t^{w73} \) mutation. To test for the involvement of \( Orct2 \) in \( t^{w73} \), YAC transgenic mice overexpressing \( Orct2 \) were used in a genetic rescue experiment. However, no rescue of \( t^{w73} \) homozygous embryos was obtained. This result not only discards \( Orct2 \) as a candidate gene, but also leads to a further reduction of the \( t^{w73} \) critical region by excluding a 300 kb region contained on the YAC transgenes spanning from \( Orct2 \) to \( Mas \). Thus, the \( t^{w73} \) critical region is now reduced to 200 kb within the \( t^{wLub2} \) deletion on chromosome 17.

Results

\( Orct3 \) and \( Orct2 \) are expressed in early post-implantation embryos
Chapter 3

We previously reported that Orct3 is absent in midgestation embryos but shows very high levels of expression in placenta, a tissue derived from the trophectoderm lineage, that is affected in $t^{73}$ mutants (28). To examine its expression in early embryogenesis at the developmental stage affected in the $t^{73}$ mutant, we analyzed expression in the ectoplacental cone (epc) and embryonic region (ER) of 7.5 dpc embryos by RT-PCR (Fig. 1). Using intron-spanning primers, Orct3 expression is detected in both the ectoplacental cone and embryonic region of day 7.5 pc embryos, whereas no signal is obtained in the minus RT control lanes. In addition, Orct3 expression was also observed in 6.5 dpc total egg cylinder samples (data not shown). Corresponding with our previous Northern blot analyses, Orct3 expression could also be detected in 12.5 days pc placenta, but not in liver (Fig. 1). These data show that Orct3 fulfills one criteria for being a $t^{73}$ candidate gene.

A similar expression analysis was done for Orct2 (Fig. 1). No expression was detected in 7.5 days pc ectoplacental cone, whereas expression in the embryonic region of 7.5 days pc embryos was seen, which could be more readily detected upon hybridization with an internal oligonucleotide (data not shown). RT-PCR analysis of Orct1 showed expression in adult liver but revealed no expression in either the ectoplacental cone or embryonic region, confirming a previously reported analysis of Orct1 gene expression during mouse embryogenesis (23). Therefore, both Orct2 and Orct3 represent possible $t^{73}$ candidate genes.

Orct3 gene organization on wildtype chromosomes

<table>
<thead>
<tr>
<th>Gene</th>
<th>7.5 dpc epc</th>
<th>7.5 dpc ER</th>
<th>12.5 dpc placenta</th>
<th>liver</th>
<th>RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orct3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Orct2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Orct1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 1 Orct1, 2, and 3 RT-PCR expression analysis.
Ethidium bromide stainings are shown of RT-PCR reactions performed on 7.5 dpc ectoplacental cone (epc) and embryonic region (ER) tissue, as well as 12.5 dpc placenta and adult liver with primers specific for the Orct1, Orct2, Orct3 and Actin genes. Actin PCR reaction was performed as a control for the RT reactions. In neither of the PCR reactions a product was observed in the minus RT control samples (- RT lanes). The Orct3 expression in both the ER and epc of 7.5 dpc embryos was confirmed by hybridization with an internal oligomer (data not shown). Oligonucleotide hybridizations of the RT-PCR reactions failed to detect a signal in the 7.5 dpc and placenta samples for Orct1, whereas Orct2 expression was detected in placenta and in 7.5 dpc ER (the ethidium bromide signal is faint but clearly visible in the panel), but not epc samples.
Reduction of the \( t \) haplotype mutant \( t^{w73} \) critical region

In wildtype chromosomes the 3' end of the Orct3 gene is separated by less than 1 kb from the 3' end of the Plg gene ((28) and Fig. 5). Plg is a single copy gene on wildtype chromosomes, but is contained in a multicopy repeat array on \( t \) haplotype alleles (19). To investigate the Orct3 gene organization in \( t \) haplotype alleles, and to look for possible \( t^{w73} \)-specific polymorphisms within the Orct3 gene, we first determined the exon-intron structure in wildtype chromosomes. We used Orct3 cDNA oligonucleotides for identification and direct sequencing of DNA subclones of the Orct3 BAC contig. The Orct3 gene is organized in 11 exons. Table 1 presents the sizes and cDNA positions of the Orct3 exons, as well as an overview of the splice donor and acceptor sites. The sequences of these junctions precisely match the GT-AG rule for the splice acceptor \((c_{65}a_{100}g_{106}/g_{100}a_{100}g_{106})\) and splice donor \((a_{64}g_{73}/g_{64}a_{73})\) consensus sequences. Exon 1 is embedded in the CpG-island that originally led to the identification of the gene, and includes the 5'UTR as well as the translation start site and the first 413 bp of the open reading frame (ORF). Almost half of the complete Orct3 cDNA is contained within the last exon. It contains the last 58 bp of the ORF and the 3'UTR of 1463 bp. The number of exons and the exon sizes precisely match the recently published exon structure of the human gene homologue EMTISLC22A3 (32). Hybridization of the cDNA oligonucleotides to BAC restriction enzyme digestion panels revealed that the Orct3 gene spans a total region of 110 kb, with a large first and third intron of approximately 45 kb and 50 kb (Fig. 2A).

**Orct3 is repeated in \( t \) haplotype alleles, and carries a \( t^{w73} \)-specific polymorphism**

To analyze the Orct3 genomic organization in \( t \) haplotype alleles, single copy fragments were isolated from the gene locus and used as probes in genomic DNA blot experiments. Figure 2B shows a TaqI digest of wildtype, \( t \) haplotype heterozygous, and partial \( t \) haplotype homozygous DNA hybridized with an exon 11 probe (see Fig. 2A). A common 6.5 kb fragment is detected in all samples. However, much stronger signals were observed in each lane containing \( t \) haplotype DNA (lanes 2, 3, 4, 6, 8, 9, 10). To verify that this was not due to loading differences, the same panel was hybridized with a single copy probe of the Oct6 transcription factor gene, which is located on mouse chromosome 4 (33). Phospholimager quanti-
Chapter 3

**Figure 2** Mouse Orc3 gene organization.

**A** Schematic overview of the exon-intron organization of the mouse Orc3 gene is shown. The exons are shown as bars, where black represents the coding parts and gray represents the 5' and 3' UTR sequences. The arrows indicate the direction of transcription. The probe that is indicated represents a 1.2 kb PCR product of the most 3' end of the Ocr3 cDNA that is contained within the last exon (exon 11). The neighbouring Plg gene is schematically depicted as a white box. **B** Genomic DNA blot of wildtype and various t haplotype heterozygous and homozygous DNAs digested with with TaqI and hybridized with an exon 11 probe of Orc3 (top panel), a 616 bp PCR amplified Orc2 cDNA fragment (middle panel), and with a 520 bp Orc6 DraI fragment (bottom panel). lane 1, C3H wildtype; lane 2, r\(^{57}/+\); lane 3, r\(^{57}/+\) t\(^{f/d}/+\) tf; lane 4, r\(^{57}/+\) t\(^{f/d}/+\) tf; lane 5, 129Ola wildtype; lane 6, r\(^{57}/+\); lane 7, T f\(^{low}\)/+; lane 8, r\(^{57}/+\) t\(^f/d\) T; lane 9, r\(^{57}/+\) T; lane 10, r\(^{57}/+\) t\(^f/d\) T.

Tavative analysis of the 6.5 kb fragment with respect to Orc6 revealed 4- to 5-fold stronger signals in t haplotype heterozygous versus wildtype DNA (data not shown). This amplification was observed with both 5' and 3' probes of the Orc3 locus, indicating that the complete Orc3 gene has amplified 4 to 5 times on a t haplotype allele (data not shown). This observation is reminiscent of the finding that the neighboring Plg gene is contained in a multicycopy repeat array on t haplotype alleles (19). To analyze the extent of this repeat array the same panel was hybridized with a 3' cDNA fragment of the Orc2 gene whose 5' end is located 50 kb upstream of the Orc3 promoter (28). A 1.6 kb wildtype and a polymorphic 3 kb t haplotype fragment were detected (Fig. 2B). However, these fragments appeared in equal ratios in t haplotype heterozygous DNAs, suggesting that Orc2 is present in a single copy on t haplotype alleles.

60
Reduction of the t haplotype mutant t\textsuperscript{73} critical region

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>t\textsuperscript{73}/+</th>
<th>+/+</th>
<th>t\textsuperscript{73}/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg RNA</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Orct3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pai1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orct3/Pai1 (%)</td>
<td>98</td>
<td>100</td>
<td>70</td>
<td>68</td>
</tr>
</tbody>
</table>

**Figure 3** Orct3 expression in t\textsuperscript{73}, and t\textsuperscript{6} and t\textsuperscript{32} t haplotype heterozygous placentas. A 10 µg and 20 µg of total RNA from wildtype (+/+) and t\textsuperscript{73} heterozygous (t\textsuperscript{73}/+) 12.5 dpc placentas were hybridized with Orct3 (top panel) and Pai1 (bottom panel) cDNA probes. B 10 µg placental RNA from wildtype and t\textsuperscript{6} or t\textsuperscript{32} 12.5 dpc heterozygous embryos hybridized with Orct3 (top panel) and Pai1 (bottom panel) cDNA probes. +/+, wildtype; t\textsuperscript{6}/+, t\textsuperscript{6} +/- T tf; t\textsuperscript{32}/+, t\textsuperscript{32}/ T qk tf. In both A and B the relative levels of Orct3 expression with respect to Pai1 are indicated in percentages, in which the level of Orct3 expression in the 10 µg wildtype samples is set to 100%.

These data indicate that the multicopy repeat array present on t haplotype alleles includes both the Plg and Orct3 genes, but not Orct2.

Whereas the amplification was present on all t haplotype alleles, a 1.2 kb polymorphic fragment could be detected with the Orct3 exon 11 probe in t\textsuperscript{73} heterozygous DNA (lane 2 in Fig. 2B), but not in t\textsuperscript{5}, t\textsuperscript{12}, t\textsuperscript{32}, or t\textsuperscript{6} DNA. No t\textsuperscript{73}-specific fragment was observed with the same probe in any other digest that was tested, nor were additional t\textsuperscript{73}-specific polymorphisms found with other probes from the Orct3 locus (data not shown). These data suggest that the 1.2 kb t\textsuperscript{73}-specific TaqI fragment is a polymorphism within one of the multiple Orct3 gene copies present on a t\textsuperscript{73} allele and may reflect a DNA modification of one gene copy.

**Orct3 gene expression in t\textsuperscript{73}, t\textsuperscript{6}, and t\textsuperscript{32} heterozygous placentas**

To investigate Orct3 expression from the t\textsuperscript{73} allele Northern blot experiments were performed with RNA isolated from 12.5 dpc placentas of wildtype and t\textsuperscript{73} heterozygous embryos. Figure 3A shows two panels of two pairs of RNA samples prepared from independent litters. Each panel was hybridized with an Orct3 cDNA probe, as well as a probe for the plasminogen activator inhibitor 1 (Pai1) gene as a loading control. To introduce an internal control for the accuracy of the quantification, 10 and 20 µg of each sample was loaded. The
Orct3 gene showed reduced levels of expression in \( \texttt{r}^{\texttt{w}32} \) heterozygous compared to wildtype placentas. The relative levels of Orct3 expression with respect to Pail are indicated, in which the levels of expression in 10 µg of wildtype placentas is set to 100 percent (%). The two panels are representative of the results from a total of ten independent wildtype and \( \texttt{r}^{\texttt{w}32} \) heterozygous samples that have been analyzed, and in which the levels of Orct3 expression in \( \texttt{r}^{\texttt{w}32} \) heterozygous placentas range from 50% to 70% compared to wildtype. Furthermore, no Orct3 mRNA of alternative size was detected from the \( \texttt{r}^{\texttt{w}73} \) allele. These data strongly suggest that expression of the Orct3 gene is severely reduced, or completely absent from the \( \texttt{r}^{\texttt{w}32} \) allele.

To determine if the reduction in expression is specific for the \( \texttt{r}^{\texttt{w}32} \) haplotype allele, Orct3 gene expression was also studied in RNA isolated from the placentas of 12.5 dpc \( \texttt{r}^{\texttt{w}73} \) and \( \texttt{r}^{\texttt{w}32} \) heterozygous embryos and their wildtype litter mates (Fig. 3B). When corrected for the loading differences in each lane, a reduction in Orct3 expression of 40% to 60% was observed from both the \( \texttt{r}^{\texttt{w}73} \) and \( \texttt{r}^{\texttt{w}32} \) haplotype alleles. Thus, although Orct3 expression is reduced in 12.5 dpc \( \texttt{r}^{\texttt{w}32} \) heterozygous placentas compared to wildtype, reduced levels of expression are also observed in other \( \texttt{r} \) heterozygous placentas, suggesting that the reduction of Orct3 expression at this stage of placental development is \( \texttt{r} \) haplotype-specific, rather than \( \texttt{r}^{\texttt{w}32} \)-specific.

A targeted inactivation of Orct3 complements the \( \texttt{r}^{\texttt{w}32} \) mutation

The previous findings suggest that repression of the Orct3 gene occurred on all \( \texttt{r} \) haplotype alleles despite the amplification of the genomic locus. Thus, identification of a \( \texttt{r}^{\texttt{w}32} \)-specific DNA polymorphism in the Orct3 gene may not indicate its involvement in the \( \texttt{r}^{\texttt{w}32} \) phenotype. To directly test this, we performed a genetic complementation experiment by breeding \( \texttt{r}^{\texttt{w}73} \) heterozygous mice (\( \texttt{r}^{\texttt{w}73/1} \) in Table 2) with mice heterozygous for an Orct3 targeted-inactivation allele (\( \texttt{03/}^{+} \) in Table 2). The Orct3 targeted allele carries a 4.3 kb deletion of exon 1 and the CpG-island that forms the transcription start site. This deletion resulted in a complete absence of mRNA in adult mice homozygous for the targeted allele. In homozygous mutant placentas, however, an aberrant, non-coding transcript was observed, that is expressed from 12.5 dpc to term (R. Zwart et al., submitted). Mice born from these crosses were genotyped for the presence of the \( \texttt{r}^{\texttt{w}73} \) and the Orct3 targeted alleles, and \( \texttt{r}^{\texttt{w}73/03} \) double heterozygotes were identified in Mendelian ratios when male Orct3 heterozygotes were crossed with \( \texttt{r}^{\texttt{w}73} \) heterozygous females (Table 2). When the \( \texttt{r}^{\texttt{w}73} \) allele was transmitted through males, a non-Mendelian inheritance of the \( \texttt{r}^{\texttt{w}73} \) allele was observed that is caused by the male-specific meiotic drive present on complete \( \texttt{r} \) haplotype alleles (Table 2). These results show that a wildtype allele carrying an inactive Orct3 gene can complement the \( \texttt{r}^{\texttt{w}73} \) allele and therefore Orct3 is not responsible for the \( \texttt{r}^{\texttt{w}32} \) phenotype.
Reduction of the t haplotype mutant t^w73 critical region

A

+ + - + + - YAC-T1/P
Orct2

1.8 2.2 1 2.1 2.0 1 Orct2/GAPDH

B

<table>
<thead>
<tr>
<th>cross</th>
<th>stage</th>
<th>number</th>
<th>genotype offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+/+</td>
</tr>
</tbody>
</table>
| r^w73 YAC-T1/P /++ | postnatal 36 | 4       | 32   | 0      | -
| x     | 7.5 dpc 11  | 2       | 6    | 0      | 3
| r^w73/+ | 8.5 dpc 11 | 5       | 4    | 0      | 2
|       | 9.5 dpc 11  | 0       | 4    | 0      | 7

Figure 4 Transgenic rescue with the YAC transgenic line YAC-T1/P.
A YAC-T1/P overexpresses Orct2. 5 μg of total RNA isolated from the kidney of wildtype and YAC-T1/P heterozygous transgenes were hybridized with the Orct2 (top panel) and human GAPDH (bottom panel) cDNA probes. The relative level of Orct2 expression with respect to the GAPDH loading control is given below the lanes and was set to 1 for the wildtype samples. B Table presenting the genotypes of offspring from female r^w73 YAC-T1/P /++ and male r^w73/+ crosses.

Orct2-YAC transgenic rescue

To test the role of Orct2 and to further delineate the t^w73 critical region, we performed a genetic rescue experiment with the YAC transgenic mouse line, called line b. This line had previously been generated in our laboratory to study the imprinting mechanism of the neighboring Igf2r gene (34). It contains two intact copies of YAC T1/P that spans the Orct2 to Mas region, including the Igf2r locus (Fig. 5). The Mas gene was not expressed from YAC T1/P, and Orct3 was not present ((34) and Fig. 5). It was determined that in line b the YAC transgenic DNA had not integrated on chromosome 17, allowing a genetic rescue experiment for the t^w73 mutation. To investigate if the line b YAC transgene would allow a direct genetic test for Orct2, we analyzed the expression levels of Orct2 in the kidney of transgenic and wildtype mice. A 2-fold increase was found in the line b transgenes, which is in agreement with the two transgene copies present in these mice (Fig. 4A). Furthermore, when Orct2 expression was examined in a panel of adult organs of the YAC-T1/P transgenic line b, no expression was observed in any other organ that was tested (data not shown). This corresponds with the Orct2 expression pattern from the endogenous locus ((31), and our own unpublished observations), indicating that all elements necessary for tissue-specific expression are present on the trans-
Figure 5 Schematic representation of the \( \textit{t}^{\text{w}73} \) critical region. 

**Top** Overview of the relative positions of the genes located in the \( \textit{t}^\text{Lub2} \) deletion region. The genes are depicted as boxes corresponding to the size of the gene loci. The boxes under the line represent overlapping genes. **Below** the \( \textit{t}^\text{Lub2} \) deletion is indicated by a black line. Also, the alignment of YAC-T1/P with the locus is shown. Its proximal end maps between Orct2 and Orct3, and its distal end maps in the Mas coding exon. The transgene spans the Orct2 gene, as well as the Orct1, Igf2r, and the Air RNA. Based on the genetic data presented in this report, the \( \textit{t}^\text{w}73 \) critical region can now be reduced to two domains of 50 kb between Orct2 and Orct3 and 150 kb between Mas and Sod2. Sizes in kilobases (kb) are indicated below.

To perform the genetic rescue experiment, line b YAC-T1/P transgenes were crossed into the \( \textit{t}^\text{w73} \) genetic background and females that carried both the \( \textit{t}^\text{w73} \) and transgenic alleles were crossed with a \( \textit{t}^\text{w73} \) heterozygous male. Figure 4B shows a table presenting the genotyping results of this cross. No \( \textit{t}^\text{w73} \) homozygous mice were identified among a total of 36 postnatal offspring that were genotyped. However, because of the meiotic drive in \( \textit{t}^\text{w73} \) heterozygous males, 7 or 8 were expected if overexpression of the Orct2 gene could fully rescue the \( \textit{t}^\text{w73} \) phenotype. It has been reported that a secondary, later acting lethal gene, named \textit{cld}, is present on the \( \textit{t}^\text{w73} \) allele (17,35). Thus, overexpression of the Orct2 gene may rescue \( \textit{t}^\text{w73} \) homozygous embryos only to a later stage in embryonic or early postnatal development. To investigate if any early developmental rescue occurred, embryos were isolated and genotyped at different stages. No \( \textit{t}^\text{w73} \) homozygous embryos were found in litters isolated at 7.5, 8.5, or 9.5 dpc (Fig. 4B). However, of a total of 33 implantation sites that were isolated, 12 contained reabsorbed embryonic material. These could not be accurately genotyped, however, taking into account the 85% transmission ratio distortion (TRD) routinely observed in our \( \textit{t}^\text{w73} \) breeding colony, this number closely matches the expected number (0.425 x 33) of homozygous embryos to be present in a \( \textit{t}^\text{w73} \) heterozygous cross. From this result we conclude that not only overexpression of Orct2 fails to rescue the lethality of \( \textit{t}^\text{w73} \) homozygous embryos, but that the total region of 300 kb present in YAC T1/P can be excluded from the \( \textit{t}^\text{w73} \) critical region (see Fig. 5).

**Discussion**
Reduction of the t haplotype mutant \( t^{w73} \) critical region

**Orct3 gene organization in t haplotype alleles**

We have investigated two organic cation transporter genes as possible candidates for the \( t^{w73} \) mutation. Both genes mapped to the critical region, and we have shown that they are expressed in early post-implantation embryos. Southern blot analyses revealed a genomic amplification of the Orct3 gene, but not Orct2, in the haplotype chromosomes (Fig. 2). Based on quantification of the hybridization signals, we estimate that 4 to 5 copies of the Orct3 gene are present in haplotypes. A similar amplification had previously been reported for the Plg gene (19). As the 3' ends of Plg and Orct3 are separated by approximately 1 kb, it is therefore most likely that both genes are part of a single genetic region that has amplified in the haplotypes. This region is at least 150 kb in size as the Plg and Orct3 genes span 43-47 kb and 110 kb, respectively. Genetic analysis of Plg on the partial t haplotype allele \( t^{wLab2} \) showed that it contains a complete wildtype copy of the gene in addition to several complete t haplotype copies recognized by 3' and 3' sequences of the Plg gene (19). This amplification, however, is not associated with increased expression, as similar Plg mRNA levels were found in livers of adult wildtype and \( t^{w73} \) heterozygous mice (19). This raised the suggestion that t haplotype alleles carry one functional copy of the Plg gene and multiple silent ones. In contrast, Orct3 expression analysis in placentas of \( t^{w73} \), \( t^{e} \), and \( t^{e32} \) heterozygous embryos showed a reduction to approximately 50 % compared to wildtype. This result would argue that in placenta all Orct3 gene copies on the haplotype alleles are silent. However, in kidneys of postnatal day 16 mice, Orct3 expression is strongly elevated in \( t^{w73} \) heterozygotes compared to wildtype mice (data not shown). Thus, it appears that regulation of Orct3 expression is disturbed on the haplotype alleles in a tissue-specific fashion.

A single, \( t^{w73} \)-specific polymorphism was identified that distinguishes one copy of the Orct3 gene in \( t^{w73} \). A 1.2 kb polymorphic fragment was detected in a Taq1 digest with a probe derived from the Orct3 3'UTR (Fig. 2B). To determine the nature of this fragment, it was isolated from an agarose gel and sequenced. The analysis showed that it was identical with the 3' end of the wildtype Orct3 gene, except for a 5 bp mismatch within the 3'UTR that introduced a Taq1 restriction site (data not shown). This polymorphism is probably part of a minor DNA alteration as a probe located 5' of the polymorphic Taq1 site detected a 5.3 kb fragment, that would complement for the size of the non-polymorphic 6.5 kb Taq1 fragment (data not shown).

**Definition of the \( t^{w73} \) critical region**

Genetic complementation of the \( t^{w73} \) allele with an Orct3 null allele or introduction of a YAC transgene overexpressing Orct2 showed that a defect in either of these genes is not involved. The Orct3 null allele has a 4.3 kb deletion of the Orct3 locus, removing exon 1 and 3.5 kb of surrounding sequences (R. Zwart et al. Submitted). This resulted in a complete null for Orct3. We previously reported the isolation of a BAC contig spanning the Orct3 gene locus that was used as a tool for gene searching (28). This resulted in the isolation of Orct3, but yielded no further evidence for the presence of additional genes. Therefore, we consider that Orct3 is the only gene located within the proximal 80 kb of the in \( t^{wLab2} \) deletion region.

The YAC transgene spans the region from Orct2 to Mas (Fig. 5). We have shown that the Orct2 gene and its regulatory sequences are contained on this YAC. This is also true for the Orct1/Sle22a1 gene locus that maps between Orct2 and the 3' end of Igf2r (Fig. 5), and these data confirm our previous conclusion based on its expression profile that Orct1 is not involved in \( t^{w73} \). Furthermore, the possible presence on the YAC transgene of genes, which
remained unidentified until now, also excludes them as \( r^{\text{w7i}} \) candidate genes. Thus, based on the genetic experiments reported here, we can now redefine the \( r^{\text{w7i}} \) critical region, as consisting of two separate domains: a 50 kb region from the 5’ end of \( \text{Orc}t3 \) to 5’ \( \text{Orc}t2 \), and a 150 kb region from the 3’ end of \( \text{Mas} \) to \( \text{Sod}2 \) (Fig. 5).

The \( \text{Mas} \) to \( \text{Sod}2 \) interval spans 150 kb and contains in addition the chaperonin gene \( \text{Tcpl} \) (formally called t complex polypeptide 1), and the acetylCoA acetyltransferase 1 gene (\( \text{Acat}1 \)). These genes overlap in their 3’ regions and they have also duplicated to form the closely linked \( \text{Tcplx/Acat}2 \) gene cluster (36-38). \( \text{Acat}1 \) and \( \text{Acat}2 \) are expressed in kidney and brain, which corresponds with the reported Acat activity in rat (37). \( \text{Tcpl} \) was originally isolated from testis. However, cDNA clones for both \( \text{Tcpl} \) and \( \text{Tcplx} \) have also been isolated from a 8.5 dpc embryo cDNA library, indicating that both genes are expressed in early embryogenesis (39). Furthermore, \( \text{Tcpl} \) mRNA was found in 7.5 days pc total embryo samples that included the maternal decidua (40). \( \text{Tcpl} \), and possibly also \( \text{Tcplx} \), thus represent two possible \( r^{\text{w7i}} \) candidate genes.

The second interval of the \( r^{\text{w7i}} \) critical region spans the \( \text{Orc}t3-\text{Orc}t2 \) intergenic region. With the isolation of the mouse \( \text{Orc}t2 \) gene, a recessive embryonic lethality associated with the enhancer-trap transgenic line TKZ736 was linked to the region (31). No homozygous embryos were found in midgestation litters of heterozygous crosses. Furthermore, the transgene integration was mapped in the first intron of the \( \text{Orc}t2 \) gene. Since no reduction in \( \text{Orc}t2 \) expression levels were observed in adult TKZ736 heterozygous mice compared to wildtype, this casts doubt on the involvement of \( \text{Orc}t2 \) in the enhancer trap embryonic lethal phenotype. Although an intriguing possibility, it is at present unclear whether the transgene integration resulted in the same gene defect as in \( r^{\text{w7i}} \). A further characterization of the embryonic lethality in the TKZ736 transgenic mice will be required to address this. Also, it can not be excluded that complex DNA rearrangements have accompanied the transgene integration, that would affect the function of more distal and possibly multiple genes.

Physical mapping of the position of the \( r^{\text{w7i}} \) embryonic lethal mutation with partial \( t \) haplotype alleles previously defined a critical region of 500 kb from \( \text{Plg} \) to \( \text{Sod}2 \) (21). Our experiments reported here have minimized the critical region to two intervals of 50 kb and 150 kb between the \( \text{Orc}t3 \) and \( \text{Orc}t2 \) genes and the \( \text{Mas} \) and \( \text{Sod}2 \) genes, respectively. Genomic contigs spanning these two regions are available and sequence analysis already in progress for the mouse \( t \) complex (41) will soon lead to a completion of the gene map for the \( r^{\text{w7i}} \) critical region.

### Materials and Methods

#### Mice

All mouse strains used in this study were maintained under SPF conditions. The identity of the \( r^{\text{w7i}} \) chromosome was verified by hybridization of \( \text{Taq}1 \) digested genomic DNA with probe \( \text{Au}9 \) that detects a 4.0 kb \( r^{\text{w7i}} \)-specific polymorphic fragment present in the fourth \( t \) complex inversion (42). Conservation of the integrity of the \( r^{\text{w7i}} \)-chromosome was followed by the TRD percentage that is observed upon male transmission that requires loci mapping to all four inversions that comprise the \( t \) complex. TRD for the \( r^{\text{w7i}} \) chromosome in our breeding colony was 85%. The line b YAC transgenic mice were kept on a FVB/N genetic background. Integration of the YAC T1/P transgenic DNA into chromosome 17 was excluded by the generation of YAC T1/P transgenic mice that also carried two different alleles for the \( \text{lg}2\text{r} \) gene.
Reduction of the t haplotype mutant t<sup>e73</sup> critical region

**RT-PCR**

Ectoplacental cone tissue and embryonic region were isolated from 7.5 dpc egg cylinder stage embryos by mechanical dissection and the integrity of the sample was confirmed by expression analysis of the embryonic region-specific marker Oct4, and the ectoplacental cone-specific marker 43II (data not shown). Reversed transcriptase (RT) reaction was done in a 20 μl reaction with 1 μg of total RNA and 0.5 μg oligo-dT primer and 200 U SuperScript<sup>™</sup> Reverse Transcriptase (GibcoBRL). For the minus RT controls all components were added except for the enzyme. 1 μl of RT product was used in a 20 μl PCR reaction with intron-spanning primers for Orct1 (F, 5'-cttgacgaagatgcctcagag-3'; R, 5'-tagaagtgtctgggaag-gcaa-3'), Orct2 (F, 5'-agccatgaaaatcattaagc-3'; R, 5'-caaccacageaaatacgac-3'), and Orct3 (F, 5'-ttcagagtttacccaacgac-3'; R, 5'-taacctgggacctgggac-3').

**Sequence analyses**

For sequencing 0.1-0.5 μg was used in a Bigdye Terminator (Perkin-Elmer) sequence reaction and analyzed with the Perkin-Elmer ABI 373 sequencing device.

**Southern blot analyses**

10 μg of genomic DNA was digested with 15U TaqI and run overnight on a 1% TAE-agarose gel. After blotting to Hybond-N+ (Amersham) hybridization and washing was performed under standard Church conditions (43).

**Northern blot analyses**

12.5 dpc embryos and placentas were isolated from t<sup>e73</sup>, t<sup>e</sup>, or t<sup>e32</sup> heterozygous females that were time-mated with a wildtype male. Placentas were processed with the LiCl/Urea method for total RNA isolation (44), and the corresponding embryos were used for DNA isolation and genotyping. The RNA samples were run out on a 0.8 % formaldehyde gel in MOPS running buffer and blotted in 50 mM NaPi to Hybond-N+ nylon membrane (Amersham). Hybridizations were performed in Church hybridization buffer at 65°C. Short time exposures with the Fujix Phosphoimager were used for quantification.

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Chapter 3

References

Reduction of the \( t \) haplotype mutant \( t^{u73} \) critical region


69