

FIG. 3 Evidence that the isodicentric chromosome of A.G. is subject to X inactivation. Reverse-transcribed RNA (RT-RNA) from somatic cell hybrids containing an  $X_1$ , and  $X_a$ , and the idic(Xp) chromosome (tAG), as well as from a human female lymphoblast and a mouse line (the parental mouse line for the hybrids) was used for amplification by the polymerase chain reaction (PCR) with primers for the *MIC2*, *TIMP*, *POLA*, and *XIST* genes. The *TIMP* and *POLA* genes have previously been demonstrated to be subject to X inactivation, while the *MIC2* gene is known to escape X inactivation<sup>22,23</sup>. The *MIC2* primers amplified a 360-bp product for all RT-RNAs except for the mouse. The *TIMP* primers were used in a duplex reaction with the *MIC2* primers. They amplified a 150-bp product in the human RT-RNA and the  $X_a$  hybrid RT-RNA. The *POLA* primers also only amplified their 320-bp product in the female and  $X_a$  hybrid RT-RNA. The 180-bp *XIST* product was amplified from the female, the  $X_1$  hybrid, and the tAG hybrid. Therefore, the tAG hybrid behaved identically to the  $X_1$  hybrid.

**METHODS.** RT-PCR was performed as described<sup>9,22</sup>, except that 10 times the input RT-RNA was used for the *POLA* primers. The *MIC2* and *TIMP* primers have been described<sup>22</sup>. The *POLA* primers are 5'-TGCCATTTCAT-CACCCAGT-3' and 5'-ACTGCCATACTGAAATACAT-3' which amplify a predicted 320-bp product<sup>33</sup>. The *XIST* primers were 1 → and 2 ← as described in the accompanying article<sup>9</sup>. The  $X_a$  and  $X_1$  hybrids used were as described<sup>9,22</sup>.

single *XIC* on the human X chromosome and, accordingly, should significantly refine efforts to clone and analyse this locus. That the *XIC* region is coincident (at the current level of mapping resolution) with the location of the *XIST* gene<sup>9</sup>, whose expression pattern is specifically and uniquely affected by the inactivation status of the X chromosome on which it lies, strongly implicates *XIST* in some aspect of the X inactivation process. □

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## The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the *Tme* locus

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**T-ASSOCIATED maternal effect (*Tme*) is the only known maternal-effect mutation in the mouse<sup>1,2</sup>. The defect is nuclear-encoded<sup>3</sup> and embryos that inherit a deletion of the *Tme* locus from their mother die at day 15 of gestation<sup>4</sup>. There are many genomically imprinted regions known in the mouse genome<sup>5,6</sup>, but so far no imprinted genes have been cloned. The *Tme* locus is absent in two chromosome-17 deletion mutants, *T<sup>hp</sup>* and the *t<sup>Lub2</sup>*, and its position has been localized using these deletions to a 1-cM region<sup>7-10</sup>. We report here that the genes for insulin-like growth factor type-2 receptor (*Igf2r*) and mitochondrial superoxide dismutase-2 (*Sod-2*) are absent from both deletions. Probes for these genes and for plasminogen (*Plg*) and T-complex peptide 1 (*Tcp-1*) were used in pulsed-field gel mapping to show that *Tme* must lie within a region of 800-1,100 kb. We also demonstrate that embryos express *Igf2r* only from the maternal chromosome, and that *Tcp-1*, *Plg* and *Sod-2* are expressed from both chromosomes. Therefore *Igf2r* is imprinted and closely linked or identical to *Tme*.**

The position of the *T<sup>hp</sup>* and *t<sup>Lub2</sup>* deletions relative to cloned DNA markers<sup>1,8-10</sup> is shown in Fig. 1. Of these marker loci, only *Tcp-1* is deleted in the *t<sup>Lub2</sup>* chromosome, so the distance between the flanking marker loci *D17Rp17* and *D17Leh66D* gives the closest approximation of the limits of the region containing the *Tme* gene. Cumulative mapping data indicate that these markers are separated by at least one centimorgan<sup>7</sup>.

*Tcp-1* (ref. 11) and a gene from the *D17Leh66D* locus, *Tcp-10* (ref. 12), map in the human to chromosome 6q21-27 in close linkage to the plasminogen, insulin-like growth factor type-2 receptor and superoxide dismutase-2 loci<sup>13</sup>. These last three genes have been assigned to mouse chromosome 17 (refs 14-16) but not mapped with respect to the *Tme* locus. Figure 2 shows the mapping of these three genes in the *t<sup>Lub2</sup>* deletion chromosome and the *Tt<sup>Orl</sup>* chromosome that is presumed to contain a duplication of the *Tme* locus (see refs 10 and 17 for a description

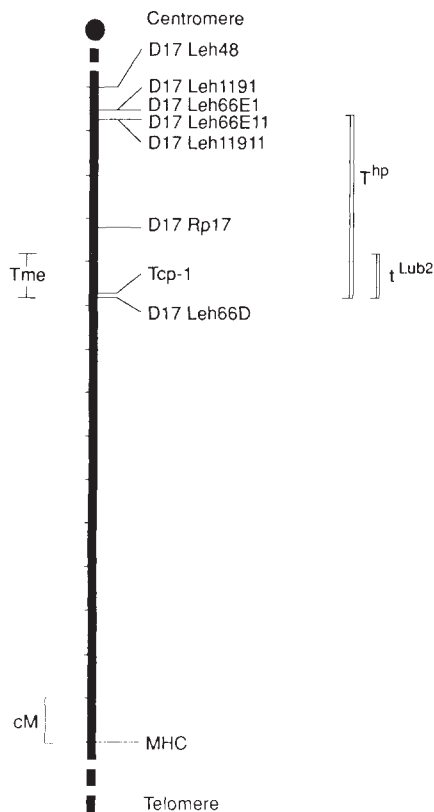
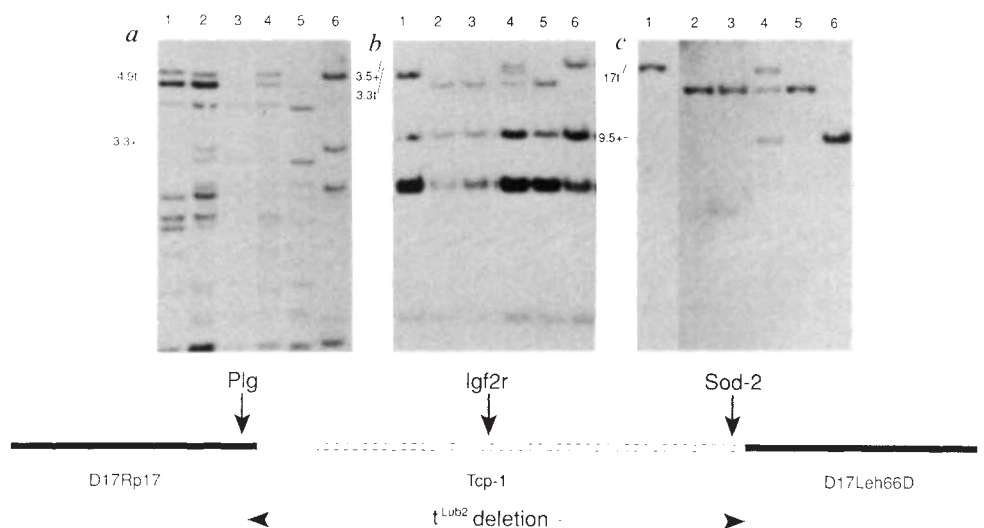


FIG. 1 Genetic map<sup>7</sup> of the proximal part of mouse chromosome 17 showing the position of the  $T^{hp}$  and the  $t^{Lub2}$  deletions used to define the limits of the region containing the  $Tme$  locus<sup>9,10</sup>.

of the structure and origin of the  $t$  haplotype and partial  $t$  haplotype chromosomes). Our results show that the  $Igf2r$  and  $Sod-2$  loci are deleted in  $t^{Lub2}$  and duplicated in  $Tt^{Ori}$ . The locus for  $Plg$  is present in two copies in  $t^{Lub2}$ , one of which is partially deleted and present once in  $Tt^{Ori}$ . Subsequent work has identified the  $Sod-2$  and  $D17Leh66D$  loci as residing on the same cosmid clone and thus the distal end of the  $t^{Lub2}$  deletion can be localized to within 45 kilobases (kb) of the  $Sod-2$  locus (K.S. and D.P.B., data not shown). In addition, further study of the  $Plg$  locus in the  $t^{Lub2}$  and  $Tt^{Ori}$  chromosomes<sup>9</sup> has shown that this locus forms the proximal breakpoint of the  $t^{Lub2}$  deletion (N.S. and D.P.B., data not shown). Thus the region that contains the  $Tme$  locus can be exactly defined as lying between the  $Plg$  and  $Sod-2$  loci. Genetic mapping has separated these two loci<sup>13</sup>, but the estimated genetic distance of  $\sim 1$  cM does not differ from that estimated for  $D17Rp17$  and  $D17Leh66D$ . Recombination frequencies are not an accurate measure of physical distance and we therefore constructed a long-range restriction map using DNA separated by pulsed-field gel electrophoresis<sup>18</sup> in order to size the region containing the  $Tme$  locus. Figure 3 shows a long-range restriction map including the  $D17Rp17$  and the  $D17Leh66D$  loci and we have used this to estimate the separation of the  $Plg$  and  $Sod-2$  loci as 0.8–1.1 megabases (Mb).

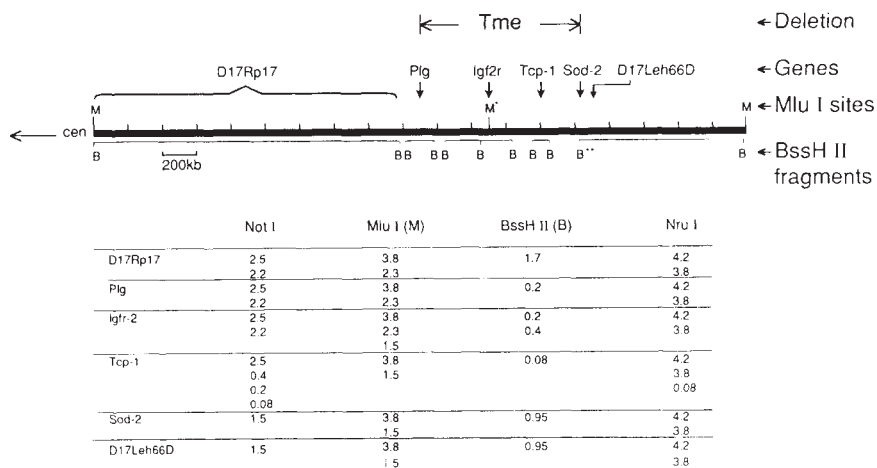
These results and the previous mapping of the  $Tcp-1$  gene<sup>9</sup> thus assigns four genes to the region known to contain the  $Tme$  locus. Next we decided to test these four genes for evidence of imprinting. We reasoned that the  $Tme$  gene would only be functionally expressed from the maternal chromosome. Therefore the  $Tme$  embryonic-lethal phenotype could be explained by the absence or alteration of the  $Tme$  transcript when the embryo inherits a deletion of the maternal locus and contains only the paternal  $Tme$  locus. The normal transcript would be present in the converse situation, when the embryo inherits a deletion of the paternal locus and contains only the maternal locus. Non-imprinted genes would be expressed from both

FIG. 2 Mapping using deletion chromosomes. *a*, *TaqI*-digested mouse genomic DNAs hybridized with a mouse plasminogen cDNA clone (MP33B; provided by S. J. F. Degan<sup>14</sup>); *b*, *PvuII*-digested DNAs hybridized with an insulin-like growth factor type-2 receptor cDNA clone (mouse partial cDNA; provided by A. Ullrich); *c*, *Bam*HI-digested DNAs hybridized with a mitochondrial superoxide dismutase-2 mouse cDNA clone (provided by G. I. Bell<sup>15</sup>). The lane order is the same for all three panels: lane 1,  $t^{w12}/t^{w12}$ ; lane 2,  $t^{Lub2}/M.spretus$ ; lane 3,  $T^{hp}/M.spretus$ ; lane 4,  $Tt^{Ori}/M.spretus$ ; lane 5,  $M.spretus$ ; lane 6,  $C57Bl/6$ . The three deletion chromosomes ( $T^{hp}$ ,  $t^{Lub2}$  and  $Tt^{Ori}$ )<sup>9,10</sup> were bred over the *spretus* chromosome because of the ease with which polymorphisms can be found between *Mus spretus* and *Mus musculus*; all other chromosomes in the panels have a *Mus musculus* origin, referred to as + or wild-type. Lanes 1, 5 and 6 are control lanes that identify the specific restriction fragments for these three DNA types. The *Plg* panel shows that a 4.9-kb *t*-specific fragment is present in  $t^{Lub2}$  and  $Tt^{Ori}$ , and a 3.3-kb + specific fragment is absent in  $T^{hp}$  and  $Tt^{Ori}$  but present in  $t^{Lub2}$ . These and other data (not shown here) place *Plg* at the proximal border of the  $t^{Lub2}$  deletion. The *Igf2r* panel shows that a 3.3-kb *t*-specific fragment, and a 3.5-kb + specific fragment are absent in  $t^{Lub2}$  and  $T^{hp}$  respectively, but are both present in  $Tt^{Ori}$ . The  $Tt^{Ori}$  chromosome contains a duplication of the *Tcp-1* locus, and is presumed to contain a duplication of the region that is deleted in the  $t^{Lub2}$  chromosome, including the  $Tme$  locus<sup>9</sup>. The data here place the *Igf2r*



locus within the  $t^{Lub2}$  deletion and within the duplicated region in  $Tt^{Ori}$ . The *Sod-2* panels shows that a 17.0-kb *t*-specific fragment and a 9.5-kb + specific fragment, are absent in  $T^{hp}$  and  $t^{Lub2}$  but are both present in  $Tt^{Ori}$ . These data place the *Sod-2* locus within the  $t^{Lub2}$  deletion and within the region duplicated in  $Tt^{Ori}$ , further analysis of cosmid clones (data not shown) places the *Sod-2* locus at the distal border of the  $t^{Lub2}$  deletion. The positions of the *Plg*, *Igf2r* and *Sod-2* loci relative to the  $t^{Lub2}$  deletion and existing markers for this region<sup>9,10</sup> are shown underneath. Preparation of genomic DNA, enzyme digestion, DNA blots and hybridizations were all achieved using standard techniques<sup>10</sup>.

FIG. 3 Physical mapping using pulsed-field gels. Genomic DNA from the BALB/c inbred strain was prepared in agarose blocks, digested with rare cutter enzymes and DNA fragments in the 1–5 Mb range were separated by pulsed-field gel electrophoresis. Fragment sizes obtained with four enzymes are given. The map was constructed by sequential hybridization of one filter with the probes listed, and is centred on two neighbouring *Mlu*I fragments. The *Igf2r* gene spans the central *Mlu*I (*M\**) site, which was partially cut in genomic DNA. *D17Rp17* is located on a 1.7-Mb *Bss*HI fragment contained within the 2.3-Mb *Mlu*I fragment. *Sod-2* spans a *Bss*HI site (*B\*\**) and is located on a 0.95-Mb fragment which is contained within the 1.5-Mb *Mlu*I fragment. If these two *Bss*HI fragments are positioned at the ends of the two *Mlu*I fragments, then the *Plg* and *Sod-2* loci are separated at most by 1.1 Mb, and this is the maximum size of the region which contains *Tme*. The minimum distance separating these two markers (~0.8 Mb) is estimated by summation of the *Bss*HI and *Not*I fragments recognized by *Igf2r* and *Tcp-1* but not by the flanking markers. METHODS. Pulsed-field gel DNA preparation using the spleen from BALB/c female mice, enzyme digestion, and electrophoresis were as described<sup>24</sup> (detailed protocols available on request). DNA fragment sizes estimated after pulsed-field gel separation are given in megabases and listing of more than one fragment size indicates partial digestion. The *Mlu*I, *Not*I and *Nru*I fragments were sized on pulsed-field gels separating 1–5 Mb over 5 cm



using *S. pombe* chromosomes as size markers<sup>25</sup>. The *Bss*HI fragments were sized on gels separating 0.1–1.5 Mb over 12 cm, using *S. cerevisiae* YP148 chromosomes as size markers (P. Heiter, personal communication). Probes: as described in Fig. 2, plus *D17Rp17* (DNA segment Roswell Park 17) provided by R. Elliott<sup>26</sup>, *Tcp-1* (t-complex protein-1) provided by K. Willison<sup>11</sup> and *D17Leh66D* (DNA segment Lehrach 66D) provided by H. Lehrach<sup>10</sup>.

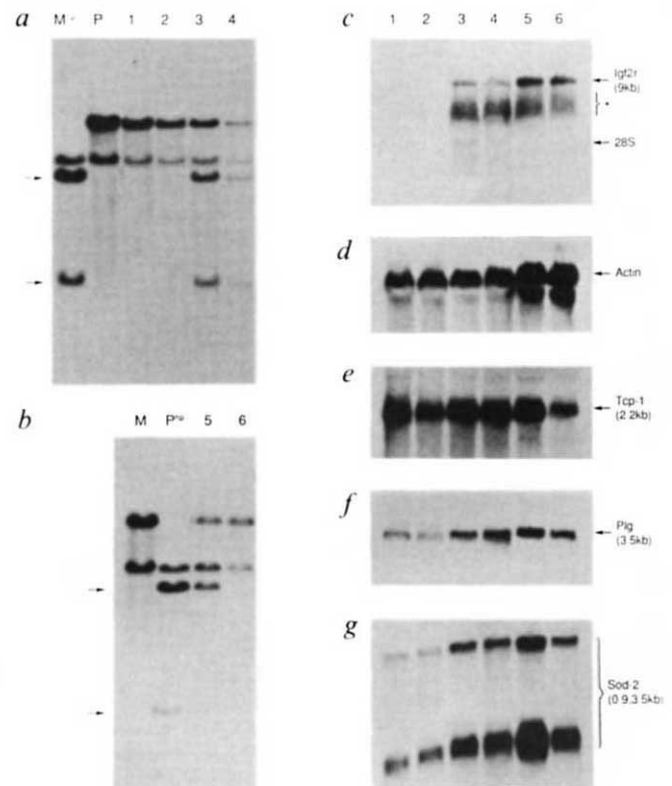
chromosomes in proportion to the number of chromosomes present.

Figure 4 shows the results of an analysis of steady-state messenger RNA levels of the *Igf2r*, *Tcp-1*, *Plg* and *Sod-2* genes in 15-day-old embryos (E15) that inherited a deletion of *Tme*

from their mother (embryos 1 and 2) or their father (embryo 6). The parental and embryo genotypes were identified using a marker from the *D17Rp17* locus (Fig. 4a and b). From the argument presented above, an imprinted gene would show no expression in embryos 1 and 2, but would show expression in

FIG. 4 Genotype (a and b) and mRNA expression analysis (c–g) of E15 embryos. a, Maternal inheritance of the *T<sup>hp</sup>* deletion. M<sup>hp</sup>: mother, *T<sup>hp</sup>*/C3H.P: father, BALBc/BALBc. Lanes 1 and 2: embryos of genotype *T<sup>hp</sup>*/BALBc. Lanes 3 and 4: embryos of genotype C3H/BALBc. b, Paternal inheritance of the *T<sup>hp</sup>* deletion. M: mother, BALBc/BALBc; P<sup>hp</sup>: father, *T<sup>hp</sup>*/C3H. Lane 5: embryo of genotype C3H/BALBc. Lane 6: embryo of genotype *T<sup>hp</sup>*/BALBc. The absence, in embryos 1, 2 and 6, of the two *Dra*I C3H-specific restriction fragments (indicated by the arrows) shows that they have inherited the *T<sup>hp</sup>* deletion chromosome. c–g, Messenger RNA expression in embryos 1–6. c, Hybridization of RNA blots using *Igf2r*, and d, hybridization of the filter shown in c with an actin probe. e, f and g. Three separate RNA blots prepared from the same samples as in c, hybridized with *Tcp-1*, *Plg* and *Sod-2* cDNAs respectively. This mRNA analysis shows that expression of *Igf2r* mRNA is absent in embryos that inherit a deletion of the *Tme* locus from their mother (embryos 1 and 2) and compared with their wild-type litter mates (embryos 3 and 4), when equivalent amounts of RNA can be detected using the actin probe (d). Embryos that inherit a deletion of the *Tme* locus from their father (embryo 5) express *Igf2r* at levels similar to wild-type control litter mates (embryo 6). These results have been confirmed in a second cross analysing three maternally derived deletions and two paternally derived deletions (data not shown) and identify *Igf2r* as being expressed from only the maternal chromosome. The expression of *Plg*, *Tcp-1* and *Sod-2* mRNAs are independent of the parental origin of the chromosome, embryos 1 and 2 show hemizygous levels of expression for these three genes compared with wild-type embryos 3 and 4. The same hemizygous levels of expression are seen for these genes when embryo 6 is compared with its littermate control, embryo 5.

METHODS. Embryos were killed at day 15 and separated from extra-embryonic tissue. Embryonic tissue was minced and half was used to prepare DNA<sup>10</sup> and half to prepare RNA<sup>27</sup>. DNA was digested with *Dra*I to prepare blots which were hybridized with probe RpB2, a *Bam*HI fragment from a cosmid clone isolated using *D17Rp17* marker (D.P.B., unpublished data). The *D17Rp17* locus is deleted from the *T<sup>hp</sup>* chromosome<sup>26</sup>. *T<sup>hp</sup>*/C3H male and female mice were provided by E. Eicher. RNA was analysed on formaldehyde gels<sup>27</sup> and hybridized as shown. The migration position of 28S ribosomal RNA is indicated (c). *Tme* mutant embryos die at E15 and normally show a generalized body oedema before death, but no other abnormality is evident<sup>1,2,4</sup>. To preserve the integrity of the tissues, embryos were killed before the onset of visible oedema. However in c, *Igf2r* mRNA is slightly



degraded, producing a band of ~9 kb (indicated by arrow) and a smear (\*) that extends down to the 28S ribosomal band. This degradation was a feature of many RNA preparations and may be a result of the large size of the mRNA. Hybridization of this filter with actin cDNA (d) shows roughly equal RNA loading for embryos 1, 2, 3 and 4, but embryos 5 and 6 contain more RNA.



embryo 6, at levels comparable to wild-type embryos. Non-imprinted genes would show expression in embryos 1, 2 and 6 that would be equal to half that in wild-type controls. From the RNA blots in Fig. 4, it can be seen that only *Igf2r* shows the predicted expression pattern of an imprinted gene. The *Plg*, *Tcp-1* and *Sod-2* genes all have the predicted expression pattern of non-imprinted genes.

Our results show that the *Igf2r* gene maps to the *Tme* locus and is maternally imprinted. The expression of three other genes that also map to the same region is not apparently influenced by parental origin. Although confirmation of *Igf2r* as the *Tme* gene awaits rescue or inactivation experiments, immediate use can be made of the results described here to examine the role of the *Igf2* receptor in development and the molecular basis of imprinting. The *Tme* mutation is lethal at day 15 of embryogenesis, and embryos are oedematous but do not show any growth defects<sup>4</sup>. If the *Igf2* receptor is the *Tme* gene, this phenotype contrasts with that generated by inactivation of the *Igf2* ligand gene<sup>19</sup> which resulted in small but viable mice. Because the *Igf2* receptor lacks signal-transducing properties and is identical to the mannose-6-phosphate receptor<sup>20</sup>, its role in mediating the growth-regulating properties of the *Igf2* ligand is not clear but may be better understood by studies with the *Tme* mutant. The molecular basis of genomic imprinting is unknown but we are now in a position to use the *Igf2r* gene to identify the mechanisms through which parental origin can modify gene expression in development. Our immediate goals are to examine the regulatory regions of the *Igf2r* genomic locus for evidence of parental-specific epigenetic modification. The only known type of epigenetic modification in mammalian DNA is cytosine methylation in the CpG dinucleotide<sup>21</sup>. Evidence from several sources indicates that methylation can interfere with gene expression and the availability of a genomic clone will allow us to test this and other possible mechanisms of imprinting. Whether genomic imprinting exists as a specific mechanism to regulate gene expression in development or whether it is a nonspecific mechanism, the consequence of which would prevent asexual reproduction, is still not clear<sup>22,23</sup>. The identification here of the *Igf2* receptor as an imprinted gene, and the recent report that the *Igf2* ligand also shows parental-specific differences in gene expression<sup>19</sup> mean that molecular tools are now available to answer questions about genomic imprinting. □

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City \_\_\_\_\_

State \_\_\_\_\_ Zip \_\_\_\_\_

Phone ( ) \_\_\_\_\_

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