

A genetic study of the human T gene and its exclusion as a major candidate gene for sacral agenesis with anorectal atresia

Charalambos Papapetrou, Felicity Drummond, William Reardon, Robin Winter, Lewis Spitz, Yvonne H Edwards

Abstract

Sacral agenesis is a heterogeneous group of congenital anomalies in which most cases are sporadic but rare familial forms also occur. Although one gene has been mapped to chromosome 7q36 in families with hemisacrum, associated with anorectal atresia and presacral mass, it is clear that the genetic aetiology of these disorders is complex and other genes remain to be discovered. Some years ago, the idea of T (Brachyury) as a candidate gene for sacral agenesis was raised, because tail abnormalities associated with T and the t complex, on mouse chromosome 17, resemble spinal defects seen in man. The recent cloning and mapping of the human T gene prompted us to re-evaluate this idea. T is a transcription factor essential for the normal development of posterior mesodermal structures. Although the sequence and function of T are highly conserved in evolution, our genetic study shows that the coding region of the human gene is highly polymorphic. Three common variable amino acid sites in known functional domains have been identified: Gly356Ser, Asn369Ser, and Gly177Asp. For the latter variant, functional studies have shown that the presence of Asp at residue 177 reduces the stability of T dimer formation. A search for rare mutations of T in 28 selected patients with sacral agenesis/anorectal atresia identified a novel, rare variant in one patient and her mother. This mutation leads to an amino acid change within a conserved activation domain. While the functional significance of this single mutation requires further investigation, we can conclude from our studies that if T has a role in the aetiology of sacral agenesis, its contribution is small in this particular set of patients. However, we cannot exclude a more major role in other forms of sacral defect.

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Sacral agenesis (caudal regression syndrome) is a heterogeneous group of congenital anomalies in which most cases are sporadic but rare familial forms have been described (OMIM 182940, 176450, 312800).^{1,2} Sporadic cases are often associated with maternal diabetes.^{3,4}

A wide spectrum of phenotypes occur, ranging from simple sacral abnormalities to sacral agenesis with accompanying musculoskeletal, urogenital, cardiovascular, and pulmonary anomalies; neurological disorders which affect the pelvic region or lower limbs are also found.

Familial forms of sacral agenesis can show complete or partial loss of the sacrum, or hemisacrum (sickle shaped sacrum), with or without anterior meningocele or presacral teratoma. The presence of a presacral mass increases the risk of neonatal or childhood meningitis and of complications during labour, chronic constipation, rectal fistula, or abscess. Imperforate anus/rectum is another commonly associated anomaly.^{5,6} The incidence of various types of sacral agenesis is uncertain. Radiographic studies suggest that 0.43% of the general population show partial or complete absence of the sacrum while the incidence of hemisacral dysgenesis with anterior meningocele is around 0.14%.^{1,7-9}

Apart from sacral agenesis, some other forms of sacral anomaly are very common, for example, dorsomedial failure of ossification in the lumbosacral region, a benign form of spina bifida occulta, is found in one fifth of the normal adult population.^{10,11} There is some evidence to suggest an overlapping aetiology between sacral agenesis and spina bifida occulta/cystica from families segregating both clinical features.¹²⁻¹⁴

The task of defining the genetic components of this group of disorders is made difficult by the phenotypic and aetiological heterogeneity. In families, segregation of the disorder is compatible with an autosomal dominant form of inheritance. Some progress towards identifying genes has been made by selecting patients for study based on careful delimitation of clinical features. For example, for cases with the so-called Currarino triad of symptoms, that is, autosomal dominant sacral agenesis with anorectal atresia and presacral mass, a gene was located to chromosome 7q36 by linkage analysis.^{15,16} Subsequently, this region of human chromosome 7 has been implicated cytogenetically in various forms of sacral agenesis.¹⁷⁻¹⁹ The 7q36 region contains the sonic hedgehog gene (hSHH)¹⁷ which shows mutations in patients with holoprosencephaly, but, so far, no mutations implicated in this group of disorders have been found in patients with sacral agenesis.²⁰ Despite this important advance, it is clear that other genes involved in this group of disorders remain to

MRC Human
Biochemical Genetics
Unit, University
College London,
Wolfson House,
4 Stephenson Way,
London NW1 2HE, UK
C Papapetrou
F Drummond
Y H Edwards

Institute of Child
Health, 30 Guilford
Street, London,
WC1N 1EH, UK
W Reardon
R Winter

Department of
Surgery, Great
Ormond Street
Hospital for Children
NHS Trust, Great
Ormond Street,
London WC1N 3JH,
UK
L Spitz

Correspondence to:
Professor Edwards.

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Table 1 Primers and annealing temperatures used in the amplification of exons from the human T gene. F indicates the forward (sense) and R the reverse (antisense) primers. * indicates the 5' UTR of exon 1 and † exon 1 coding sequence

Primer	Sequence (5' → 3')	Annealing temp	Size of PCR product
TX1Fb*	CCAGAGACCTACACTAGTAGAGCC	58°C	228 bp (exon 1)
TX1Rb*	AAGCCCAGACTCGCTACCTGAG		
TX1F†	ATCTCAGGTAGCGAGTCTGGGCTTC	61°C	269 bp (exon 1)
TX1R†	ACCCACCTGCGGTTCTTGGTCAC		
TX2F	TGGCGCCGCCCCGTGTTTCCAG	68°C	309 bp (exon 2)
TX2R	TGGCGGGCTCCTCACACCTACCT		
TX3F	TGCCACCAATCCTGTATCTGTCTC	63°C	191 bp (exon 3)
TX3R	AAGCGATCCGCCTCTGTCTCTCA		
TX4F	GGTGCCAAATTAATCCCCTG	53°C	173 bp (exon 4)
TX4R	CTGCATACTTCTTTGCCAAGG		
TX5F	CCTTGGCAAAGAAGTATGCAG	53°C	192 bp (exon 5)
TX5R	TGACAGAGTGCACAAACCG		
TX6F	CTCAGCACTGTCTTCTCACAG	58°C	223 bp (exon 6)
TX6R	GGCAGAAATGAGGCTGAGGACTC		
TX7F	TGTTCTTGAAGACACCATC	53°C	221 bp (exon 7)
TX7R	TCCCAGGATGCTTTGCACCA		
TX8F	CCCTGCTCACTGGTGTCTTTC	56°C	338 bp (exon 8)
TX8R	AGTCACTGCATCTTTCGGGAC		
TX8Fb	AGGCAGCAGCCGTGTCCAACG	57°C	256 bp (exon 8, 3' end)
TX8Rb	ATGGGTGAGGGGTGTGTAGTG	58.5°C	159 bp (exon 8, 5' end)

be discovered; for example, Nagai *et al.*²¹ have described a Currarino patient with no abnormality of 7q36 who shows partial trisomy of chromosomes 13q and 20p.

In this study, we have investigated T Brachyury as a candidate gene for sacral agenesis. The idea that T might be implicated in this disorder was raised some years ago, because tail abnormalities associated with the t complex on chromosome 17 in the mouse were thought to resemble spinal defects seen in man.^{9, 13} In the mouse, the T gene is situated within the t complex and determines a short tail/tailess phenotype. Genetic studies were carried out using HLA as a marker, since in the mouse, the H-2 major histocompatibility complex (MHC) is located within the distal end of the t complex. These studies failed to show linkage to T. However, it has become clear that, in man, the t complex region has undergone rearrangement within the genome such that HLA is not a useful marker. We have recently mapped the human T gene to 6q27, some considerable distance from the MHC locus at 6p21.3.²² This new information stimulated us to reinvestigate T as a candidate locus for sacral agenesis.

There are a number of other factors which influence the choice of T as a reasonable candidate gene. T protein is a transcription factor which is essential to the normal development and differentiation of the notochord²³ and this fits well with the proposal that the primary defect in sacral agenesis lies in the notochord.¹⁵

Table 2 Sequence variation identified in the human T gene. * indicates that the mutation lies 17 bp from the 5' end of intron 7 and † that exons 2 and 3 were analysed in larger populations (n=105 and n=231, respectively)

Polymorphism	Amino acid	Variant allele frequency			Enzyme
		SA (n=28)	Controls (n=60)	Total	
5' UTR	G*68A	—	0.46	0.50	BsrBI
Exon 2	C363T	Ser121Ser	0.19	0.14	PvuII
Exon 3	G530A	Gly177Asp	0.46	0.49	BamHI
Intron 7	A17G*	—	0.13	0.15	HaeIII
Exon 8	G1066A	Gly356Ser	0.13	0.16	BamI
Exon 8	A1106G	Asn369Ser	0.04	0.10	Cac8I
Exon 8	G1176A	Ala392Ala	0.27	0.31	HhaI

The notochord controls cell migration and differentiation in those tissues most often involved in sacral agenesis, the hindgut, the neural tube, and the sclerotomal cells which form vertebrae.^{24, 25} T is expressed abundantly, not only in the definitive notochord, but also in the remnant primitive streak in the tailbud. The tail bud gives rise to all non-epidermal structures caudal to the first sacral vertebrae. T^{-/-} embryos die in mid gestation, fail to develop a notochord, and display severe defects in posterior mesodermal structures. T^{+/-} heterozygotes and T^{-/-} chimeras show anomalies which resemble those which occur in sacral agenesis in man, fusions between gut and neural tube, malformations of the sacral/lumbar vertebrae, and split notochords.^{26, 27} In human patients the presence of split notochords is associated with cleft posterior vertebral column, meningocele, teratoma, and large bowel fistula.²⁸

T is a member of the T box family of transcription factors, each member of which has a unique function in embryogenesis. Mutations in other T box genes are associated with complex developmental disorders in man; for example, mutations in TBX3 and TBX5 cause ulnar-mammary²⁹ and Holt-Oram³⁰ syndromes, respectively.

As a starting point in our investigations, we have examined a well defined subset of patients with sacral malformation which is associated with anorectal atresia, but no other congenital abnormalities. Furthermore, within this group, cytogenetic anomalies of 7q36 and the involvement of maternal diabetes were excluded.

Material and methods

CLINICAL SAMPLES

Patients with anorectal atresia and radiographic evidence of sacral agenesis were selected from a consecutive series seen in the clinic. Metaphase spreads of blood lymphocytes were examined for cytogenetic abnormalities. Twenty eight subjects who showed no chromosome abnormalities and whose mothers were non-diabetic were included in this study. Genomic DNA from patients and their parents was extracted from whole blood lymphocytes.

PCR AMPLIFICATION

A total of 100–250 ng of DNA was amplified by the polymerase chain reaction (PCR) using primer pairs (Oswel, UK) specific for exons 1 to 8 of the human T gene. With the exception of exons 1 and 2, primers were designed to include the exon/intron boundaries.²² The SSCP banding pattern from the 338 bp of exon 8 was complex and it was necessary to design additional internal primers, TX8Fb and TX8Rb, which could be used in combination with the primers TX8F and TX8R to generate two overlapping PCRs which spanned the entire exon. The primer sequences, sizes of PCR products, and reaction annealing temperatures are shown in table 1. PCR conditions were 15 seconds at 95°C, 30 seconds at the appropriate annealing temperature, and 30 seconds at 72°C for a total of 35 cycles.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

SSCP was carried out using 1 μ l PCR product mixed 1:1 with loading buffer (95% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue) in a total volume of 8 μ l, heated to 97°C

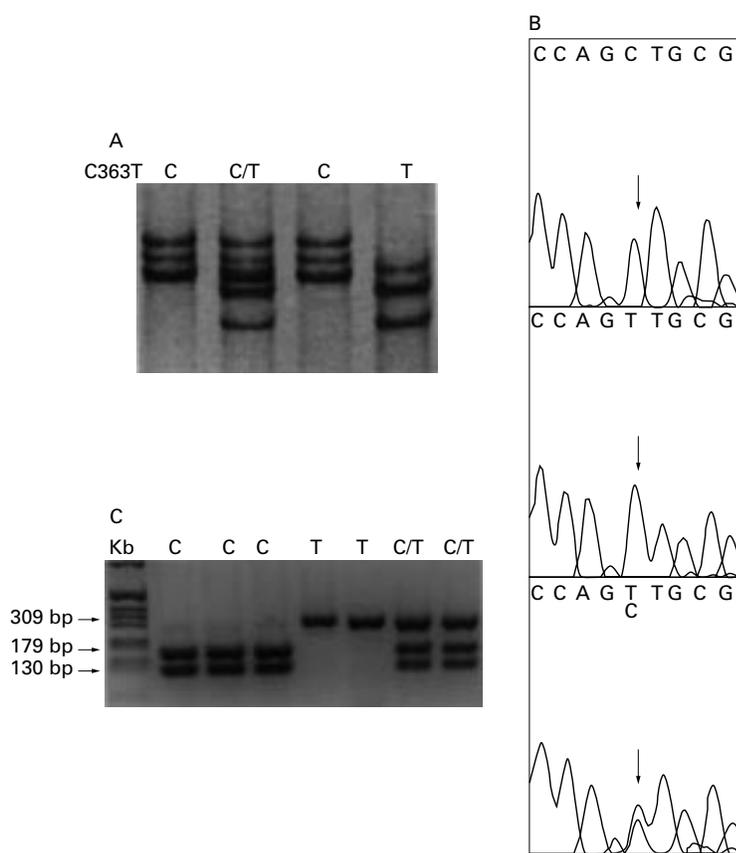


Figure 1 Analysis of human *T*, exon 2. The common C363T variant identified by SSCP (A) and characterised by DNA sequence analysis (B). The base change leads to a loss of a *Pvu*II site and this facilitates rapid genotyping (C). DNA from homozygotes and heterozygotes are shown together and indicated as T, C, and C/T.

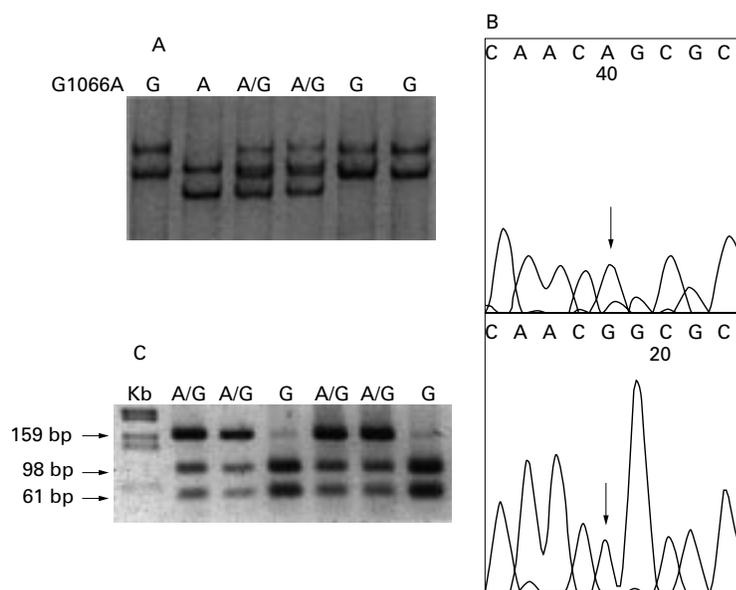


Figure 2 Analysis of human *T*, exon 8. The common G1066A variant was identified by SSCP (A) and characterised by DNA sequence analysis (B). The base change leads to a loss of a *Ban*I site and this facilitates rapid genotyping (C). DNA from homozygotes and heterozygotes are shown together and indicated as A, G, and A/G.

for 10 minutes, and snap cooled on ice. The conditions of electrophoresis for each PCR fragment were optimised by varying the acrylamide:biscrylamide ratio (range 40:1 to 66.7:1), glycerol content, and electrophoresis conditions (range 150 V to 310 V for 2.5 to 20 hours) in order to maximise resolution and band sharpness. For all gels, 0.5 \times TBE running buffer (45 mmol/l Tris, 45 mmol/l borate, 1 mmol/l EDTA) was used and the electrophoresis carried out at either 4°C or at room temperature. After electrophoresis, the gels were washed/fixated in ethanol/acetic acid and stained using silver nitrate and sodium borohydride, as described by Harvey *et al.*³⁹

DNA SEQUENCE ANALYSIS

PCR products were sequenced from both strands, using the Cycle Sequencing Dye Terminator Kit according to the manufacturer's instructions (Amersham Life Sciences) and the ABI PRISM 373 automated DNA sequencer. Primers used for sequencing were the same as those used in the PCR reaction (table 1).

Results

GENETIC ANALYSIS OF T IN SACRAL

AGENESIS/ANORECTAL ATRESIA PATIENTS

DNA from 28 patients with sacral agenesis/anorectal atresia was analysed together with DNA from 60 normal, unrelated controls of white UK origin. SSCP analysis of PCR products containing each of the eight exons of the *T* gene led to the identification of seven variant T alleles. All of these occurred in both the patient and control populations with no significant difference in frequencies between the two groups.

Direct sequence analysis was used to determine the basis of the polymorphism at each variant site. In all instances, this showed that the SSCP was the result of a single nucleotide change and in three cases this gave rise to an amino acid substitution. All the mutations involved either the abolition or creation of a restriction enzyme site and this allowed the rapid genotyping of the remaining samples. The results are summarised in table 2. Figs 1 and 2 show, as examples, the analyses of exon 2 and exon 8. DNA sequencing of the variant alleles identified a C to T change at nucleotide 363 in exon 2 and a G to A change at nucleotide 1066 in exon 8 of the coding sequence (fig 1B and fig 2B, respectively). These mutations lead to the abolition of a *Pvu*II enzyme site in exon 2 (fig 1C) and a *Ban*I site in exon 8 (fig 2C) such that restriction enzyme digestion and electrophoresis can be used to distinguish homozygotes and heterozygotes.

SSCP did not show any unusual variants among the patient samples. However, full sequence analysis of all eight exons identified a single novel rare variant in a female patient (arrowed in fig 3A and C). This is a C to T transition at nucleotide 1013 in exon 7 which results in an amino acid change, Ala338Val, within the functionally conserved transcription

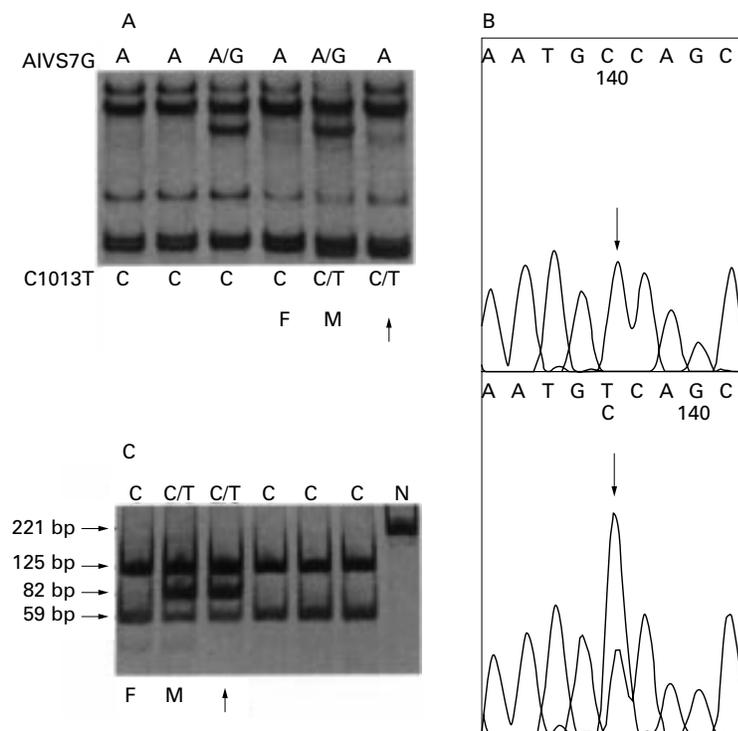


Figure 3 Analysis of human T, exon 7. The rare C1013T variant was identified and characterised by DNA sequence analysis (B). The base change leads to a loss of a *Cac8I* site and this facilitates rapid genotyping (C). (A) SSCP analysis of DNA from the proband, her parents, and several controls. The common polymorphism in intron 7, AIVS7G, is well resolved in these SSCP electrophoretic conditions, but the rare C1013T variant is more difficult to visualise (genotyping indicated as C or C/T). DNA from homozygotes and heterozygotes are shown. The proband is indicated with an arrow and her parents as M, mother and F, father.

activation domain (TA2 in fig 4) of the protein. None of the other patients, or the 60 UK controls, exhibit this variant. However, the mother of the patient is heterozygous for the same variant (M in fig 3A and C). Fig 3B shows the sequence chromatogram of the affected subject and an unaffected control. The mutation abolishes a *Cac8I* restriction site and fig 3C shows the *Cac8I* digests in the patient, her parents, and controls. Close inspection of an SSCP analysis of the same PCR products (fig 3A) shows minor differences in electrophoretic mobility which correspond to the C1013T

change. This analysis also clearly resolves the two alleles at an adjacent polymorphic site in intron 7, AIVS,G, which is only 38 bp downstream.

POLYMORPHIC VARIATION IN THE HUMAN T GENE

The human T (Brachyury) gene comprises eight exons and encodes a transcription factor of 435 amino acids (fig 4). Exons 1 to 4, and part of exon 5, encode the DNA binding domain (amino acids 1 to 229), containing the core consensus T box (amino acids 43 to 227) which defines the family of T box transcription factors.^{31 32} Based on amino acid sequence similarity with the mouse T gene, exons 5 to 8 are expected to encode two transcription activation domains (TA1 and TA2) and two repression domains (R1 and R2, fig 4²³).

The positions of the polymorphisms identified in our study are shown in fig 4. Polymorphic sites are scattered throughout the gene, both in the DNA binding domain and the transcription activation domains. Of particular interest are those variations which result in an amino acid substitution. For example, in exon 3, within the highly conserved T box, a G to A transition occurs that changes a conserved glycine residue to aspartic acid, Gly177Asp (table 2). This variant occurs at high frequency in the UK population and in other ethnic groups. Functional studies of the proteins encoded by this variant allele have shown that the aspartic acid residue reduces the stability of T dimer formation.³³

In exon 8, two further polymorphisms, Gly356Ser and Asn369Ser, were identified. Since these substitutions involve amino acid changes and map to the region corresponding to the conserved transcription activation domain, TA2 (fig 4),^{23 34} they are potentially functional.

Haplotype analysis was incomplete, but it was possible to determine that the rare Val338 mutation found in the sacral agenesis patient occurs on a chromosome carrying the common alleles encoding Gly356 and Asn369.

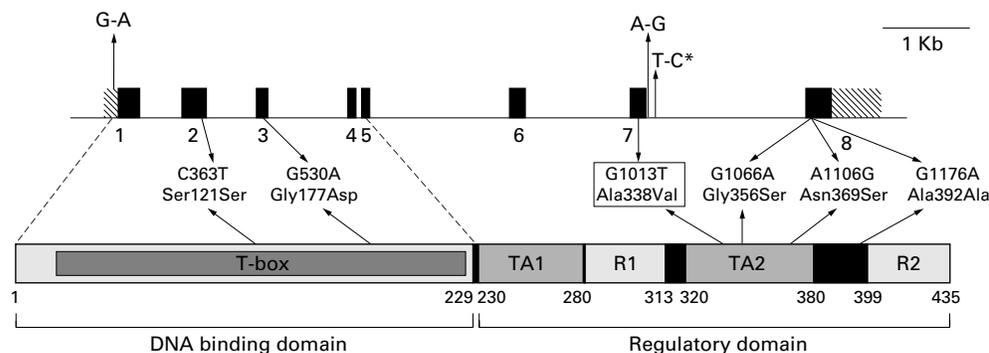


Figure 4 The structure of the human T gene (top) and protein (bottom). Exons are shown as boxes and numbered. The relative positions of the various polymorphic sites in the gene and in the protein are indicated by arrows. The rare variant found in exon 7 of a sacral agenesis patient is boxed. The domains of the T protein shown correspond in position to those described for the mouse protein.²³ The T box is the conserved core of the DNA binding domain which defines the T box gene family, TA1 and TA2 are transcriptional activation domains, and R1 and R2 are putative repressor domains. The nucleotide numbers are given for positions in the coding sequence, rather than the T transcript, since the transcription start site of the human T gene has not yet been ascertained with certainty.

Discussion

The T gene encodes a transcription factor, essential for normal mesoderm differentiation, and the T sequence is highly conserved across vertebrate species, from protochordates to man. There is, for example, 91% amino acid identity between human and mouse T, and 75% between human and *Xenopus* T.²² It was surprising, therefore, to find that the coding sequence of this gene is highly polymorphic in man, leading to amino acid variation in both the DNA binding domain and transcription regulatory domains. The positions corresponding to the Gly177Asp and Asn369Ser variants in the human sequence are conserved in other species and are Gly and Asn respectively in T from mouse, chicken, and *Xenopus*; zebrafish T differs by having Gly at 369. The Gly356Ser position is less strongly conserved, with Gly at this position in the mouse, and either Ser or Thr in other species.^{23 35}

The three variant amino acid sites of the human T gene lie within a 7 kb genomic region and are likely to be in linkage disequilibrium. This might have some bearing on the outcome for protein function, since particular amino acid changes will occur in combination on the same polypeptide. For example, it can be envisaged that the dimer instability conferred by the change from Gly to Asp at amino acid 177³⁵ may be influenced by the presence of other variant amino acids on the same polypeptide. Our preliminary studies show that there is linkage disequilibrium between the Gly177Asp site and other variant sites, such that Asp177 occurs most often on a chromosome carrying the common alleles Gly at 356 and Asn at 369.

A search for rare mutation at the T gene locus in 28 selected patients with sacral agenesis/anorectal atresia identified a novel, rare variant in one patient and her mother. It remains feasible that the mother has some mild sacral abnormality and we hope to investigate this further. This rare mutation leads to an amino acid change, Ala338Val, within a conserved transcription activation domain. Ala is conserved in this position in human and mouse T but Thr occurs at the same position in chicken, *Xenopus*, and zebrafish T. At present the functional significance of this mutation, if any, is unknown. If T has a role in the aetiology of sacral agenesis, its contribution is small in this particular subset of patients.

The genetic aetiology of sacral agenesis seems certain to be heterogeneous. This study has focused in the first instance on a selected group of patients which define a small subset of sacral anomalies. While the results appear to exclude T as a major gene for this particular set of anomalies, we cannot exclude a role for it in other forms of sacral agenesis and related sacral defects. In this context, it may be significant that there is some evidence to suggest that T is a minor gene associated with susceptibility to spina bifida.³⁶ One possibility which remains to be explored is that T underlies relatively mild sacral anomalies, often referred to as spina bifida occulta, and which sometimes occur in families segregating neural tube defects. It may

be that the presence of spina bifida occulta increases susceptibility to neural tube abnormalities in the region of the lower spine. This might explain the concurrence of spina bifida and sacral anomalies in the same family and accords with the view that spina bifida occulta and spina bifida aperta are different in origin.^{37 38}

Note added in proof

Since the acceptance of this manuscript, mutations in the HB9 homeobox gene have been identified in patients with the Currarino form of sacral agenesis.⁴⁰ HB9 is distantly related to the *Drosophila* gene *proboscipedia* and maps to human chromosome 7q36. The panel of patients comprising the data reported here are being screened for mutation at this locus.

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