ORIGINAL ARTICLE

Array comparative genomic hybridisation analysis of boys with X linked hypopituitarism identifies a 3.9 Mb duplicated critical region at Xq27 containing *SOX3*

N M Solomon, S A Ross, T Morgan, J L Belsky, F A Hol, P S Karnes, N J Hopwood, S E Myers, A S Tan, G L Warne, S M Forrest, P Q Thomas

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See end of article for authors' affiliations

Correspondence to: Dr P Thomas, Pituitary Research Unit, Murdoch Children's Research Institute, Tenth Floor, Royal Children's Hospital, Parkville, Victoria, Australia, 3052; paul.thomas@mcri.edu.au

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Introduction: Array comparative genomic hybridisation (array CGH) is a powerful method that detects alteration of gene copy number with greater resolution and efficiency than traditional methods. However, its ability to detect disease causing duplications in constitutional genomic DNA has not been shown. We developed an array CGH assay for X linked hypopituitarism, which is associated with duplication of Xq26–q27.

Methods: We generated custom BAC/PAC arrays that spanned the 7.3 Mb critical region at Xq26.1–q27.3, and used them to search for duplications in three previously uncharacterised families with X linked hypopituitarism.

Results: Validation experiments clearly identified Xq26–q27 duplications that we had previously mapped by fluorescence in situ hybridisation. Array CGH analysis of novel XH families identified three different Xq26–q27 duplications, which together refine the critical region to a 3.9 Mb interval at Xq27.2–q27.3. Expression analysis of six orthologous mouse genes from this region revealed that the transcription factor *Sox3* is expressed at 11.5 and 12.5 days after conception in the infundibulum of the developing pituitary and the presumptive hypothalamus.

Discussion: Array CGH is a robust and sensitive method for identifying X chromosome duplications. The existence of different, overlapping Xq duplications in five kindreds indicates that X linked hypopituitarism is caused by increased gene dosage. Interestingly, all X linked hypopituitarism duplications contain *SOX3*. As mutation of this gene in human beings and mice results in hypopituitarism, we hypothesise that increased dosage of *Sox3* causes perturbation of pituitary and hypothalamic development and may be the causative mechanism for X linked hypopituitarism.

Congenital pituitary hormone deficiencies (hypopituitarism) occur in approximately 1 in 4000 births,[1] and have many associated clinical features that reflect the identity and degree of pituitary hormone deficiency. The key phenotypic features (and their causes) are short stature (growth hormone deficiency), infertility and delayed puberty (gonadotropin deficiency), secondary hypothyroidism (thyroid stimulating hormone deficiency), and poor stress response (adrenocorticotropin deficiency). Many cases of congenital pituitary hormone deficiency result from a failure of the hormone secreting cell types in the pituitary to differentiate. Alternatively, aberrant morphogenesis of the hypothalamus, which controls pituitary hormone secretion through the release of peptide signals, may also cause hypopituitarism.

Most familial forms of congenital hypopituitarism are autosomal recessive, and are associated with mutations in transcriptional factors including HESX1, PROP1, and PIT1.[2] However, an X linked form of hypopituitarism, (OMIM 312000) has also been reported.[3–8] This disorder only affects men, who display profound growth hormone deficiency from birth, and most of whom also have deficiencies in other anterior pituitary hormones. In some cases, patients are deficient in all anterior pituitary hormones (panhypopituitarism). Mild, non-specific mental retardation is also a feature of most patients with X linked hypopituitarism.

Although the precise genetic basis for X linked hypopituitarism remains to be identified, our previous studies indicate that it results from increased gene dosage. Through cytogenetic and fluorescence in situ hybridisation analysis of a large Australian X linked hypopituitarism pedigree,[6] we mapped a 7.3 Mb interstitial duplication (previously estimated to be 9 Mb) spanning Xq26.1–q27.3 that cosegregates with the disease.[8] Importantly, this duplication was shown to be smaller and completely encompassed by a 13 Mb interstitial duplication that we mapped in an independent X linked hypopituitarism kindred.[7 8] These data define a 7.3 Mb critical region for X linked hypopituitarism at Xq26.1–q27.3.

Whilst fluorescence in situ hybridisation has proved to be a useful technique for mapping X linked duplications, it has several disadvantages including low throughput, a labour intensive methodology and a necessity to use the patient's chromosomes or interphase nuclei. An alternative technology that has recently been developed and that circumvents these limitations is array comparative genomic hybridisation (array CGH).[9–14] This technique has been applied extensively in cancer genomics and has proved to be a fast and reliable method for detecting severalfold changes in gene copy number within tumour samples.[15 16] Furthermore, proof of principle studies using chromosome specific arrays have recently shown that array CGH is a sensitive and accurate method for the high resolution mapping of heterozygous autosomal deletions.[17 18] However, the ability of array CGH to detect autosomal and X chromosome duplications has not been examined in detail.

Abbreviation: array CGH, array comparative genomic hybridisation

Here we report the development of an array CGH methodology for detecting duplications that span the X linked hypopituitarism critical region. X linked duplications were detected in three previously uncharacterised families with X linked hypopituitarism. These data allow us to refine the critical region for X linked hypopituitarism to 3.9 Mb and support our hypothesis that increased gene dosage results in X linked hypopituitarism. Expression analysis of critical region genes showed that *Sox3* is expressed in the infundibulum of the developing pituitary, the presumptive hypothalamus, and other regions of the central nervous system, implicating this gene in the aetiology of X linked hypopituitarism.

DATA ACCESS

The Ensemble Genome Browser (v16.33.1, 2 July 2003; www.ensembl.org/) was used to obtain the chromosomal position of clones and to identify candidate genes.

METHODS

Array comparative genomic hybridisation analysis

We produced arrays and performed hybridisation and analysis using the MicroCASTer handheld micro-array system and MicroHybridisation kit protocol (Schleicher and Schuell). We prepared DNA from BAC and PAC genomic clones using the modified QIAGEN protocol obtained from the BACPAC Resources Center (http://bacpac.chori.org/). 10 μg of target BAC and PAC DNA were sonicated into 1.5–15 kb fragments, ethanol precipitated and resuspended in 5 μl of 80% DMSO. DNA samples were printed manually onto Superfrost Plus microscope slides (ESCO, Biolab Scientific). One array, consisting of 24 clones, was printed per slide in quadruplicate. Prior to hybridisation, DNA quality was tested using POPO-3, a reversible DNA dye.

1.5 μg of test and reference DNA was labelled by nick translation with CY3-dCTP or CY5-dCTP for 7 h at 15℃. Samples were then left overnight at 4℃. Labelled test and reference probes were combined with 50 μg of Cot-1 DNA, precipitated with ethanol and dissolved in 10 μl of hybridisation mix containing 50% formamide, 10% dextran sulphate and 2×SSC. Samples were denatured at 95℃ for 5 min and incubated at 37℃ for 1–2 h to block repetitive sequences. Labelled probes were then added to slides, coverslips sealed with rubber cement and placed in a humidified sealed chamber at 37℃ for 48 hours. Following hybridisation, slides were rinsed in 1×SSC and washed in 1×SSC twice for 10 min at 60℃. After draining excess fluid, the slides were air dried and scanned using the GenePix4000B scanner (Axon Instruments). DNA clones were automatically located based on their signal intensity. The CY3 and CY5 fluorescence intensities for each spot were calculated and the local background was subtracted. We used the median of the ratios measurement for our studies, defined as the median of pixel by pixel ratios of pixel intensities (GenePix Pro3). All features had a signal intensity >30 (as defined by the %B635/B532+2SD value[14 17]), which allowed us to obtain reliable fluorescence values for every clone in the array. The four fluorescence values for each clone were averaged and then normalised using the average fluorescence values of the four autosomal reference clones. This generated a normalised fluorescence intensity ratio for each clone in the array. For all arrays the standard deviations for each spot were calculated, the upper and lower range of which are listed in the figure legends. In all array experiments, the standard deviation was <20%.

Fluorescence in situ hybridisation analysis

Fluorescence in situ hybridisation experiments were performed using the method of Solomon et al[8] using affected male lymphoblast cell lines from the families with X linked hypopituitarism described by Hol et al[7] and Lagerström-Fermér et al.[6]

X inactivation

This assay was performed using the androgen receptor methylation assay on peripheral blood lymphocyte genomic DNA as previously described.[8]

RT-PCR

Pituitary tissue was dissected from mouse embryos from 11.5 days after conception through to 17.5 days after conception and RNA was extracted using TRIzol Reagent (Gibco BRL). RNA was DNAase treated by incubation with 1 U DNAase I (Roche) per μg of RNA at 37℃ for 30 min. 10 μg of DNAase I-treated RNA was primed with hexamers and reverse transcribed using AMV reverse transcriptase (Roche). PCR was performed using 1/20 of the volume of the RT reaction as template, 0.5 mM each primer, 0.2 mM dNTPs, and 0.5 U *Taq* polymerase. All of the primers used were designed to amplify under the following conditions: 94℃ for 30 s followed by 35 cycles of 94℃ for 30 s; 55℃ for 30 s; 72℃ for 30 s; followed by a 10 min extension at 72℃.

The sequences for forward and reverse primers are:

- AI526776, 5′TCCCTTGGCAGGTCATTTTA, 5′CTTTGCCTCC TTCACGGTAG;
- BG920442, 5′CAGAAAGAGTGGGACCTTGC, 5′CAGCACC TTCTCCAGATTCG;
- AI324160, 5′AGAACCCCAAGATGCACAAC, 5′CGGCGTTC ATGTAGCTCTG;
- AW456110, 5′TTTTTGTGGCAGACGTTGAT, 5′TGTCTCCCA ACAACCTCAGAT;
- BG077501, 5′TTCCTGCTCAGCCTCTCTGT, 5′CATCATCTTC GTCGTCGGTA;
- AV256964, 5′GCCATTACAAAAAGCCCAGA, 5′GTGGCTGT TGTGAGAATCCA.

In situ hybridisation

Mouse embryos were fixed overnight in 4% paraformaldehyde in phosphate buffered saline at 4℃ and then placed in 20% sucrose in phosphate buffered saline overnight at 4℃. Embryos were then embedded in OCT and 16 μM sections prepared using a cryostat. Gene expression of *Sox3* was detected by hybridising digoxigenin labelled antisense riboprobes generated from the *Sox3* IMAGE clone 388723 (GenBank). T3 RNA polymerase was used to generate the sense (control) and T7 was used to generate the antisense riboprobe. For the *Atp11c* gene, the primers were designed to amplify a 427 bp fragment which was cloned into pCR II-TOPO (Invitrogen). T7 RNA polymerase was used to generate the sense (control) and SP6 RNA polymerase was used to generate the antisense riboprobe. The procedure for in situ hybridisation was performed as described previously.[19]

Clinical history

This study was approved by the Royal Children's Hospital Ethics in Research Committee and informed consent was obtained from all participants.

Family A

Subject II.1 was born at 37 weeks gestation weighing 2.84 kg to a 32 year old multiple gravida female (three miscarriages, one ectopic pregnancy, two induced abortions, and two live births) who was 162.5 cm tall (50th percentile). She smoked cigarettes and took anticonvulsant medication during pregnancy for a grand mal seizure disorder. The Hispanic father was of normal stature (1.68 m) and had no evidence of

physical or mental abnormalities. APGAR scores were 8 (1 min) and 9 (5 min). As a neonate, II.1 had transient feeding difficulties and bradycardia, a brief spell of hypoglycaemia, and was treated with antibiotics for group B streptococcal infection. A computed tomography scan of the brain was normal. He was referred on discharge from the neonatal intensive care unit to receive physical therapy for hypertonic posturing, which continued until 4½ months of age. Speech delay was diagnosed at 3 years, and at this time his IGF-1 level was low (11 ng/ml; normal 22–87 ng/ml) and his bone age was delayed (1 year 3 months, less than two standard deviations). At age 4, his baseline IGF-1 level was 5 ng/ml (normal 22–87 ng/ml) and IGFBP-3 level was 0.5 mg/l (normal 1.4–3.0 mg/l). A clonidine stimulation test was proposed at this time, but was not performed because of missed appointments. Further testing at age 5 revealed low baseline levels of IGF-1 (7 ng/ml, normal 17–248 ng/ml), IGFBP-3 (0.8 mg/l, normal 1.4–3.0 mg/l) and thyroid stimulating hormone (<0.04 mIU/l, normal 0.34–5.6 mIU/l), prompting continued thyroxine treatment. At 7 years 8 months, his height was 1.02 m (−4.37 standard deviations), weight 18.6 kg. (−2.00 standard deviations), and head circumference 52.5 cm (50th percentile for age). Endocrine testing at this time revealed low to normal thyroxine levels (0.4 ng/dl, normal 0.16–1.6 ng/dl), low to normal thyroid stimulating hormone levels (1.6 mIU/l, normal 0.36–5.6 mIU/l) low to normal triiodothyronine levels (2.6 pg/ml; normal 2.3–4.2 pg/ml), and normal baseline cortisol levels (14 μg/dl; normal 8–24 μg/dl). His baseline growth hormone level was 0.4 ng/ml and rose to 5.1 ng/ml after vigorous exercise. Growth hormone therapy commenced at 7 years 10 months, and resulted in a marked increase in height growth rate (11.3 cm height gain over the first 13 months of treatment). His short stature is symmetric, and there is no facial dysmorphism. His posture was lordotic. His penile length was 5 cm (25th percentile) and testicular volume was 2 cm^3 (50th percentile). He is at the appropriate grade level but has received some special education for learning problems; however no formal IQ testing has been performed.

Subject II.2 was the 2.66 kg product of a 38 week pregnancy to his 33 year old mother. His African-American father was of normal stature (1.63 m). He spent 3 weeks in the neonatal intensive care unit for poor feeding, bradycardia, and hypoglycaemia. A physical examination revealed normal genitals. At age 2, his IGF-1 level was low (<10 ng/ml, normal 20–126 ng/ml) and thyroid stimulating hormone and thyroxine levels were borderline (thyroid stimulating hormone = 0.62 IU/ml; normal 0.32–5.0 IU/ml; thyroxine = 0.7 mg/ml; normal 0.7–1.8 mg/ml). Multiple tests in the following years revealed consistently low baseline levels of IGF-1 (12 ng/ml at age 4, 13 ng/ml at 5 years 10 months, 13 ng/ml at 6 years 6 months, normal 17–248 ng/ml) and IGFBP-3 (1 mg/l at 3 years 3 months, 1 mg/l at 4 years, normal 1.4–3.0 mg/l), indicating growth hormone deficiency. Similarly, low to normal values for baseline thyroid stimulating hormone (0.58 IU/mL at 5 years 10 months, 0.79 IU/ml at 6 years 3 months, normal 0.34–5.6 IU/ml), thyroxine (0.5 ng/dl at 6 years 3 months, normal 0.6–1.6 ng/dl) and triiodothyronine (2.5 pg/ml at 6 years 3 months, normal 2.3–4.2 pg/ml) levels indicated borderline thyroid stimulating hormone deficiency, and thyroxine therapy was initiated shortly before age 4. Cortisol levels were normal (13 μg/dl at 6 years 3 months, 14 μg/dl at 6 years 3 months, normal 8.0–24 μg/dl). An appointment for a clonidine stimulation test was not kept. At age 6 years 8 months, his height was 1.00 m (−3.90 standard deviations), weight 17.5 kg (−1.85 standard deviations), and head circumference was 51 cm (25th percentile). Penile length was 5 cm (25th percentile) and testicular volume was 2.5 cm^3 (50th percentile). On physical examination, he has exaggerated lordosis, symmetric short stature, and no facial dysmorphology. A markedly delayed bone age was documented on a hand X ray (bone age 4 years, chronological age 7½ years). Growth hormone therapy was initiated at 6 years 10 months, and resulted in a dramatic increase in growth rate (11.9 cm height gain over the first 13 months of treatment). II.2 is at the appropriate grade level but has received some special education for learning problems and was diagnosed with attention deficit disorder.

Family B

Subject II.1 was born at 39 weeks gestation to a healthy 25 year old mother of normal height (1.65 m), weighing 3.47 kg. Labour and delivery were uncomplicated and he went home at one day old. At three days old, he presented to the hospital with jaundice, hyperbilirubinaemia, and persistent hypoglycaemia. His random growth hormone level was <1 ng/ml, serum thyroxine level was 13.0 μg/dl, and 11 am cortisol level was 16.9 μg/dl. He was sent home after an investigation for sepsis yielded negative results. His hypoglycaemia was apparently resolved by frequent feedings and careful monitoring. At 12 months of age, he presented with hypoglycaemia and short stature; and was found to have a bone age of 9 months. At 20 months, he was seen by another endocrinologist for persistent hypoglycaemia, short stature, and delayed growth. He was 73.6 cm tall, with a weight of 8.9 kg and head circumference of 44.5 cm, all below the 5th percentile for age. His bone age was 18 months. On physical examination, it was noted that he had a microphallus and undescended testes. Endocrine testing revealed an adequate cortisol response to adrenocorticotropin (peak cortisol 21 μg/dl). However, there was evidence for secondary hypothyroidism (thyroid stimulating hormone <5, total thyroxine 3.4 μg/dl, repeat total thyroxine 4.9 μg/dl, normal 5–10 μg/dl). His IGF-1 level was undetectable at <0.2 U/ml. Two provocative tests, one with L-dopa and another with glucagons, revealed consistently undetectable levels of growth hormone after maximum provocation (growth hormone levels <0.5 ng/ml, criterion for diagnosis is <10 ng/ml). He also continued to have hypoglycaemic episodes. A spontaneous hypoglycaemic event with a serum glucose level of 34 mg/dl yielded a low growth hormone level at 1.0 ng/ml and a normal cortisol level of 25 μg/dl. He was then started on human growth hormone and thyroid hormone replacement. Interestingly, he has had persistent episodes of hypoglycaemia despite being on growth hormone and thyroid hormone replacement. He was also started on and maintained on cortisone acetate despite ''adequate'' cortisol levels for a period of time, which helped his hypoglycaemia. II.1 was maintained on growth hormone replacement until the age of 18 years. He is currently doing well, having achieved a final height of 1.78 m. He is also on thyroid hormone replacement.

II.2 was the 3.37 kg product of a normal, term pregnancy. His mother was 27 years old. His father is 1.93 m and healthy. He was referred to a paediatric endocrinologist at 5 months for failure to thrive, at which time a history of neonatal hypoglycaemia (serum glucose 20 mg/dl) was elicited and a small penis (2.5 cm) was noted. He was started on L-thyroxine at 14 months for a thyroxine level of 6.3 μg/dl (thyroid stimulating hormone level, 0.5 μU/ml) and growth hormone at 16 months for growth hormone levels of <0.7 ng/ml after provocation with L-dopa/arginine and glucagon. Growth hormone therapy was discontinued at 15½ years at a height of 1.80 m, with a subsequent IGF-I level of 69 ng/ml (reference range 286–627 ng/ml). Peak cortisol levels after stimulation with Cortrosyn have consistently been over 20 μg/dl. Low dose testosterone therapy was prescribed for pubertal delay from 14½ until 16½ years,

when early testicular enlargement was noted. At 18 years, II.2 has 15–20 cm^3 testicular volumes, Tanner V genitalia and pubic hair, no facial hair, and a testosterone level of 358 ng/dl (reference range 241–827 ng/dl). He has attended special education classes since middle school. His maternal uncle, I.3, is currently treated with L-thyroxine and hydrocortisone, having had previous growth hormone therapy as a child.

Family C

Clinical details of this family have been described previously.[4]

RESULTS

Validation of array CGH assay

Fourteen BAC/PAC genomic clones spaced at regular intervals within the 7.3 Mb X linked hypopituitarism critical region at Xq26.1–q27.3 were selected for the array (clones 4–17; table 1, fig 1). These clones contain approximately 30% of the critical region sequence. Three BAC/PAC clones that map proximal (clones 1–3) and distal (clones 18–20) to the 7.3 Mb critical region were also included in the array, as were four autosomal control BACs (clones 21–24). The chromosomal location of all clones was confirmed by fluorescence in situ hybridisation (data not shown).[8] We first tested whether the twofold difference in X chromosome dosage in XY males against XX females could be detected using this array. As shown in fig 1A, the average normalised fluorescence intensity ratio for the X chromosome clones in this experiment was 1.943. Therefore, the expected 1:2, male:female X chromosome copy number ratio was readily detected. Next, we tested the ability of the array to detect interstitial X chromosome duplications. Control male DNA was cohybridised with DNA from an affected male from the X linked hypopituitarism family described by Lagerström-Fermér et al.[6] The normalised fluorescence intensity ratio for X chromosome clones that flank the 7.3 Mb duplication (clones 1–3 and 18–20) was 0.990 (fig 1B). In contrast, the normalised fluorescence intensity ratio of clones located within the 7.3 Mb duplication clones was 1.956, approximately double the value of non-duplicated clones. Array CGH analysis of an affected male from the X linked hypopituitarism family described by Hol et al[7] produced similar results, and indicated that this duplication encompasses the proximal clones 1–3 and the distal clone 18 (fig 1C). Fluorescence in situ hybridisation analysis of each of these clones in the Hol et al[7] male cell line confirmed their duplicated status, as indicated by the two closely spaced signals in interphase nuclei (fig 2A, B, C). In contrast, clones 1, 3, and 18 generated a single signal in interphase nuclei from the Lagerström-Fermér et al[6] family (fig 2 D, E, F). These data demonstrate for the first time that array CGH is a robust and sensitive method for identifying interstitial X chromosome duplications.

Identification of novel duplications in families with X linked hypopituitarism

Array CGH was then used to investigate whether duplicated X chromosome material was present in two novel families (families A and B) and one previously published family (family C;[4] fig 3 A, B, C) with X linked hypopituitarism. The phenotypes of affected males in families A and B are summarised in table 2. All affected males have profound growth hormone deficiency, and some of them have thyroid stimulating hormone deficiency. Mild developmental delay may be present in these families, but has not been diagnosed. Unaffected carrier females in families A and B showed skewed X chromosome inactivation (approximately 80%), as was observed in the Lagerström-Fermér et al[6] and Hol et al[7] X linked hypopituitarism families (fig 3D).

Array CGH analysis of family A was performed by comparing affected male DNA with normal male DNA, and identical results were obtained for each affected male. Affected males contain a novel Xq27 chromosome duplication that overlaps with the Lagerström-Fermér et al[6] and Hol et al[7] X linked hypopituitarism duplications (fig 1D and supplementary figure). The family A duplication includes clones 11–20 which span a 5.3 Mb region at Xq27.2–q27.3. The proximal breakpoint lies between clones 10 and 11, which are linked by a single small clone, RPCI-3 523M5 (23.4 kb). The distal breakpoint is less well defined as the duplication includes clone 20, which is the most distal X

Table 1 Address and position of array CGH BAC and PAC genomic clones

Clone number	Clone name, library	Chromosomal position (Mb)	Accession number
1	RP1–136O17	125.9	Z72001
2	RP3–473B4	131.9	Z83826
3	RP11–481F23	132.6	AL357384
4	RP11–535K18	133.2	AL078638
5	RP11–528F22	133.6	AL445427
6	RP11–462H12	135.1	AL019212
7	RP11–389N12	135.3	AL359703
8	RP1–260J9	135.7	Z82193
9	RP1–227P17	136.2	Z81007
10	RP1–36J3	136.3	Z82975
11	RP1–93C23	136.4	AL008713
12	RP11–189F12	137.1	AL449184
13	RP11–51C14	137.5	AL121875
14	RP3–507I15	138.1	Z98950
15	RP1–85A12	139.0	AL049858
16	RP1–142F18	139.3	AL031073
17	RP3–357K22	140.1	AL022720
18	RP1–231L4	140.3	AL159988
19	RP11–514L15	141.0	AL022719
20	RP11–480M11	141.7	AC007015
21	RP11–89N12 (A)		AL135920
22	RP11–91G21 (A)		AC026304
23	RP11–3F4 (A)		AC096886
24	RP11–71O21 (A)		AC022291

Array CGH X chromosome clones (1–20) spanning Xq26–q28, listed in centromeric-telomeric order. Clones 21–24 are autosomal (A).

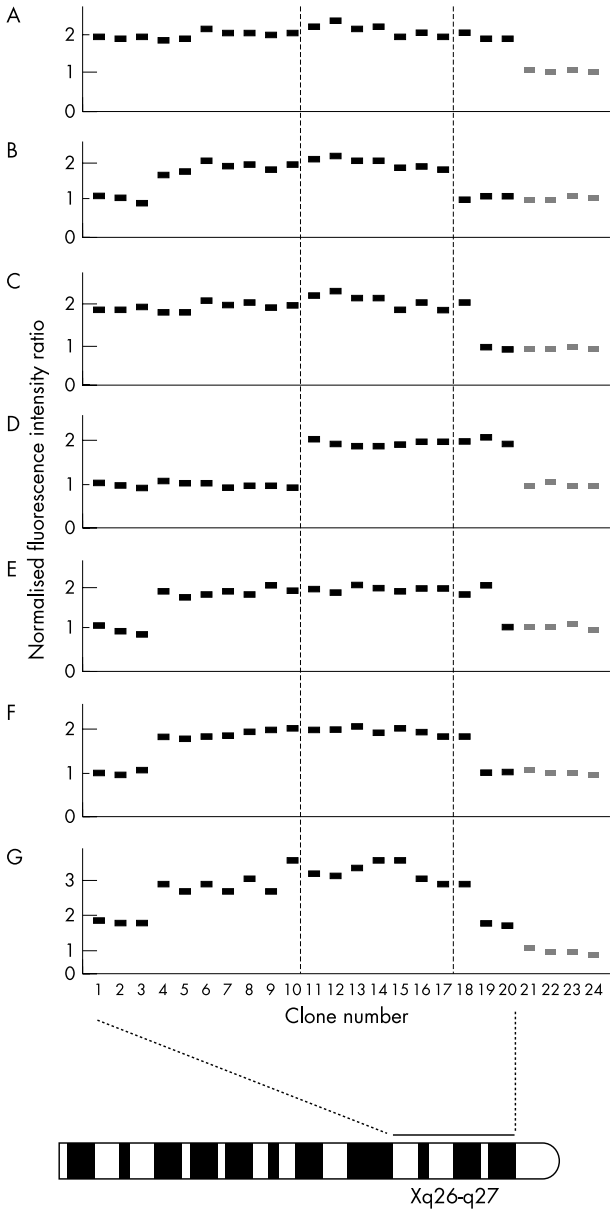

Figure 1 Identification of Xq26–q27 duplications in families with X linked hypopituitarism using array CGH. Each array CGH experiment was conducted twice and the means of the eight replicates were averaged. The relative positions of the X chromosome PAC/BAC clones (1–20) are indicated at the bottom of the figure. Normalised fluorescence intensity ratio values for X chromosome clones and autosomal control clones (21–24) are shown in black and grey, respectively. (A) Comparison of normal female versus normal male DNA. (B) Lagerström-Fermér et al[6] family X linked hypopituitarism male versus normal male. (C) Hol et al[7] male versus normal male. Note that normalised fluorescence intensity ratio values are approximately 2 for clones within the previously characterised Lagerström-Fermér et al[6] and Hol et al[7] duplications. (D) Comparison of family A affected male (II.1) versus normal male showing duplication of 5.3 Mb interval at Xq26–q27 spanning clones 11–20. The average normalised fluorescence intensity ratio for non-duplicated clones and duplicated X clones was 1.037 and 1.879, respectively. (E) Family B affected male (II.1) versus normal male. An 8.4 Mb interstitial duplication spanning clones 4–19 is present. The average normalised fluorescence intensity ratio for non-duplicated and duplicated X clones was 0.988 and 1.879, respectively. (F) Array CGH analysis of pooled DNA from family C affected males reveals a 7.7 Mb duplication spanning clones 4–18. The average normalised fluorescence intensity ratio for non-duplicated and duplicated X clones was 1.02 and 1.897, respectively. (G) Cohybridisation of family C carrier female DNA with normal male DNA. The duplicated interval spans clones 4–18, as indicated by the approximately threefold increase in normalised fluorescence intensity ratio for these clones compared with autosomal control clones. Non-duplicated X chromosome clones gave a normalised fluorescence intensity ratio of approximately 2, reflecting the presence of two X chromosomes. These data are consistent with array CGH analysis of family C affected males shown in F. The reduced 3.9 Mb X linked hypopituitarism critical region is indicated by the region between the dotted lines. The standard deviation for all array CGH experiments was well within the $\pm$0.2 inclusion threshold used by other researchers.[14 17] The standard deviation ranges for each experiment were as follows: A, 0.0173–0.1290; B, 0.0165–0.1246; C, 0.0102–0.1606; D, 0.0124–0.1312; E, 0.0151–0.140; F, 0.0157–0.1481; G, 0.0147–0.1351.

chromosome clone on the array. Based on these data, we estimate that the family A duplication is at least 5.3 Mb and includes Xq27.2–q27.3. Importantly, the position of the proximal breakpoint in family A lies approximately 3.4 Mb telomeric to the proximal breakpoint of the Lagerström-Fermér et al[6] 7.3 Mb duplication (fig 1B). Therefore, a 3.9 Mb interval at Xq27.2–q27.3 is duplicated in family A, the Lagerström-Fermér et al[6] family, and the Hol et al[7] family, which defines a reduced critical region for this disorder.

Array CGH analysis of families B and C also revealed duplications in affected males (fig 1E, F). The proximal breakpoint was localised in each family to an interval of approximately 0.6 Mb between clones 3 (132.6 Mb) and 4 (133.2 Mb) that also contains the Lagerström-Fermér et al[6] family breakpoint. The distal breakpoint for family B lies between clones 19 and 20, indicating that the duplication spans approximately 8.4 Mb. Identical results were obtained for each affected male in family B. The family C duplication is slightly smaller, and lies between clones 18 and 19. However, because of the limited quantity of DNA that was available from affected males in family C, we pooled an equal amount of DNA from each available affected male (IV.1, IV.3, IV.4, and IV.9) to obtain sufficient genomic material for analysis. As the normalised fluorescence intensity ratio of clones 4 to 18 was approximately twice that of the autosomal control BACs, it appears that all four affected males have the duplication. To confirm the existence of this duplication, we compared family C carrier female DNA (III.4) with a normal male control. As shown in fig 1G, clones 4 to 18 all had normalised fluorescence intensity ratios that were approximately three times the autosomal controls and therefore provide additional evidence for the presence of a duplication spanning these clones in this family. Therefore, family C contains a novel Xq26.1–q27.3 duplication of approximately 7.7 Mb which overlaps with other X linked hypopituitarism family duplications (fig 2D).

The critical region gene *Sox3* is expressed in the developing pituitary and hypothalamus

The identification of five different, overlapping duplications in families with X linked hypopituitarism provides strong evidence for increased gene dosage as the causative mechanism for this disorder. The refined 3.9 Mb critical region for X linked hypopituitarism contains 18 annotated transcripts (table 3). Ten of these are published genes, and the remaining eight putative genes are supported by multiple ESTs, Unigene clusters, and gene prediction programs. As X linked hypopituitarism is a congenital disease, we reasoned that the causative gene or genes would be expressed in the embryonic pituitary. As human embryonic pituitary tissue is not readily available, and the genetic program that controls pituitary morphogenesis is well conserved,[20 21] we performed an expression analysis using orthologous mouse genes. Nine out of 18 genes had mouse orthologues that shared greater than 80% sequence identity with the corresponding human gene and were located on the X chromosome (table 2). Six of

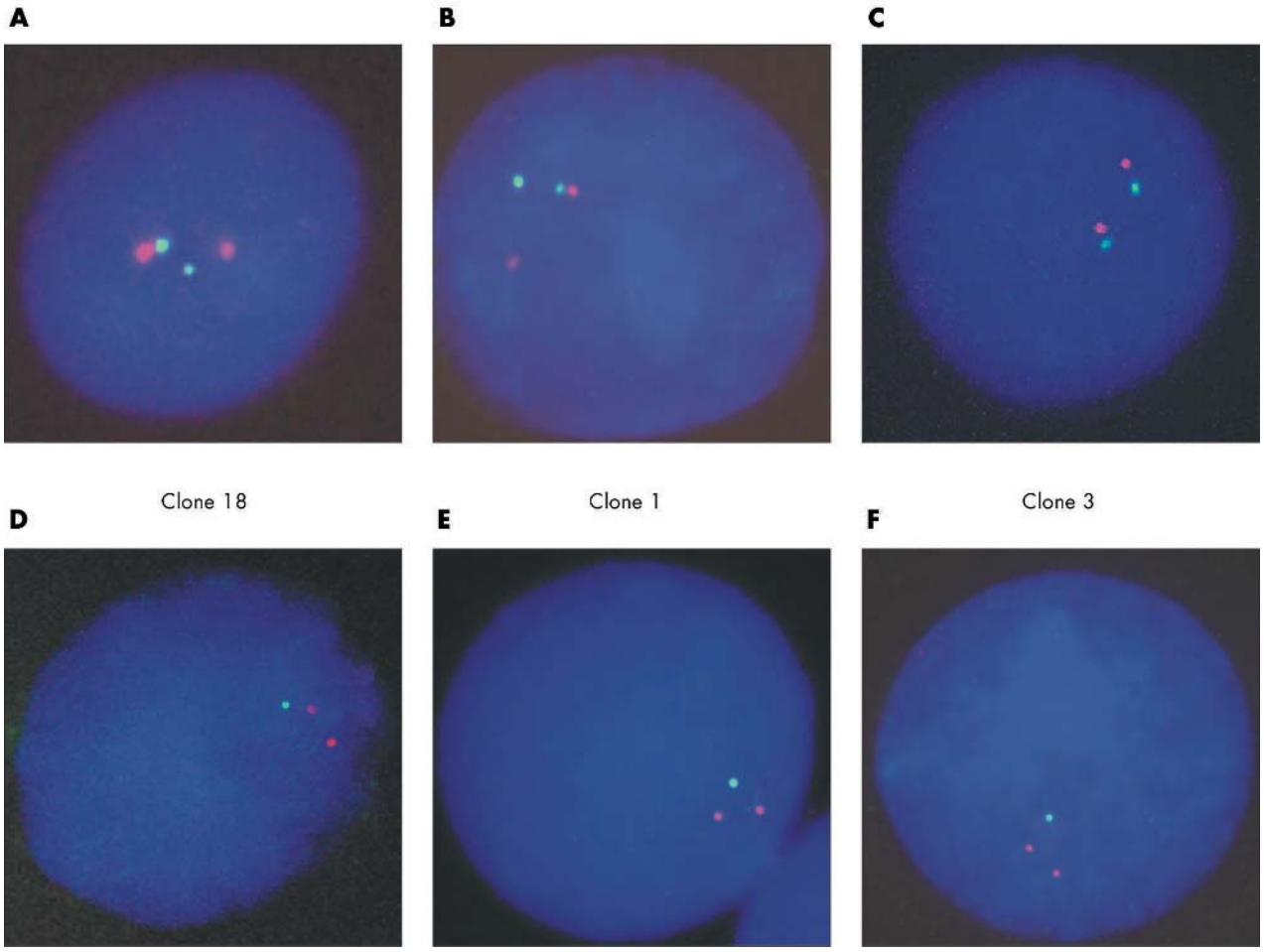

Figure 2 Confirmation of array CGH results using interphase fluorescence in situ hybridisation analysis of affected male lymphoblast cell lines from the Hol et al[7] (A, B, C) and Lagerström-Fermér et al[6] (D, E, F) X linked hypopituitarism families. Signals from ''test'' probes are in green and signals from a duplicated Xq26.3 reference probe (RP1–260J9) are in red. Note that none of the test clones are contained within the Lagerström-Fermér et al[6] family duplication whereas all three clones are duplicated in the Hol et al[7] cell line.

these were screened for expression in the developing pituitary using RT-PCR on mouse embryonic pituitary cDNA. The integrity of the embryonic pituitary cDNA was tested and confirmed using the pituitary specific markers *Hesx1* and *Prop1* (data not shown). Only two were found to be expressed in the developing pituitary; the transcription factor, *Sox3*,[22 23] and the phospholipid transporting ATPase, *Atp11c*. To determine the precise spatial and temporal regulation of these genes in the developing pituitary, further expression analysis was performed using in situ hybridisation. Gene expression was also assessed in the developing central nervous system, as many men with X linked hypopituitarism exhibit mild mental retardation. At 12.5, 14.5, and 16.5 days after conception, *Atp11c* expression was not detected in the developing pituitary and hypothalamus by in situ hybridisation, suggesting that this gene may be expressed at very low levels. In contrast, *Sox3* expression was developmentally regulated, as has been shown in previous expression studies.[23 24] At 10.5 days after conception, *Sox3* is expressed throughout the central nervous system and by 12.5 days after conception is expressed in the dorsal aspect of the lateral ventricle, the ventral diencephalon (including the presumptive hypothalamus), and the roof of the midbrain (fig 4A). Importantly, in the developing pituitary, *Sox3* is expressed at high levels in the infundibulum at 11.5 days after conception and 12.5 days after conception (fig 4A, B). At 13.5 days after conception, *Sox3* expression in the infundibulum is down regulated and is maintained at a low level until 17.5 days after conception (data not shown).

DISCUSSION

As X linked hypopituitarism does not appear to result from gene mutation, standard mutation scanning methods cannot be employed to identify the causative gene. X linked hypopituitarism candidate genes, identified by their expression in the developing pituitary and hypothalamus, must be tested functionally for their ability to generate a hypopituitarism phenotype through overexpression in animal models such as the mouse. As this process is laborious and time consuming, we sought to reduce the size of the critical region for X linked hypopituitarism through the identification of additional X linked hypopituitarism duplications. Therefore, we generated a high resolution X linked hypopituitarism critical region array and performed a series of validation experiments.

Array CGH analysis of males with X linked hypopituitarism having Xq26–q27 duplications clearly showed the expected normalised fluorescence intensity ratio value of ≈2 for BAC/PAC clones located inside the duplicated region.[7 8] Furthermore, analysis of affected males from previously uncharacterised families with X linked hypopituitarism also revealed a doubling of normalised fluorescence intensity ratio

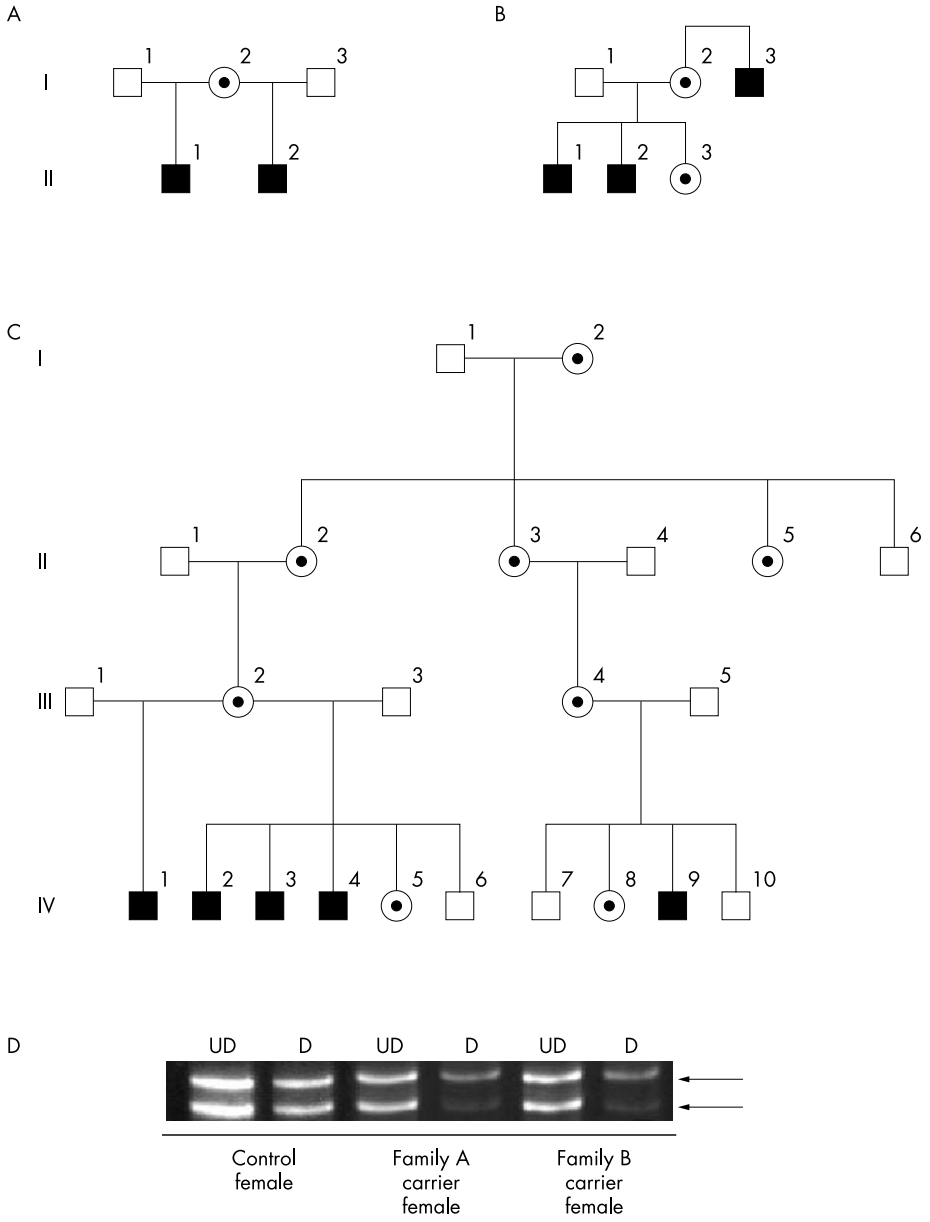

Figure 3 Pedigrees for families A, B, and C. (A, B) Note that individual I.1 in family B is probably affected. (C) Family C was previously published by Zipf et al.[4] This revised pedigree has been extended to include four generations, containing five affected males. DNA used in this study was obtained from affected individuals IV.1, IV.3, IV.4, and IV.9 and from the carrier female III.4. (D) Skewed X inactivation in family A and B carrier females. The two androgen receptor alleles are indicated by the arrows on the right. UD, undigested; D, *Hpa* II digested.

values in consecutive BAC/PAC clones across Xq26–q27, allowing us to define three novel duplications associated with this disorder. Interestingly, when we cohybridised carrier female DNA from families A and C with control male DNA, we observed the expected 3:2 normalised fluorescence intensity ratio values for duplicated BACs versus non-duplicated

Table 2 Summary of clinical features for families A and B

Patient	Hormone deficiencies	Mental retardation	Craniofacial dysmorphology
Family A II.1	growth hormone, thyroid stimulating hormone	No*	No
Family A II.2	growth hormone	No*	No
Family B II.1	growth hormone, thyroid stimulating hormone	No	No
Family B II.2	growth hormone, thyroid stimulating hormone	No*	No

*Has received special education for learning problems.

Table 3 Summary of X linked hypopituitarism critical region genes, mouse orthologues, and pituitary expression analysis

Human gene name	Position (Mb)	Human gene symbol	Genbank mouse accession	Mouse identity (%)	Expression in pituitary
ENSG00000181753 (novel)	136.2	nd	?	?	?
ENSG00000173954 (novel)	136.4	nd	?	?	?
Coagulation factor IX	136.6	F9	AI526776	87.9	no
Proto-oncogene DBL	136.6	MCF2	BG920442	85.5	no
Atpase, class VI, type 11C (NM_173694)	136.8	ATP11C	AV256964	91.3	yes*
ENSG00000177219 (novel)	137.1	nd	ENSG00000047561	?	?
Transcription factor SOX3	137.5	SOX3	AI324160	94.1	yes
Cerebellar degeneration-related protein	137.8	CDR1	AW456110	94.1	no
SPANX family, member F1	138.0	SPANXF1	?	?	?
ESNG00000184649 (Novel)	138.1	nd	?	?	?
Ribosomal Protein L44	138.2	O60801	?	?	?
Leucine zipper, down regulated in cancer1	138.2	LDOC1	BG077501	81.3	no
SPANX family, member C1	138.3	SPANXC	?	?	?
ENSG00000171099 (novel)	138.3	nd	?	?	?
ENSG00000184242 (Q8N9U9)	138.5	nd	?	?	?
SPANX family, member A2	138.6	SPANXA2	?	?	?
SPANX family, member E	138.7	SPANXE	?	?	?
Melanoma antigen, family E/C, 1	139.2	MAGEE1 MAGEC1	ENSMUSG00000045330	?	?

*RT-PCR only; ?, unknown; nd, not determined.

BACs on the X chromosome. Our array CGH method may therefore also provide a rapid and definitive assay for screening potential carrier females in X linked hypopituitarism families. Furthermore, the ability to detect a 50% increase in signal ratio intensity using this technique indicates that array CGH may be a useful tool for detecting autosomal duplications. This method may prove useful for screening patient DNA for chromosomal imbalances such as trisomy 21 or regional duplications such as are observed in Charcot Marie Tooth Syndrome[25] and could be performed with minimal DNA and in the absence of cell lines or chromosomes.

A major difference between our array and the majority of published studies is the larger size of the features, which in our study were approximately 500 μm in diameter. Consequently, our arrays had a relatively large quantity of target DNA, which may provide greater sensitivity by increasing the signal to noise ratio. Furthermore, the relatively small number of BAC/PAC clones on the array and large quantity of target DNA printed onto the slide enabled us to include 100% of the features for analysis. This is a significant improvement over other studies in which 10–20% of the features were excluded from the analysis due to factors such as poor quality printing and low signal intensity.[11 13 15–17]

The existence of different duplications spanning Xq27.2–q27.3 in all five families with X linked hypopituitarism who have been investigated to date provides strong support for our hypothesis that increased dosage of a gene or genes within this region is causative for X linked hypopituitarism.[8] To identify X linked hypopituitarism candidate genes, we performed an expression analysis of genes that map within the newly defined 3.9 Mb critical region. The marked congenital hypopituitarism in affected males indicates that

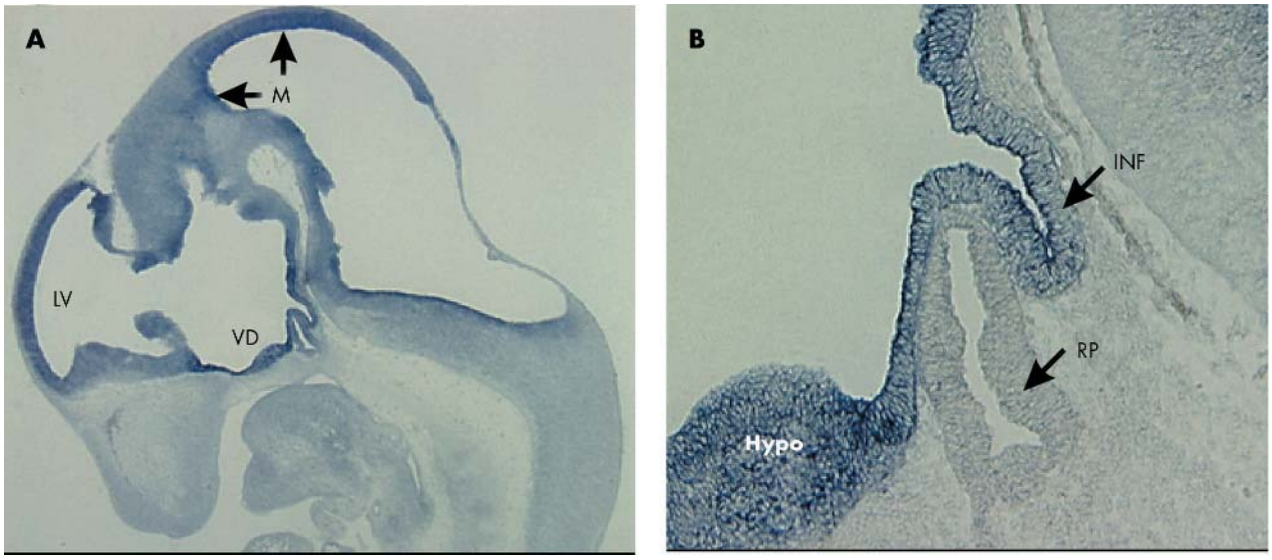

Figure 4 *Sox3* is expressed in the developing pituitary and central nervous system. Panel A represents a sagittal section from a mouse embryo 12.0 days after conception showing strong expression in the infundibulum (IFN), the dorsal aspect of the lateral ventricle (LV), the ventral diencephalon (VD), and the roof and wall of the midbrain (M). Panel B represents a sagittal section from a mouse embryo 11.5 days after conception, showing strong expression in the IFN and presumptive hypothalamus (Hypo). No signal was detected in the Rathke's pouch (RP).

a severe defect in pituitary organogenesis occurs during the commitment or differentiation of the five types of hormone secreting cell. We screened six critical region genes for expression in the pituitary during these developmental stages, which occurs from 11.5 to 16.5 days after conception in the mouse embryo.[20] The *Sox3* gene[22 23] and the *Atp11c* gene were the only genes found to be expressed in the developing pituitary by RT-PCR analysis. In situ hybridisation analysis of *Atp11c* however did not detect any expression in the embryo at 12.5 days after conception, indicating that this gene is expressed at very low levels. In contrast, *Sox3* expression was readily detected and was restricted to the developing pituitary and central nervous system. *Sox3* has not previously been shown to be expressed in the pituitary and adds to the growing list of transcription factors that are expressed in this gland, many of which are critical for its development and have conserved roles in mice and human beings.[2 20 21] Furthermore, expression of *Sox3* within the central nervous system also implicates this gene in neuronal development or function, which is impaired in the majority of males with X linked hypopituitarism.[6]

Several genetic and classical embryological studies have established that the infundibulum and ventral diencephalon provide critical signals for the growth and differentiation of the anterior pituitary primordium, Rathke's pouch.[26–29] In addition, mutations that cause perturbation in hypothalamic development, also result in hypopituitarism.[30] A role for *Sox3* in the development of the hypothalamic pituitary axis is supported by a recent study by Laumonnier et al,[31] who identified *SOX3* polyalanine expansion and deletion mutations in two pedigrees with growth hormone deficiency and mental retardation.[31 32] The endocrine and neurological features of affected males in these families fall within the phenotypic spectrum of X linked hypopituitarism, although the mild craniofacial anomalies are not typical of X linked hypopituitarism. While the functional consequence of the Laumonnier et al[31] mutations is unclear, the phenotypic overlap of affected males with increased dosage and polyalanine expansion of SOX3 suggests a common molecular pathology. These studies also suggest that the structural components of the hypothalamic pituitary axis may be sensitive to the levels of SOX3 protein during morphogenesis. This hypothesis is strongly supported by the phenotype of *Sox3* null and heterozygous mice, which includes growth retardation, hypopituitarism, and significant morphological defects in the pituitary and central nervous system.[33 34] Taken together, these data suggest that increased dosage and consequent overexpression of *SOX3* in the infundibulum and central nervous system of human embryos may be the causative mechanism for X linked hypopituitarism. Generation of transgenic mice containing additional copies of the *SOX3* gene would provide a method for testing the affect of increased *SOX3* dosage and may ultimately provide a mouse model for X linked hypopituitarism.

The identification of Xq26–q27 duplications in five independent X linked hypopituitarism kindreds indicates that X linked hypopituitarism is a genetically homogeneous condition. We have shown that array CGH is a robust and sensitive assay for the detection and fine mapping of X chromosome duplications. This technique will allow us to test the possibility that sporadic cases of growth hormone deficiency, which are significantly more common in males than in females,[35] are due to submicroscopic duplication of Xq26–q27. Our initial array CGH analysis of five such male patients indicates that Xq26–q27 duplications are not present. However, we cannot exclude the possibility that microduplications, too small to be detected by array CGH, are present in these individuals.

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Authors' affiliations

N M Solomon, S A Ross, S M Forrest, P Q Thomas, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, VIC, 3052, Australia
N M Solomon, Department of Paediatrics, University of Melbourne, Melbourne, VIC, 3052, Australia
T Morgan, J L Belsky, Yale University School of Medicine, Department of Genetics, 333 Cedar Street, New Haven, CT 06520 USA
J L Belsky, Danbury Hospital, 24 Hospital Avenue, Danbury, CT 06810 USA
F A Hol, Department of Human Genetics, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands
P S Karnes, Mayo Clinic, Department of Medical Genetics, 200 First Street SW Rochester, Minnesota 55905, USA
N J Hopwood, University of Michigan Medical Center, Ann Arbor, MI 48109-0718, USA
S E Myers, Saint Louis University, 1465 S Grand Blvd St Louis, MO 63122, USA
A S Tan, Division of Endocrinology, Diabetes, and Metabolism, University of Missouri-Columbia, One Hospital Drive D110A, Columbia, Missouri 65212, USA
G L Warne, Department of Endocrinology, Royal Children's Hospital, Melbourne, VIC, 3052, Australia
S M Forrest, Australian Genome Research Facility, Flemington Road, Parkville, Melbourne, VIC, 3052, Australia

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