# Molecular characterisation of the *Hyp* deletion and an improved assay for its detection

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## Abstract

The *Hyp* mouse is a commonly used model for the study of the phosphate wasting disease X-linked hypophosphataemia. The defect in this mouse line is a deletion that includes exons 16 to 22 of *Phex*, although the exact extent of this X chromosome deletion remains unknown. This complicates genotyping which increases costs, time and difficulty of working with this important model. We aimed to determine the molecular breakpoints of this deletion in order develop a robust assay for its detection. We designed short mapping PCRs around the *Phex* locus to refine the putative breakpoint locations, then used gap PCR to amplify a product containing the breakpoint junction. DNA sequencing showed the deleted region was approximately 297 kb, significantly larger than previous reports, but did not contain any genes other than *Phex*. DNA sequence analysis revealed that this deletion may be the result of microhomology-mediated end joining. Finally, we designed a multiplex PCR assay for genotyping *Hyp* colonies and validated it using a panel of *Hyp* colony mice. This study provides confirmation of the *Hyp* phenotype as a single gene defect, a potential mechanism for its formation and an improved method for genotyping that will make working with this strain significantly easier.

## Keywords

X-linked hypophosphataemia; *Hyp* mouse; deletion

## Introduction

X-linked hypophosphataemia (XLH) is the most common inherited form of hypophosphataemic rickets, a dominant disorder that affects phosphate homeostasis. XLH is characterised by abnormal bone mineralisation, abnormal renal vitamin D metabolism, osteomalacia, and hyposphosphataemia. XLH is caused exclusively by mutations in *PHEX* [1], a 22 exon gene that encodes a protein called phosphate regulating endopeptidase homolog, X-linked. While the specific function of this protein is unknown, it is thought to exert its effects on phosphate balance via increased fibroblast growth factor 23 signalling [2]. The *Hyp* mouse strain arose from a spontaneous mutation and its phenotypic similarity

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to human XLH patients has led to wide use as a model of the human disease [3]. Several attempts to map the *Hyp* defect have been made. Initial studies showed that the *Hyp* phenotype mapped to the distal end of the long arm of chromosome X [3]. Later studies showed that the *Phex* mRNA lacked exons 16 to 22, due to a deletion that had one breakpoint in intron 15 and another in the region to the 3' of the gene [4][5]. The most comprehensive mapping of the deletion was performed by Sabbagh *et al.* who used PCR to refine the location of the breakpoint in intron 15 and determined the other was approximately 10 kb downstream of *Phex*, consistent with a deletion of between 53 and 58 kb [6]. Despite this, they were unable to amplify a product containing the deletion breakpoints and so the precise extent of the deletion remains to be determined.

As the exact boundaries of the *Hyp* deletion are unknown, genotyping it is problematic. The deletion is generally genotyped using either PCR or Southern hybridisation with primers or a probe in the known deleted region, respectively [5][7]. While these methods can be used to determine male genotypes by the absence of amplification or hybridisation, this may lead to misclassification due to methodological errors and, in the case of Southern hybridisation, is slow and technically demanding. This is also unsuitable for discriminating between heterozygous and wild-type (WT) females due to the presence of at least one normal X chromosome. These factors complicate breeding and increase both the number of mice used and the associated costs.

In this study, we aimed to determine the molecular extent of the *Hyp* deletion to provide an improved method for genotyping this strain. We designed PCR primers to amplify short products around the proposed breakpoints of the deletion which allowed us to refine the breakpoints sufficiently so that we were able to amplify a product containing the breakpoint junction. DNA sequencing showed the *Hyp* deletion was approximately 297 kb, significantly larger than previously thought, but does not affect any genes other than *Phex*. Molecular characterisation of the deletion enabled us to design a rapid multiplex PCR assay for *Hyp* genotyping that will simplify work with this important mouse model of XLH.

## Methods and Materials

### Animals

*Hyp* (stock number 000528) and C57BL/6J (stock number 000664) mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All studies were performed with IACUC approval.

### DNA Extraction

DNA was extracted from C57BL/6J and *Hyp* mouse tail clips using a DNeasy Blood and Tissue kit according to manufacturer's instructions (QIAGEN, Hilden, Germany).

### Mapping PCRs

The mouse genome sequence from exon 15 of *Phex* to exon 6 of *Sat1* on chromosome X was retrieved from the University of California Santa Cruz mouse genome browser (http://genome.ucsc.edu; April 2011, assembly NCBI37/mm9) and this sequence was filtered for putative repeat DNA sequences using RepeatMasker (www.repeatmasker.org) and CENSOR (www.girinst.org/censor/) [8]. PCR primer pairs were designed in repeat-free regions to amplify products of between 114 and 337 bp. PCRs were performed using Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's specifications, at an annealing temperature of 60°C. Products were analysed by electrophoresis through a 2% agarose gel and stained with ethidium bromide. All mapping PCRs were performed in duplicate, one with *Hyp* male DNA and one with wild-

type (WT) C57BL/6J DNA. Sequences for all primers used in this study are provided in Supplementary Table 1.

### Gap PCR

PCR primer pairs were designed in regions closely flanking putative breakpoints, shown to be intact in the *Hyp* mouse by mapping PCR. PCRs were performed using Platinum *Taq* High Fidelity DNA Polymerase (Invitrogen), according to manufacturer's specifications, at an annealing temperature of 62°C.

### DNA Sequencing

PCR products were purified by centrifugation at $750\times g$ through a Montage MultiScreen PCR$_{96}$ microtitre plate (Millipore, Billerica, MA, USA), then resuspended in nuclease free water. Purified PCR products were sequenced using BigDye Terminator v. 3.1 chemistry (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing data was analysed using SeqScape v. 2.6 software (Applied Biosystems).

### Multiplex genotyping PCR Assay

*Hyp* genotyping PCRs contained 1× Hot Start Supermix (Biopioneer Inc, San Diego, CA, USA) with primers WT-F (62.5 nM), WT-R (62.5 nM), *Hyp*-F (500 nM) and *Hyp*-R (500 nM), according to manufacturer's specifications, at an annealing temperature of 60°C.

## Results and Discussion

The location of the *Hyp* deletion on the X chromosome makes mapping it by PCR significantly easier than an autosomal deletion where the normal chromosome would mask the deletion. We exploited this property by designing short PCRs, which enabled mapping of the deletion by the presence or absence of a product from male *Hyp* DNA. We used the findings of the most extensive previous attempt to characterise the deletion as a starting point [6]. In that study, Sabbagh *et al.* refined breakpoint locations to between ~10 to 13 kb into intron 15 and between ~ 8 to 10 kb from the 3' end of *Phex*. We were able to reproduce their results (not shown) and subsequently designed further primers in proximity to these to refine the deletion boundaries. Using this approach, we found the intron 15 breakpoint was between PCRs 5 and 6 (Figure 1A and B), which corresponds to a region of approximately 2.0 kb between nucleotides 153,645,729 and 153,647,703. We were unable to amplify any further products (primer pairs A, B, C and D) in the vicinity of the primer pair used by Sabbagh *et al.* (designated F6R6) to delineate the boundary of the deletion. Gap PCR, with multiple combinations of primers designed to amplify across the deletion junction, also failed to amplify any *Hyp* specific products.

We reviewed the primer sequences and putative products described in Sabbagh *et al.* and found that the primer pair F6R6 (filled square in Figure1B) amplifies a 620 bp fragment of a LINE element, which occurs 202 further times within the mouse genome with 98% or higher sequence identity. This indicates a false positive and that the *Hyp* deletion is likely more extensive than predicted in that study. We designed further PCR primer pairs (E, F, G, H, I and J) extending approximately 300 kb into the intergenic region. These showed a breakpoint between PCRs H and I (between nucleotides 153,336,911 and 153,376,320) Further mapping PCRs resolved the breakpoint to between K and L (between nucleotides 153,347,029 and 153,348,980), a region of approximately 2.3 kb. PCR primers were designed between PCRs 5 and 6 and between K and L to amplify across the breakpoint junction. A single product of 518 bp was amplified from *Hyp* DNA, but not from WT DNA, and was subsequently sequenced (Figure 2A).

DNA sequencing showed unique sequence ending at nt 153,348,569 and restarting at nt 153,646,285 with 48 base pairs of AT microsatellite sequence common to both breakpoints in between (Figure 2B). This represents a deletion of 297,752 bp, significantly greater than the 53 to 58 kb previously predicted. Despite the greater extent of the deletion, it does not include any genes other than *Phex*, as the next closest gene (*Ptchd1*) is located approximately 1.8 Mb from the end of the deletion. This confirms the *Hyp* mouse phenotype is due solely to a *Phex* defect and is not a contiguous gene phenotype.

Examination of breakpoints in genomic rearrangements can yield interesting insight into the mechanisms by which they form. While the most common explanations for simple genomic deletions are non-homologous end joining or unequal homologous recombination (reviewed in [9]), the DNA sequence at the *Hyp* breakpoint junction does not suggest either of these mechanisms. The most parsimonious explanation is microhomology-mediated end joining (MMEJ), a more recently described alternative mechanism of DNA double strand break (DSB) repair (reviewed in [10]). MMEJ is a multistep DSB repair process that begins with resection to expose microhomologous regions at both breakpoints, which allows annealing of single stranded DNA. After error-prone DNA polymerases fill in gaps the processed, annealed ends are ligated and the DSB is repaired, albeit with loss of the interstitial sequence. The defining feature of MMEJ is short tracts of homology between the ends of the deletion, such as the AT microsatellites we observed. MMEJ junctions also usually have inserted nucleotides, introduced by the low fidelity polymerases involved in repair. Here we found a short AAATAA sequence (underlined in Fig 2B) in the otherwise perfect AT microsatellite repeat. This is the likely site of ligation, indicating the true break points are after nucleotide 153,348,586 in intron 15 and before 153,646,258 in the region downstream of the gene.

Having characterised the *Hyp* deletion, we were able to design a multiplex PCR assay for rapid genotyping. In this assay, a mutant specific primer pair amplifies a product of 346 bp, while a second primer pair located in the deleted region amplifies a product of 234 bp for the WT allele. We used this assay to genotype a series of 11 mice from our *Hyp* colony and were able to assign genotypes to all mice in this group, validating the assay (Figure 3). This provides a robust, rapid, direct detection of all genotypes, in contrast to existing methods in which a genotype is inferred by absence of amplification or hybridisation [5][7].

The *Hyp* mouse is a widely used model of human XLH, however the exact extent of the deletion causing this defect was not defined. Here we exploited the X-linked nature of the mutation to clearly map and delineate the *Hyp* deletion and provide some insight into its mechanism of formation. We also report an improved assay for its direct detection, which will simplify breeding and colony management for this important mouse model of XLH.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## Abbreviations

**XLH**          X-linked hypophosphataemia

**WT**           wild-type

**MMEJ** microhomology-mediated end joining

**DSB** double strand break

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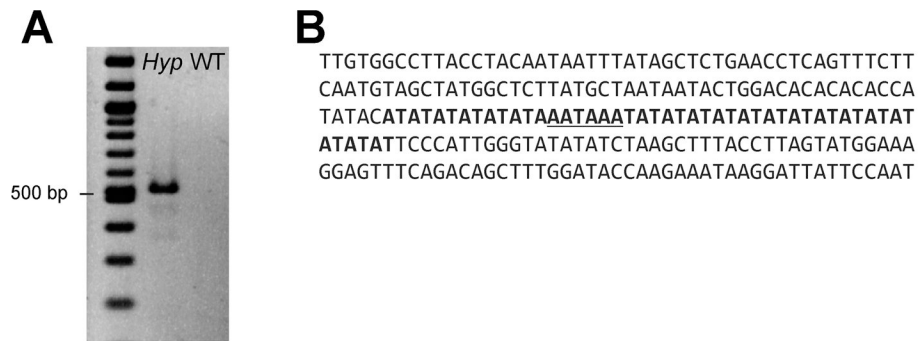
## Highlights

The deletion in the *Hyp* mouse model of X-linked hypophosphataemia was fully characterised.

The *Hyp* deletion was more extensive than previously described but only affects *Phex*.

A genotyping method for direct detection of the *Hyp* deletion was established.
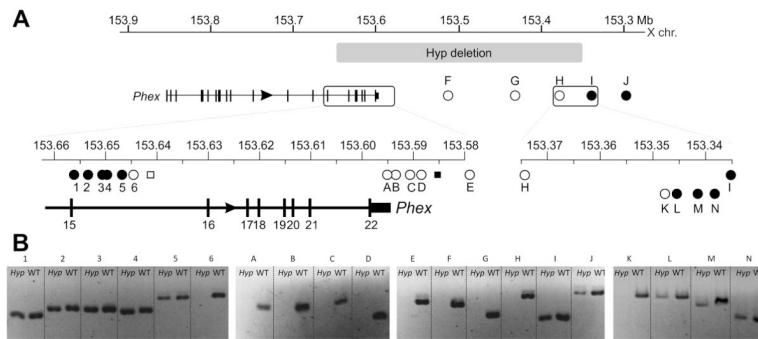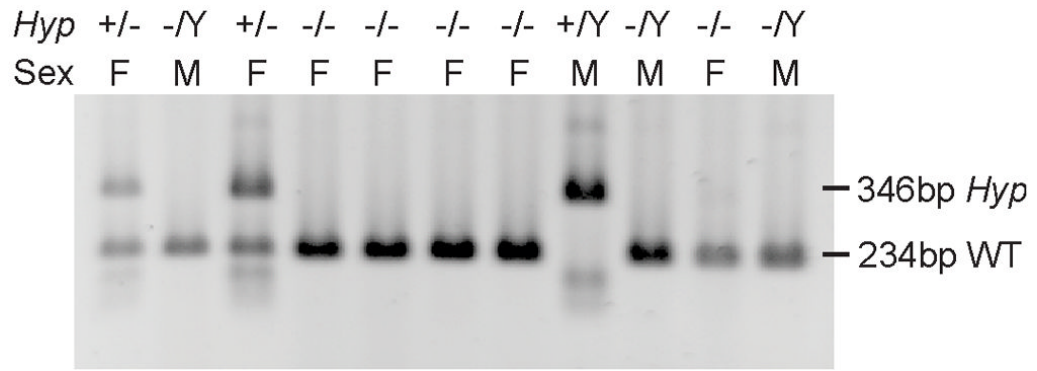
**Figure 1.**
(A) Mapping PCRs of *Hyp* deletion locus. Numbers (1 to 5) and letters (A to N) indicate PCRs in the telomeric (intron 15) and centromeric (intergenic) breakpoint regions respectively. Absence of a product for PCR 6 indicates a breakpoint between PCRs 5 and 6. Lack of a product for H denotes a breakpoint between H and I, which was narrowed to between K and L in a subsequent round of mapping PCR. (B) Summary of *Hyp* deletion mapping PCRs showing the extent of the *Hyp* deletion (grey box). Boxed regions are enlarged in the lower panels. Circles represent PCRs used in this study and squares denote results of Sabbagh et al for comparison (not to scale). Filled and unfilled shapes represent presence or absence of PCR products from male *Hyp* mouse DNA, respectively.

**A**



**B**

TTGTGGCCTTACCTACAATAATTTATAGCTCTGAACCTCAGTTTCTT
CAATGTAGCTATGGCTCTTATGCTAATAATACTGGACACACACACCA
TATAC**ATATATATATATA**AATAAA**TATATATATATATATATATATAT**
**ATATAT**TCCCATTGGGTATATATCTAAGCTTTACCTTAGTATGGAAA
GGAGTTTCAGACAGCTTTGGATACCAAGAAATAAGGATTATTCCAAT

**Figure 2.**
Gap PCR and DNA sequence of a product containing the *Hyp* deletion junction. (A) PCR
with primers flanking the putative deletion breakpoints amplified a 518 bp *Hyp* specific
product that was not observed with wild-type (WT) DNA. The left lane contains 2 log DNA
marker. (B) DNA sequence of the PCR product from (A) revealed the location of the *Hyp*
deletion junction. Arrows show the X chromosome nucleotide position of the unique
sequence flanking the junction. Bold sequence shows AT microsatellite sequence common
to both ends of the deletion and underlined sequence represents the proposed junction.

**Figure 3.**
Genotyping PCRs of *Hyp* mouse colony DNA. Bands at 346 bp and 234 bp represent the *Hyp* (+) and wild type (−) alleles, respectively. All mice are the offspring of female (F) Hyp +/− (inferred) and male (M) -/Y parents.