# Detailed mapping of a congenital heart disease gene in chromosome 3p25

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**Abstract**

**Distal deletion of chromosome 3p25-pter (3p− syndrome) produces a distinct clinical syndrome characterised by low birth weight, mental retardation, telecanthus, ptosis, and micrognathia. Congenital heart disease (CHD), typically atrioventricular septal defect (AVSD), occurs in about a third of patients. In total, approximately 25 cases of 3p− syndrome have been reported world wide. We previously analysed five cases and showed that (1) the 3p25-pter deletions were variable and (2) the presence of CHD correlated with the proximal extent of the deletion, mapping a CHD gene centromeric to D3S18. To define the molecular pathology of the 3p− syndrome further, we have now proceeded to analyse the deletion region in a total of 10 patients (five with CHD), using a combination of FISH analysis and polymorphic markers, for up to 21 loci from 3p25-p26. These additional investigations further supported the location of an AVSD locus within 3p25 and refined its localisation. Thus, the critical region was reduced to an interval between D3S1263 and D3S3594. Candidate 3p25 CHD genes, such as *PMCA2 (ATP2B2)*, fibulin 2, *TIMP4*, and *Sec13R*, were shown to map outside the target interval. Additionally, the critical region for the phenotypic features of the 3p− phenotype was mapped to D3S1317 to D3S17 (19-21 cM). These findings will accelerate the identification of the 3p25 CHD susceptibility locus and facilitate investigations of the role of this locus in non-syndromic AVSDs, which are a common form of familial and isolated CHD.**

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Congenital heart defects (CHD) are an important cause of morbidity and mortality affecting ~8 in every 1000 children. Although most cases are sporadic, the role of genetic factors in CHD has received increasing attention because of the major advances in paediatric cardiac surgery, such that now more than 85% of children with CHD survive into adulthood. Overall, the recurrence risk for most types of isolated congenital heart defect is ~5% but atrioventricular septal defects (AVSD) appear to have a greater genetic contribution.[1] Thus, follow up of adults with AVSD suggests that at least 20% of apparently isolated cases result from a dominant gene. AVSD is classically associated with Down syndrome, but genetic linkage analysis of large AVSD families have excluded linkage to the Down syndrome critical region on chromosome 21, so establishing locus heterogeneity in the genetic pathogenesis of AVSD.[2 3] In view of the complexity of cardiac development, such heterogeneity is expected. Linkage analysis in a large extended family mapped an atrioventricular septal defect susceptibility locus to chromosome 1p31-p21.[4] In this kindred inheritance was observed to be autosomal dominant with incomplete penetrance. However, large AVSD families are rare, and so the potential for identifying AVSD genes using linkage is limited.

Cytogenetic deletion syndromes are rare, but can provide important clues to the localisation of developmental genes. Classical examples of this approach include the association of cardiac and other developmental defects with chromosome 22q11 deletions, and the occurrence of Hirschsprung disease (HSCR) with chromosome 10 deletions, leading to the identification of *RET* as a HSCR susceptibility gene. Cytogenetic deletion syndromes associated with AVSD include del(8)(p23)[5 6] and del(3)(p25).[7] Analysis of del(8)(p23) cases mapped the critical deletion region for heart defects and microcephaly to a 6 Mb region of 8p23.1.[8] Distal deletion of the short arm of chromosome 3 (3p− syndrome) is a rare disorder (~25 reported cases) associated with developmental and growth retardation as well as a characteristic dysmorphology (including ptosis, telecanthus, and micrognathia). Additional, but variable, developmental defects include postaxial polydactyly, renal anomalies, cleft palate, and gastrointestinal anomalies. Congenital heart defects occur in about a third of patients[7 9] and are typically AVSDs. To date, all patients with 3p− syndrome have had large cytogenetically visible deletions, although there are also reports of patients with large deletions and a normal phenotype[10] (unpublished observations).

We and others have reported that the extent of distal 3p deletions in 3p− syndrome patients is variable.[7 9] Furthermore, we found that of five patients with 3p− syndrome studied, the three with CHD had the most proximal breakpoints. Subsequently, Drumheller *et al*[11] reported data on five patients (including one case studied previously by us[7]) that provided further evidence for a chromosome 3p25 CHD gene. To confirm and extend these findings we have collected a total of 10 cases of 3p− syndrome and performed molecular cytogenetic and molecular genetic studies to define the extent of the 3p deletions and correlate this with the presence or absence of CHD.

*Table 1 Details of FISH probes and microsatellite markers used in the study. The probes marked by * are PAC clones derived from RPCI1 library, and those probes starting with AC are cosmid probes isolated from the library LLO3NCO1, both obtained from the UK HGMP resource centre*

| *Locus* | *FISH probe* | *Polymorphism* |
|---|---|---|
| D3S1304 | — | Microsatellite |
| D3S18 | cLIB 1 | — |
| D3S1597 | — | Microsatellite |
| D3S1317 | — | Microsatellite |
| D3S601 | cos 7 | — |
| D3S1038 | cos 14 | Microsatellite |
| D3S3589 | 61P20* / AC50K12 | Microsatellite |
| D3S587 | cLIB 12-48 | — |
| D3S3601 | — | Microsatellite |
| D3S3594 | 226D20 | Microsatellite |
| D3S1263 | 283J16* | Microsatellite |
| NIB1677 | 201I11* | Microsatellite |
| D3S3714 | 183M12* / AC12J24, AC19C22 | Microsatellite |
| D3S3680 | 183M12* | Microsatellite |
| D3S3088 | 225B20* / AC38A7 | — |
| D3S1259 | 248M7* / 275B20* | Microsatellite |
| D3S3701 | — | Microsatellite |
| D3S3602 | — | Microsatellite |
| D3S3693 | — | Microsatellite |
| D3S3610 | — | Microsatellite |
| D3S1585 | 289I19* | Microsatellite |

## Materials and methods

### PATIENTS AND SAMPLES

Lymphoblastoid cell lines were available for nine patients: P1, P2 (CUMG3.1), P3 (GM 10922), P4, P5 (CUMG3.4), P6, P7, P8 (GM 10985), and P9, but not P10 (CUMG3.10). DNA was collected from the affected child and both parents in seven families (P1, P2, P5, P6, P7, P9, and P10), from the affected child and mother in one case (P4), and only the affected child for P3 (GM 10922) and P8 (GM 10995).

### P1 ARTIFICIAL CHROMOSOME (PAC) ISOLATION AND MAINTENANCE

P1 clones were isolated by PCR based screening using polymorphic marker primers (3p25-26) from the UK HGMP resource centre RPCI1 library.[12] P1 clones were maintained in LB media supplemented with 25 μg/ml Kanamycin and PAC DNA was isolated from the host strains by column purification using the Qiagen Maxi kit (Qiagen). The manufacturer's recommended protocol adaptations for optimal recovery of PAC DNA were used. The P1 clones isolated are shown in table 1.

### ISOLATION OF COSMIDS

Cosmids were isolated for the markers D3S3088, D3S3714, and D3S3589 from the chromosome 3 library LLO3NO3 obtained from the UK HGMP resource centre. Hybridisation probes were prepared from the amplified PCR products from YAC 753F7 and 70D6 DNA, purified by Qiagen PCR product kit and radiolabelled by random priming ~25 ng using the Rediprime kit (Amersham). Repetitive sequences were blocked by preannealing with Cot-1 DNA (Gibco BRL) and hybridising under standard conditions[12] at 65°C. The identified clones (table 1) were obtained from the UK HGMP resource centre then maintained in LB media supplemented with ampicillin. Cosmid DNA was isolated using the Qiagen Midi kit.

### FLUORESCENCE IN SITU HYBRIDISATION (FISH)

Metaphase chromosome spreads were prepared from EBV transformed cell lines using standard methods. Before chromosome harvest, cells were blocked in metaphase by the addition of colcemid to a concentration of 10 μg/ml. Probes (PACs, cosmids, and YACs) were biotinylated by nick translation with biotin-11dUTP (Bionick labelling system, Gibco BRL). Chromosomal in situ suppression (CISS) hybridisation was performed to improve the specificity of the hybridisation, with Cot-1 DNA (Gibco BRL) added to the biotin labelled probe at a ratio of 50:1. For all hybridisations, a biotin labelled chromosome 3 alpha satellite centromere probe (Oncor) was used as a control to identify chromosome 3.

Hybridisation signals were visualised by a three layer avidin-fluorescein isothiocyanate (FITC), biotinylate anti-avidin detection system. Analysis was carried out using a BX50 Olympus microscope and images captured with an automated image analysis system (Cytovision, Applied Imaging). At least 20 metaphases were analysed for each probe.

*Table 2 3p– deletion mapping results*

| *Locus* | *P1* *(CHD)* | *P2* *CUMG 3.1* *(CHD)* | *P3* *GM10922* *(CHD)* | *P4* *(CHD)* | *P5* *CUMG 3.4* *(CHD)* | *P6* | *P7* | *P8* *GM10985* | *P9* | *P10* *CUMG 3.10* |
|---|---|---|---|---|---|---|---|---|---|---|
| D3S1304 | ND | ND | ND | ND | ND | ND | ND | ND | ND | – |
| D3S18 | ND | – | ND | – | – | ND | ND | – | – | ND |
| D3S1597 | ND | – | NI | ND | ND | NI | ND | ND | ND | NI |
| D3S1317 | ND | ND | ND | ND | ND | ND | ND | ND | ND | + |
| D3S601 | – | ND | – | – | – | ND | ND | + | ND | ND |
| D3S1038 | – | – | – | – | – | – | – | + | + | + |
| D3S3589 | NI | – | – | – | – | – | – | ND | ND | ND |
| D3S587 | – | – | – | – | – | – | – | + | + | ND |
| D3S3594/D3S3601 | – | NI | NI | NI | – | – | – | ND | ND | ND |
| D3S1263 | – | – | – | – | + | + | + | + | + | ND |
| NIB1677 | – | – | – | – | + | + | + | ND | ND | ND |
| D3S3680/D3S3714 | NI | – | NI | NI | + | + | + | ND | ND | ND |
| D3S3088 | – | – | – | – | + | + | + | ND | ND | ND |
| D3S1259 | – | – | NI | NI | + | + | + | ND | ND | ND |
| D3S3701 | NI | + | + | ND | + | NI | + | ND | ND | ND |
| D3S3602 | – | + | + | ND | + | + | + | ND | ND | ND |
| D3S3693 | + | + | + | NI | + | NI | + | ND | ND | ND |
| D3S3610 | + | + | + | NI | + | + | ND | ND | ND | ND |
| D3S1585 | + | + | + | + | + | + | + | ND | ND | ND |

+ = locus retained. – = locus deleted. NI = non-informative. ND = not done.

DIRECT DNA SEQUENCING OF PACS

The ends of the PACs were directly sequenced by automated fluorescence chain termination sequencing. At least 2 μg of PAC DNA was required for the sequencing reaction, with 25 pmol vector primer (T7 or Sp6), 8 μl di-rhodamine kit reaction mix (Applied Biosystems) to a final volume of 20 μl, overlaid with mineral oil, and thermal cycled under the following conditions: 98°C for 20 seconds, 50°C for 20 seconds, and 60°C for four minutes for 35 cycles on a Omn-E thermal cycler (Hybaid). Samples were precipitated, washed, and resuspended in denaturing buffer before denaturing and loading onto the ABI 377 sequencer according to the manufacturer's instructions (Applied Biosystems).

YEAST ARTIFICIAL CHROMOSOME (YAC) ISOLATION AND MAINTENANCE

YACs were obtained from the UK HGMP resource centre. YAC DNA was isolated following a 36 hour culture in YEPD media, using a standard yeast DNA mini-prep protocol.[13] FISH analysis was performed using YAC DNA to confirm that the YACs were not chimeric. Overlaps between YACs were confirmed by PCR

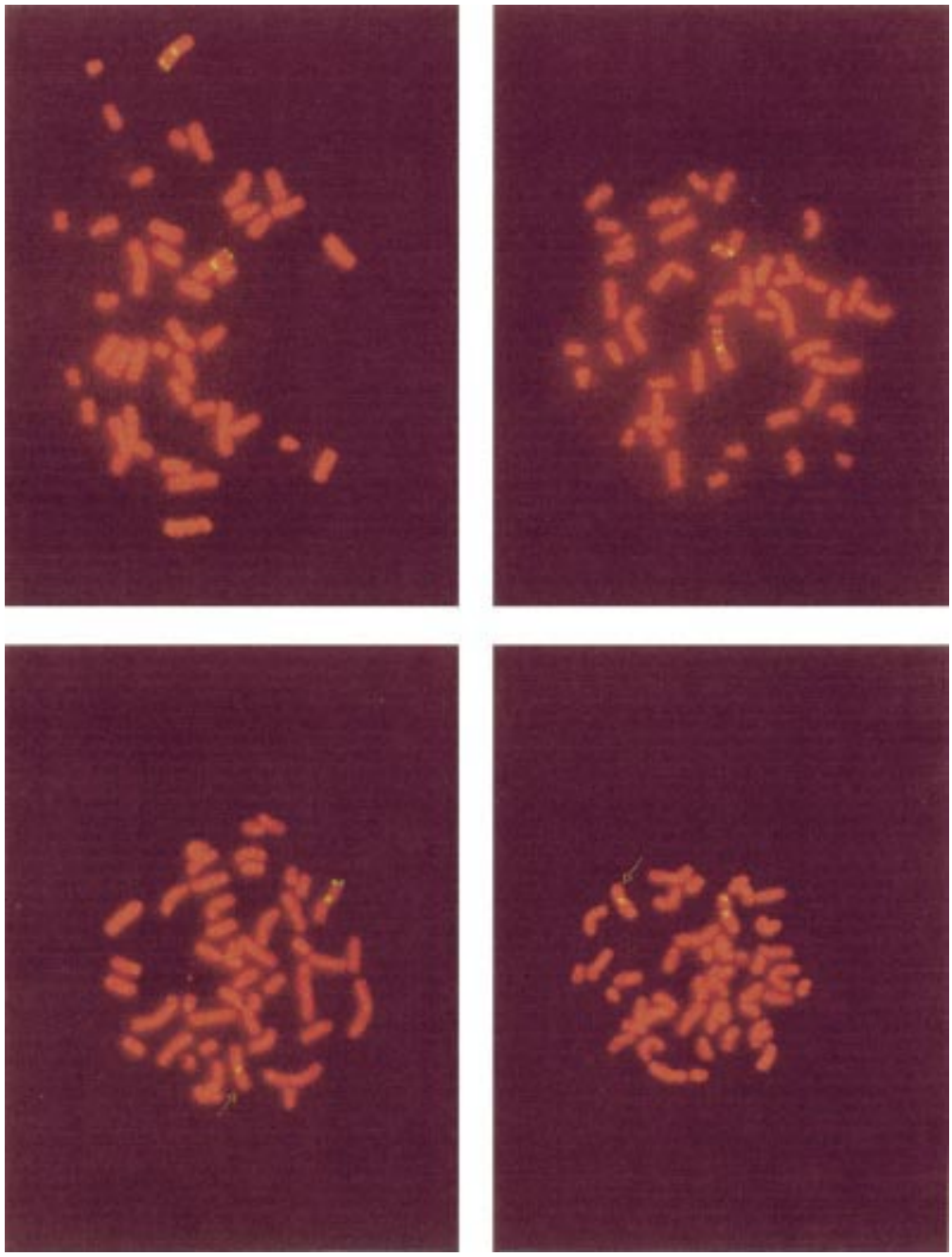

*Figure 1 FISH mapping studies. (Top left) P5 hybridised with PAC 283J16 for D12S1263 and centromeric probe D3Z1. Both chromosome 3 homologues are identified with both probes. (Bottom left) P5 hybridised with PAC 226D20 for D3S3594 and D3Z1. Both chromosome 3 homologues were identified by D3Z1, but only one homologue by PAC 226D20. (Top right) P6 hybridised with PAC 283J16 for D12S1263 and centromeric probe D3Z1. Both chromosome 3 homologues are identified with both probes. (Bottom right) P6 hybridised with PAC 226D20 for D3S3594 and D3Z1. Both chromosome 3 homologues were identified by D3Z1, but only one homologue by PAC 226D20.*

using primers for the polymorphic and STS markers positioned on each of the YACs.

### POLYMORPHIC MARKER ANALYSIS

Dinucleotide polymorphic microsatellite markers (table 1) from chromosome 3p25-26 were analysed on the patients and (when available) their parents. Markers were identified through the Genome Database (GDB) and CEPH. A standard PCR was performed using the forward primer 5' end labelled with $\gamma^{32}$P ATP and thermal cycled on an Omn-E thermal cycler (Hybaid). The PCR products were separated in a 6% denaturing polyacrylamide gel and subjected to autoradiography. To establish whether a polymorphic locus was deleted, patient alleles were compared to parental allelotype. For those patients for whom neither or only one parental DNA sample was available (P3 (GM10922), P8 (GM10985), and P4), heterozygosity for a marker was indicative of absence of a deletion.

### GENE MAPPING ON CHROMOSOME 3p25

Genes known to map to 3p25 were precisely positioned by PCR amplification using gene specific primers on available YACs and PACs. PCR primers were designed for either the 5'UTR of gene sequence, as for KIAA0121, or the 3'UTR, as for *TIMP4* (tissue inhibitor of metalloproteinases 4) and *Sec13R*. The STS WI6061 in *ATP2B2* (plasma membrane calcium transporter gene) was amplified, as was an intragenic dinucleotide repeat polymorphism in fibulin 2.[14]

### VECTORETTE PCR AND DNA SEQUENCING OF YAC CLONE INSERT ENDS

Vectorette PCR was used to facilitate sequencing of YAC insert ends. YAC DNA was digested with either *Eco*RV or *Rsa*I restriction enzyme and ligated to the vectorette.[15] A nested PCR amplification was performed using the vectorette primer and YAC vector primers.[15] Vectorette PCR products were gel purified using the Qiagen Gel Extraction kit and were sequenced directly on an ABI 377 semiautomated sequencer using standard methods.

## Results

### CLINICAL STUDIES

In addition to the five cases described previously,[7] five new patients (P1, P4, P6, P7, and P9) were ascertained. All patients had mental retardation and classical dysmorphological features of 3p− syndrome. A total of five cases (three reported previously) had evidence of congenital heart disease (P1, P2, P3, P4, and P5).

### CHROMOSOME 3p DELETION MAPPING: INITIAL EVALUATION

In order to confirm the cytogenetic diagnosis, FISH studies and microsatellite marker analyses were performed. The latter method was also used to determine the parent of origin in the six informative cases (table 1). Analysis at up to 21 loci showed that seven cases had deletion breakpoints extending centromeric to D3S3594 (table 2). The three cases with more distal breakpoints (P8 (GM10985), P9, and P10 (CUMG3.10)) did not have CHD. The deletion breakpoint for P8 (GM 10985) was previously mapped to between D3S601 and D3S18.[7] Patient P10 (CUMG3.10) had been shown to have a 3p deletion by cytogenetic analysis and had classical phenotypic features of 3p− syndrome, although previous molecular analysis had not detected a chromosome 3p deletion.[7] We have now shown the breakpoint interval for P10 (CUMG3.10) to be between D3S1317 and D3S1304. In P9 the breakpoint was shown to be telomeric to D3S1038.

### CHROMOSOME 3p DELETION MAPPING: DETAILED ANALYSIS OF THE CHD CANDIDATE REGION

The seven patients with the most centromeric deletion breakpoints were analysed in detail, including three of the cases reported previously (P2 (CUMG3.1), P3 (GM10922), and P5 (CUMG3.4)). Congenital heart disease was present in five of the seven patients. A total of 16 loci from D3S1585 to D3S1038 were analysed (tables 1 and 2, figs 1 and 2). The four patients with the most extensive deletions (P1, P2, P3, and P4), with deletion breakpoints between D3S3693 and D3S1263, all had CHD. A further three patients, one with CHD and two without (P5 (CUMG3.4), P6, and P7), had deletion breakpoints distal to D3S1263 and were informative for mapping the CHD gene. All three cases were deleted at

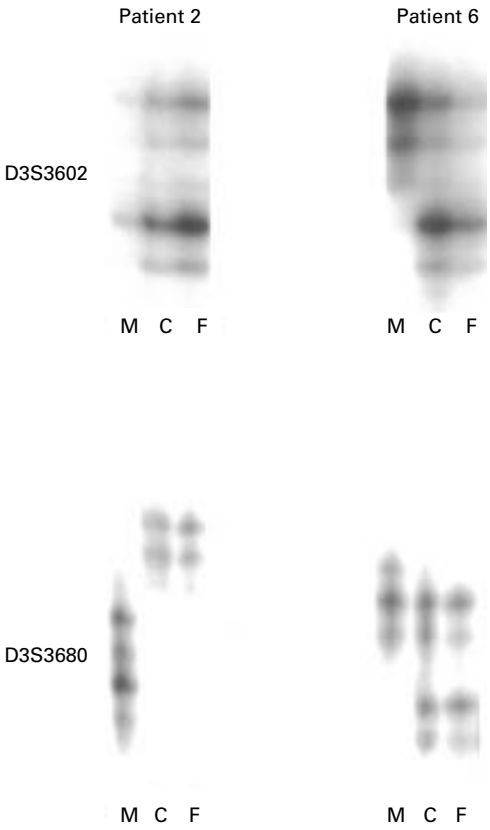


*Figure 2 Polymorphic marker analysis of 3p− syndrome patients. Results for D3S3602 and D3S3680 are shown for P2 and P6. Both patients are heterozygous at D3S3602 and therefore not deleted for this marker. At D3S3680, P6 is also heterozygous, but P2 shows failure to inherit a maternal allele. M=mother, C=child (proband), and F=father.*

D3S3594. These results hence mapped the CHD gene to the D3S1263 to D3S3594 interval, assuming complete penetrance of CHD, a distance of 3.7 cM.

### PHYSICAL MAPPING OF 3p25 CHD SUSCEPTIBILITY LOCUS INTERVAL AND MAPPING OF CANDIDATE GENES

To facilitate (1) establishment of the correct mapping order, (2) estimates of physical distance, and (3) precise mapping of genes on 3p25, we isolated YAC and PAC clones from the region of interest. Five YACs were isolated (753F7, 949C12, 70D6, 52A10, and 736G10) which contained markers from the region of interest as it was defined at the beginning of this investigation (fig 3). In all seven patients, YACs 70D6 and 52A10 were deleted by FISH analysis. Multiple attempts to complete a YAC contig between YACs 949C12 and 70D6 were unsuccessful. This suggests that the interval between these two YACs may contain repeat sequences, as YACs spanning this region are unstable, and contain interstitial deletions or are chimeric.

Genes on chromosome 3p25 were precisely mapped by PCR amplification of the YACs and

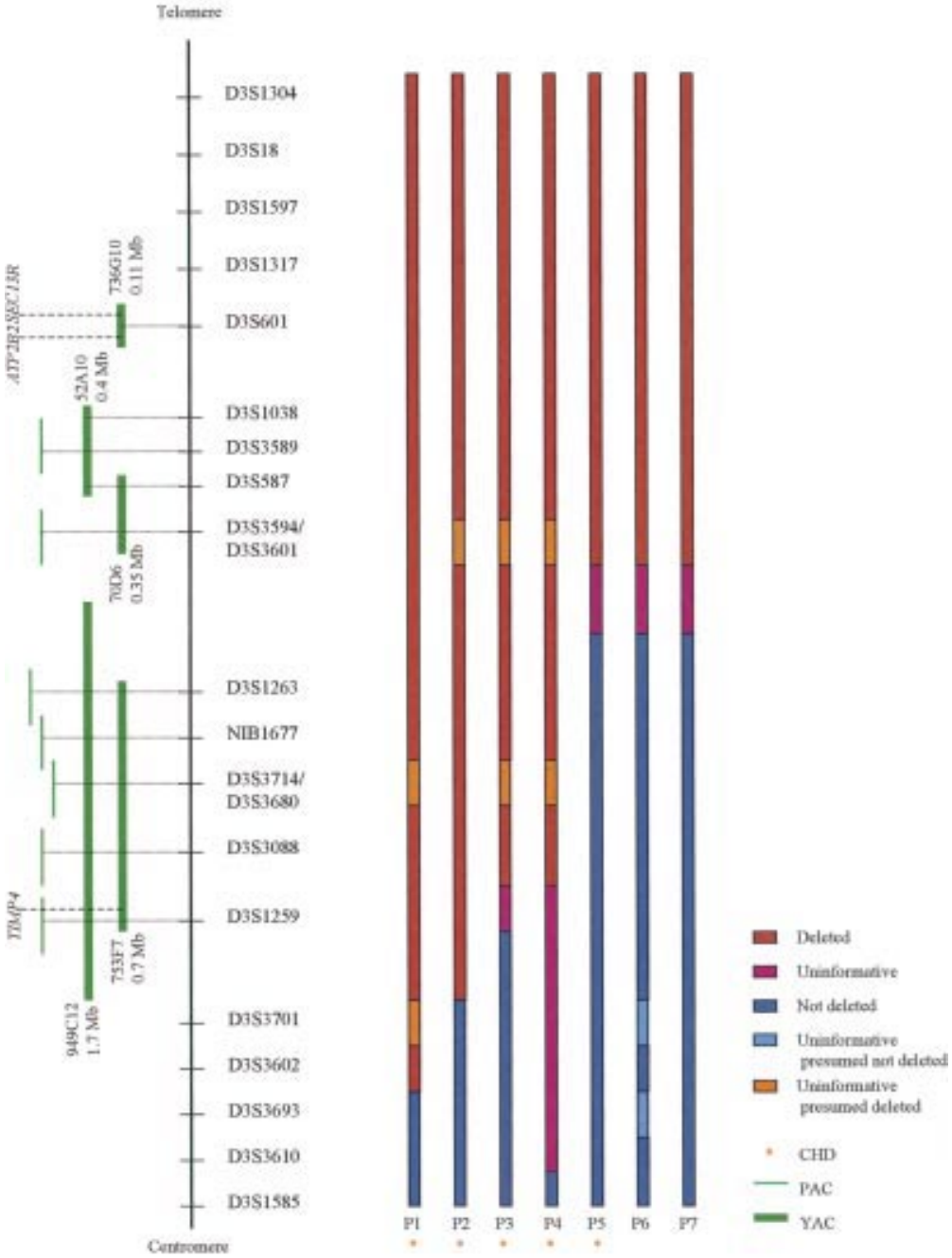


*Figure 3 YAC and PAC physical map of the 3p25 CHD critical interval. Chromosome 3p25-26 is represented by a horizontal line. YAC and PAC clones are positioned to the side and the marker content of each clone is indicated by dashed horizontal lines. The critical interval for the heart developmental gene is D3S1263 to D3S3594. Genetic distances between markers are shown below: D3S1585_1.6 cM_D3S3701_0.5 cM_D3S1259_0.5 cM_D3S1263_3.7 cM_D3S3594_2.6 cM_D3S1597_7.6 cM_D3S1304.*

PACs available using gene specific primers. *TIMP4* mapped close to D3S1259 on PAC 248M7, centromeric to the critical interval. Genes *ATP2B2* and *Sec13R* were both positioned on YAC 736G10, distal to D3S3594, and so these two genes were also excluded. The fibulin 2 gene *(FBLN2)* was excluded as a candidate by typing a highly polymorphic dinucleotide repeat polymorphism and showing heterozygosity in 3p− patients with CHD (results not shown).

## Discussion

In our previous analysis of 3p− syndrome we suggested that a CHD susceptibility gene mapped to 3p25.[7] However, this analysis was based on only five cases and the mapping interval was very large (D3S1250 to D3S18). Subsequently, Drumheller *et al*[11] investigated five 3p− cases (four new cases), and also found that the only patient (GM10922 (P3)) with a CHD had the most extensive deletion. In doing so they refined the CHD target interval slightly to D3S1585 to D3S1317. In mapping the breakpoints of the seven patients with the largest deletions, we have provided further evidence for a CHD locus in 3p25 and significantly reduced the target interval. By assuming that the absence of CHD in patients P6 and P7 is not the result of non-penetrance, it is likely that haploinsufficiency of a gene or genes in the critical D3S1263 to D3S3594 interval disrupts cardiac development. Although incomplete penetrance has been described in familial AVSD, the mapping data in 3p− syndrome patients is, so far, consistent with complete penetrance. However, a normal phenotype has been observed in patients even with large 3p deletions,[10] and if P6 or P7 are both non-penetrant the candidate interval would extend more telomerically.

To evaluate candidate genes known to map to 3p25, we performed PCR amplification of the YACs and PACs with gene specific primers. In our previous studies[7] and investigations by Drumheller *et al*,[11] the plasma membrane calcium transporter gene (*ATP2B2)* was contained within the target interval. However, by showing that it mapped distal to D3S3594, we were able to exclude *ATP2B2* as the CHD susceptibility locus. Similarly, the *SEC13R* gene[16] was shown to map close to *ATP2B2* outside the critical CHD region. Another candidate gene from 3p24-p25 is fibulin 2 *(FBLN2)*, which is prominently expressed in mouse heart but present in low levels in other tissues.[17–19] Nonetheless, we were able to show retention of *FBLN2* in 3p− patients with CHD. The tissue inhibitor of metalloproteinases 4 (*TIMP4*) (which is highly expressed in the heart[20]) maps proximal to D3S1263 since it is situated on both YAC 949C12 and the PAC containing D3S1259. It too can therefore be excluded as a CHD susceptibility gene. Also excluded is the human caveolin-3 gene, suggested as a candidate 3p25 CHD susceptibility gene but mapping close to D3S18, distal to the critical region.[21] Recently a candidate extracellular matrix protein, cirrin, has been suggested as the CHD locus. However, detailed mapping data are not available for cirrin so it is not possible to exclude this gene.[22]

Although the primary objective of this study was to delineate the 3p25 CHD susceptibility region, our investigations have also refined the critical region for the major features of 3p− syndrome. In previous studies of 3p− syndrome,[7 9 11] the critical region for the 3p− phenotype was mapped to the D3S601 to D3S17 interval (21 cM). In the current study, analysis of P10 showed that D3S1317 was retained, so reducing the target region for the phenotypic features of 3p− syndrome to the D3S1317 to D3S17 interval. D3S1317 lies in a 2 cM interval between D3S601 and D3S18, with D3S17 lying a further 19 cM telomeric to D3S18.

The parental origin of the chromosome 3p deletion in the six informative cases studied by us was maternal. Combining our results with those of Mowrey *et al*,[9] the parent of origin of 3p deletions is maternal in six cases and paternal in two. Devriendt *et al*[8] found that all of four instances of del(8p) syndrome had a maternal origin, and a preponderance of maternally derived deletions has also been reported in 1p36 deletion syndrome.[23] This maternal excess is in contrast to deletions of distal 4p and 5p (Wolf-Hirschhorn and Pitt-Rogers-Danks syndromes and cri du chat syndrome respectively), in which ~80% of de novo deletions arise in the paternal germline.[24–26] It has been suggested in studies of 1p36 deletions that the predominance of maternally derived deletions versus those paternally derived may be apparent because the deletion preferentially occurs during maternal gametogenesis. It may, however, be possible that there is a survival bias owing to imprinted genes in the region.[23] There is no evidence that the phenotypic variability of 3p− syndrome patients is influenced by genomic imprinting effects, as we did not detect phenotypic differences between those 3p− syndrome patients with maternally derived deletions and those of paternal origin.

The isolation of the 3p25 CHD susceptibility gene will provide further insights into the mechanisms of normal cardiac development and enable the genetic pathology of familial and isolated AVSDs to be investigated. The detailed mapping results and localisation of the CHD gene to a 3.7 cM interval, already partially covered by a YAC, will facilitate the identification of the chromosome 3p25 AVSD susceptibility gene.

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