

Isolation of a new marker and conserved sequences close to the DiGeorge syndrome marker HP500 (D22S134)

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Abstract

End fragment cloning from a YAC at the D22S134 locus allowed the isolation of a new probe HD7k. This marker detects hemizyosity in two patients previously shown to be dizygous for D22S134. This positions the distal deletion breakpoint in these patients to the sequences within the YAC, and confirms that HD7k is proximal to D22S134. In a search for coding sequences within the region commonly deleted in DGS we have identified a conserved sequence at D22S134. Although no cDNAs have yet been isolated, genomic sequencing shows a short open reading frame with weak similarity to collagen proteins.

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Hemizyosity for a region within 22q11 can be associated with a wide range of phenotypes from no detectable abnormality to severe dysmorphism. The clinical diagnoses given in these cases have included DiGeorge syndrome (DGS),¹⁻⁷ velocardiofacial⁸⁻¹⁰ (Shprintzen) syndrome (VCFS), Noonan syndrome,¹¹ conotruncal face anomaly, and familial congenital heart defect.^{12,13} In these cases one or more of the following abnormalities may be observed: absent or hypoplastic thymus, absent or hypoplastic parathyroids, conotruncal heart defect, and cleft palate and abnormal facies. Besides these main features many other defects have been reported in association with DGS and VCFS.¹⁴⁻¹⁹ Several lines of evidence point to the major abnormalities having a similar underlying embryological basis, namely a deficiency in the neural crest's contribution to the development of the branchial arch/pharyngeal pouch system.²⁰⁻²³

We have previously reported the marker HP500 for the locus D22S134 which is hemizygous in most cases of DGS/VCFS. This locus was also used to show deletions in the familial congenital heart disease study. Although HP500 is not deleted in all cases of DGS known to have chromosome 22 abnormalities, the possibility that DGS is a contiguous gene deletion syndrome²⁴ led us to attempt the identification of potential coding sequences in the region. Our previous mapping studies had indicated that HP500 might detect cross hybridising sequences in rodents. This was investigated further using 'zoo' blots and DNA sequencing.

We have previously described a YAC isolated with HP500 and the isolation of cosmids

corresponding to the terminal sequences of the YAC insert; D0832 is one of these cosmids. The terminal sequence cosmids have been orientated by FISH,²⁵ with D0832 mapping proximal to sc4.1, a cosmid containing HP500.²⁶ In this work we report the isolation of a single copy probe from D0832 for use as a Southern hybridisation probe, and the detection of potential conserved sequences at HP500.

Materials and methods

DNA PROBES, SUBCLONING, AND SEQUENCING

Cosmid D0832 has been described previously and was isolated from library ICRF library 106(L4/FS22) constructed by D Nizetic. Cosmid DNA was digested with the enzyme *Hind*III and fragments shotgunned into *Hind*III digested pBluescript vector.²⁷ Subclone inserts were isolated from LMP agarose gels, labelled by random oligonucleotide priming,²⁸ and hybridised to strips of *Hind*III digested human genomic DNA. In this way single copy sequences could be identified. Similarly, *Sau*IIIa fragments of HP500 were shotgunned into M13mp18 for sequencing, which was conducted using a kit (Sequenase II, USB). Some sequence was also obtained from the original HP500 clone in pUC13 using universal primers and an automated ALF sequencer (Pharmacia). XV2c is a genomic clone from chromosome 7 close to CFTR²⁹ and was used as a control probe in quantitative Southern hybridisation experiments.

SOUTHERN HYBRIDISATION

Genomic DNA from a variety of species was digested with *Eco*RI and Southern blotted according to standard procedures. HP500 was labelled by random oligonucleotide priming and hybridised to the resulting 'zoo' blot. The filter was washed at varying stringencies and the results are shown in fig 1.

Quantitation of Southern hybridisations with probe HD7k was conducted according to our previously published protocol.⁶ However, in place of autoradiography and densitometry a Molecular Dynamics phosphorimager was used to quantitate hybridisation signals on the filter. The Molecular Dynamics Imagequant software was used throughout. This system eliminates those errors secondary to the non-linear response of film to radiation intensity. Signal strength ratios were calculated as described previously⁶ and as shown in table 1.

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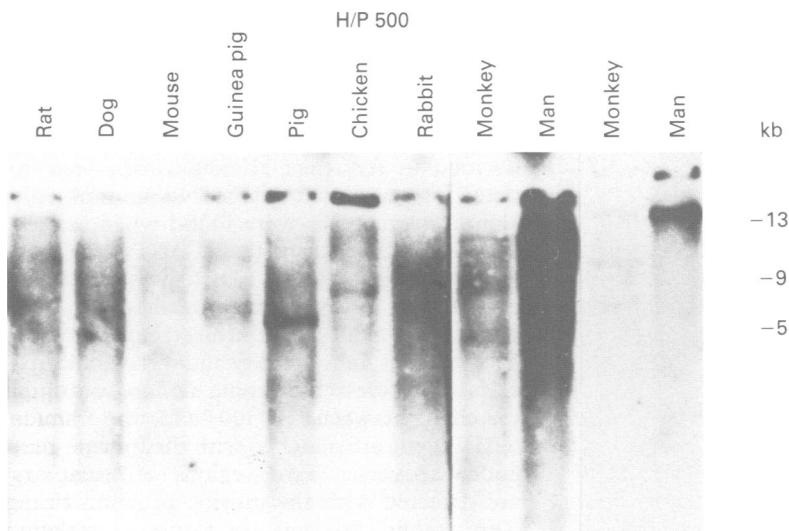


Figure 1 HP500 detects potentially conserved sequences. *EcoRI* digested genomic DNA was hybridised with HP500 and the filter washed at $1 \times$ SSC/0.1% SDS, 65° initially, then at $0.1 \times$ SSC to increase the visibility of fragments in the human and monkey lanes. Sizes given are determined from a 1 kb ladder which was run alongside the genomic DNA.

Hemizyosity is defined as signal strength ratio of < 0.6 on three occasions.

Results

A subclone of D0832 called HD7k was found to give a single copy hybridisation signal in *HindIII* digested genomic DNA. It was mapped to 22q11 by somatic cell hybridisation analysis (not shown). Quantitative Southern analysis showed that it was deleted in all patients already known to be hemizygous for HP500 (not shown). It was also shown to be hemizygous in two patients (mother and daughter) previously found to be dizygous for HP500 (fig 2A and B); the daughter was previously shown to have a more proximal deletion.¹⁰ An example of a signal strength ratio calculation is given in table 1. It can also be seen from inspection of the greyscale printouts that signal strength for the control probe is higher in the patients than the controls, for example, fig 1A, left panel. Conversely, the HD7k hybridisation signal is greater in the controls than the test lanes giving a graphic illustration of the reduced dosage. The probe was also used to detect deletions within 22q11 in patients not investigated previously (see clinical case report by Holder *et al*, this issue).

HP500 was hybridised to a zoo blot (fig 1). Hybridisation could be seen in a range of mammals including rodents, dog, and pig, and also in chick. In order to isolate possible coding sequences responsible for this cross hybridisation an 8.5 day pc whole mouse em-

bryo and human fetal brain libraries were screened, but no positives were detected. Therefore, we initiated DNA sequencing of HP500 in a search for open reading frames. Sequencing of *SauIIIa* shotgun subclones of HP500 yielded no undoubtedly significant database homologies, but one potential open reading frame with borderline homology to various collagen α chains was found (Poisson $p = 0.0015$ under BLAST versus the procollagen $\alpha 2(\text{IV})$ chain precursor) (table 2).

Discussion

Despite the advent of FISH based diagnosis for DGS/VCFS there is still a need for quantitative Southern analysis in cases where only stored blood samples are available, or where a laboratory does not have access to the relevant cytogenetic facilities. Single copy probes useful for Southern analysis may also be used in chromosome mapping experiments, for instance, in screening genomic libraries. Since our previous experiments had allowed us to orientate a YAC containing the HP500 marker,²⁵ and a gap in our genomic clone contig exists proximal to this YAC, we decided to subclone the cosmid D0832 which lies at the proximal end of the YAC. Marker HD7k was isolated and shown to be deleted in several previously untested patients with DGS and VCFS.

Patients known to be dizygous for HP500 were also examined with HD7k, for example, patient NY9 who is hemizygous for the more proximal marker 22.71.¹⁰ The patient's affected mother NY10 was also included in the analysis. Both samples were frozen, anticoagulated blood and therefore FISH analysis was not possible. Both mother and daughter were found to be hemizygous for HD7k indicating that, at least in some cases, HD7k is a more sensitive marker for hemizyosity than HP500. Additionally, it can be inferred that the distal deletion breakpoint in these subjects can be localised to the HP500-HD7k interval, that is, within the TM-YAC. Attempts to clone the sequences at the breakpoint are underway as we are interested in determining whether there are any particular sequence motifs associated with chromosome breakage within 22q11. One possibility is that there is a predisposition to deletion associated with the low copy repeat families present in this region,²⁶ with intrachromosomal recombination between such repeats leading to deletions.

It has been postulated that the haploinsufficiencies associated with hemizyosity for 22q11 are the result of deletion of contiguous genes. We tend to the view that the major features of the condition are the result of haploinsufficiency of one gene (or family of genes) which controls the rostral neural crest's contribution to development. However, it is quite possible that some of the less frequent features of the condition are the result of insufficiency of different genes, or that deletion of additional genes exacerbates the phenotype. Although there is no evidence (as yet) for correlation of extent/position of deletion with

Table 1 Calculation of signal strength ratios for fig 2, L.

Average counts XC2c in control lanes	A + D = (326 + 343)/2 = 334.5
Average counts HD7k in control lanes	A + D = (233 + 217)/2 = 225
Counts XV2c lane B = 490	Counts XV2c lane C = 458.5
Counts HF7k lane B = 160	Counts HD7k lane C = 142
Ratio lane B (NY9) = $160 / (490 / 334.5 \times 225) = 0.49$	
Ratio lane C (NY10) = $142 / (458.5 / 334.5 \times 225) = 0.46$	

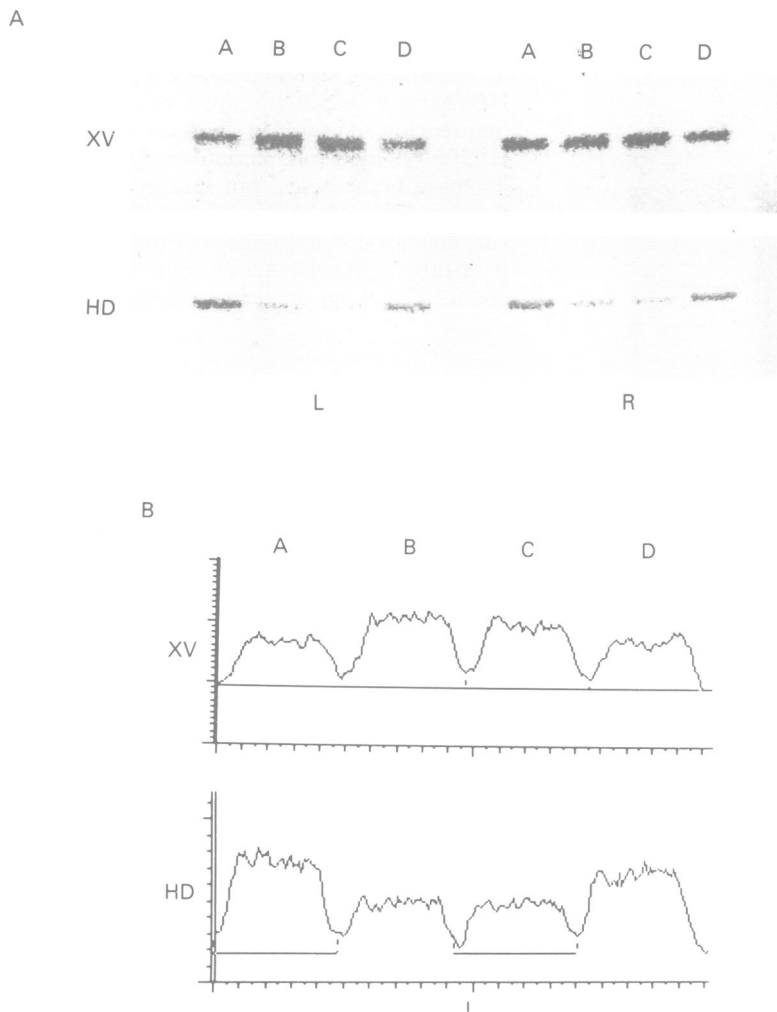


Figure 2 Patients NY9 and NY10 are both hemizygous for HD7k. HindIII digests of control and test DNA were hybridised with HD7k and XV2c giving single fragments of 8 kb and 3 kb respectively. Panel A shows greyscale printer output of scanned images, panel B shows the densitometric profiles obtained for the left hand set of digests (L). Lane A=control; lane B=NY9; lane C=NY10; lane D=control.

phenotype, the large size of the deletions associated with DGS (at least 500 kb, and in most cases 2 Mb)²⁵ is consistent with the involvement of more than one gene.

Previous somatic cell hybrid panels had shown that HP500 cross hybridised with sequences in rodent DNA. This was confirmed by zoo blot analysis which indicated that HP500 detected sequences in a range of mam-

mals and chick. Such a result is typical of probes containing conserved sequences potentially encoding a gene. Several screens of mouse embryo and human fetal brain cDNA libraries proved negative and it was therefore decided to sequence HP500 with a view to detecting potential ORFs. No undoubtedly significant matches were found when searching the databases using the BLAST program.²⁸ The most significant match was with the procollagen $\alpha 2(\text{IV})$ chain precursor in both human and mouse. Over a stretch of 38 amino acids there is 42% identity and 68% similarity. It is of interest that some amino acids not matched between HP500 and the human $\alpha 2(\text{IV})$ gene are matched with the murine gene and vice versa. Two regions of homology are detected with the murine protein. There were other relevant (but non-significant, $p < 1 > 0.05$) matches for other collagens. It is now known that collagens are likely to have roles in development unrelated to chondrogenesis. For instance, collagens can promote avian neural crest migration *in vivo*³⁰ and collagen $\alpha 1(\text{II})$ is expressed transiently in the heart, fetal brain, and neural tube early in mouse embryo development.³¹ It has also been proposed that type II collagen, or a coexpressed matrix molecule, functions as a morphogenetic signal at the epithelial-mesenchymal tissue interface.³² Therefore, we are attempting to clone expressed sequences containing this possible collagen-like sequence using RT-PCR of early embryo RNA.

The HD7k/D0832 probes are available for diagnostic use.

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Table 2 Sequence matches of derived HP500 ORF with collagens. Identical amino acids are shown between the two sequences. Conservative matches are designated +. The HP500 sequence is shown above the collagen sequence.

Human procollagen $\alpha 2(\text{IV})$ chain p=0.0013

HP500 GPAGSQGSTGEVLGAGKVGVSANSPLCPLPRGKRAKPG
GP +G++G+GE + G G++A SP +L++G R+PG
329 GPDGPRGPKGEAGDPGPPGLPAYSPHPSLAKGARGDPG 366

Murine procollagen $\alpha 2(\text{IV})$ p=0.0015

Match 1
HP500 GPAGSQGSTGEVLGAGKVGVSANSPLCPLPRGKRAKPG
GP+G++G+GE G G S SP +L++G R+PG
327 GPSGPRGPKGEAGDPGPPGSPVSPHPSLAKGARGDPG

Match 2

HP500 GPAGSQGSTGEVLGA
GP+G+G GEVLGA
760 GPPGDRGIPGEVLGA 774

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