ORIGINAL ARTICLE

Organisation of the pericentromeric region of chromosome 15: at least four partial gene copies are amplified in patients with a proximal duplication of 15q

J A Fantes, S K Mewborn, C M Lese, J Hedrick, R L Brown, V Dyomin, R S K Chaganti, S L Christian, D H Ledbetter

J Med Genet 2002;**39**:170–177

See end of article for authors' affiliations

Correspondence to: Dr D H Ledbetter, Department of Human Genetics, The University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA; dhl@genetics.uchicago.edu

Revised version received 15 November 2001
Accepted for publication 19 November 2001

Clinical cytogenetic laboratories frequently identify an apparent duplication of proximal 15q that does not involve probes within the PWS/AS critical region and is not associated with any consistent phenotype. Previous mapping data placed several pseudogenes, *NF1*, *IgH D/V*, and *GABRA5* in the pericentromeric region of proximal 15q. Recent studies have shown that these pseudogene sequences have increased copy numbers in subjects with apparent duplications of proximal 15q. To determine the extent of variation in a control population, we analysed *NF1* and *IgH D* pseudogene copy number in interphase nuclei from 20 cytogenetically normal subjects by FISH. Both loci are polymorphic in controls, ranging from 1-4 signals for *NF1* and 1-3 signals for *IgH D*. Eight subjects with apparent duplications, examined by the same method, showed significantly increased *NF1* copy number (5-10 signals). *IgH D* copy number was also increased in 6/8 of these patients (4-9 signals). We identified a fourth pseudogene, *BCL8A*, which maps to the pericentromeric region and is coamplified along with the *NF1* sequences. Interphase FISH ordering experiments show that *IgH D* lies closest to the centromere, while *BCL8A* is the most distal locus in this pseudogene array; the total size of the amplicon is estimated at ~1 Mb. The duplicated chromosome was inherited from either sex parent, indicating no parent of origin effect, and no consistent phenotype was present. FISH analysis with one or more of these probes is therefore useful in discriminating polymorphic amplification of proximal pseudogene sequences from clinically significant duplications of 15q.

Molecular cytogenetic analysis has shown that apparent duplications of proximal 15q identified by G banding are not usually associated with a duplication of DNA probes in the Prader-Willi/Angelman critical region. The majority of these cases are not duplicated for *SNRPN* and have a normal phenotype or inconsistent phenotypes.[1–4] They are usually inherited from a phenotypically normal parent and have been interpreted as euchromatic variants.

Two recent reports[5 6] used molecular cytogenetic techniques to show that amplification of a pseudogene cassette, mapping close to the centromere of 15q, causes the visible appearance of a duplication in banded chromosomes. The pseudogene cassette contains truncated gene sequences from a minimum of three genes transposed from other sites in the genome. One such sequence was shown to be a partial copy of the neurofibromatosis-1 (*NF1*) gene from chromosome 17q11.2.[5 7–10]

To determine whether increased dosage of this *NF1* pseudogene correlated with observed cytogenetic duplications of 15q11.2, Barber *et al*[5] used FISH to show that the signals from a PAC probe containing the *NF1* pseudogene were increased in intensity in two unrelated cases of chromosome 15 proximal duplications and estimated the degree of amplification from measurements of fluorescence intensities on metaphase chromosomes.

Ritchie *et al*[6] found that a truncated copy of the *GABRA5* (γ-aminobutyric acid type A receptor α 5 subunit) gene, the full length copy of which maps within the Prader-Willi/Angelman critical region, mapped to the pericentromeric region of chromosome 15, and was present in multiple copies on proximal duplication chromosomes. An estimate of the degree of amplification was made from the number of FISH signals in interphase nuclei and from released chromatin preparations. By isolating P1 clones from the region, they found that both the *GABRA5* duplication sequence and one of the *NF1* non-processed pseudogenes were present in the same P1 clone in the contig. A third sequence, the immunoglobulin heavy chain diversity segment gene (*IgH D*), had previously been mapped to proximal 15q[11–13] and was shown to be amplified in proximal duplications, but was not part of the same contig.

Ritchie *et al*[6] reported variation in the number of signals from *GABRA5* and flanking probes in cytogenetically normal controls. In this paper, we present a more detailed study of the variation in copy number of *NF1* and *IgH D* sequences in 20 cytogenetically normal controls and in eight proximal duplication cases. We quantified the degree of amplification by interphase FISH and established the normal range in controls. In addition, we show that a fourth pseudogene for *BCL8* (Dyomin and Chaganti, unpublished data) is part of the amplified cassette. *BCL8* was originally identified at the breakpoint of a translocation (t(14;15)(q32;q11-13)) in a diffuse large cell lymphoma patient.[14] Recent work has shown that the copy on chromosome 15 lacks several exons, now termed *BCL8A*; the full length copy, *BCL8B*, maps to 13q11-q12 (Dyomin and Chaganti, unpublished data). By interphase FISH mapping we have established the order of the pseudogenes within the cassette and estimated the size of the region amplified.

MATERIALS AND METHODS

Patient material

The control population consists of 20 cytogenetically normal subjects from the University of Chicago Clinical Cytogenetics

Abbreviations: PWS, Prader-Willi syndrome; AS, Angelman syndrome

Table 1 Copy number variation in 20 cytogenetically normal subjects

Controls	Ascertainment*	1st chromosome 15†		2nd chromosome 15	
		NF1 (P1–4)	*IgH D* (c13c6)	*NF1* (P1–4)	*IgH D* (c13c6)
1	Coriell	**2** (1–3)	**2** (1–2)	**2/3** (2–4)	**2** (1–3)
2	DD	**1** (1)	**1** (1)	**4** (2–5)	**1 (1)**
3	DD	**2** (1–2)	**2** (1–2)	**2** (2–3)	**3** (2–4)
4	DD	**2** (1–3)	**2** (1–3)	**3** (1–4)	**2** (1–4)
5	LIS	**1** (1–3)	**1** (1–2)	**3** (2–4)	**3** (2–4)
6	RM parent	**1** (1–2)	**1** (1–2)	**3** (2–3)	**2** (2–3)
7	RM parent	**3** (2–3)	**1** (1–2)	**3** (2–4)	**2** (1–3)
8	RM parent	**2** (1–3)	**1** (1–2)	**3** (2–3)	**2** (1–2)
9	RM parent	**1** (1–2)	**1** (1–2)	**2** (1–3)	**2** (2–3)
10	RM parent	**2** (1–3)	**2** (1–2)	**3** (2–4)	**2** (2–3)
11	RM parent	**2** (1–2)	**2** (1–2)	**3** (2–3)	**2** (2–3)
12	RM parent	**2** (1–3)	**2** (1–3)	**2** (2–3)	**3** (2–4)
13	RM parent	**2** (1–2)	**1** (1–2)	**2** (1–3)	**2** (1–2)
14	RM parent	**2** (1–3)	**1** (1–2)	**3/4** (2–5)	**2** (1–2)
15	Inv(9) parent	**2** (1–2)	**2** (1–2)	**3** (2–3)	**2** (2–3)
16	Inv(9) parent	**1** (1–2)	**1** (1–2)	**4** (2–4)	**3** (2–3)
17	Ins trans parent	**2** (1–3)	**2** (1–3)	**3** (2–4)	**2** (2–3)
18	LIS/MCA parent	**3** (1–4)	**2** (1–3)	**4** (3–5)	**3** (1–5)
19	LIS del parent	**2** (1–3)	**2** (1–3)	**4** (3–5)	**3** (2–3)
20	LIS del parent	**2** (1–3)	**2** (1–3)	**3** (2–4)	**3** (2–4)

*Ascertainments for each control: DD, developmental delay; LIS, lissencephaly; RM, recurrent miscarriage; MCA, multiple congenital abnormalities.
†For each subject the domain with the smaller number of signals is on the left; the mode of the number of signals is shown in bold and the range is in parentheses.

Laboratory. Details of the control population's ascertainment are given in table 1.

The patient population was divided into two groups: subjects in group I are phenotypically normal and were ascertained during routine cytogenetic analysis by the presence of a proximal duplication on chromosome 15, or as the parent of a fetus with a proximal duplication, while subjects in group II were ascertained on the basis of an abnormal phenotype and were found by cytogenetic analysis to have a proximal duplication on chromosome 15.

Group I

Case A was a 31 year old, phenotypically normal female, who was found to have a proximal duplication of one chromosome 15 after identification of a similar chromosome in a pregnancy loss. No evidence of a duplication was found by FISH using probes for D15S11, *SNRPN*, and *GABRB3*. Cytogenetic analysis of her parents determined that the chromosome with the duplication was inherited from her mother.

Case B was a 21 week fetus that showed extra material on chromosome 15q during routine prenatal diagnosis by amniocentesis. No evidence of duplication was found using FISH with *SNRPN* and the same duplication was subsequently found in the phenotypically normal mother.

Cases C and E are phenotypically normal mothers whose amniocentesis showed a proximal duplication of one chromosome 15 in the fetus. Case D is the father of a fetus found to have a proximal duplication of 15q11-13. Subsequent chromosome analysis showed that subjects C, D, and E had a similar proximal duplication. No duplication of *SNRPN* was seen by FISH in any of these cases.

Group II

Case F was a 5 year old child with autism. Cytogenetic analysis showed extra material on one chromosome 15q, but *SNRPN* and more distal probes were not duplicated.

Case G was a 9 year old child with moderate mental retardation, developmental delay, hyperactivity, and a proximal duplication of 15q11-12. Subject G's parents are first cousins. The *SNRPN* probe was not duplicated.

Case H was a 7 year old child with a diagnosis of autism and mild dysmorphic features. A maternal cousin is mentally retarded. Previous cytogenetic analysis had shown extra material on chromosome 15, but *SNRPN* was not duplicated.

Genomic clones

A P1 clone (P1-4) for the *NF1* pseudogene on chromosome 15 was described by Purandare *et al*.[8] The chromosome 15

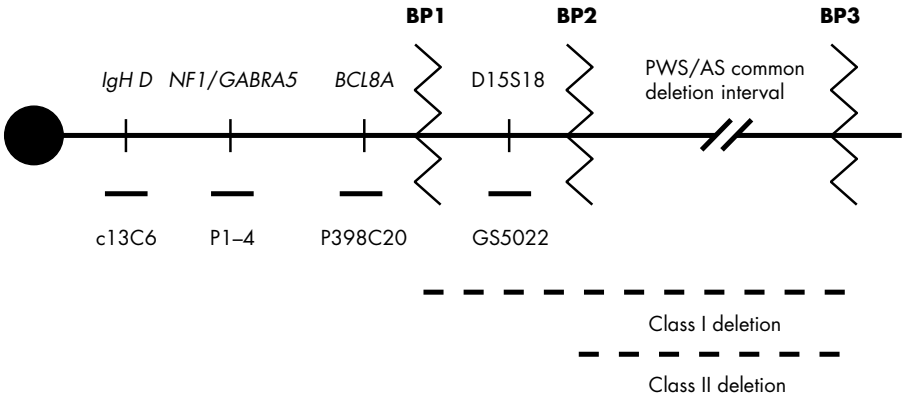

Figure 1 A schematic diagram showing the map order of the pseudogenes and their position relative to the common PWS/AS proximal breakpoints (BP1 and BP2). Genomic clones used for FISH are shown below the line.

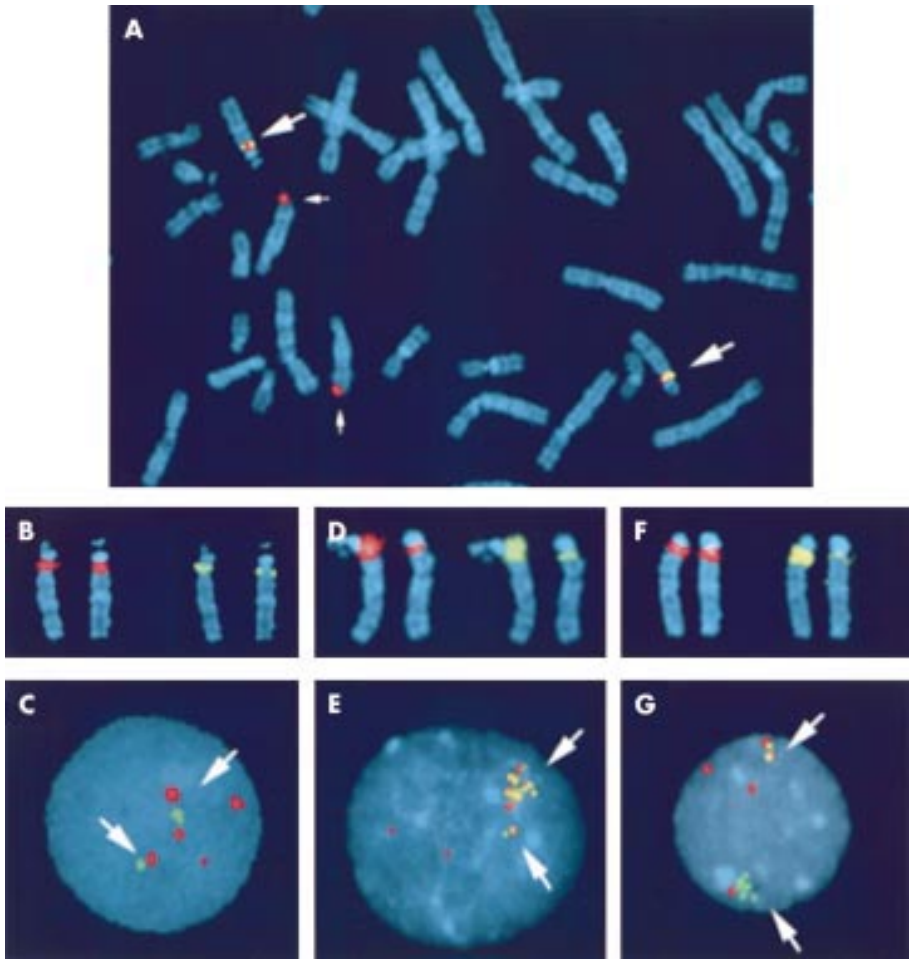

Figure 2 (A, B, C) Hybridisation of metaphase and interphase cells from a cytogenetically normal control with the *NF1* P1 clone (green) and the *IgH D* cosmid (red). In (A), signals from each probe appear similar in size on both chromosome 15 homologues (large arrows). Note the cross hybridisation of the *IgH D* probe to 14q32 (small arrows). In (B), an expanded view of the same pair of chromosomes is shown with separation of the signals from each probe. (C) Variation in copy number between the two chromosome 15 domains (arrowed); the left domain has one signal from each probe while the right domain has two red signals and one or two fused green signals. (D, E) Hybridisation of metaphase and interphase cells from a subject with a visible cytogenetic duplication (case C) with the *IgH D* cosmid (red) and the *NF1* P1 clone (green). Both probes give large or multiple signals on the chromosome 15 homologue with a visible duplication (E). Multiple signals from both probes are seen in the interphase nucleus. In the upper domain (arrowed), there are 10 green NF1 signals and seven red *IgH D* signals. (F, G) Hybridisation of *NF1* (green) and *IgH D* (red) probes to metaphase chromosomes and interphase nuclei from a second subject with a visible duplication (case G). In (F), the *IgH D* (red) signals are similar in size on the two 15 homologues, but the *NF1* signal is much larger on one chromosome 15. In the interphase nucleus (G), the lower chromosome 15 domain has one red *IgH D* signal but seven green *NF1* signals.

specific *IgH D* cosmid (c13C6) was described by Nagaoka *et al*.[11] GS5022 is a P1 clone containing the STS D15S18, described by Christian *et al*.[15] A plasmid clone (pMC15) for the more distal of the two chromosome 15 specific alphoid sequences (D15Z3) is described by Finelli *et al*.[16] The *SNRPN* probe was obtained commercially (Vysis Inc, Downers Grove, IL). Two PACs (P398C20, P406K8) and a phage clone (SS283) containing *BCL8A* have been described by Dyomin *et al*.[14] Primers derived from the 5 kb *GABRA5* duplication sequence (GenBank AF061786) were used to screen a total human BAC library (Genome Systems, St Louis, MO) by PCR and clone GS35F21 was isolated. DNA from genomic clones was isolated using an automated DNA isolation system (AutoGen 740, Autogen Inc, Framingham, MA).

FISH

Chromosome preparations were made from peripheral blood cultures or lymphoblastoid cell lines using standard methods. In dual colour FISH experiments, probe labelling, DNA hybridisation, and antibody detection were carried out using methods described previously.[17] In three colour ordering experiments, probes were labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche, Indianapolis, IN) or directly labelled with Spectrum Orange-dUTP (Vysis Inc, Downers Grove, IL). Biotin labelled probes were detected with avidin-Cy5 (Amersham Pharmacia Biotech Inc, Piscataway, NJ) and digoxigenin labelled probes with FITC anti-digoxigenin (Roche, Indianapolis, IN). FISH slides were analysed using a Zeiss Axiophot microscope with the appropriate filters (No 83000 for DAPI, FITC, and rhodamine; No 84000 for DAPI, FITC, Spectrum Orange, and Cy5; Chroma Technology, Brattleboro, VT), a cooled CCD camera (Nu 200; Roper Scientific, Tucson, AZ), and Quips PathVysion software (Applied Imaging, Santa Clara, CA).

The copy number for each probe in both control and proximal duplication subjects was determined by counting the number of signals in each chromosome 15 domain in at least 30 interphase nuclei and calculating the mode for each domain.

Interphase ordering and distance measurements

The order of the probes was scored in at least 20 interphase nuclei. The distance between the two probe signals in an interphase nucleus was measured using IPLab software

Table 2 Copy number variation in eight proximal duplication cases and parent

Case	Ascertainment*	1st chromosome 15†		2nd chromosome 15	
		NF1 (p1–4)	*IgH D* (c13c6)	*NF1* (p1–4)	*IgH D* (c13c6)
Class I					
A	Pregnancy loss with prox dup	**2** (1–3)	**2** (1–3)	**9/10** (6–12)	**5/6** (5–9)
B	Fetus with prox dup	**2** (1–3)	**2** (1–3)	**5** (4–6)	**4** (3–5)
Mother of B		**2** (1–2)	**2** (1–2)	**5** (4–6)	**4** (2–6)
C	Mother of fetus with prox dup	**1** (1–2)	**2** (1–2)	**10** (6–11)	**6** (5–7)
Father of C		**2** (2–3)	**2** (1–2)	**9** (7–11)	**7** (5–8)
Mother of C		**1** (1–2)	**1** (1–2)	**2** (1–3)	**2** (1–3)
D	Father of fetus with prox dup	**3** (2–5)	**2** (1–3)	**9** (8–13)	**7/8** (7–9)
E	Mother of fetus with prox dup	**2** (1–2)	**2** (1–3)	**6** (3–8)	**4** (3–5)
Class II					
F	Autism, prox dup	**1** (1–2)	**1** (1–2)	**5** (3–6)	**4** (2–6)
Father of F		**2** (1–3)	**2** (1–3)	**4** (3–5)	**3** (2–3)
Mother of F		**1** (1)	**1** (1–2)	**3** (2–5)	**2** (1–2)
G	MR, DD, prox dup	**2** (1–3)	**2** (1–3)	**7** (5–10)	**2** (1–2)
Mother of G		**2** (1–4)	**1** (1–2)	**7** (6–10)	**2** (2–3)
H	Autism, prox dup	**2** (1–3)	**1** (1–2)	**6** (5–7)	**2** (1–3)
Father of H		**2** (1–2)	**1** (1–2)	**2** (2–3)	**2** (2)
Mother of H		**2** (1–3)	**2** (1–3)	**6** (4–6)	**2** (1–4)

*Ascertainment of each subject: MR, mental retardation; DD, developmental delay.
†For each subject, the domain with the smaller number of signals is on the left; the mode of the number of signals is shown in bold and the range is in parentheses.

(Scanalytics, Fairfax, VA). Probes were hybridised to interphase nuclei from a subject previously shown to have two copies of the *NF1* and *IgH D* probes in one chromosome 15 domain. At least 20 interphase distances were measured for each pair of probe signals. Genomic distance was estimated from a calibration curve.[18 19]

RESULTS

NF1 pseudogene and *IgH D* gene segments map proximal to PWS/AS deletions

Using fluorescence in situ hybridisation (FISH), we mapped an *NF1* pseudogene P1 clone (P1-4) and a cosmid (c13C6) containing an *IgH D* segment in relationship to the common proximal breakpoints (BP1 and BP2) found in PWS/AS deletion patients and subjects with small supernumerary inv dup(15) chromosomes[20 21] (fig 1). When these probes were hybridised to metaphase chromosomes from a class I deletion patient (deletion extending from BP1 to BP3), signals were present on both the normal chromosome 15 and the deleted chromosome 15 (data not shown), placing both clones between the centromere and BP1, the most proximal breakpoint in PWS/AS deletions. Both clones showed signals on class I as well as class II small inv dup(15) marker chromosomes, confirming their position proximal to BP1. This result is consistent with the data obtained by Ritchie *et al*.[6]

Polymorphic variation in controls

In our initial hybridisations with the *NF1* and *IgH D* clones on metaphase chromosomes in normal subjects, we observed differences in signal size and intensity between the two chromosome 15 homologues. To investigate this variation, we hybridised both clones to metaphase chromosomes and interphase nuclei from 20 cytogenetically normal subjects. An example is shown in fig 2A-C. In metaphase cells (fig 2A, B), there is a slight difference in the size of the signal on the two chromosomes 15 with both probes. However, in the interphase nucleus, where the DNA is less condensed (fig 2C), several distinct signals for each probe are clearly present. In one chromosome 15 domain, there is one green signal and one red signal, while the second domain has one green or possibly two fused green signals and two red signals. We assume that each signal represents one copy of the sequence but note that any contiguous duplication of sequence would not be detected. The additional signals on two D group chromosomes in fig 2A represent cross hybridisation of the *IgH D* cosmid to related sequences on 14q32, the site of the functional *IgH D* gene locus.[11] This cross hybridisation can also be seen in interphase nuclei (fig 2C). In the nucleus in fig 2C, the copy number for the *IgH D* probe on each chromosome 15 is (1, 2) with a similar result for the *NF1* probe.

The number of signals per chromosome 15 domain for each probe was analysed in 30 G1 nuclei from each control. Nuclei were classified as G1 nuclei both by their size and by the absence of signal doublets from the additional *IgH D* hybridisation sites on 14q32, which indicates replication status. We used the modal number of signals as a measure of the copy number of each probe because of the known variation in signal number in interphase nuclei caused by overlapping signals, split signals, or variation in hybridisation efficiency.[22]

These results are summarised in table 1. We found that the copy number for *NF1* varies from one to four signals; *IgH D* generally has fewer copies and varies from one to three signals. These results show that there is variation in copy number of both probes in our control population and provide base levels for further studies.

Copy number in subjects with a proximal duplication

To test the hypothesis that these sequences are amplified in proximal duplication patients, we hybridised both probes to chromosome preparations from the eight proximal duplication cases, A-H. An example is shown in fig 2D, E. On metaphase chromosomes (fig 2D), both the *NF1* and *IgH D* probes give large or multiple FISH signals in the pericentromeric region of one chromosome 15, the region that appears duplicated by conventional G banding analysis. In the interphase nucleus (fig 2E), the difference between the two chromosome 15 domains is striking: in one domain there are 10 green signals from the *NF1* probe and seven red signals from *IgH D*, in the other domain there are two *NF1* signals and one *IgH D* signal. Although the signals sometimes colocalise to give yellow spots, there are also separate green and red signals with intervening unlabelled chromatin, suggesting that other sequences in this region may also be amplified.

Table 2 summarises the copy numbers per chromosome for the proximal duplication cases and any available family members. In each case, one chromosome 15 domain clearly has a higher number of copies of the *NF1* probe than the second domain. The copy numbers for *NF1* on the duplicated

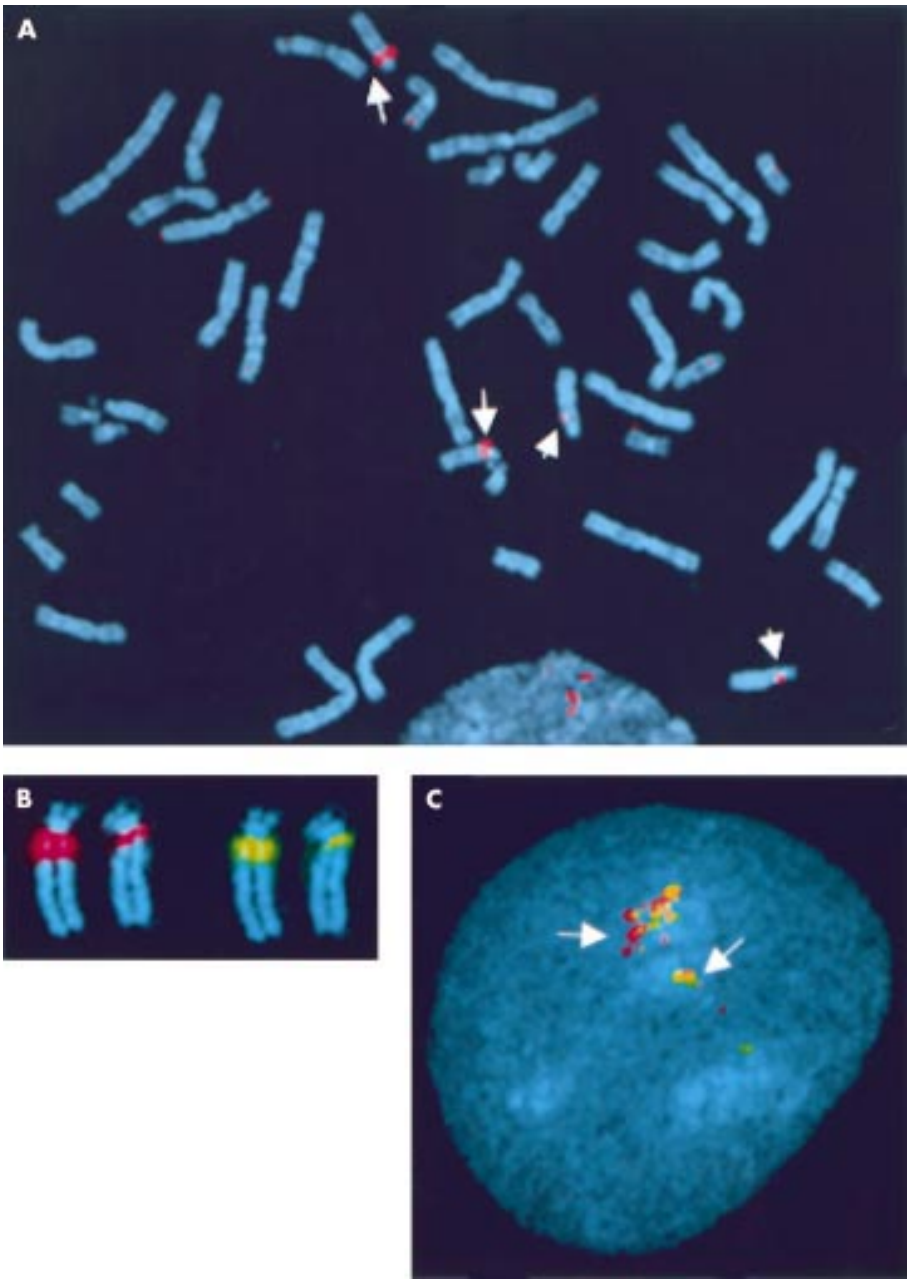

Figure 3 (A) Hybridisation of *BCL8A* PAC (red) to metaphase chromosomes from a subject with a class I PWS/AS deletion. Signals are present on both chromosome 15 homologues (arrows). (B) Hybridisation of *BCL8A* (red) and *IgH D* (green) probes to metaphase chromosomes from a subject with visible duplication. Note the larger signal on one homologue for each probe. (C) Variation in copy number between the two chromosome 15 domains in the interphase nucleus (arrowed): the upper domain has seven *BCL8A* signals and six *IgH D* signals, while the lower domain has only one *BCL8A* signal and two *IgH D* signals.

chromosomes (5-10) were higher than those in the control population (1-4) (Mann-Whitney test, $p<0.0001$). In six subjects, *IgH D* is also increased in copy number on the duplicated chromosome; the modal copy number for *IgH D* ranges from 4-8, again higher than the copy number of 1-3 seen in the control population (Mann-Whitney test, $p<0.0001$). In all six cases, there are fewer copies of *IgH D* than *NF1*, consistent with the controls.

Surprisingly, in two subjects (cases G and H) the copy number for *NF1* was increased but the copy number for *IgH D* was within the range for normal subjects (fig 2F, G). Thus, the apparent duplication seen by routine cytogenetic analysis in these two cases is associated with polymorphic amplification of only part of the pseudogene cassette.

Inheritance of proximal duplication chromosomes

To determine the inheritance of the proximal duplication chromosome, we obtained samples from additional family members for five cases and analysed them as described previously. The results are summarised in table 2. In three subjects (cases B, G, and H), the phenotypically normal mothers also carried similar proximal duplication chromosomes. One subject (case C) inherited the proximal duplication chromosome from the phenotypically normal father. Although FISH studies were not done on the parents, previous cytogenetic analysis had clearly shown that case A inherited a proximal duplication chromosome from her phenotypically normal mother. Case F has inherited a chromosome 15 with one copy of each probe maternally, but the second chromosome appears to have a slightly higher modal copy number for both *NF1* (5)

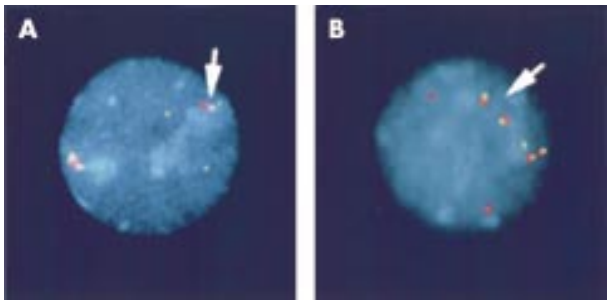

Figure 4 (A) Orientation of *NF1* (purple) and *IGH D* (green) clones in relation to a more distal probe, GS5022 (red). The interphase nuclei are from a control where one chromosome 15 domain has only a single copy of each proximal sequence (arrowed); the other domain has two to three copies. The order in the single copy domain (arrowed) is green-purple-red suggesting that *IgH D* is closer to the centromere than *NF1*. (B) Estimation of the size of the pseudogene cassette by hybridising *NF1* (green) and *IgH D* (red) clones to a control with two copies of each proximal sequence in one of the chromosome 15 domains (arrowed); the other domain has two to three copies. The measured interphase distance between the two red signals is similar to that between the two green signals, suggesting a tandem rather than an inverted duplication (arrowed).

and *IgH D* (4) probes than either paternal chromosome 15, although the ranges clearly overlap.

Three probands have an abnormal phenotype. Two of these inherited the duplication chromosome from their phenotypically normal father and one from the phenotypically normal mother. Thus, no evidence of parent of origin effects was observed in the population studied here.

BCL8A pseudogene copy number is increased in proximal duplication chromosomes

BCL8A sequences were previously mapped close to the centromere by STS mapping of the chromosome 15 YAC contig.[23] Two PAC clones were available for mapping to PWS/AS deletions by FISH: P406K8 contains the entire *BCL8A* sequence, while P398C20 lacks the 3′ end. Neither clone contained *NF1* or *GABRA5* pseudogene sequences by PCR. Both clones mapped by FISH to the 15q pericentromeric region and gave weak cross hybridisation to the pericentromeric regions of other chromosomes (P406K8 to 13q12, the location of the functional gene; P398C20 to 14q11.2, 18p11.2, and 21q11.2). In interphase nuclei from controls, each PAC gave a cluster of two to three signals close to the pMC15 (D15Z3) signal on chromosome 15. This pattern suggested that the *BCL8A* sequence was another member of the cassette of sequences amplified in proximal duplications. To confirm this, hybridisation to metaphases from a class I deletion patient showed that the *BCL8A* PAC maps proximal to the PWS/AS critical region (fig 3A). Hybridisation to metaphase chromosomes from a proximal duplication patient showed large FISH signals on the duplicated chromosome in metaphase (fig 3B) and multiple signals in interphase (fig 3C). The copy number for *BCL8A* probes was comparable to *NF1* and higher than *IgH D*. Additionally, in case G, the proximal duplication where *IgH D* was not increased in copy number, *BCL8A* probes were coamplified with *NF1*.

Orientation of the amplification cassette

We performed three colour FISH on interphase nuclei to determine the order of three of the pseudogenes, *NF1*, *IgH D*, and *BCL8A*, on chromosome 15. As the truncated *GABRA5* sequence is within 80 kb of *NF1*,[6] it was not included because it would be unlikely to give a separate signal in interphase nuclei.[24] To simplify the analysis, we used preparations from a subject who has a single copy of *NF1* and *IgH D* on one chromosome 15 (control 16 in table 1). *NF1* and *IgH D* were ordered with respect to a centromeric alphoid probe, pMC15 (D15Z3), and to a distal probe, GS5022 (D15S18), which maps between BP1 and BP2 (fig 1). *BCL8A* and *NF1* were ordered with respect to GS5022 only. Fig 4A shows the results of hybridisation of GS5022, *NF1*, and *IgH D* in an interphase nucleus. In the single copy domain (arrowed), the order is *IgH D*-*NF1*-GS5022, which indicates that *IgH D* lies closer to the centromere than *NF1*. This was confirmed by a second hybridisation with pMC15 (D15Z3), where the order was pMC15-*IgH D*-*NF1*. Although signals from *IgH D* and *NF1* overlapped on metaphase chromosomes, *IgH D* was consistently more centromeric whenever order could be scored. *BCL8A* was also present as a single copy in this domain and was found to map distal to *NF1*. Thus, the most likely order for these probes is cen-*IgH D*-(*NF1*/*GABRA5*)-*BCL8A*-BP1-GS5022-tel.

Size of the amplified region

We estimated the size of the amplified cassette from interphase distance measurements. If every probe within the cassette gave two signals in one domain, we assumed that this represented a duplication of the amplification cassette. In a tandem duplication, the distances between the two signals from each probe should be similar and reflect the size of the cassette. In contrast, in an inverted duplication, the distance should vary between probes, depending on the position of the probe within the cassette. We hybridised probes for *IgH D*, *NF1*, *BCL8A*, and *GABRA5* pairwise to interphase nuclei from a subject with two copies of *IgH D* and *NF1* in one domain (control 15 from table 1) and measured the interphase distance between the two copies of each probe. A representative result is shown in fig 4B. Table 3 shows the mean interphase distance and estimated genomic distance for each probe. Estimates of the size of the amplification cassette from each probe were similar (800 kb to 1.1 Mb) and suggest a tandem, rather than an inverted, duplication. Note that the *NF1* and *GABRA5* probes, which map within 80 kb of each other, give very similar estimates of genomic distance.

DISCUSSION

Our results indicate that the pericentromeric region of chromosome 15q, between the centromere and BP1, contains truncated or non-functional copies of genes transposed from other pericentromeric regions (*NF1*, 17q11.2 and *BCL8A*, 13q11), from a telomeric region (*IgH D* and *IgH V*, 14q32.3) and from a more distal region of the same chromosome (*GABRA5*, 15q12). Pericentromeric regions of human chromosomes have acquired duplicated gene segments from elsewhere in the genome by a

Table 3 Size of the duplicated pseudogene cassette

Probe	No of measurements	Mean ID* (μ)	(Mean ID)	Distance (kb)
IgH D (c13C6)	56	1.04	1.08	1100
NF1 (p1–4)	31	0.94	0.88	850
GABRA5 dup (35F21)	28	0.86	0.74	800
BCL8A (398C20)	22	1.01	1.02	950

*Mean interphase distance.

process referred to as pericentromeric directed gene duplication[10 25–27] and the centromeric region of 15q is a prime example.

Detailed analyses of pericentromeric regions have previously been performed on chromosomes 10, 16, and 21.[28–30] In all three cases a similar pattern of complex juxtaposition of arm specific sequences, stable duplications, and unstable sequences with homologies to telomeric and centromeric sequences have been observed. Additionally, amplification of a pseudogene cassette has been observed in the pericentromeric region for chromosome 16 without phenotypic effects, similar to the amplification observed on chromosome 15.[31]

Our interphase FISH results indicate the following order for sequences in proximal 15q: cen-*IgH D*-(*NF1/GABRA5*)-*BCL8A*-BP1-tel. In silico analysis of data from the UCSC Golden Path (http://genome.cse.ucsc.edu) for 15q11 is consistent with this order (data not shown). However our results were obtained from one subject and there may be polymorphism in the population. The four pseudogenes are part of a large cassette or amplicon that varies in copy number in subjects with a normal karyotype and is increased in copy number in those with a visible proximal duplication of chromosome 15q. From interphase distance measurements, we estimated the size of this cassette to be ~1 Mb, a result comparable to an earlier estimate derived from the number of copies found in a subject with a cytogenetic band sized duplication.[6] We have also shown that the pseudogene cassette is duplicated in a tandem, or direct, orientation.

Our data show that the copy number of the cassette has a polymorphic distribution in the normal population, with 3- to 4-fold differences in copy number for the *NF1* and *IgH D* probes between chromosome 15 homologues. This observation was reflected in differences in signal size and intensity on metaphase chromosomes and may contribute to subtle differences in banding patterns in the 15q proximal region. At one end of this distribution are subjects with a cytogenetically visible proximal duplication where one 15 homologue has copy numbers of 6-10 for several probes.

We analysed a larger number of proximal duplication subjects than previous studies and found that all eight proximal duplications involved significant amplification of *NF1* pseudogenes, while only some also involved coamplification of *IgH D*. In both controls and subjects with proximal duplications, we found that the copy number for *IgH D* was the same or less than that of *NF1*. These two observations suggest that the length of the amplification cassette is variable and sometimes does not include *IgH D*.

For six of the subjects with proximal duplications, we obtained parental material and were able to identify the parental origin of the duplication chromosome by cytogenetic and/or FISH analysis. We observed both maternal and paternal transmission and found that the copy number of each probe was similar from parent to offspring. In one case we found a higher modal copy number of both probes than in either of the parents, and while this may reflect differences in hybridisation or overlap of signals, it may also represent an increase in copy number.

Five of the subjects with proximal duplications had a normal phenotype and were ascertained by the presence of a proximal duplication during routine cytogenetic investigations. Three subjects were ascertained on the basis of an abnormal phenotype (autism or mental retardation) and subsequent cytogenetic analysis indicated the presence of a proximal duplication. Does a proximal duplication of chromosome 15 have any phenotypic effect? These three cases have some phenotypic overlap (autism and mental retardation) but additional phenotypes (talipes, hernia, short stature, epilepsy) as well as developmental delay have been reported in earlier publications.[5 6] Maternal and paternal transmission from phenotypically normal parents was also reported. We suggest that there is no evidence of parent of origin effects, unlike the maternally derived duplication of the PWS/AS critical region which is associated with autism.[2 32] The link between autism and chromosome 15 duplications may lead to an increased ascertainment rate of subjects with a proximal polymorphism owing to the close scrutiny of subtle differences between homologues. Consistent with this is the fact that three of our group II cases have less total amplification in proximal 15q than group I cases: two cases were not amplified for *IgH D* sequences and the third has fewer copies of the amplified sequences than other proximal duplications with a normal phenotype. We therefore conclude that the presence of a proximal duplication is unlikely to cause any phenotypic consequences.

The new BAC and PAC clones identified in this study will allow a more accurate definition of the proximal breakpoints of deletions and marker chromosomes, which may contribute to our understanding of the underlying mechanisms of the frequent chromosome 15 rearrangements. For clinical testing purposes, it is recommended that any person with a cytogenetically visible proximal 15q duplication, which does not include the probes within the PWS/AS critical region, be analysed by FISH for the presence of an amplification of the pseudogene cassette. As *NF1* is amplified in all reported proximal duplications, P1-4 appears to be the single most effective FISH probe for detecting proximal duplications in metaphase or interphase nuclei. The use of P1-4 will verify the amplification of these sequences as the cause of the cytogenetic variation observed by G banding. Although no consistent phenotype has been observed in subjects with an increased copy number of these pseudogenes, further studies will help to define genotype/phenotype correlations.

ACKNOWLEDGEMENTS

We thank Drs Mark Pasquarette, Sau W Cheung, J T Mascarello, Arturo Anguiano, Ed Cook, Osama Kamal Zaki, and Bennett Leventhal for providing patient material and clinical information. We thank Drs David Viskochil and Tasuku Honjo for providing genomic clones. This work was supported by NIH grants HD36111 (DHL) and CA72669 (RSKC).

Authors' affiliations

J A Fantes, S K Mewborn, C M Lese, J Hedrick, R L Brown, D H Ledbetter, Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA
J A Fantes, Medical Genetics Section, Department of Medical Sciences, University of Edinburgh, Edinburgh EH4 2XU, UK
V Dyomin, R S K Chaganti, Cell Biology Program and the Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
S L Christian, Department of Psychiatry, University of Chicago, Chicago, IL 60637, USA

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ECHO

Genes versus environment in ocular refraction

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Variation in ocular refraction is determined genetically rather than environmentally—that is, according to a Danish study of twins. The question of genes versus environment has been complicated previously by possible natural selection in populations in epidemiological studies and differences in age or environment in family studies. Previous studies of twins have shown a high degree of heritability for ocular refraction—that is, the proportion of biological variation caused by genetic variation—but even these have had major drawbacks.

Lyhne *et al* set out to put the record straight with a population based study of same sex twins. In all, they studied 114 pairs of identical (53 pairs: 23 male, 30 female) and non-identical (61 pairs: 27 male, 34 female) twins aged 20–45 years, measuring within each pair refraction and measures of the eye that determine refraction. After correcting for any age/sex dependence, they determined best fit for each measure in a model estimating the proportion of biological variation due to additive genetic effects, dominant genetic effects, common environmental effects, and unique environmental effects.

Heritability was high, between 0.89 and 0.94 (95% confidence interval 0.82 to 0.96), for refraction, total refraction, axial length, and radius of corneal curvature, and variation was due to additive genetic effects. Similar values, between 0.88 and 0.94 (0.81 to 0.96), were obtained for anterior chamber depth and lens thickness, and variation was due to dominant genetic effects. Signs of a gene-environment interaction, evident for refraction, suggested that genetically liable individuals might develop myopia under certain conditions—for example, from near work..

▲ *British Journal of Ophthalmology* 2001;**85**:1470–1476.