Allele specificity of DNA replication timing in the Angelman/Prader-Willi syndrome imprinted chromosomal region

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DNA replication within chromosome 15q11–q13, a region subject to genomic imprinting, was examined by fluorescence *in situ* hybridization. Asynchronous replication between homologues was observed in cells from normal individuals and in Prader-Willi (PWS) and Angelman syndrome (AS) patients with chromosome 15 deletions but not in PWS patients with maternal uniparental disomy. Opposite patterns of allele-specific replication timing between homologous loci were observed; paternal early/maternal late at *D15S63*, *D15S10* and the γ-aminobutyric acid receptor β3 subunit gene (*GABRB3*); and maternal early/paternal late at the more distal γ-aminobutyric acid receptor α5 subunit gene (*GABRA5*). At the most distal locus examined, *D15S12*, both patterns of allele-specific replication timing were detected.

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Chromosome replication is under stringent temporal and spatial control to ensure accurate duplication of the genome. It is initiated from discrete loci in mammalian chromosomes and proceeds in an ordered manner during S phase[1–4]. High resolution cytogenetic studies have revealed more than 1000 temporal chromatin domains or replication bands[5] with replication bands appearing synchronously in autosomal homologues[5–7]. Molecular studies suggest that each band represents 10–20 replicons and that adjacent replicons are replicated coordinately[8,9].

Replication timing and genetic activity have been correlated at the chromosomal level. Inactive X chromosomes in eutherian females replicate later in S phase than their active counterparts[10,11] and tissue specific genes generally replicate earlier when they are expressed than when they are not[12–14]. Housekeeping genes whose products are necessary for cell maintenance replicate early in S phase while constitutive heterochromatic regions (thought to be genetically inactive) replicate late in S phase[12].

A relationship between replication timing and activity of genes that display allele-specific expression — that is, they are imprinted — has recently been suggested[15,16]. One of the best examples of parental imprinting in man occurs in chromosome 15q11–q13. The absence of a paternal contribution to this region either by deletion or through uniparental maternal inheritance results in Prader-Willi syndrome (PWS)[17–19] while lack of maternal contribution to this region results in Angelman syndrome (AS)[20–22]. The two syndromes are clinically distinct. A gene (*SNRPN*, small ribonucleoprotein N), whose murine homologue shows preferential expression of the paternal allele, has recently been mapped to 15q11–q13 (ref. 23) and differential timing in the appearance of chromosome replication bands in the PWS region has been described[15]. Kitsberg *et al.* observed recently that loci in the AS/PWS region and in other imprinted regions replicate asynchronously[16]. For all loci examined, the paternally derived locus replicates earlier than the maternal one[16]. To investigate further the potential relationship between imprinting and DNA replication, we have used an approach, described recently and involving fluorescence *in situ* hybridization (FISH)[24], to examine the replication behaviour of maternal and paternal alleles at several loci within 15q11–q13. Here we show that there are at least three different asynchronous patterns of allele-specific replication within this chromosomal region.

Results

Asynchronous replication pattern of 15q11–q13

FISH is a sensitive method for assaying replication[24]. Single sequence hybridizations result in two discrete signals in G1 cells, two pairs of discrete signals in G2 cells and one single and one pair of signals in G1–G2 cells (if both homologues have not replicated; Fig. 1). Results of the replication pattern of sequences in 15q11–q13 are listed in Table 1. Asynchronous replication for markers spanning the PWS and AS minimum region of deletion overlap is observed in 25–40% of synchronized PHA-stimulated lymphocytes or lymphoblastoid cell lines from normal individuals. The relative timing of the sequences as determined by the frequency of cells with G1–G2 pattern is similar for the sequences examined. This asynchronous replication extends across the region commonly deleted in patients with AS and PWS.

Hybridization analysis of the control phage clone for

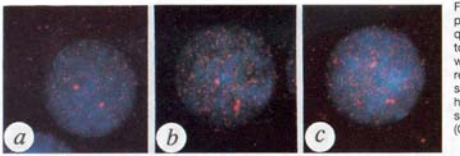

Fig. 1 Representative replication patterns of one chromosome 15q11–q13 sequence. Hybridization of α5-39 to lymphoblasts revealed cells in which *a*, the sequence has not yet replicated (G1 pattern); *b*, the sequence has replicated on only one homologue (G1–G2 pattern); c, the sequence has completed replication (G2 pattern).

CD7 revealed synchronous replication of the homologues with 20–30% of the cells in G2, 5–10% with asynchronous replication and the remaining cells in G1. The level of asynchronous replication of *CD7* could be attributed to the efficiency of the hybridization and/or the position of the hybridization within the nucleus. The level of asynchronous replication of chromosome 15q11–q13 was reduced to that of *CD7* when cells from PWS patients with maternal uniparental disomy were examined (Table 2). These results strongly suggest an association between replication asynchrony of the maternal and paternal homologues and imprinting in this chromosomal region.

Parent of origin differences in replication

In order to establish whether replication timing is allele specific, cells from patients with small deletions of known parental origin were examined (Fig. 2*a*). Using a two colour/two sequence hybridization protocol with one sequence from within the deletion and the other adjacent but outside the deletion (Fig. 2*b*, *c*), we were able to assess the replication timing on the two homologues (Table 3, Fig. 3). In the cells of patient II-2, the paternal chromosome is deleted and hybridizes only to JP3 whereas the intact maternal chromosome hybridizes to both JP3 and 3-21-12 (Fig. 2*b*, *c*). JP3 replicates early on the paternal (deleted) chromosome in the majority of cells of II-2 (Table 3). For III-2, the reverse is true: the maternal chromosome is deleted and hybridizes only to JP3 while the paternal chromosome hybridizes to both JP3 and 3-21-12. In III-2, JP3 also replicates early on the paternal (intact) chromosome in the majority of cells (Table 3). Combined, these results indicate that the JP3 locus of paternal origin replicates before the maternally derived locus and that the deletion does not influence replication.

FISH replication analysis at the distal extent of the deletion revealed that probes from the 5' end (α55-24) and 3' end (α5-39) of *GABRA5* replicate earlier on the maternal chromosome than on the paternal chromosome 15 (Table 3). Once again, this finding was consistent for both II-2 and III-2. For II-2, probes for *GABRA5* hybridized only to the paternal chromosome while probes for both *GABRA5* and *GABRB3* hybridized to the maternal chromosome. For III-2, the reverse was true. The replication pattern of *GABRB3* could not be determined in II-2 and III-2 as one homologue had a deletion. It could however be examined directly in HS32.5 (Fig. 2*b*) by simultaneous hybridization with JP3 and probes from *GABRB3*. HS32.5 is derived from a PWS patient bearing a *de novo* unbalanced translocation which is of paternal origin and which is hemizygously deleted for markers proximal of *D15S10* (refs 25,26). Probes from the central region (16β3) and 5' end (β3-61 of *GABRB3*, which are intact in HS32.5, replicate early on the paternal chromosome (Table 3). *GABRB3* and *GABRA5* are located within 100 kb of each other in a head to head array[27] and both show asynchronous replication (Table 3). However, the parental replication pattern of the two genes is different. Both probes from *GABRB3* replicate early on the paternal chromosome and late on the maternal chromosome, while the two probes from *GABRA5* replicate early on the maternal chromosome and late on the paternal chromosome (Table 3). Furthermore, sequences proximal to *GABRB3* (JP3 and 3-21-12) replicate in a coordinated manner (paternal early/maternal late) and one sequence from the distal 3' end of *GABRA5* (α5-39) replicates with the same asynchronous pattern as the 5' end of *GABRA5* (α55-24), that is maternal early/paternal late. Representative examples of two sequence/two colour asynchronous replication are presented in Fig. 3.

Table 1 Replication pattern of chromosome 15q11–q13 sequences in normal individuals

		No. of cells (%)					
Individual	Pattern[b]	JP2	JP3	3-21-12	16β3	α5-39	1R10-1-45
1	G1	68 (54.8)	76 (52.4)	118 (51.5)	142 (52.4)	84 (52.8)	132 (61.1)
	G1–G2	42 (33.9)	50 (34.5)	83 (36.2)	98 (36.2)	52 (32.7)	61 (28.2)
	G2	14 (11.3)	19 (13.1)	28 (12.2)	31 (11.4)	23 (14.5)	23 (10.7)
2	G1	110 (59.5)	85 (51.5)	83 (48.3)	65 (54.6)	75 (54.3)	81 (63.3)
	G1–G2	58 (31.4)	62 (37.5)	68 (39.5)	42 (35.3)	48 (34.8)	35 (27.3)
	G2	17 (9.1)	18 (10.9)	21 (12.2)	12 (10.8)	15 (10.9)	12 (9.4)
3[a]	G1	91 (61.5)	81 (59.1)	95 (57.9)	100 (62.9)	72 (51.8)	105 (68.2)
	G1–G2	44 (29.7)	42 (30.7)	51 (31.1)	41 (25.8)	39 (31.7)	39 (25.3)
	G2	13 (8.8)	14 (10.2)	18 (11.3)	18 (11.3)	12 (9.7)	10 (6.5)

[a]lymphoblastoid cell line analysis, other analyses are from lymphocytes.
[b]G1, G1–G2 and G2 refer to cells that have not replicated (two singlet hybridization signals), have replicated only one homologue (one singlet and one doublet hybridization signal) and have replicated both homologues (two doublet hybridization signals), respectively.

Mosaic asynchronous replication of *D15S12*

IR10-1 (*D15S12*), the most distal characterized plasmid probe observed in 15q11–q13, is beyond the minimum region of deletion for AS and PWS[26]. It recognizes a cDNA that has close homology to the murine pigmentation gene, *p* (ref. 28). Like other sequences within the region, IR10-1-45 (the genomic phage of IR10-1) replicates asynchronously but in contrast to the other sequences examined, its replication is random with respect to parental origin. IR10-

Table 2 Replication pattern of chromosome 15q11–q13 sequences in PWS patients with maternal uniparental disomy

Patient	Pattern[a]	No. of cells (%) JP2	JP3	3-21-12	16β3	α55-24
WJK75	G1	106 (90.6)	114 (90.5)	102 (88.7)	131 (86.8)	116 (95.9)
(isodisomy)	G1–G2	6 (5.1)	4 (3.2)	8 (7.0)	10 (6.6)	3 (2.5)
	G2	5 (4.3)	8 (6.3)	5 (4.3)	10 (6.6)	2 (1.7)
WJK64	G1	129 (92.1)	142 (88.2)	122 (87.1)	120 (90.9)	154 (95.6)
(heterodisomy)	G1–G2	8 (5.7)	9 (5.6)	10 (7.1)	8 (6.1)	3 (1.9)
	G2	3 (2.1)	10 (6.2)	8 (5.7)	4 (3.0)	4 (2.5)
WJKB173[b]	G1	–	–	94 (88.9)	79 (80.6)	–
(heterodisomy)	G1–G2	–	–	8 (7.1)	6 (6.1)	–
	G2	–	–	10 (8.9)	13 (13.3)	–

[a]See Table 1.
[b]Lymphocyte analysis, other analyses are from lymphoblastoid cell lines

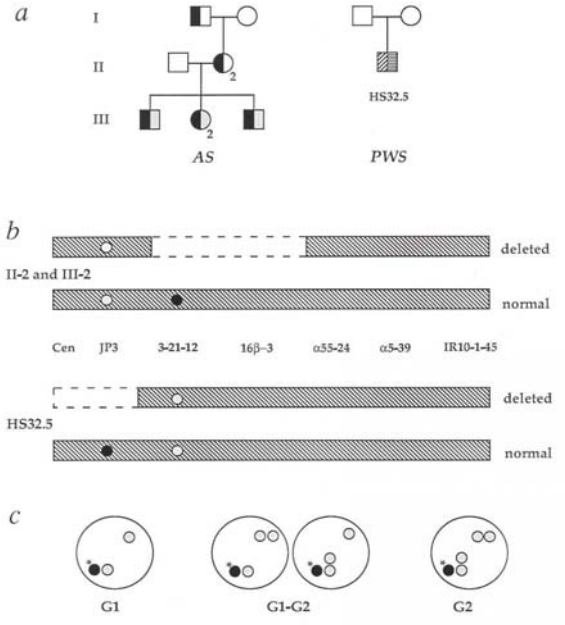

Fig. 2 *a*, Pedigrees of deletion patients used to establish the pattern of replication asynchrony. Circles, females; squares, males. Left pedigree: darkened left symbol, submicroscopic deletion of chromosome 15; shaded right symbol, Angelman syndrome (AS). Right pedigree: cross hatched symbol, *de novo* 9;15 unbalanced translocation and Prader-Willi syndrome (PWS). *b*, Chromosomes 15 in II-2 and III-2 and HS32.5. Deletions (dashed lines) of probes in the AS family (II-2, III-2) and in the PWS patient (HS32.5). The parental origins of the deletion in II-2, III-2 and HS32.5 are paternal, maternal and paternal, respectively. Simultaneous hybridization of cells to probes from within (●) and adjacent to the deletion (○) permits determination of parental origin of replication asynchrony of the nondeleted sequence (○). *c*, FISH replication assay of two sequence hybridizations as illustrated in (*b*). The sequence (●*) from within the deletion serves only as a marker of parental origin and its replication pattern is not scored.

1-45 replicates early on the paternal chromosome 15 in some cells and early on the maternal chromosome in other cells in about equal proportion in both HS32.5 and III-2 (Table 3, Fig. 3*e*, *f*).

Discussion

There have been few investigations of frequency of asynchronous DNA replication in mammalian genomes. Isumikawa *et al.*[14] compared the replication timing of R-bands in 15q11–q13 to five X chromosome bands and found that about 40% of metaphase cells demonstrated asynchronous replication at 15q11–q13. The level of asynchrony was 5–10% at five other autosomal chromosome bands (4p14, 4q12, 13q32, 13q34 and 15q26) which were examined[15]. Using the high resolution FISH assay with 15q11–q13 specific probes on interphase cells, we observed a similar proportion of cells displaying asynchronous replication of 25–40% (Table 1). *CD7*, a chromosome 17-specific gene, displayed replication asynchrony in only 5–10% of cells.

Replication asynchrony of 15q11–q13 is not restricted to peripheral T lymphocytes, as similar results were obtained in EBV transformed B lymphoblasts (Table 1). The reason that only 25–40% of cells are subject to allele-specific replication asynchrony within 15q11–q13 is

Table 3 Asynchronous replication pattern of chromosome 15q11–q13 sequences in patients with distinguishable parental chromosomes 15

Patient (origin)	Probes[a]	G1–G2 pattern[b] M[E]P[+]	M[+]P[E]
II–2 (M[+]P[+])	JP3	6	199
	α55–24	173	31
	α5–39	37	3
III–2 (M[+]P[+])	JP3	6	73
	α55–24	160	24
	1R10–1–45	35	31
	α5–39	37	3
HS32.5 (M[+]P[+])	16β3	2	38
	1R10–1–45	41	44
	3–21–12	3	51
	α55–24	25	0
	β3–61	3	98

[a]The deleted chromosome 15 was identified using probes 3–21–12 or 16β3 in II–2 and III–2, and probe JP3 in HS32.5 for each two sequence hybridization.
[b]G1–G2 refers to cells that have replicated only one homologue (one singlet and one doublet hybridization signal).
M, maternal; P, paternal; –, deleted; +, intact; E, early replication; L, late replication.

unclear but the presence of both parental 15q homologues is required for asynchronous replication of this region (Table 2). Cells derived from three PWS patients with maternal uniparental disomy displayed a background level of replication asynchrony in interphase (2–10%) (Table 2).

Using cell lines in which the parental chromosomes 15 are distinguishable, two different allele-specific patterns of replication at *D15S63*, *D15S10*, *GABRB3* and *GABRA5* are evident. *D15S63*, *D15S10* and *GABRB3* replicate earlier on the paternal chromosome while *GABRA5* replicates earlier on the maternal chromosome (Table 3, Fig. 3). Synchronous replication of these four loci is observed in the cells of three patients displaying maternal disomy of chromosome 15 (Table 2). These results indicate that allele-specific asynchronous replication can delineate chromosomal domains containing imprinted genes.

Further evidence of an association between replication asynchrony and parental imprinting was recently reported by Kitsberg *et al.*[16], who showed that *GABRB3* displays a paternal early/maternal late replication pattern of asynchrony and that one region containing both paternally and maternally imprinted genes (*H19* and *IGF2*) display this same pattern. Our results and those of Kitsberg *et al.* are surprising if it is assumed that replication timing is related to gene activity. The PWS and AS critical regions, which are currently defined as including *SNRPN* to *D15S63* and *D15S10* to *GABRB3*, respectively, are predicted to be oppositely imprinted but yet have the same paternal early/maternal late replication patterns, a finding comparable to that of *H19* and *IFG2* which are differentially imprinted. We have also identified other patterns of allele specific replication asynchrony at loci within 15q11–q13 but outside the critical regions of AS and PWS. *GABRA5* and *D15S12*, which are distal to the AS critical region, display maternal early/paternal late and a mixed paternal early/maternal early patterns of allele specific asynchronous replication, respectively. These additional patterns of asynchronous replication suggest a complex relationship between replication asynchrony and imprinted domains. There is no evidence to suggest that all loci displaying asynchronous replication are imprinted. Replication of *SNRPN*, a paternally imprinted gene in mouse[29,30] was not determined but it is between loci (*D15S63* and *D15S10*) that display a paternal early/maternal late replication pattern. *GABRB3*, however, is also included in this same region of asynchronous replication but is not imprinted in the mouse[29]. One interpretation of our results is that allele-specific replication asynchrony is associated with or reflects a process of chromosomal imprinting which may be distinct from the process which establishes allele-specific expression or imprinting at the level of individual genes.

The zone of replication transition between the paternal early/ maternal late and maternal early/paternal late patterns of replication asynchrony encompasses about 100 kb, the approximate distance between the 5' ends of *GABRB3* and *GABRA5* (ref. 27). An origin of DNA replication, DNA controlling element or replication fork barrier could be located between *GABRB3* and *GABRA5*,

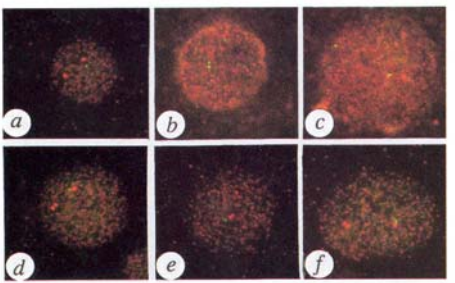

Fig. 3 Replication behaviour of chromosome 15q11–q13 sequences in chromosomes with distinguishable parental origin. *a*, G1 cell of II-2 hybridized with JP3 (red) and 3-21-12 (green). JP3 has not replicated on either the maternal (one green and one red signal) or paternal chromosome (one red signal). *b*, G1–G2 cell of II-2 hybridized with JP3 (green) and 3-21-12 (red). The paternal chromosome (green doublet) replicated before the maternal chromosome (one green and one red signal) at JP3. *c*, G1–G2 of III-2 hybridized with JP3 (green) and 3-21-12 (red). The paternal chromosome (red singlet, green doublet) replicated before the maternal chromosome (one green signal) at JP3. *d*, G1–G2 cell of III-2 hybridized with 16β-3 (green) and α55-24 (red). The maternal chromosome (red doublet) replicated before the paternal chromosome (one red and one green signal) at α55-24. *e*, G1–G2 of HS32.5 hybridized with IR10-1-45 (red) and JP3 (green). IR10-1-45 replicated on the maternal chromosome (red doublet, green singlet) before the paternal chromosome (one red signal). *f*, G1–G2 cell of HS32.5 hybridized with IR10-1-45 (red) and JP3 (green). In this case, the paternal chromosome replicated before the maternal chromosome.

which are in a head-to-head transcriptional orientation[27] such that the pattern of imprinting would bias the direction of the DNA replication fork either in the transcriptional orientation of *GABRB3* (on the paternal chromosome) or in the transcriptional orientation of *GABRA5* (on the maternal chromosome). In this regard, replication of human *c-Myc* has been observed to initiate at the 5'-end and proceed in the direction of transcription when the gene is transcriptionally active[31].

A third pattern of replication asynchrony in chromosome 15q11–q13 is observed at *D15S12* (IR10-1-45), a region containing the human homologue of the murine *p* locus. IR10-1-45 shows a mosaic pattern of replication asynchrony with a similar number of cells displaying the maternal and paternal earlier replication asynchrony patterns (Fig. 3*e*, *f*; Table 3). The *p* locus may be involved in hypopigmentation which is common to AS and PWS patients with deletions which encompass *D15S12*. A mosaic pattern of allele-specific expression at *D15S12* (and presumably the *p* locus) could explain the observation that hypopigmentation occurs in both AS and PWS patients with hemizygous deletions of IR10-1 irrespective of the parental origin of the deletion.

Allele-specific replication of 15q11–q13 may be important for normal development, and alterations/rearrangements that affect replication could result in an abnormal phenotype such as PWS or AS. Uniparental maternal disomy of chromosome 15q11–q13 is associated both with PWS and the absence of replication asynchrony (Table 2). This result not only suggests the association between chromosome 15q parental origin and asynchronous replication of chromosome 15 homologues but also implies that other abnormalities of 15q could perturb DNA replication and result in disease phenotype. Since replication timing and the activity of replication origins is profoundly influenced by chromosomal context[32], DNA rearrangements that place 15q11–q13 near other regions of the genome could affect the normal gene expression and replication pattern of this region. Similarly, point mutations in elements which regulate DNA replication timing could alter the replication timing of chromatin domains and result in some cases of AS and PWS. The identification of such elements will be crucial to further understanding how DNA replication asynchrony is involved in the regulation of the allele-specific disease genes in 15q11–q13.

Methodology

Patients. EBV lymphoblastoid cell lines from an AS patient (III-2) and her mother (II-2), both with microdeletions of chromosome 15q11–q13, were available for study. This family is hemizygously deleted for 16B3 (*GABRB3*) and 3-21 (*D15S10*)[33,34] (see Fig. 2). Lymphoblastoid cell lines from three PWS patients, HS32.5, WJK75 and WJK64 were also used. HS32.5 has a paternally derived *de novo* 9;15 translocation and is hemizygously deleted for loci proximal to 3-21 (*D15S10*)[25,26]. WJK75 and WJK64 both have maternal uniparental disomy. WJK75 has a 15;15 translocation and isodisomy. WJK64 has two normal chromosomes 15 and heterodisomy (patient PWS2[19]). Peripheral blood was obtained from a nondeletion PWS patient (WJKB173) with maternal heterodisomy and a 13;15 translocation. Peripheral blood lymphocytes and lymphoblastoid cell lines were available on normal individuals from our laboratories and from parents of AS or PWS children.

DNA probes. Most of the chromosome 15q11–q13 specific phage clones used have been described[26,27]. They include 34-10 (*D15S9*), JP2 (*D15S11*), JP3 (*D15S63*), 3-21-12 (*D15S10*), 16β3 (*GABRB3*), α55-24 (*GABRA5*), α5-39 (*GABRA5*) and B3-61 (*GABRB3*). IR10-1-45, a 16 kb phage clone, was isolated by screening a λ DASH II library constructed from flow sorted chromosomes 15 with IR10-1 (*D15S12*). A λ phage probe for *CD7*, a T-cell differentiation antigen, served as a control nonchromosome 15 probe[35]. It maps to 17qter.

FISH. Interphase cells were prepared from lymphoblastoid cell lines and PHA-stimulated peripheral blood lymphocytes using standard cytogenetic techniques. Cultures were synchronized by addition of methotrexate for 17 h followed by a 4 1/2 release with either bromodeoxyuridine or thymidine[26]. Pretreatment of slides for FISH, probe labelling, hybridization and fluorescence detection of hybridized probes were performed as described[26,27]. Probes were either labelled with biotin-16-dUTP (Boehringer Mannheim) and detected with FITC-avidin DCS (Vector Laboratories), or labelled with digoxigenin-11-dUTP (Boehringer Mannheim) followed by rhodamine anti-digoxigenin (Boehringer Mannheim) detection. Suppression of repetitive DNA sequences was achieved for each probe by preannealing with Cot-1 DNA (Gibco BRL). Two differentially labelled probes were hybridized simultaneously for each experiment.

FISH replication analysis. The replication pattern of hybridized sequences was scored in interphase cells. Hybridized sequences appeared as two single signals in cells that had not replicated (G1), as two pairs of doublets in cells in which both homologs had replicated (G2) and as a singlet and a doublet in cells where only one homolog had replicated (denoted G1–G2 cells) (Fig. 1). The relative replication timing of each sequence was determined by examining the proportion of cells in G1, G1–G2 and G2. The relative timing of the paternal and maternal chromosomes was determined by two colour/two sequence hybridization on cells of patients in which the parental origin of the two chromosome 15s could be unambiguously distinguished in interphase cells, i.e. II-2, III-2 and HS32.5 (see Fig. 2). Interphase nuclei were visualized by fluorescence microscopy using a Zeiss Axiophot microscope equipped with a dual band pass filter set (fluorescein/Texas red, Omega Optical) or triple band pass filter set (fluorescein/Texas red/DAPI, Omega Optical) and photographed on colour film (Kodak Ektar 1000 film).

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