Am. J. Hum. Genet. 57:1137–1142, 1995

Mechanisms of Small Ring Formation Suggested by the Molecular Characterization of Two Small Accessory Ring Chromosomes Derived from Chromosome 4

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Summary

Molecular cloning of a microdissected small accessary ring chromosome 4 from a moderately retarded and dysmorphic patient has been performed to identify the origin of the ring chromosome. FISH was performed with cosmids identified with the cloned, microdissected products and with other markers from chromosome 4. The present study clearly demonstrates that the small ring in this patient originates from three discontinuous regions of chromosome 4: 4p13 or 14, the centromere, and 4q31. It is suggested that the origin of the ring chromosome is a ring involving the entire chromosome 4, which has then been involved in breakage and fusion events, as a consequence of DNA replication generating interlocked rings. A second severely retarded and dysmorphic patient also had a small accessary ring derived from chromosome 4. FISH studies of this ring are consistent with an origin from a contiguous region including the centromere to band 4q12. It is apparent that there are at least two mechanisms for the formation of small ring chromosomes. This adds a further complication in any attempt to ascertain common phenotypes between patients known to have morphologically similar markers derived from the same chromosome.

Introduction

The potential risk for mental retardation or phenotypic abnormality associated with small supernumerary accessory chromosomes in the human karyotype cannot be defined precisely. Determination of the origins and structures of these chromosomes may overcome this limitation. Initial attempts utilizing such conventional chromosome-staining methods as C-banding, distamycinA/4-6-diamidino-t-phenylindole-2HCl (DAPI) staining, and nucleolus-organizing-region staining provided a first step at classification (Buckton et al. 1985). The development of in situ hybridization allowed determination of the chromosome of origin and revealed considerable complexity (Callen et al. 1990*b*, 1991, 1992; Blennow et al. 1993; Plattner et al. 1993). Small marker chromosomes originating from virtually every human chromosome have now been described.

Markers arising from the same chromosome could be associated with different phenotypes because of variation in the genetic content of each marker. The focus of recent studies has been to provide a more precise definition of the genetic content of marker chromosomes. For example, the presence of the XIST gene in small markers derived from the X chromosome results in inactivation of the marker and minimal phenotype consequences (Wolff et al. 1994). In addition, defining the region of 15q11 retained in small markers derived from chromosome 15 reveals a relationship between phenotype and genetic content (Crolla et al. 1995).

In the present study, small marker chromosomes found in two patients with mental retardation and phenotypic anomalies were characterized by molecular techniques. The aim was to precisely determine the origin and genetic content of the marker and to relate with the phenotype.

Patients, Material, and Methods

Patients and Chromosome Materials

Patient A.—This patient has moderate mental retardation and minor anomalies consisting of macrocephaly, plagiocephaly, brachycephaly, epicanthic folds, flat midface with relative prognathism, malocclusion, high arched palate, hypoplastic ala nasi, thin upper lip, short and broad neck, and small hands and feet (Callen et al. 1992). The karyotype is 46,XY/47,XY,+r(4) with the

Received May 22, 1995; accepted for publication July 24, 1995.

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0002-9297/95/5705-0021$02.00

ring being less than the width of a chromosome in size. Short-term lymphocyte cultures showed 30% of cells with the marker in 100 cells scored. Fibroblast cultures showed a similar proportion of the ring, but the ring was lost during subsequent subculture. Variation in the size of this ring was not observed in any cultures. Parental karyotypes were normal.

Patient B.—Pregnancy history and birth of this patient was unremarkable, but motor development was delayed from birth. In his late teens, he was diagnosed with insulin-dependent diabetes mellitus. Assessment at age 27 years showed severe mental retardation with no development of language skills. Height was 160 cm, weight was 76 kg, and head circumference was at 98th percentile. He had central obesity with gynecomastia and kyphosis. Minor dysmorphism includes narrow forehead and ridged occiput, downward slanting palpebral fissures, down-turned mouth, short philtrum, narrow pinna, narrow fingers with bilateral clinodactyly of the fifth finger, and syndactyly of toes 2 and 3. The karyotype was a 47,XY,+r(4). The size of the ring was less than the width of a chromosome, and the origin was chromosome 4 by FISH studies using a probe specific for the centromere of this chromosome (D4Z1, ONCOR) and by a whole chromosome library (pBS4, gift of Dr. Pinkel, University of California, San Francisco). A tentative diagnosis of partial trisomy 4q11 to q13 was established for this patient (Estop et al. 1993). The marker was de novo, as parental karyotypes were normal.

Microdissection and Amplification of Chromosomal DNA

Short-term lymphocyte cultures from peripheral blood of patient A were harvested with minimal exposure to acetic acid and spread on glass coverslips. The ring chromosome was stained with Leishman's stain and dissected under an inverted microscope with the use of fine glass needles controlled by an electronic micromanipulator (Eppendorf model 5170). Fifteen dissected chromosomes were PCR amplified by a sequence-independent amplification procedure as detailed by Bohlander et al. (1992). In brief, the procedure involves addition of buffer and the primer 5′-TGGTAGCTC-TTGATCANNNNN-3′ to the microdissected products. Two cycles of amplification with T7 DNA polymerase allow nonspecific amplification of DNA. The products of this amplification were added to a final volume of 30 μl with 1.5 μM of a universal primer: 5′-CUACUACU-ACUAGAGTTGGTAGCTCTTGATC-3′, 200 μM each dNTP, 1.5 mM $MgCl_2$, 55 mM KCl, 6.6 mM Tris-HCl (pH 9.0), 0.1 mg gelatin/ml, and 1.5 U *Taq* polymerase. The reaction was performed for 15 cycles at 94°C for 1 min, at 56°C for 1.5 min, and at 72°C for 2 min. A no-DNA control was utilized to test for possible contamination. A Southern blot using total human DNA as probe showed that the further amplified PCR products were a smear in comparison with primary PCR products, and the no-DNA control lane was blank.

Cloning and Analysis of Microdissected Products

PCR products were purified with the Magic™ PCR DNA purification system (Promega) and cloned into the vector pAMP10 (BRL). Colonies were transferred to Hybond-N^+ nylon membrane (Amersham) and probed with ^{32}P-labeled total human DNA to identify those clones containing high copy repeats. For colonies that did not contain repeats, the insert was amplified by PCR using the pAMP10 vector oligoprimers: forward, 5′-CACGACGTTGTAAAACGACGGCCAGT-3′, and reverse, 5′-TAATACGACTCACTATAGGG-3′. Insert sizes were estimated from agarose gels and those >300 bp were selected for further study. The PCR-amplified products from these selected clones were labeled with ^{32}P and used to probe filters from Southern blots of restricted DNAs from total human, mouse/human hybrids, and mouse (A9). The somatic cell hybrids used were CY170, which contains an intact chromosome 4, and CY120, which contains the region of chromosome 4 from 4pter to 4q25 (Callen et al. 1986). Since these hybrids also contain human chromosome 16, a hybrid containing only chromosome 16 (CY18), was used as an additional control.

Screening of Human Chromosome 4 Cosmid Library

PCR-amplified products that were unique and mapped to chromosome 4 were kinase labeled with $\gamma^{32}P$ and used to screen filters of a high density arrayed chromosome 4 cosmid library (Riess et al. 1994). Presumptive positive cosmids were confirmed by using the labeled DNA of the microdissected clones to probe filters of Southern blots of the restricted cosmid DNA.

FISH

The FISH procedure was as described elsewhere by Callen et al. (1990*a*). An fluorescein isothiocynate–labeled avidin/antibody step was used to amplify hybridization signal. Reverse painting was by FISH using PCR-amplified microdissected products labeled with biotin by nick translation. Biotin-labeled DNA from cosmids and a YAC were hybridized to metaphase spreads from the patients. The YAC clone, My884E7, was purchased from Research Genetics and is the most proximal member of the chromosome 4 short arm YAC contig. Counter-staining of the propidium iodide–stained chromosomes with DAPI allowed identification of chromosomes and location of signal to chromosome bands. Images of metaphase preparations were recorded on color slides or captured by a CCD camera and were computer enhanced.

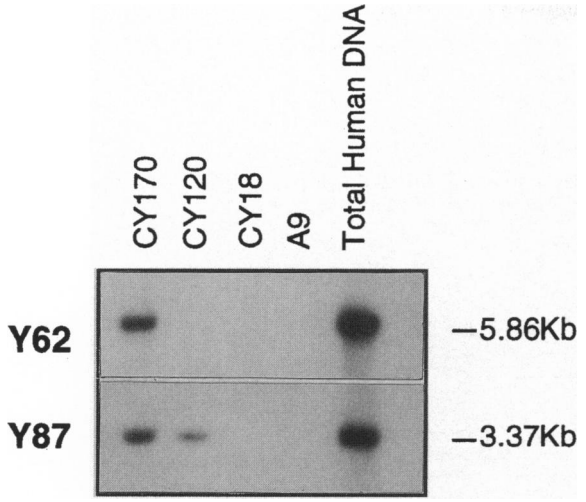

Figure 1 Southern blots using clones Y62 and Y87 as probes against *Hin*dIII-digested rodent/human hybrids CY170, CY120, and total human DNA. CY18, A9 were as control lanes.

Results

Reverse painting using the biotin-labeled microdissected PCR-amplified small ring chromosome 4 from patient A showed signal at the centromere of chromosome 4 and D group chromosomes (data not shown). In addition, signal was located at 4q31. The microdissected products were then used to construct a small library in pAMP10. Six clones (Y37, Y51, Y62, Y87, Y112, and Y136) with inserts of human DNA ranging from 300 to 600 bp were selected for further analysis. Probing DNA from somatic cell hybrids (fig. 1) demonstrated that all clones were on chromosome 4 since there was hybridization to the somatic cell hybrid CY170, which contained an intact chromosome 4. All clones but Y87 were negative for CY120. Since CY120 contains the region of chromosome 4 from 4pter to q25, that was consistent with these probes having a localization on the long arm of chromosome 4, distal to q25.

For use in FISH studies to allow the localization of the probes, cosmids were identified from a gridded human chromosome 4 library. The results are illustrated in figure 2 (*A* and *B*) and summarized in table 1. Additional FISH studies were undertaken with other cosmids and a YAC that map to various regions of chromosome 4 (table 1). These results confirm that the ring chromosome 4 of patient A consists of three discontinuous regions of this chromosome: the centromere (probe to pericentromeric repeat, D4Z1); the region of euchromatin on the long arm at 4q31 (cosmids c142B3, c234C8, c35H2, c77G3, and c69F1); and a region of the short arm at 4p13 or 14 (cosmid c269G2). The cosmid c176F1, located proximal to the centromere on the long arm at 4q12, did not hybridize to the ring of patient A but did hybridize to the ring of patient B. The ring of patient B is likely to contain a contiguous region from the centromere to q12. These results are illustrated in figure 2 (*C* and *D*). All other cosmids and the YAC (My884E7 at 4p11) did not hybridize to the ring chromosomes of the two patients.

Discussion

To investigate the structure of the accessary ring chromosome 4 in patient A, the ring was microdissected and DNA was amplified by degenerate-primer PCR. Six single-copy clones were used to isolate cosmids. FISH studies with these cosmids, and with additional markers on chromosome 4, demonstrated that the small ring in this patient consisted of three noncontiguous regions of chromosome 4. These regions were in the vicinity of 4p13 to 14 and at the centromere and at 4q31 (fig. 3). Reverse painting with PCR-amplified microdissected DNA demonstrated signal at the centromere and at 4q31. No signal could be seen at 4p13 to p14. FISH studies of the ring from patient B were consistent with an origin from a contiguous region from the centromere to 4q12 (fig. 3). However, in view of the results from patient A, it is possible that this marker may also contain noncontiguous DNA from this chromosome. This issue can only be resolved by microdissection of the ring and analysis of the cloned DNA. Both patients had in common mental retardation and similar minor dysmorphic features (brachycephaly, downward slanting eyes, and down-turned mouth) which are evident in their facial resemblance. These shared phenotypic abnormalities can only have a common genetic basis if there is pericentric euchromatin in common between the two rings, which has not been detected by the present study.

A previous approach to the analysis of marker chromosomes is by FISH using the PCR-amplified DNA from the microdissected marker as a probe (Thangavelu et al. 1994; Viersbach et al. 1994). This has been named "reverse painting." However, in patient A this technique was not sufficiently sensitive to detect a small noncontiguous region of euchromatin present in the short arm of chromosome 4, although the larger region on the long arm was detected. The microdissected DNA is a complex probe containing amplified products originating from the highly repetitive centromeric and pericentromeric repeats. The presence of these repeats may limit the sensitivity of this procedure.

There are numerous patients reported with a 46-chromosome complement where one entire chromosome is a ring. These rings are thought to arise by breaks at both ends of the chromosome and subsequent fusion of the open ends (Kosztolányi 1987). FISH studies using the consensus telomeric sequences as probes have established that such rings can form without loss of genetic

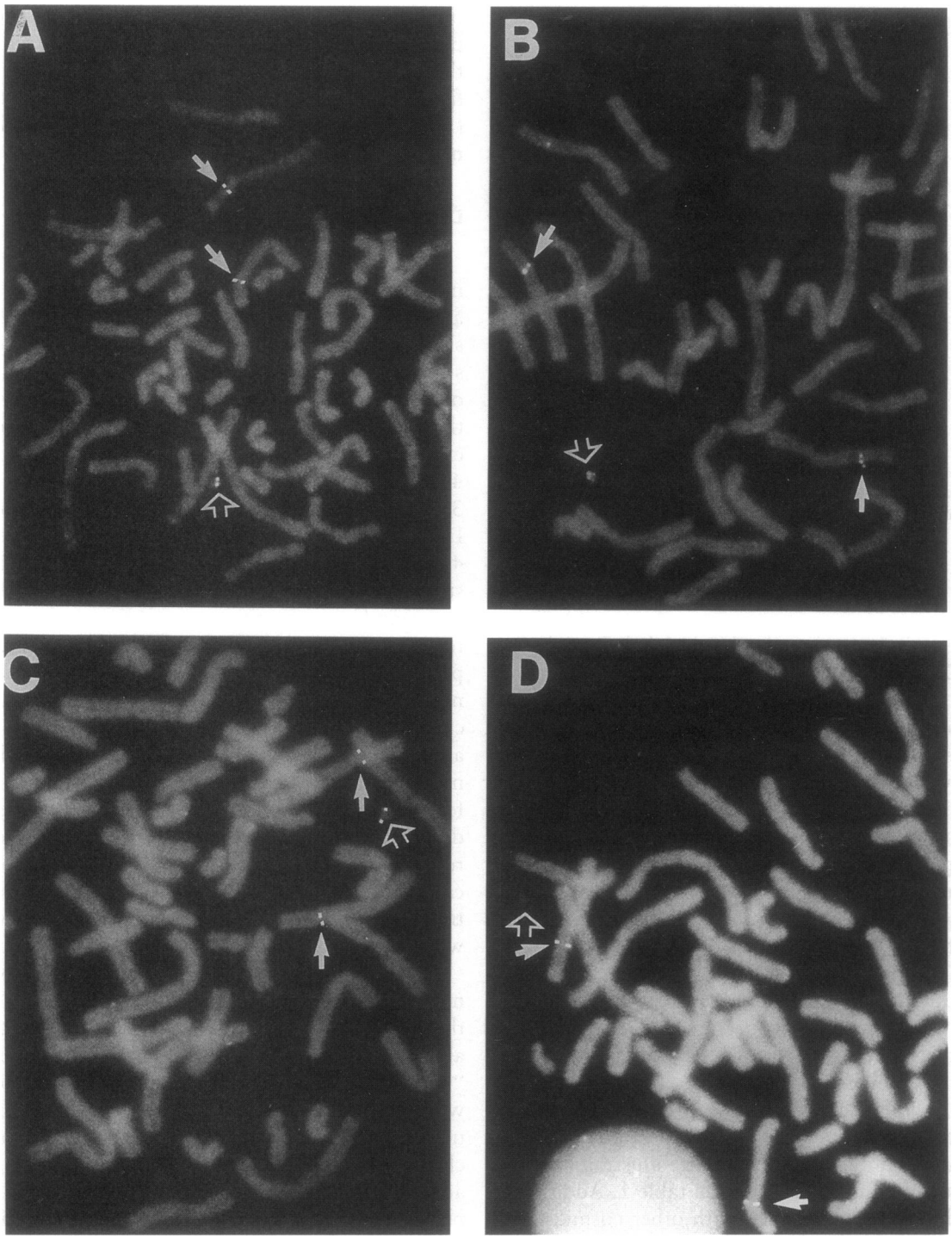

Figure 2 Two cosmids, 269G2 and 77G3, were separately located at 4p13-14 and 4q31.1 and also hybridized to the marker of patient A (*A* and *B*). The other chromosome 4 cosmid, 176F1, was located at 4q12 and hybridized to the marker of patient B (*C*) but did not hybridized to the patient A (*D*). The ring is indicated with the unblackened arrow and the normal homologues are indicated by the blackened arrows.

material, although the phenotype of patients with such rings can be abnormal, with failure to thrive and dysmorphism (Pezzolo et al. 1993). This so-called ring syndrome is thought to be caused by ring chromosome instability continuously generating aneuploid cells, which are subsequently lost. Instability of ring chromosomes in in vitro culture has been well documented. For example, Carter et al. (1969) reported a ring chromosome 4 in a

46, XY karyotype. A variety of derivatives of this ring, ranging from dicentric rings to various smaller products, were found, presumably generated by breakage and reunion of interlocked rings formed at cell division. It was found that among the different breakdown products of this large ring 4 there were 2 examples in 200 cells scored of small rings of similar size to that found in the two patients of this report (E. Baker, personal communication).

The small ring marker chromosomes found as accessary chromosomes were considered to arise from one break at the centromere, a second break in close proximity on either the long or short arm of the chromosome and with subsequent rejoining of the broken ends (Callen et al. 1991). This second break may be a "U-type" exchange. The evidence for this mechanism was based on the observation that ring chromosomes from chromosomes 1, 9, or 16 were either distamycinA/DAPI positive with a normal phenotype, or distamycinA/DAPI negative with an abnormal phenotype (Callen et al. 1990*b*, 1991). The distamycinA/DAPI-negative rings were considered to arise by one break at the centromere and a second within the euchromatin of the short arm, while the distamycinA/DAPI-positive ring originated by one break at the centromere and a second within the pericentric heterochromatin on the long arm.

Consistent with this origin is a small chromosome marker derived from chromosome 9, which was characterized by sorting, molecular cloning, and in situ hybridization by Raimondi et al. (1991). This chromosome contained the centromere and adjacent proximal region of the short arm of chromosome 9. The patient B reported here has a small ring chromosome derived from the centromere and adjacent proximal region of the long arm of chromosome 4.

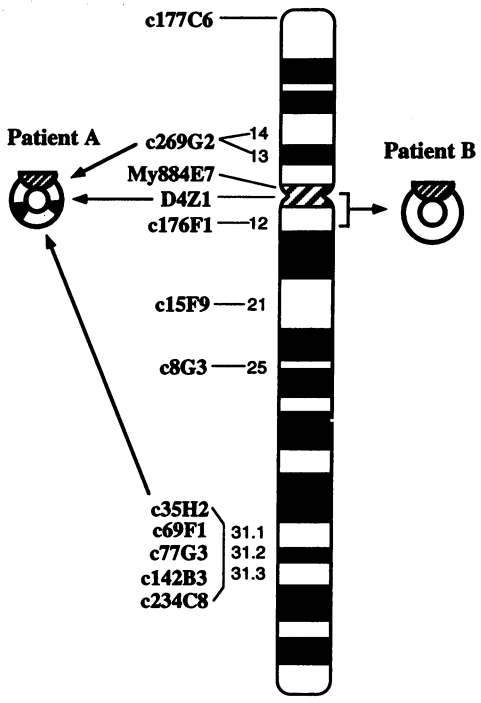

Figure 3 Ideogram of chromosome 4 showing localization of cosmids and YAC and origin of ring chromosomes in two patients.

The marker of patient A characterized in this report shows a complex structure and contains three discontinuous regions of chromosome 4 DNA. The origin of such a chromosome is consistent with the breakdown product from an original 47,+r(4) karyotype where the ring 4 was a typical large ring chromosome. It is suggested that this large ring was involved in breakage and reunion cycles as a result of the formation of interlocked rings during cell division. As a consequence, complex deletions of DNA have occurred until the stable form was generated.

In conclusion, the unexpected complex nature of a small ring derived from chromosome 4 adds a further complication to any attempt to ascertain common phenotypes between patients known to have similar markers derived from the same chromosome.

Table 1

Summary of FISH to Patients with Small Ring

Probe[a]	Cosmids	Location[b]	Ring Chromosome[c]	
			Patient A	Patient B
...	c177C6	4pter	–	–
Y87	c269G2	4p13-14	+	–
My884E7	...	4p11	–	–
D4Z1	...	Centromere	+	+
...	c176F1	4q12	–	+
...	c15F9	4q21	–	–
...	c8G3	4q25	–	–
Y37	c142B3	4q31	+	–
Y51	c234C8	4q31	+	–
Y51, Y62	c35H2	4q31	+	–
Y112	c77G3	4q31	+	–
Y136	c69F1	4q31	+	–

[a] The probes with the prefix "Y" are derived by microdissection from the ring of patient A.

[b] The location of the markers was determined from hybridization to normal chromosome 4's in metaphases of the patients.

[c] A plus sign (+) indicates that probe hybridized to ring chromosome; a minus sign (–) indicates that probe did not hybridize to ring chromosome.

Acknowledgments

We would like to thank Prof. G. R. Sutherland for support and helpful comments. This research was supported by the National Health and Medical Research Council of Australia. O.R. thanks Dr. L. L. Deaven for providing the chromosome

4-specific cosmid library. Work in the laboratory of O.R. is supported by the DFD (Ri3-1). The flow-sorted chromosome 4 library was constructed at the Human Genome Center, Los Alamos Medical Laboratory, Los Alamos, New Mexico, under the auspices of the U.S. Department of Energy.

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