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Molecular mechanism in the formation of a human ring chromosome 21

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ABSTRACT **We have characterized the structural rearrangements of a chromosome 21 that led to the *de novo* formation of a human ring chromosome 21 [r(21)]. Molecular cloning and chromosomal localization of the DNA regions flanking the ring junction provide evidence for a long arm to long arm fusion in formation of the r(21). In addition, the centromere and proximal long arm region of a maternal chromosome 21 are duplicated in the r(21). Therefore, the mechanism in formation of the r(21) was complex involving two sequential chromosomal rearrangements. (*i*) Duplication of the centromere and long arm of one maternal chromosome 21 occurred forming a rearranged intermediate. (*ii*) Chromosomal breaks in both the proximal and telomeric long arm regions on opposite arms of this rearranged chromosome occurred with subsequent reunion producing the r(21).**

Ring chromosomes in humans represent a class of aberrant chromosomes observed frequently in congenital anomalies and occasionally with normal phenotypes (1). Most ring chromosomes arise *de novo*, yet occasional familial transmission has been reported (2–5). The frequency of ring chromosomes is 1 in 25,000 recognized conceptions (6), and all human chromosomes have been observed as rings. Almost 50% of ring autosomes are derived from the acrocentric chromosomes (7). The proposed mechanism of ring formation, breakage of both short and long arms of a chromosome with subsequent end to end fusion (8), remains unproven. In several reports of acrocentric ring chromosomes, a Robertsonian translocation produced by short arm fusion has been observed in the patient, a parent, or a sibling (9–16). In each case, one or both of the chromosomes involved in the translocation were present in the ring, suggesting that ring formation was related to or derived from the translocation. In this study, we characterize a ring chromosome 21 [r(21)] and provide molecular evidence** for the formation of a rearranged chromosome 21 with a duplicated centromere and long arm that preceded ring formation.

MATERIALS AND METHODS

Sources of DNA. DNA was isolated from cultured amniocytes and fibroblasts and peripheral blood from the proband with the r(21) and his parents (17). Fibroblast and lymphoblastoid cell lines were established from the proband (GM 6137 and GM 6779) and a lymphoblastoid line was established from his mother (GM 8779) and stored in the Coriell Institute for Medical Research (Camden, NJ).

DNA fragment pPW231C of locus D21S3 is a 2.1-kilobase (kb) *Eco*RI fragment cloned in pBR328 (18, 19). Additional chromosome 21-specific probes, pPW228C of locus D21S1, pPW236B of locus D21S11, pPW245D of locus D21S8, and D21K9 of locus D21S13, were used (18). The superoxide dismutase 1 probe (SOD-1) (20) and β-amyloid probe (APP) (21) were also employed. The DNA fragment CW21A is a 650-nucleotide (nt) *Pvu* II–*Sph* I fragment, cloned in M13mp9, that spans the break point. The DNA fragment CW21B of locus D21S111 is a 530-nt *Hin*dIII–*Sph* I fragment located on the 5′ side of the break point, cloned in M13mp9 and pGEM-4.

Somatic Cell Hybrids. A somatic cell hybrid (R2-10) containing r(21) as the only detectable human chromosome was made (19). The R2-10 line was also back selected for loss of the r(21) (cell line R2-10W-S5) by growth in nonselective (purine supplemented) medium, followed by selective medium with the addition of bromodeoxyuridine and light (22). For mapping, a panel of somatic cell hybrids, 72532X-6, 153E7bx, and 2FuR1 (23), with characterized rearrangements of human chromosome 21 was used (19).

Cloning the Chromosomal Break Points. DNA from the patient's blood lymphocytes was digested with *Eco*RI and a size-selected library (7–9 kb) was made in λgtwes B. About 300,000 recombinant phage were screened (24) with pPW231C and a single clone, λ21BP (Fig. 1*B*), was purified. DNA from the region on the 3′ side of the break point was cloned (λ21pq) by using probe CW21A from a flow-sorted, *Hin*dIII-digested chromosome 21 library in Charon 21A (ID code, LL21NS02; American Type Culture Collection number, 57713).

Immunofluorescence Studies. For quantification of DNA, photographic negatives of 4′,6-diamidino-2-phenylindole (DAPI)-stained (25) images were scanned using a Loats (Westminster, MD) video densitometer equipped with a two-dimensional gel-scanning software package. Indirect immunofluorescence was performed on mitotic spreads (26). Chromosomes were first stained with DAPI and then with a monoclonal anti-centromere antibody prepared against the cloned 80-kDa human autoantigen CENP-B (27). Bound antibody was detected with a second antibody coupled to streptavidin linked to Texas red dye.

RESULTS

Cytogenetic Characterization and Origin of r(21). r(21) was observed in 90% of the cultured amniocytes, fibroblasts, and lymphocytes (28, 29); the remaining 10% of the cells were monosomic for chromosome 21. Break points were located at band 21q22.3 and in the pericentromeric region. r(21)s were variable in size, the majority (84%) being slightly larger than

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Abbreviations: r(21), ring chromosome 21; nt, nucleotide(s); DAPI, 4′,6-diamidine-2-phenylindole; MAR, matrix-association region; LINE, long interspersed repetitive element.

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**The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04504).

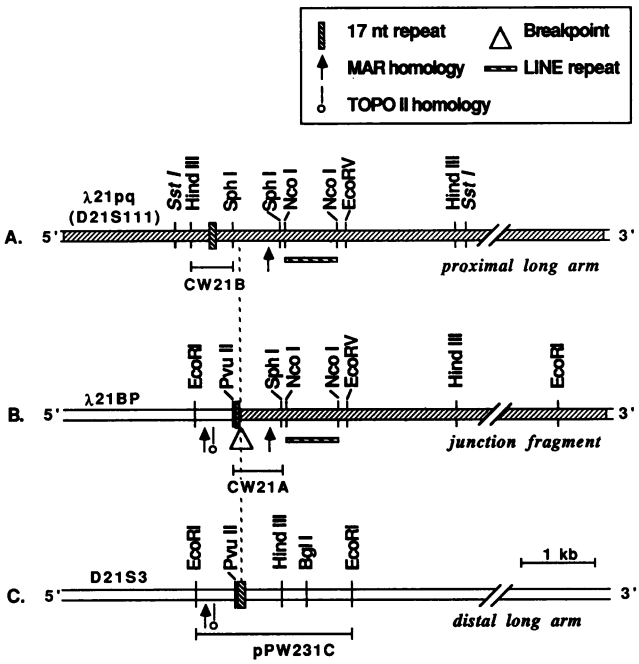

FIG. 1. Identification and cloning the ring junction fragment and surrounding normal DNA. The junction fragment λ21BP (*B*) as well as 3.5 kb from the surrounding region λ21pq (*A*) were cloned. The DNA fragment pPW231C is also shown (*C*). Restriction maps of the three cloned regions are aligned by the vertical dotted line representing the reunion site. The 5′ and 3′ orientations of the clones were arbitrarily designated. Regions of identity are indicated by the hatched or open bars. Probes CW21B and CW21A are underlined in *A* and *B*, respectively. Symbols identify DNA sequence determinants shown in Fig. 3.

the corresponding homologue. Dicentric rings approximately twice the size of the normal chromosome 21 were 12% of the r(21), and the remaining 4% of the rings appeared equivalent in size to the corresponding homologue. Densitometric scans of the r(21) from cultured fibroblasts were compared to those of the normal chromosome 21 after staining with DAPI. Optical density values for two control chromosomes 22 varied by <1%, whereas the r(21) had values 24% greater than the normal homologue in 8 of 8 metaphase spreads examined. (A total of 50 spreads were examined, and 8 were selected in which both chromosomes 21 and both chromosomes 22 were suitable for analysis.) A large region of constitutive heterochromatin was also visible at the centromeric region of the r(21) by C-banding.

The ring chromosome was absent from both parental karyotypes and no other rearrangements were observed. Parental inheritance was determined using a *Msp* I polymorphism of SOD-1 and a *Pst* I polymorphism of locus D21S58. The child inherited a paternal 6.4-kb SOD-1 allele and a maternal 4.2-kb allele. The somatic cell hybrid (R2-10) demonstrated only the maternal 4.2-kb SOD-1 allele (Fig. 2). Similarly, the somatic cell hybrid R2-10 contained the allele of the *Pst* I D21S58 polymorphism that is present only in maternal DNA. These results suggest that the r(21) arose either in maternal meiosis or very early in embryogenesis.

Cloning the Ring Junction and Corresponding Normal DNA Region. Genomic DNA from peripheral blood and fibroblasts of the child and his parents was analyzed. Probe pPW231C detected new DNA fragments in the child's DNA that were absent in the DNA of the parents with several restriction enzymes. Analysis with *Eco*RI, for example, showed a normal 2.1-kb fragment in all family members and a new 7.5-kb fragment in DNA isolated from the child's blood and fibroblasts, indicating that a chromosomal break occurred within the 2.1-kb *Eco*RI fragment pPW231C (29).

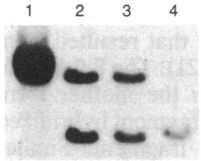

FIG. 2. Maternal derivation of the r(21). Autoradiogram of genomic DNAs digested with *Msp* I, Southern blotted, and hybridized to the SOD-1 probe. Lanes: 1, father; 2, mother; 3, child r(21); 4, r(21) hybrid R2-10.

The 7.5-kb *Eco*RI junction fragment was cloned (λ21BP) from blood lymphocytes of the child and the corresponding normal region of DNA brought into juxtaposition to pPW231C was also cloned as a 3.0-kb *Hin*dIII fragment from a chromosome 21 library. This latter fragment is λ21pq of Fig. 1*A* and has been designated locus D21S111. The restriction maps of the cloned fragments (Fig. 1 *A–C*) indicated that breakage occurred in λ21pq and pPW231C and that fusion was within λ21BP. DNA fragments on both sides of the junction were mapped to chromosome 21 by using somatic cell hybrids containing chromosome 21 (72532X-6) as the only human chromosome, thereby excluding involvement of chromosomes other than 21 in ring formation.

Nucleotide Sequence Comparisons of pPW231C, λ21BP, and λ21pq. DNA sequences of the 2.1-kb pPW231C fragment, the 2.7-kb from the 5′ end of λ21BP, and the 1.5-kb from the 5′ half of λ21pq were determined (Fig. 3). The sequences of λ21BP and pPW231C 5′ to nt 623 are identical except for a nt substitution at position 574, a probable neutral sequence polymorphism. No differences were noted between λ21BP and λ21pq in the 1.5-kb region sequenced 3′ to the junction point.

At the ring junction, 9 nt, CATTCACCA, of unknown origin were inserted (Fig. 3). Both break points occurred within single-copy sequences; pPW231C is single-copy and the first 1 kb at the 5′ end of λ21pq is also free of repetitive sequences. Two single-copy probes flanking the ring junction (CW21B, just 5′ to the break point, and CW21A, on the 3′ side) represent contiguous fragments in normal DNA, yet they were derived from two separate clones, λ21pq and λ21BP, respectively (see Fig. 1 *A* and *B*).

Further analysis demonstrated seven MARs (31) between nt −450 and +440 relative to the ring junction point. Homology to a topoisomerase II binding consensus sequence (30) is present 400 nt 5′ to the ring junction (Fig. 3). Beginning 1 kb 3′ to the break point, 750 nt of the 3′ end of a long interspersed repetitive element or LINE repeat [nt 5410–6160 of the consensus LINE sequence (32)] were also observed.

A computer search for internal homology or direct repeats within pPW231C, λ21BP, and λ21pq revealed two DNA homologies involving sequences at both break points. (*i*) A 17/17-nt match (GGTCAGAAAAGG/GTGTT) between a sequence at the break point in pPW231C and a complementary sequence in the reverse orientation located 335 nt 5′ to the break point in λ21pq was present (Figs. 1 and 3). A 17-mer probe containing this sequence detected only eight fragments in the entire human genome, at least two of which were present on chromosome 21 (data not shown). The same sequence was at least 100-fold more abundant in the hamster genome. (*ii*) A 13/13-nt match (TTT/GCTTGGCATG) involves a sequence at the break point in λ21pq and a complementary sequence 557 nt 3′ to the ring junction in λ21pq.

Chromosomal Mapping of CW21A and CW21B. Southern blot analysis using a panel of somatic cell hybrids with various chromosome 21 deletions (19) (Fig. 4*A*) showed that CW21B is located on the long arm of chromosome 21. Probe CW21B hybridized to DNA from line 153E7bx containing an intact 21q with human centromeric sequences but lacking the distal short arm. Probe CW21B was also present in cell line 2Fu^{r}1 that contains an intact 21q but little if any human centromere or short arm material. This confirms the unexpected result that

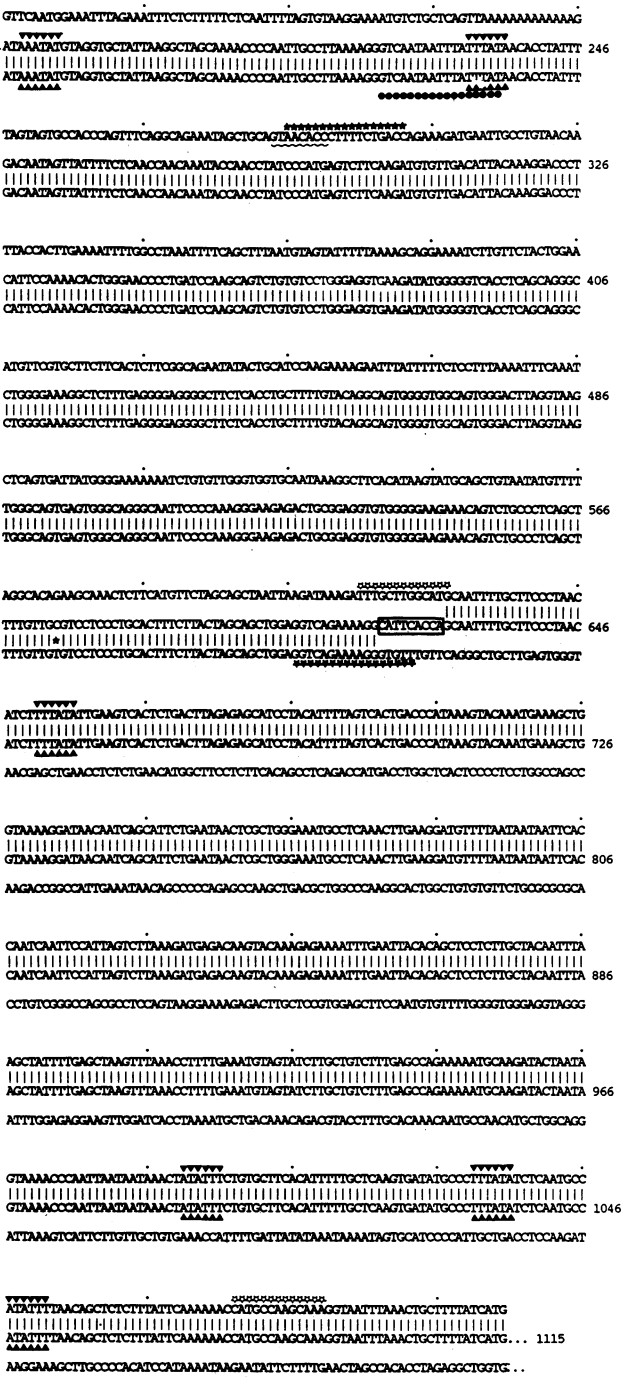

FIG. 3. DNA sequence determinants near the ring junction. Partial DNA sequences surrounding the breakage and reunion sites are aligned with homologies noted by the vertical lines. The normal proximal long arm sequence, λ21pq (sequence 1), is shown on the top line and the normal distal long arm sequence, pPW231C (sequence 3), is shown on the bottom line. The middle line of sequence, λ21BP (sequence 2), represents the reunion of the two sequences. The 9 nt inserted at the ring junction are enclosed in a box. The 17-nt repeat in pPW231C and λ21pq is noted by solid stars and the 13-nt repeat in λ21pq is shown by open stars. Matrix association region sequences (MARs) on both sides of the break point are indicated by solid triangles. The topoisomerase II homology with the consensus GT-NWAYATTNATNNR (where W is A or T, Y is C or T, and R is A or G) (30) is shown by the solid circles below the sequence. The nucleotide sequence difference between λ21BP and pPW231C is indicated by an asterisk.

the DNA fragment CW21B from locus D21S111 is located on the proximal long arm of chromosome 21.

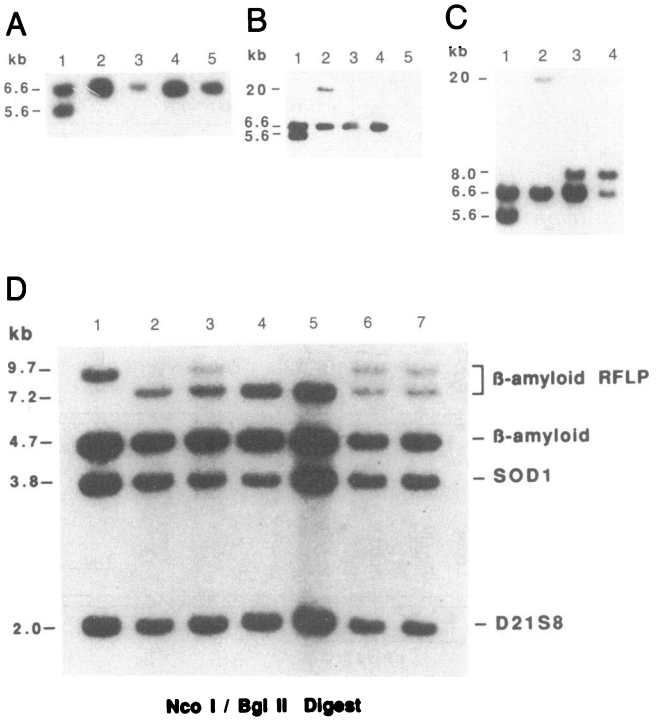

FIG. 4. Chromosomal localization of the DNA fragments CW21A and CW21B on the proximal long arm of chromosome 21. (*A*) CW21B was hybridized to a panel of DNAs isolated from hamster–human hybrid cell lines with characterized deletions of chromosome 21 and digested with *Sst* I. Lanes: 1, father; 2, child r(21); 3, r(21) hybrid; 4, 2 Fu^{r}1; 5, 153E7bx. (*B*) The DNA fragment CW21B located on the 5′ side of the break point and adjacent to CW21A in normal DNA detects the same normal *Sst* I fragments as CW21A upon Southern blot hybridization and does not hybridize to sequences across the break point (C). R2-10W-S5 is the somatic cell hybrid that has lost the r(21). Lanes: 1, father; 2, mother; 3, child r(21); 4, r(21) hybrid; 5, R2-10W-S5. (*C*) The two *Sst* I polymorphisms detected with probes CW21A and CW21B are shown by Southern blot analysis of DNA from the father (lane 1), mother (lane 2), child (lane 3), and r(21) hybrid (lane 4). Hybridization with CW21A is shown. The abnormal 8.0-kb fragment represents hybridization to sequences across the ring junction. (*D*) Southern blot analysis and gene dosage studies of DNA from the father (lane 1), mother (lane 2), patient (lane 3), r(21) hybrid (lane 4), a patient with trisomy 21 (lane 5), and normal control (lanes 6 and 7) hybridized with probes APP, D21S8, and SOD-1. DNA was digested with *Bgl* II and *Nco* I for the β-amyloid polymorphism.

Linkage analysis was also performed to map CW21A and CW21B using three DNA polymorphic sites identified by either probe: an *Sst* I site with allele frequencies in Caucasians of 0.5 and 0.5, a second *Sst* I site limited to Blacks with allele frequencies of 0.9 and 0.1, and a *Hin*cII site with allele frequencies of 0.9 and 0.1. Two- and multi-point linkage analysis placed locus D21S111 (CW21A and CW21B) ≈14 cM (1 centimorgan ≈ 1000 kb) distal to D21S1 and D21S11 (θ = 0.14; z = 17.26); D21S111 also maps ≈24 cM distal to the most proximal long arm marker D21S13 (46) (see Fig. 6 for chromosomal locations of D21S1, D21S11, and D21S13).

Duplication of Proximal 21q Loci in the r(21). DNA polymorphism analysis using probe CW21A (Fig. 4*C*) revealed that the father is heterozygous for the common *Sst* I polymorphism (6.6/5.6-kb alleles), whereas the mother is heterozygous for the rarer polymorphism (20/6.6-kb alleles). In the child's DNA, a normal 6.6-kb fragment was observed in addition to an abnormal 8.0-kb fragment that resulted from hybridization across the junction in the r(21). The 8.0-kb and the 6.6-kb fragments both derived from the mother were present in the r(21) hybrid, yet the 8.0-kb fragment hybridized with greater intensity than the normal fragment. Similar

results were obtained with a second independent r(21) hybrid line indicating that the intensity difference was not an artifact in construction of the hybrid. The DNA fragment CW21A was not present in the Chinese hamster genomic DNA nor in the cell line R210W-S5 that lost the r(21).

Similar analysis with probe CW21B revealed the normal 6.6-kb fragment in the DNA from the child and the r(21) hybrid (Fig. 4*B*). Since probe CW21B does not span the break point, hybridization across the junction is not detectable. Also, this probe is derived from λ21pq that contains only normal DNA sequences and is not present in clone λ21BP. Its presence in an unrearranged form in the r(21) hybrid suggests that distal long arm sequences including CW21B are present in one copy in the r(21), whereas proximal long arm sequences, including CW21A, are present in two copies.

Five long-arm chromosome 21 loci, all of which are more proximal than D21S111 by linkage analysis (D21S13, D21S1, D21S11, D21S8, and β-amyloid), were examined and shown to be present in approximately two copies in the r(21) hybrid, three copies in the patient, and two copies in his mother (data for D21S8 and β-amyloid are in Fig. 4*D*). When the autoradiograms were in the linear range of exposure, they were scanned using a densitometer. For example, ratios of β-amyloid (a proximal marker) to SOD-1 (a distal marker) were 1.6 in controls and the parents, 2.7 in the child, and 5.0 in the r(21) hybrid. Similarly, ratios of D21S8 (another proximal marker) to SOD-1 were 0.85 in controls and the parents, 1.58 in the child, and 2.3 in the r(21) hybrid. A β-amyloid polymorphism showed a 1:2 ratio of the 9.7-kb paternal allele to the 7.2-kb maternal allele in the child. The hybridization intensity of SOD-1 (Fig. 4*D*) and other probes (data not shown) distal to D21S111 suggests that one copy of each of these markers is present in the r(21) hybrid, whereas two copies are present in the patient and his mother. In addition, the orientation of CW21A and CW21B with respect to the centromere is evident; duplication of CW21A and all proximal sequences places this probe closer to the centromere than CW21B, which is present in only one copy.

Evidence for Increased Centromeric Material in r(21). The extra 24% of A+T-rich material detected by densitometry of DAPI-stained chromosomes and C-banding was confirmed by immunofluorescence with a monoclonal antibody directed against CENP-B (27). Double immunostaining first with DAPI and then with the anticentromere antibody was performed on unfixed chromosomes from fibroblasts from the patient. All normal chromosomes including the normal chromosome 21 showed a single pair of fluorescent dots at the centromere. However, the r(21), which was clearly larger than a single-size ring and not a double-size ring, showed a complex pattern of fluorescence (Fig. 5). At least three fluorescent regions were always observed very close together. Similar analysis in the mother demonstrated that there were no differences in the intensity or quantity of fluorescence at the centromere in the chromosomes 21.

DISCUSSION

The simplest model of ring formation involves breakage in both arms of a single chromosome followed by end to end reunion. Our data suggest, however, that the mechanism of formation of this ring chromosome is more complex. The following observations suggest that an additional chromosomal rearrangement preceded ring formation. (*i*) The DNA fragments on both sides of the ring junction were both derived from the long arm of chromosome 21. (*ii*) All DNA fragments proximal to and including CW21A were present in two copies on the r(21), and the adjacent DNA fragment CW21B was present in the r(21). (*iii*) Increased chromosomal material at the centromeric region in the r(21) was observed. We, therefore, propose that the first step in the genesis of the r(21) was the formation of a rearranged intermediate with duplication of the centromere and the long arm of a maternal chromosome 21. The r(21) contained two copies of one allele for D21S1 and D21S11 polymorphic markers; however, the mother's DNA showed both polymorphic alleles for these markers (data not shown). Therefore, duplication of the long arm of a single maternal chromosome 21 forming an isodicentric chromosome is favored. A distal break in the telomeric region of one of the arms in the rearranged chromosome in D21S3 and a break in the proximal region of the other long arm (in D21S111) (Fig. 6*B*) with subsequent fusion resulted in a dicentric ring structure ≈1.25 times larger than the normal chromosome 21 and composed of material from both long arms of the rearranged chromosome (Fig. 6*C*).

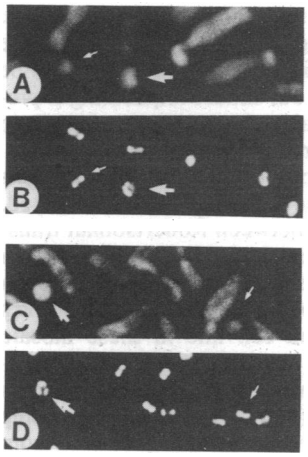

FIG. 5. Immunofluorescence analysis of the r(21) by using a monoclonal anti-centromere antibody. (*A* and *C*) Chromosomal DNA stained with DAPI. (*B* and *D*) Indirect immunofluorescence. Portions of spreads in which the r(21) (larger arrows) and normal 21 (smaller arrows) may both be seen. (×600.)

The mechanism of ring formation we propose predicts duplication of the proximal long arm region from the break point in D21S111 extending toward the centromere thereby explaining the presence of CW21A in two copies in the r(21)

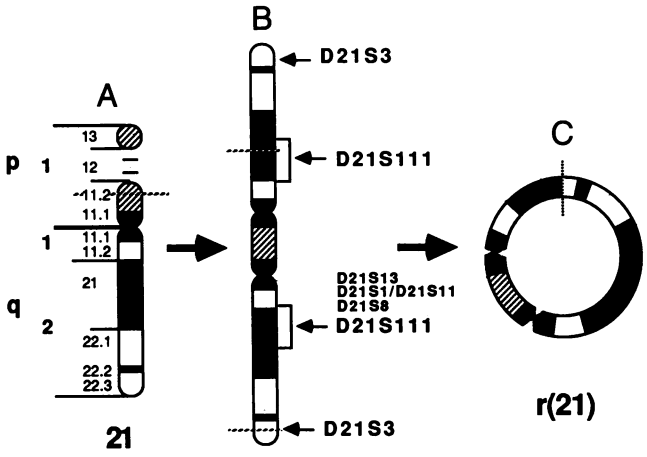

FIG. 6. Schematic representation of the two-step mechanism of r(21) formation. (*A*) Chromosome 21 is depicted with the centromere indicated by the indentation and thick line. Shaded and solid blocks correspond to the banding pattern observed after G-banding. Dotted line in the proximal p arm (arbitrarily drawn in p11.2) represents the postulated break points involved in the duplication event. (*B*) Duplication event results in the rearranged chromosome intermediate shown. Two p-arm regions are fused with retention of two centromeres but loss of telomeric p-arm regions including the stalk, ribosomal DNA and nucleotar organizing regions. The chromosomal locations of the two DNA regions directly involved in ring formation, D21S3 and D21S111, are noted by the arrows. In the second event, two additional and asymmetric breaks on opposite sides of the centromere occur as noted by the dotted lines, one in band q22.3 in D21S3 on one long arm and the second in the proximal long arm in D21S111. (*C*) Reunion of the broken arms results in the ring structure depicted. Two centromeric regions are present plus 25% more proximal long arm material. The dotted line represents the reunion site.

hybrid. The difference in intensity of hybridization of the abnormal to normal bands (Fig. 4*C*) also suggests that the entire region of DNA containing the ring junction may be duplicated. Most importantly, our model implicating a preceding duplication event explains the presence of the two centromeric regions in close proximity in the r(21).

In several reports of ring chromosomes, translocations between two acrocentric chromosomes have also been noted. In four instances, associations between Robertsonian translocations involving chromosome 21 and r(21)s have been reported (10, 11, 13). The evidence that these rings follow a t(21q;21q) Robertsonian translocation is indirect and derives from the presence of a second cell line in these mosaic individuals that was 46,−21,+t(21q;21q). In addition, a translocation between two chromosomes 21 was reported in a fetus of a normal r(21) carrier mother (33). In two reports describing a r(15) and a r(13), both rings were secondarily derived from a maternal Robertsonian translocation, t(15q;15q) or t(13q;13q) (13, 14). Three cases of Robertsonian translocations involving nonhomologous acrocentric chromosomes have also been reported, a t(13q;14q) (9), a t(13q;15q) (16), and a t(15q;22q) (34) that resulted in two r(13) chromosomes and a r(22), respectively, in the offspring. Maternal mosaicism was implicated in two of these reports.

To date, there is no direct evidence suggesting that chromosomal break points are site specific or that they occur by homologous recombination. We found no sequence similarities between the ring junction and mitotic break points from oncogene translocations (35), immunoglobulin rearrangements (36), and large deletions within the β-globin gene cluster (37–39), except for the insertion of random nucleotides at the r(21) junction, a common feature of translocation break points. However, certain distinctive sequences in the vicinity of the ring junction may have been influential in the genesis of the ring. MARs were observed within 400 bp on either side of the junction point (Figs. 1 and 3). The nuclear matrix has been proposed as an attachment site for chromatin loops (40), and preferential binding of a κ immunoglobulin gene segment to nuclear matrices by A+T-rich sequences (MARs) has been reported (31). Possibly, sequences involved in r(21) formation were contained in two adjacent chromosomal loops anchored to the nuclear matrix through MAR–matrix interactions. Furthermore, MAR sequences are also found close to consensus topoisomerase II-binding sites (30), and topoisomerase II itself is a structural element located at the bases of chromatin loops (41). A topoisomerase II consensus sequence is 400 bp from the ring junction.

In several deletions, *Alu* members were at the site of recombination and were identified as potential hot spots for illegitimate recombination (42, 43). Similar observations have been made for LINE sequences (37). Furthermore, it has been proposed that members of the LINE family participate in nuclear-matrix attachment (44). The 3′ end of a LINE repeat is located just 3′ to the junction point in the ring.

Phenotypic variation among patients with r(21) syndrome (45) suggests that the chromosomal break points and extent of deleted material are different in each patient. Additional rings of acrocentric chromosomes need to be studied to map the location and determine the nature of sequences involved in the reunion. A rearranged chromosome that contains two long arms from one or two acrocentric chromosomes appears to be a common preceding event, perhaps predisposing the chromosome structure to acrocentric ring formation.

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