Molecular Mapping and Cloning of the Breakpoints of a Chromosome 11p14.1–p13 Deletion Associated with the AGR Syndrome

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Chromosome 11p13 is frequently rearranged in individuals with the WAGR syndrome (Wilms tumor, aniridia, genitourinary anomalies, and mental retardation) or parts of this syndrome. To map the cytogenetic aberrations molecularly, we screened DNA from cell lines with known WAGR-related chromosome abnormalities for rearrangements with pulsed field gel (PFG) analysis using probes deleted from one chromosome 11 homolog of a WAGR patient. The first alteration was detected in a cell line from an individual with aniridia, genitourinary anomalies, mental retardation, and a deletion described as 11p14.1–p13. We have located one breakpoint close to probe HU11-164B and we have cloned both breakpoint sites as well as the junctional fragment. The breakpoints subdivide current intervals on the genetic map, and the probes for both sides will serve as important additional markers for a long-range restriction map of this region. Further characterization and sequencing of the breakpoints may yield insight into the mechanisms by which these deletions occur. © 1988 Academic Press, Inc.

Rearrangements of chromosome 11 are implicated in a variety of clinical disorders. Among the first cytogenetic abnormalities found were frequent deletions on the short arm involving band p13 in patients with WAGR syndrome, which consists of Wilms tumor, aniridia, genitourinary malformations, and mental retardation (Francke et al., 1979; Riccardi et al., 1980). For the formation of Wilms tumor, the most common solid tumor in childhood, a two-step model of tumorigenesis was postulated (Knudson and Strong, 1972) with the first step already inherited in the familial form of the tumor. Studies on polymorphisms in tumors and normal tissue (Fearon et al., 1984; Koufos et al., 1984; Orkin et al., 1984; Reeve et al., 1984) indicated that these two steps might result in the functional inactivation of a recessive gene through deletion or reduction to homozygosity for a mutant allele.

Recent cytogenetic studies (Douglass et al., 1985) suggest that rearrangements at chromosome 11p13 occur frequently in Wilms tumors from patients with normal phenotypes. Individuals having only the developmental defects of the AGR triad also exhibit chromosome 11p abnormalities in the absence of tumor formation. In two kindreds of familial aniridia (Simola et al., 1983; Moore et al., 1986) the disorder segregated with translocation chromosomes with chromosome 11 breakpoints at band p13 in both cases. These findings indicate that band p13 of chromosome 11 likely contains not only a tumor suppressor gene but also genes involved in differentiation and development of several organ systems. Furthermore, the frequent chromosome aberrations described for this region raise the question whether these rearrangements involve a common mechanism or specific sites which facilitate recombination.

In the present report we describe the molecular mapping of a 11p14.1–p13 deletion found in a patient with aniridia, genitourinary anomalies, and mental retardation. The breakpoints have been cloned and define new markers for a more detailed map of this region.

MATERIALS AND METHODS

Cell Lines

GM 7736 cells were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). The 6697 lymphoblastoid cells were derived from a karyotypically normal male. E36 hamster cells and hybrid cell lines G35F3, G95A4, RJK34, G156F3, G157A6, and G157A2 have been previously described (Scott et al., 1979; Bruns et al., 1984; Glaser et al., 1986).

DNA Extraction and Southern Blot Analysis

Genomic DNA was purified by SDS/proteinase K treatment and repeated extractions with phenol and chloroform followed by isopropanol precipitation

(Maniatis *et al.*, 1982). DNA concentrations were measured spectrofluorometrically (Brunk *et al.*, 1979) and restriction enzyme digests were performed according to the manufacturer's instructions with 4–10 units/µg DNA for at least 3 h. Samples were separated in 0.7% agarose gels and transferred to Gene Screen membranes (NEN, Boston, MA) with 0.5 N NaOH, 1.5 M NaCl overnight. Filters were then neutralized in 50 mM NaPO$_4$, pH 7.2, and baked for 2 h at 80°C, and nucleic acids were crosslinked to the membrane by uv irradiation (Church and Gilbert, 1984).

Hybridization Conditions

Purified DNA fragments were labeled according to Feinberg and Vogelstein (1984). Probes containing repeated sequences were competed with human DNA prior to hybridization as follows (Sealey *et al.*, 1985). Labeled DNA was boiled with 1 mg sheared placental DNA in 5× SSC in a total volume of 100 µl and allowed to reanneal for 10–15 min at 68°C before being mixed with hybridization buffer. After prehybridizing for 1 h in 0.5 M NaPO$_4$, pH 7.2, 7% SDS, 1 mM EDTA at 65°C (Church and Gilbert, 1984), filters were hybridized overnight in fresh buffer with 50 µg/ml salmon sperm DNA and 2–10 × 10^7 cpm radioactive probe. Filters were washed in 40 mM NaPO$_4$, pH 7.2, 1% SDS at 65°C. Filters could be reused after stripping of the probe by incubating in 0.1× TE, 0.1% SDS at 75–80°C for 30 min.

Gene Dosage Studies

Equal amounts of *Eco*RI-digested DNA from GM 7736 and control cell lines were separated electrophoretically and transferred to Gene Screen membranes. After hybridization and exposure to X-ray film, the autoradiograms were evaluated independently by two investigators. Relative band intensities were measured with an LKB laser scanning densitometer.

PFG Analysis

Embedding of DNA and subsequent manipulations were performed according to protocols provided by D. Barlow and H. Lehrach (EMBL, Heidelberg, FRG). In brief, cells were mixed with 1% LMP-agarose (BRL) in PBS giving a final concentration of 1–2 × 10^7/ml and poured into 100-µl block formers. After incubating in ESP (0.5 M EDTA, pH 8, 1% Na-laurylsarcosine, and 1 mg/ml proteinase K) for 2 days at 50°C, the blocks were washed extensively in TE at room temperature, twice at 50°C including 0.04 mg/ml PMSF (BRL), and were finally stored in 0.5 M EDTA, pH 8 at 4°C.

For restriction enzyme digests the blocks were cut into halves and equilibrated in the appropriate buffer. Digests were performed in 120 µl total volume with 20

units of enzyme for at least 6 h. Electrophoresis was carried out in an LKB pulsaphor apparatus using the double inhomogeneous field electrodes (Schwartz and Cantor, 1984; Carle and Olsen, 1984) or the CHEF electrode array described by Chu *et al.* (1986). DNA transfer and hybridizations were done as previously described with an extended blotting time of 48 h to allow transfer of the large DNA molecules.

Phage λ multimers and *Saccharomyces cerevisiae* chromosomes were used as size standards. Phage multimers were prepared by embedding PEG-precipitated Charon 21A in LMP-agarose, digesting in ESP, and annealing of the cos ends in 0.1 M EDTA, pH 8 at 50°C for 2 days. Chromosomes from *S. cerevisiae* were prepared as described by Carle and Olson (1985).

Genomic Cloning

A *Mbo*I partial digest library of normal human lymphoblast DNA in EMBL 3 phage was generously provided by Dr. S. Orkin. For the GM 7736 complete *Bam*HI digest library, 10 µg of DNA was digested with 100 units of *Bam*HI for 3 h followed by phenol and ether extraction and dialysis against TE. One microgram of digested DNA was ligated with 2 µg of *Bam*HI cut EMBL 3 DNA (Stratagene, La Jolla, CA) at 4°C overnight and packaged *in vitro* with Gigapack gold (Stratagene). After plating on *Escherichia coli* strain P2392, 400,000 clones were obtained.

Screening of phage libraries and subcloning of DNA fragments into plasmid vectors pUC19 and pBLUESCRIPT (Stratagene) were done according to published procedures (Maniatis *et al.*, 1982). Phage DNA minipreps were carried out essentially as described by Helms *et al.* (1985).

RESULTS

Detection of the Rearrangement

To obtain probes from the region of chromosome 11 that is frequently deleted in WAGR patients, we screened phage inserts from the complete *Hind*III digest flow-sorted chromosome 11 library of the National Gene Library Project (LL11NS01) against a panel of human–rodent somatic cell hybrid lines containing chromosome 11 homologs with different WAGR deletions (Bruns *et al.*, in preparation). Single-copy pieces were sought for clones that mapped to deleted areas, and we created long-range restriction maps by digestion of agarose-embedded genomic DNA with rare-cutting restriction enzymes and separation of fragments with pulsed field gradient gel electrophoresis (Schwartz and Cantor, 1984; Carle and Olsen, 1984). With these maps as a reference we examined DNA from four cell lines with cytogenetically described chromosome 11 aber-

rations for indications of rearrangements within the mapped regions around 12 probes.

The first alteration found was that in DNA from the cell line GM 7736 which was derived from an individual with bilateral aniridia, genitourinary anomalies, mental retardation, and a deletion described as 11p14.1–p13. Probe HU11-25, which hybridizes to a SfiI fragment of 660 kb in DNA from normal cells, detected an additional band of about 600 kb for the cell line GM 7736 upon PFG analysis (Fig. 1). Subsequent digests with additional rare-cutting enzymes revealed more abnormal fragments which hybridized with intensities comparable to those of the unrearranged fragments in the same DNA. A SacII band of 900 kb was shortened by 150 kb, and a 1.2-Mb NotI fragment appeared under conditions where the normal fragment would not have entered the gel (not shown). These multiple aberrations seen with probe HU11-25 only in GM 7736 and not in any other cell line tested made it very likely that this probe detected the rearranged fragments of the 11p14.1–p13 deletion. The restriction map around HU11-25 from a normal cell line (Fig. 1) puts this presumed breakpoint between the SfiI and BssHII sites, as digestion with BssHII did not produce an additional fragment in GM 7736.

A second random probe, HU11-164B, could be linked to HU11-25 by shared SacII and SfiI fragments of 900

and 660 kb (data not shown) and is localized in the SfiI/BssHII interval to which the GM 7736 breakpoint was assigned. Gene dosage studies showed that both alleles for this probe were present in GM 7736. As expected, all the altered bands previously detected by HU11-25 were again seen with HU11-164B. This probe also identified a rearranged BssHII fragment, confirming its position closer to the putative breakpoint.

Cloning of the Breakpoint

Several restriction enzymes (ApaI, BamHI, BclI, BglII, EcoRI, HindIII, XhoI) that cut more frequently in human DNA were then used to estimate the distance from HU11-164B to the site of the GM 7736 rearrangement. KpnI was the enzyme that produced the shortest fragments among those detecting altered restriction patterns. The sizes were calculated as 11 kb for the normal and 13 kb for the altered band. Probe HU11-164B was used to screen a genomic MboI partial digest library in EMBL 3 phage, and two of the overlapping clones, λ164-5 and λ164-8, spanning 25 kb were analyzed further. One of the subclones, 0.6ES, detected rearranged fragments in GM 7736 DNA with every enzyme tested upon conventional Southern blot analysis (Fig. 2C). Only the regular bands light up on the same blot (Fig. 2B) with probe 0.2SB, which is immediately
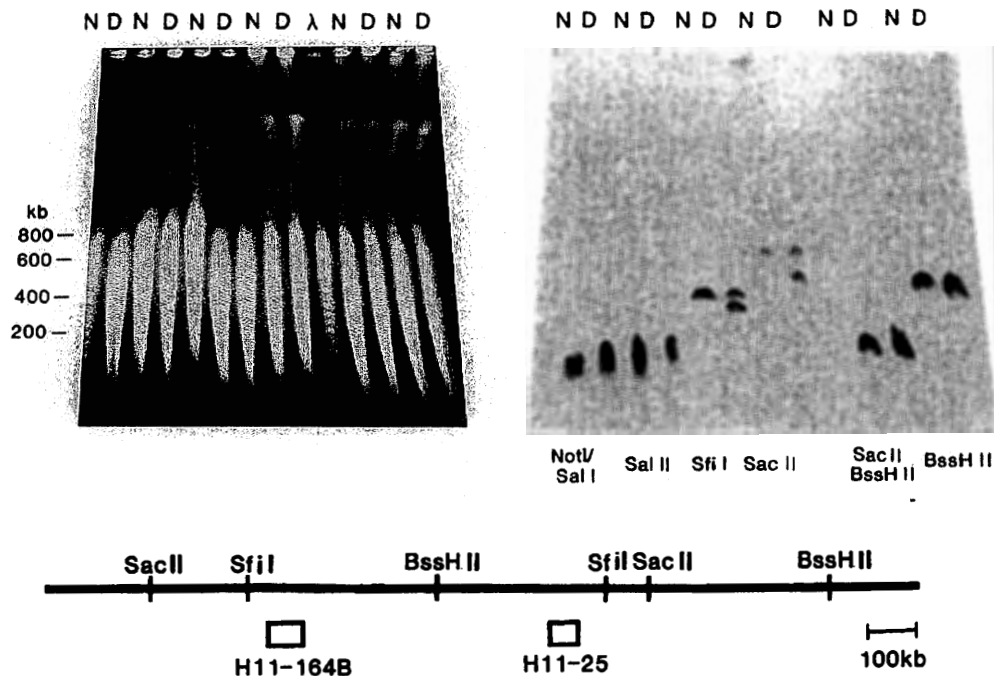

FIG. 1. Detection of the GM 7736 rearrangement with probe HU11-25. Agarose blocks with GM 7736 (D) and 6697 control (N) DNAs were digested with the enzymes indicated and separated in a 1% agarose gel using the CHEF electrode array (Chu et al., 1986) at 170 V with 80 s switching time and 11°C buffer temperature. **Left:** The ethidium bromide staining with Charon 21A multimers as size markers. **Right:** The hybridization pattern with probe HU11-25. The restriction map around probes HU11-25 and HU11-164B is indicated with the presumed breakpoint between HU11-164B and the flanking SfiI site.
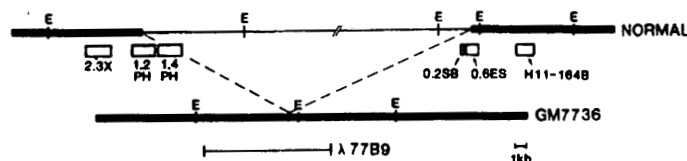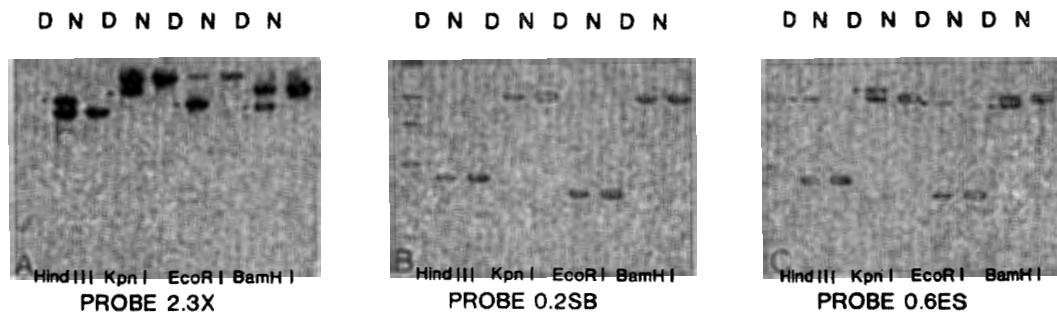
FIG. 2. Analysis of the GM 7736 breakpoints. Four micrograms of GM 7736 (D) and 6697 control (N) DNA digested with the enzymes indicated below was separated electrophoretically and transferred to Gene Screen membrane. The same filter was used for hybridization with probes 2.3X (A), 0.2SB (B), and 0.6ES (C). Arrows indicate the rearranged fragments detected by probes 2.3X and 0.6ES. Size markers in the left lanes are radioactively labeled λ-HindIII fragments. The restriction map of the normal and deleted allele of GM 7736 is shown. EcoRI (E) restriction site as well as the hybridization probes used and the location of the cloned fragment from the deleted chromosome are indicated.

adjacent to 0.6ES in normal DNA. Furthermore, gene dosage analysis showed that probe 0.2SB is present only on one allele in this cell line. This confirms that probe 0.6ES contains the site of the deletion breakpoint in GM 7736, whereas probe 0.2ES is deleted from one chromosome 11 homolog.

To obtain the deletion junction, contained within the rearranged BamHI fragment of 9.5 kb (Fig. 2C), we prepared a complete BamHI digest library of GM 7736 DNA in EMBL 3 phage without prior phosphatase treatment of the insert to ensure clonability of this fragment. The library was screened with probe 0.6ES, and the BamHI insert of one of the resulting nine clones, λ77B-9, showed restriction patterns compatible with the rearranged allele. A 2.3-kb XbaI fragment (2.3X) which was absent from clones λ164-5 and λ164-8 suggested that λ77B-9 contained the junction fragment. All the altered bands detected with probe 0.6ES upon Southern blot analysis of GM 7736 DNA were also seen with probe 2.3X (Fig. 2A). The bands for the normal allele, also present in control DNA, were of a different size in all cases. Finally, mapping with somatic cell hybrids confirmed its localization to chromosome 11 (data not shown).

Clones for the normal allele from the other deletion boundary were obtained by screening the BamHI library with probe 2.3X. Two identical phage clones (λ77N1+2) could be retrieved. The deletion breakpoint could be narrowed down to a 1.2-kb PvuII/HindIII fragment. An adjacent PvuII/HindIII piece of 1.4 kb was missing from the junction clone and detected only

DNA fragments corresponding to the normal allele upon Southern blot analysis of GM 7736. The intensity of the hybridization signal was reduced compared to that of control DNA. Hybridization against a human–rodent hybrid panel confirmed the assignment to chromosome 11p (Fig. 3). The 1.4-kb PvuII/HindIII fragment also detected a HindIII polymorphism in 6697 DNA which segregated in the hybrid panel. Among 28 chromosomes, the allele frequencies were p = 0.61 and q = 0.39. Mendelian inheritance of the RFLP was observed.

Chromosomal Mapping

The orientation of the cloned DNA relative to the centromere was established by mapping the probes against two previously characterized human–hamster somatic cell hybrid lines containing chromosome 11 alleles with deletions of different size from WAGR patients (Bruns et al., 1984). The catalase gene had been shown to be absent in the larger deletion (G156F3, N.W.) but to be present on the other WAGR deletion chromosome with a smaller deletion (G157A6, M.J.) where the gene was mapped proximal to the breakpoint (Glaser et al.,, 1986). Gene dosage studies on GM 7736 indicated that the catalase gene lies within the deleted region and is present only on the normal allele (Fig. 4). Probes HU11-25 and HU11-164B, which are missing from both hybrid lines, therefore represent the distal end of the deletion. The proximal boundary falls in the interval between the catalase gene and the lower
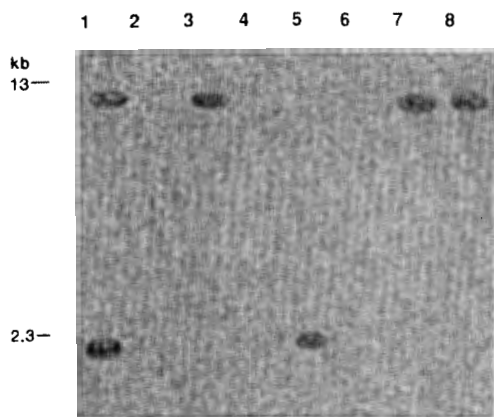
FIG. 3. Chromosome mapping of the proximal breakpoint site. Probe 1.4PH was hybridized to a mapping panel of HindIII-digested DNAs from (1) human 6697 cells; (2) Chinese hamster E36 cells; (3 and 8) human–hamster hybrids with chromosome 11; (4) human–hamster hybrid with the X as the sole human chromosome; (5) human–mouse hybrid with an 11pter–q23::Xq26–qter translocation chromosome; (6) the N.W. deletion hybrid G156F3; (7) the M.J. deletion hybrid G157A6. Chromosome 11 and Xq26–qter were the only human chromosomal elements shared by the hybrids in lanes 3, 5, 7, and 8. The absence of hybridization to the DNA in lane 4 indicates that probe 1.4PH can be assigned to chromosome 11. A HindIII polymorphism is observed with this probe.

breakpoint of the larger N.W. WAGR deletion isolated in G156F3 (Figs. 3 and 4). Additional gene dosage studies with the cell line GM 3118 (J.K.) confirmed these results (not shown).

Molecular Mapping

PFG analysis for the new probes with the enzymes SacII, SfiI, and BssHII confirmed their localization. Probe 0.6ES identified the same normal and altered fragments as HU11-164B in GM 7736 DNA (Fig. 5C). With probe 0.2SB only the bands representing the normal allele were seen on the same blot (Fig. 5B). Probe 2.3X detected not only the same rearranged fragments as 0.6ES but also new and different sized ones derived from the normal allele (Fig. 5A). Hybridization of probes from the lower breakpoint to NotI-digested DNA showed a band of about 1.2 Mb in size. A fragment of the same size was seen when the blot

was probed with the catalase cDNA clone (data not shown). This suggests that the proximal breakpoint in GM 7736 is located less than 1.2 Mb away from the catalase gene on the centromeric side of the gene. Hybridization patterns of NruI and MluI digests which produce larger fragments have confirmed this linkage. The size of the deleted region can be estimated only from the restriction maps around clones that are located within this area such as the FSHB gene and several of our random probes isolated from the flow-sorted chromosome 11 library (Gessler et al., in preparation). At least 4 million base pairs seem to be missing from the deleted chromosome. More exact measurements, however, can be made only after complete link-up of the internal clones with PFG analysis.

DISCUSSION

Chromosomal rearrangements are associated with a growing number of malignancies and developmental defects. Among the most extensively studied abnormal breaks are the chromosome translocations seen in Burkitt lymphoma (Dalla-Favera et al., 1982; Taub et al., 1982) and chronic myeloid leukemia (de Klein et al., 1982; Groffen et al., 1984) where a cellular proto-oncogene is joined with one of the immunoglobulin genes or in the latter case with the bcr gene. These translocations were found in almost every case, but were restricted to tumor cells as part of the malignant process.

The deletion described in the present report, however, belongs to another group of cancer-associated rearrangements in which the malignant phenotype appears to be a recessive trait. This group includes the WAGR syndrome, retinoblastoma, small cell lung carcinoma, and neuroblastoma (Francke et al., 1979; Riccardi et al., 1980; Balaban et al., 1982; Benedict et al., 1983; Whang-Peng et al., 1982; Brodeur et al., 1981). The chromosomal deletions are generated mostly as a somatic mutation in the affected individual, but in some instances they can also be inherited through the germ line. The characteristics of the breakpoints have not been analyzed to date in any of the cytogenetically described rearrangements. Only the recent isolation of

FIG. 4. Genetic map of the deletions. Probes CAT, FSHB, and HBVS1 have been previously mapped on hybrids G156F3 and G157A6 (Glaser et al., 1986). The cloned deletion breakpoints of GM 7736 subdivide the intervals described and orient the breakpoints relative to the centromere.
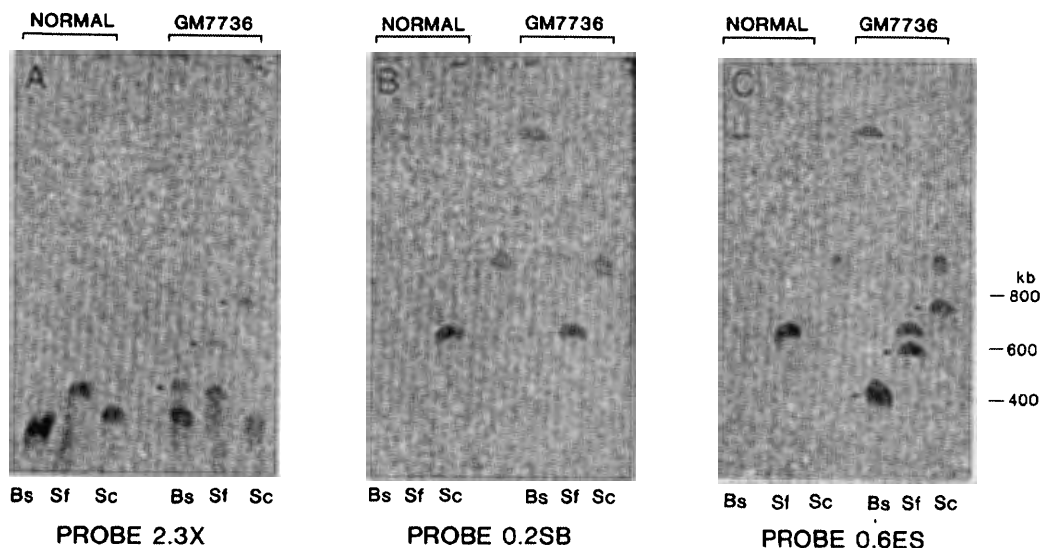
FIG. 5. PFG analysis of the GM 7736 deletion. Agarose blocks with 6697 control and GM 7736 DNA were digested with the enzymes BssHII (Bs), SfiI (Sf), and SacII (Sc) and separated in an LKB pulsaphor apparatus at 330 V with 80 s switching time and 11°C buffer temperature. After transfer, the filter was sequentially hybridized with probes 2.3X (A), 0.2SB (B), and 0.6ES (C). Arrows indicate the bands corresponding to the rearranged allele.

the retinoblastoma gene (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987) has led to the detection of a number of breakpoints in constitutive and tumor DNAs which can now be cloned and studied in detail.

The present mapping and cloning of the breakpoints of a deletion 11p14.1–p13 from an individual with aniridia, genitourinary anomalies, and mental retardation represent the first characterization of a WAGR-related deletion. The individual, now 5 years old, has not developed Wilms tumor. It is nevertheless likely that one allele of the Wilms tumor gene was included in the deletion, as the flanking markers CAT and FSHB (Glaser et al., 1986) were both missing from the deleted chromosome. With the normal alleles for both breakpoints now available, it may be possible to obtain more insight into the mechanisms for these rearrangements by sequence analysis and comparison with similar breakpoints from other cancer-related deletions and translocations.

The molecular characterization of the GM 7736 deletion also helps to establish a physical map of the WAGR region by providing additional breakpoints to subdivide present intervals of the genetic map (Glaser et al., 1986), which allows more precise mapping of any new probe. The breakpoint clones described will provide valuable additional landmarks for a complete linkup long-range restriction map of chromosome 11p14–p13.

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