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Somatic cell hybrid and long-range physical mapping of 11p13 microdissected genomic clones

(microdissection/microcloning/Wilms tumor/aniridia/*WAGR*)

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ABSTRACT **Microdissection and microcloning of banded human metaphase chromosomes have been used to construct a genomic library of 20,000 clones that is highly enriched for chromosome 11p13 DNA sequences. Clones from this library have been mapped on a panel of human–rodent somatic cell hybrids that divides the region from distal p12 to proximal p14 into seven physical intervals. A total of 1500 clones has been isolated, 250 clones have been characterized, and 58 clones have been mapped. Six of the clones were used to complete a long-range physical map of 7.5 megabases through the region. Two of the clones are localized to the Wilms tumor (WT) region, three are localized to the aniridia (AN2) region, and two are localized to the region between WT and AN2. The library represents DNA sequences spanning a distance of $\approx 13 \times 10^6$ base pairs, with an average density of one clone per 37,000 base pairs.**

Wilms tumor is a childhood nephroblastoma that appears to result from uncontrolled growth of the undifferentiated metanephric blastema (1). Aniridia is complete or partial absence of the iris, frequently accompanied by other ocular deformities and usually leading to blindness (2). A genetic correlation between Wilms tumor and aniridia was established with the observation that the frequency of Wilms tumor increases from 1:10,000 in the general population (3) to 1:3 in children born with aniridia (4). Conversely, the frequency of aniridia among children who have had a Wilms tumor increases from 1:50,000 to 1:73 (2, 5). Many children with aniridia also suffer from an array of genitourinary abnormalities, including ambiguous genitalia, cryptorchidism, hypogonadism, and fused kidneys (5) and mental retardation (the AGR triad) in addition to Wilms tumor (the WAGR complex). The observation that most of these WAGR and AGR children are born with a hemizygous interstitial deletion of 11p13 (6, 7) has led to the speculation that chromosome band 11p13 houses not only the aniridia gene and a gene predisposing to Wilms tumor but also an unknown number of additional genes necessary for normal development of the kidney, iris, genitourinary tract, and mental function. In fact, a discrete, apparently autosomal dominant locus in 11p13 that may be responsible for complete renal agenesis has been described (8).

In recent years, significant progress has been made toward the molecular analysis of this region. When somatic cell hybrids established from WAGR patients with deletions or translocations were assayed with the two closest known flanking genes, erythrocyte catalase (*CAT*) on the proximal side and the β subunit of follicle-stimulating hormone (*FSHB*) on the distal, the location of the genes responsible for the WAGR complex could be restricted to the region between the two genes, in the distal one-third of 11p13 (9–11).

Subsequently, a number of laboratories, attempting to isolate the genes responsible for this complex syndrome, have isolated many region-specific clones. For some, the source of clones was a Livermore Laboratory chromosome 11 library (12–15); others generated reduced chromosome human–rodent somatic cell hybrids (8, 16, 17), from which genomic libraries were constructed and human clones were identified by hybridization to human repetitive sequence probes. Both of these probe isolation strategies require that the markers be mapped to the region of interest on a panel of somatic cell hybrids retaining deletions or translocations in the region of interest and share the disadvantage of only recovering probes at the same percentage as the region of interest is to the total starting material. A further disadvantage is that when the library screen depends upon identification of human clones by the presence of repetitive sequences, the resulting clones tend to be clustered in repetitive-rich regions of the genome.

Nonetheless, these strategies have been adequate to isolate clones at densities ranging from 1:750,000 base pairs (bp) (17) to 1:500,000 bp (18), which is sufficient to construct long-range physical maps through the region. In the cases of Wilms tumor and aniridia, the translocation or deletion breakpoints of a few critical patients or tumors have been positioned on the physical map; consequently the aniridia gene has been localized to a region of <125 kilobases (kb) (19, 20), and the Wilms tumor gene has been localized to 2.5 megabases (Mb) in one case (18), to 800 kb in another (17), and more recently to a region of <350 kb (21–24). When the position of a gene is known to within a few hundred kilobases, strategies to identify additional clones directly at the presumptive locus can be devised, such as those undertaken to clone a candidate Wilms tumor gene (22, 23). However, there are apparently additional genes in 11p13 that have profound developmental effects on the kidney, urogenital tract, eye, and mental function, and progress in identifying those genes has been hampered by lack of discrete chromosome aberrations correlated with specific phenotypes. Markers identified by the currently available random strategies are of insufficient density to identify these genes.

To circumvent the problems of low percentages of 11p13-specific clones in entire chromosome 11 libraries and low frequencies of human clones in libraries constructed from reduced chromosome hybrids, we have used a microdissec-

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Abbreviations: Mb, megabase(s); WAGR, Wilms tumor, aniridia, genitourinary abnormalities, and retardation.

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tion and microcloning protocol described by Lüdecke *et al.* (25) to construct a library enriched for sequences from 11p13. These markers should improve the probability of isolating a number of expressed genes in 11p13. This report describes characterization of the library, mapping of the first 58 clones, and a 7.5-Mb physical map through the region.

MATERIALS AND METHODS

Construction of the 11p13 Genomic Library. Chromosome 11p13-specific DNA probes were obtained by microdissection and microcloning of banded human metaphase chromosomes, described in detail elsewhere (25–27). Briefly, mitotic cells were harvested from normal amniotic fluid cell cultures using the pipette method (28), which reduces the fixation time to 10–20 sec. Fixed metaphases were spread onto precleaned, wet coverslips, and immediately after evaporation of the fixative, metaphase spreads were washed and stored in 70% ethanol at −20°C overnight. Air-dried preparates were trypsin–Giemsa banded under sterile conditions.

Banded chromosomes were viewed at 1250× magnification on an inverted microscope (Zeiss). Band 11p13 was microdissected from 40 metaphase chromosomes with extended glass needles, moved by an electronically controlled micromanipulator (MR MOT, Zeiss). Dissected material (about 0.5 pg) was transferred to a 1-nl collection drop on a siliconized coverslip in a small moist chamber, digested with *Rsa* I, and ligated to the *Sma* I site of a pUC derivative, which has a single *Sma* I site flanked by *Eco*RI sites. The inserts were then amplified *in vitro* by the polymerase chain reaction (PCR) using the Klenow fragment of DNA polymerase and the universal M13/pUC forward and reverse sequencing primers as primers for the PCR reaction. After 26 amplification cycles, the DNA was digested with *Eco*RI and ligated to the *Eco*RI site of pUC13.

Analysis of Clones. Competent DH5α cells (Max Efficiency, BRL) were transformed with an aliquot of the ligation product and plated on NZ medium or Luria broth plates with 100 μg of ampicillin per ml and 50 μg of 5-bromo-4-chloro-3-indolyl β-D-galactoside per ml. Plasmid DNA from white colonies was isolated from 2-ml cultures either on Quiagen-20 tips according to the manufacturer's protocol (Quiagen, Studio City, CA) or using the alkaline lysis method (29). Inserts were excised from vectors with either *Eco*RI or *Pvu* II (the latter adds ≈300 bp of vector sequence to the insert), separated electrophoretically, and recovered either from low-melting agarose or by electroelution (IBI Electroeluter, model UEA).

In some cases, insert sizes were determined by filling in recessed *Eco*RI ends with [α-^{32}P]dATP (50 mM Tris, pH 7.5/10 mM $MgCl_2$/100 μM dithiothreitol/50 μg of bovine serum albumin per ml and Klenow DNA polymerase) at 20°C for 2 hr. Fragments were separated electrophoretically through 8% polyacrylamide and identified by autoradiography. Sizes were estimated by comparison to pBR322 digested with *Msp* I, end labeled with [α-^{32}P]dCTP. In other cases insert sizes were estimated by comparison to pBR322 digested with *Hae* III on a 1.2% agarose gel.

WAGR Deletion Mapping Panel. The microdissected clones were subregionally mapped on our 11p13 deletion mapping panel, which consists of six human–rodent somatic cell hybrids and human and mouse controls. J1-11 is a human–hamster hybrid that contains only the pter-q13 region of the normal chromosome 11 as its only human DNA (30).

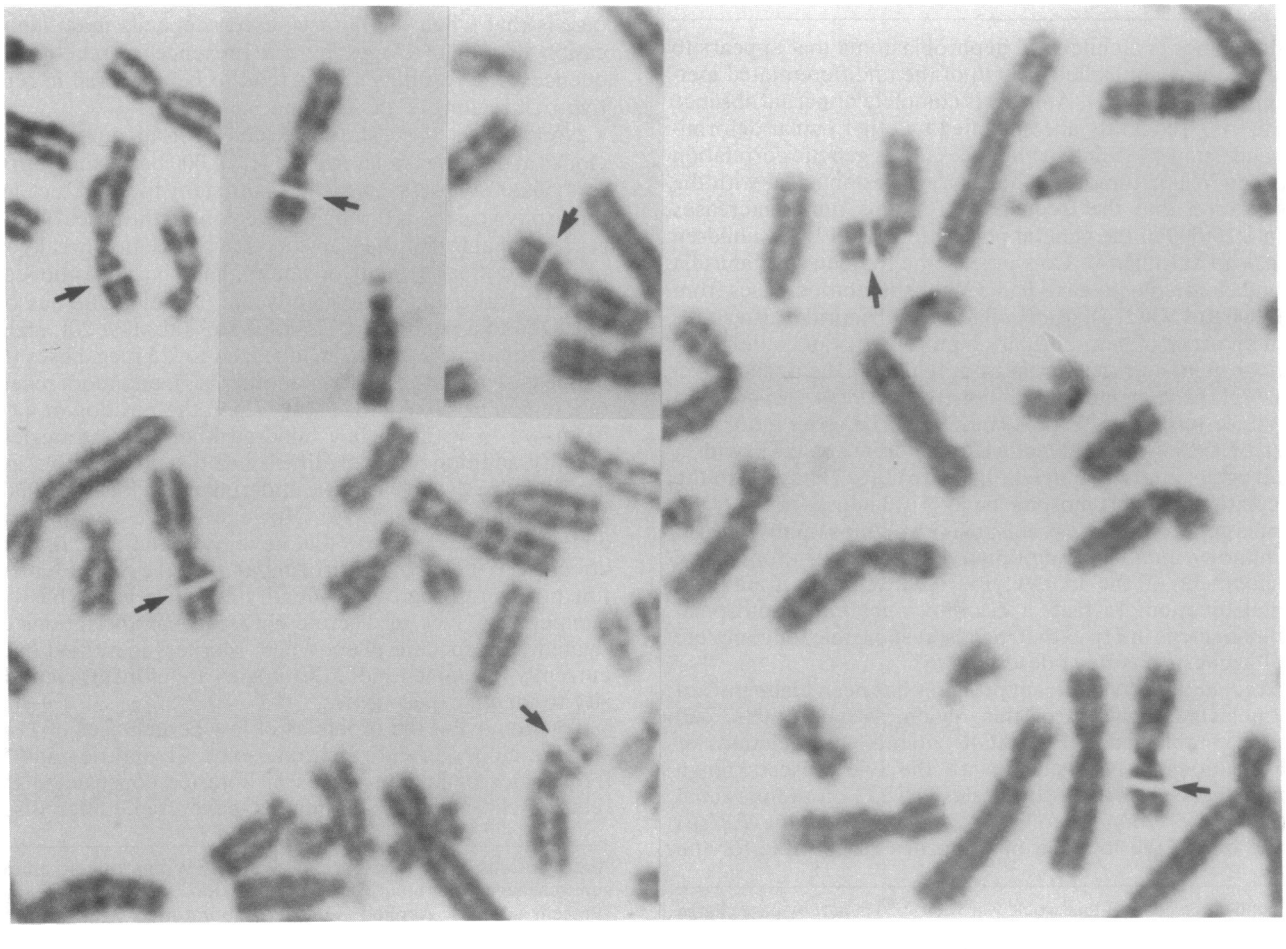

FIG. 1. Examples of Giemsa-banded metaphase chromosomes following dissection of 11p13 (see arrows).

RSR hybrids were established from a patient, RIST, who has aniridia, hypogonadism, Wilms tumor, and a deletion of distal 11p13-p14.1 (11). DAR15-14 is a human–mouse hybrid established from a patient who had Wilms tumor, but not aniridia, and a deletion of 11p12-p13 (31). DGL hybrids were established from a patient, DG-85, with familial aniridia and a cytologically balanced translocation between chromosomes 11 and 22 (32); the translocation involving chromosome 11 was located at 11p13 with an associated deletion of at least 600 kb (11, 20). The patients, their clinical histories, and the cell hybrids have been described (11).

Hybridizations. Southern blots were prepared from 20 μg of hybrid and 5 μg of human and rodent control DNA digested with *Hin*dIII or *Taq* I, electrophoretically separated, and transferred either to nylon membrane (Genatran, Plasco, Woburn, MA) under alkaline conditions or to nitrocellulose under neutral conditions (29). Usually, 20–50 ng of purified insert (excised with either *Eco*RI or *Pvu* II) was radiolabeled with 30 μCi (1 Ci = 37 GBq) of either [α-^{32}P]dTTP (Amersham, 3000 Ci/mmol) or [α-^{32}P]dCTP (Amersham, 300 Ci/mmol), for 30 min or more, using the random primer method (33). Hybridizations and washes were according to Church and Gilbert (34), and filters were exposed to XAR-5 film (Kodak) at −70°C with an intensifying screen for 3–10 days.

Pulsed Field Gel Electrophoresis. Preparation of high molecular weight DNA from lymphocyte cells, digestion with rare-cutting restriction enzymes, and electrophoresis through either transverse alternating, field inversion, or contour-clamped homogeneous field electrophoresis have been described (20). After electrophoresis, gels were stained for 20–40 min with ethidium bromide, exposed to shortwave UV light (300 nm) for 5 min, transferred to nylon under alkaline conditions, and hybridized as described above.

RESULTS AND DISCUSSION

Examples of the metaphase chromosomes following microdissection of 11p13 are shown in Fig. 1. Cell hybrid mapping data for the first 58 clones are summarized in Fig. 2 *Upper*, with representative Southerns from the 11p13 somatic cell hybrid mapping panel in Fig. 2 *Lower*. The long-range physical map through 11p13 is summarized in Fig. 3 *Upper*, with representative pulsed field gel blots shown in Fig. 3 *Lower*. DNA segment numbers assigned by the HGM10 DNA committee are listed in Table 1 for some of the clones.

Chromosome 11 origin of the clones was verified by hybridization to J1-11, a cell hybrid whose only human component is the short arm of chromosome 11, and the clones were subregionally mapped on a somatic cell hybrid mapping panel retaining the chromosome abnormalities of three patients with WAGR-related symptoms (Fig. 2 *Upper*). One patient, RIST, suffered from Wilms tumor and aniridia, whereas patient DAR had a Wilms tumor but not aniridia. Presumably the *WT* locus is located in the deleted region shared between them (see cell hybrids RSR-1 and DAR15-14, Fig. 2 *Upper*). Conversely, patient DG-85 has a complex chromosome rearrangement involving a translocation accompanied by an interstitial deletion and suffered from aniridia but not Wilms tumor. Presumably the aniridia locus is within the deleted region shared between RIST and DG-85 (compare RSR1, DGL-17, and DGL-21, Fig. 2 *Upper*). Twenty-six clones were assigned to the two intervals most proximal to the *WAGR* locus, and two clones were assigned to the same interval as WT. Two clones were assigned to the region between WT and AN2, and four additional clones were assigned to the AN2 region. Twenty-four clones were assigned to the interval distal to the AN2 region.

In previous reports describing these patients, the relationship between the distal boundary of the DAR deletion and the proximal boundary of the DG-85 deletion was unknown (11, 15). With the markers in the interval between WT and AN2 (20E and 68E), a region of non-overlap between the deletions

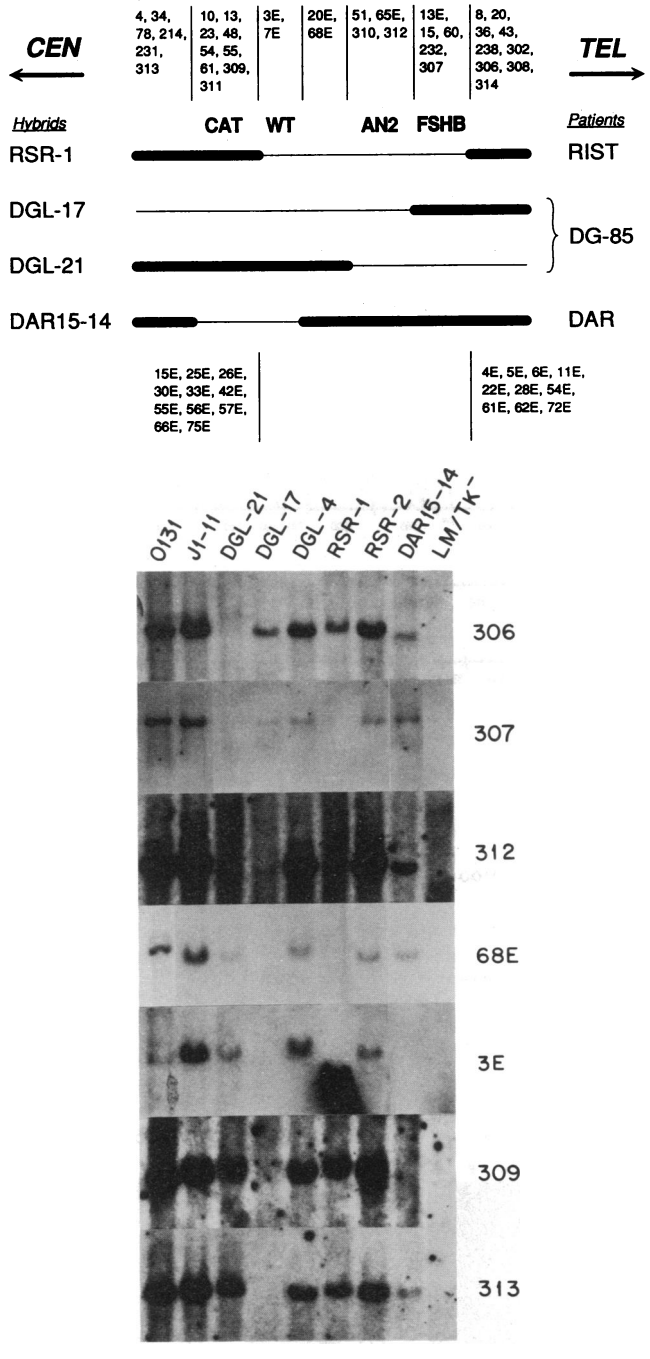

FIG. 2. Somatic cell hybrid mapping. (*Upper*) Map locations for the microdissection clones are summarized. RSR-1 and DAR15-14 are somatic cell hybrids retaining the RIST and DAR deletions, respectively. DGL-17 and DGL-21 retain the der-22 and the der-11 translocation chromosomes, respectively, of DG-85. The clones at the top were analyzed on the entire WAGR panel, and those at the bottom were analyzed only on RSR-1 and DGL-21. Locations for catalase (CAT), the β subunit of follicle-stimulating hormone (FSHB), Wilms tumor (WT), and aniridia (AN2) are indicated. (*Lower*) Representative Southern blots of the hybrid mapping panel are shown. 0131 is a normal human DNA control. J1-11 is a hybrid retaining only the short arm of chromosome 11. DGL-21 and DGL-17 retain the derivative 11 and derivative 22 translocation chromosomes of DG-85, respectively, whereas DGL-4 retains the normal chromosome 11. RSR-1 retains the RIST deletion chromosome and RSR-2 retains the normal. DAR15-14 retains the deletion chromosome of DAR. LM/TK⁻ is the mouse control. The clones are labeled on the right.

is finally established (see cell hybrids DGL-21 and DAR15-14, Fig. 2 *Upper*).

The physical map shown in Fig. 3 *Upper* was constructed primarily from single and double digests of normal control and patient cell lines with deletion or translocation breakpoints in 11p13. Several control DNAs were analyzed because different, but overlapping, sets of restriction fragments were identified among them; these differences are highly reproducible and may reflect either methylation or true DNA sequence polymorphisms. These polymorphisms were used advantageously in compiling the map and are marked by asterisks in Fig. 3 *Upper*. Patient cell lines that were critical to construction of the map included WT-21, a cultured Wilms tumor cell line, and two aniridia patients (11, 20), DG-85 and SIMO; all three cell lines have 11p13 translocations, with the first two including deletions at the translocation breakpoints.

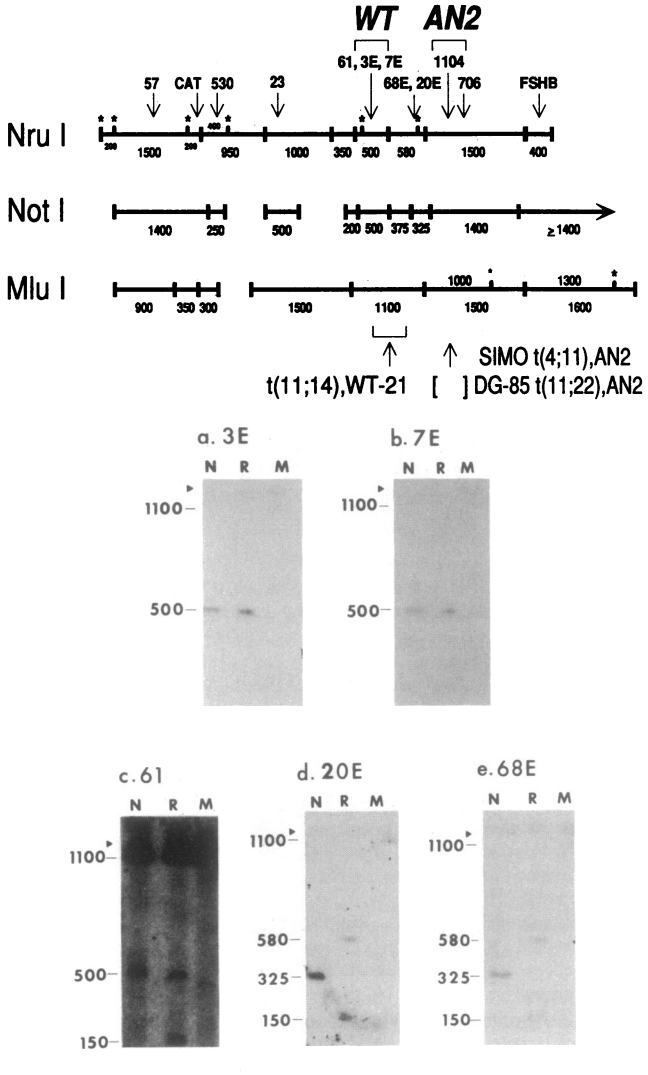

FIG. 3. (*Upper*) The long-range restriction mapping data through 11p13 are summarized. Clones 57 and 530 are in the same interval as *CAT* of Fig. 2 *Upper*, and clones 706 and 1104 are in the interval defined by the DG-85 deletion. The map was assembled from single and double digests of controls showing polymorphisms (represented by the asterisks). The aniridia region, defined by patients DG-85 and SIMO (11, 20) is marked with a bracket and arrow, and the Wilms tumor region, defined by a translocation found in a cultured Wilms tumor cell line, WT-21, is marked by a bracket. Sizes are in kb. (*Lower*) Representative Southern blots of pulsed field gels are shown, hybridized with the microdissection clones. *Not* I (N), *Nru* I (R), and *Mlu* I (M) digests of control DNAs hybridized with probes 3E (a), 7E (b), 61 (c), 20E (d), and 68E (e) are shown. The arrowheads designate the region of limiting mobility. Sizes are in kb.

Table 1. DNA assignment numbers for some of the clones

Clone	"D" number
pWT3E	D11S340
pWT7E	D11S341
pWT4	D11S342
pWT15	D11S343
pWT23	D11S344
pWT34	D11S345
pWT36	D11S346
pWT43	D11S423

The map was assembled using six clones (23, 3E, 7E, 61, 20E, and 68E) from the microdissection library, four clones (57, 530, 706, and 1104) previously isolated from the Livermore Laboratory chromosome 11 library (14), and probes for the *CAT* (35) and *FSHB* (36) genes. The 7.5-Mb map extends from the centromere proximal side of *CAT*, through the WAGR region, to the distal side of *FSHB*. Locations for the Wilms tumor gene, as defined by the translocation/deletion breakpoint of cell line WT-21 (unpublished data), and the aniridia gene, as defined by the translocation of SIMO and the translocation/deletion of DG-85 (11, 20), are indicated by the brackets. A detailed map of the aniridia region has been presented (20).

The library represents a total of 20,000 recombinant clones. DNA has been isolated from 1500 clones, and analysis of the first 250 clones permits an accurate description of the microdissection library. Of the first clones analyzed, ≈73% (182 clones) contained detectable inserts. Of the clones with inserts, 27% (49 clones) contained repetitive sequences, 32% (58 clones) detected no signal on hybridization, and 41% (75 clones, ≈30% of all of the clones) detected single-copy sequences. Hybridization signals on the hybrid mapping panel with 58 single-copy clones were unambiguous (see Fig. 2 *Upper*), predicting conservatively that 23% of the clones should be useful for mapping purposes. Only 1 clone did not originate from chromosome 11, and in only two cases were identical clones found. The inserts ranged in size from 75 bp to 400 bp, with the average size being 160 bp. A preliminary description of the microdissection library, based on analysis of 56 clones with inserts, was reported by Lüdecke *et al.* (26). They also found that 27% of the insert-containing clones contained repetitive sequences, but only 10% detected no signal on hybridization, and 63% detected single copy sequences. Whether the difference in percentages of single-copy clones reflects sample size differences or whether radiolabeling with [α-^{32}P]dCTP is more efficient than labeling with [α-^{32}P]dTTP is unknown. Presumably the insert-containing clones detecting no signals are either too small to radiolabel well or they are a cloning artifact (26).

We estimate, by comparing our pulsed field gel map of the region to our hybrid mapping data (compare Figs. 2 *Upper* and 3 *Upper*), the physical distance from the proximal boundary of the RIST deletion and the distal boundary of the DG-85 deletion/translocation to be ≈1.8 Mb. A total of eight clones was mapped to this region, predicating an average density of one clone per 260,000 bp. Assuming that the clones showed in Fig. 2 *Upper* are randomly spaced, the total physical distance included in the microdissection library can be estimated at 13 × 10^6 bp, somewhat larger than the estimated size for band 11p13. Finding clones outside the proximal boundary of the DAR deletion, reported to include distal p12, and clones beyond the distal boundary of the RIST deletion, which includes subband p14.1, supports the conclusion that the library does contain some DNA sequences from outside of 11p13. This conclusion is consistent with the result of Gessler and Bruns (18), who presented a 15-Mb physical map spanning distal p12 through proximal p14.

Nonetheless, this library provides a considerable enrichment of 11p13 sequences over currently available sources and should be representative of any microdissection library throughout the genome.

If only 23% of the 1500 clones already isolated are applicable for mapping purposes, then 350 clones should be obtained. If the library contains clones spanning a distance of 13×10^6 bp, an average density of 1:37,000 is expected. These clones can be expanded into λ or cosmid-size inserts, providing a sufficient number of single-copy sequences to screen "zoo blots" for conserved sequences and ultimately identify the expressed genes in 11p13. These clones can also be positioned on the long-range physical map of 11p13, where their density and small size should facilitate the construction of a saturated sequence tagged site map of 11p13, supporting the Human Genome Initiative as described by Olson *et al.* (24).

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