Cloning of Breakpoints of a Chromosome Translocation Identifies the AN2 Locus

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Chromosome translocations involving 11p13 have been associated with familial aniridia in two kindreds highlighting the chromosomal localization of the AN2 locus. This locus is also part of the WAGR complex (Wilms tumor, aniridia, genitourinary abnormalities, and mental retardation). In one kindred, the translocation is associated with a deletion, and probes for this region were used to identify and clone the breakpoints of the translocation in the second kindred. Comparison of phage restriction maps exclude the presence of any sizable deletion in this case. Sequences at the chromosome 11 breakpoint are conserved in multiple species, suggesting that the translocation falls within the AN2 gene.

THE PHENOTYPIC FEATURES OF THE WAGR syndrome (Wilms tumor, aniridia, genitourinary abnormalities, and mental retardation) indicate the presence of a constellation of genes important in human developmental processes within a small region of chromosome 11p13. Aniridia is a congenital developmental disorder of the eye, characterized by complete or partial absence of the iris, cataracts, lens anomalies, early onset glaucoma, and progressive loss of vision (*1*). It occurs in sporadic or autosomal dominant familial form without other phenotypic features at a frequency of 1:64,000 to 1:96,000. Many of the sporadic cases likely represent new mutations as aniridia is frequently inherited as an autosomal dominant in subsequent generations. Two genetic loci have been identified. In one large kindred, Ferrell *et al.* (*2*) observed linkage of aniridia (AN1) and the erythrocyte acid phosphatase (ACP1) locus on chromosome 2p. The presence of a second aniridia locus, the AN2 locus, on chromosome 11p is supported both by the consistent association of aniridia with the constitutional 11p13 deletions of the WAGR syndrome (*3*) and by the different inherited translocations with 11p13 breakpoints associated with familial aniridia in two kindreds (*4, 5*). The breakpoints of both translocations were localized by somatic cell hybrid analysis between the genes encoding the beta subunit of follicle-stimulating hormone (FSHB) and catalase (CAT) within the critical region of overlap of WAGR deletions (*6*). Mapping and cloning of chromosomal translocation breakpoints has been a powerful tool for characterization of *myc*-

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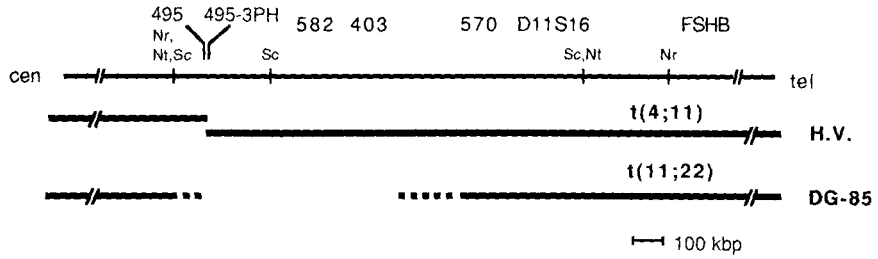


Fig. 1. Long-range chromosomal map around the aniridia locus. The location of probes used in this study and cutting sites for the enzymes Not I (Nt), Nru I (Nr) and Sac II (Sc) were determined by pulsed-field gel analysis (*12*). The position of the breakpoints for the t(4;11) translocation (H.V.) and the approximate size of the deletion associated with the t(11;22) translocation (DG-85) are indicated. The centromeric breakpoint of t(11;22) is likely between the Not I site and 495 as a rearrangement of the adjacent Not I fragment was not detected by Compton *et al.* in the DG-85 cell line (*16*). Probe 495 is located on the centromeric and probe p495-3PH on the telomeric side of the H.V. translocation breakpoint.

related oncogenesis (*7*), the bcr region on chromosome 22 (*8*), and the Duchenne muscular dystrophy locus (*9*). Molecular analysis of the breakpoints of the two aniridia translocations would be expected to identify the gene and the nature of the translocation-related mutational events.

A cytogenetically undetected deletion involving two random chromosome 11p13 DNA probes was found to be associated with the translocation t(11;22)(p13;q12.2) in the cell line DG-85 by Davis *et al.* (*10*). Similarly, three probes (403, 495, and 582) from our panel of WAGR deletion probes are included in this deletion (*11*), which is postulated to encompass the aniridia gene or to disrupt its integrity leading to the aniridia phenotype in one kindred (*5*). To determine whether a similar deletion had occurred at the 11p13 breakpoint of the second aniridia translocation, DNA from H.V., an affected individual with the translocation t(4;11)(q23;p13), was analyzed for gene dosage with the three probes deleted in DG-85. No deletion of these or other adjacent probes could be detected.

To precisely locate the translocation breakpoint in H.V., we used the detailed long-range restriction map of the WAGR region that had been previously constructed (*12*). In normal DNA, a series of probes (403, 495, 582, 570, and D11S16) recognize a 1.4-Mbp Not I fragment (Fig. 1). The telomeric end of this fragment is located approximately 0.5 Mbp centromeric to FSHB. In DG-85, altered Not I, Sac II, and Nru I fragments were seen with D11S16 and 570. A rearranged Not I fragment was also detected in H.V. DNA with these probes, suggesting that this translocation involves the same region. When probe 495 was used, however, the set of altered fragments in H.V. DNA differed from those seen with other more distal probes like 582 and D11S16, suggesting that 495 is centromeric to the breakpoint.

The breakpoint in H.V. was defined more exactly by using short-range pulsed-field gel electrophoresis (PFGE) to analyze the immediate surrounding of probes 495 and 582 (Fig. 2a). The enzymes Sac II, Sfi I, and Sal I, which produce fragments of 330 to 410 kbp with probe 495 in normal DNA, yielded additional altered fragments in H.V. DNA. The presence of a new 140-kbp Sal I fragment, derived from the der(11) chromosome, implied that the breakpoint had occurred within this limited area.

Probe 495 itself detected no rearrangement in H.V. DNA on conventional Southern blots with the enzymes Eco RI, Bam HI, Kpn I, and Hind III. However, a chromosome walking step from 495 led to the isolation of λ495-3 (*13*). After preannealing with human placental DNA to remove repetitive sequences, the complete insert of λ495-3 detected altered fragments for Eco RI, Bam HI, and Hind III in H.V. DNA. On PFGE blots, subclones p495-3ES and p495-3PH recognized the same normal fragments as 495, but the altered fragments were of different size (Fig. 2b). The detection of a completely different set of altered fragments indicated that the translocation breakpoint had been crossed. Probe p495-3ES was used to isolate a recombinant phage λHV-1 (Fig. 3a), containing the 13-kb Bam HI fragment of the rearranged der(4) allele (*13*). Restriction maps for λ495-3 and λHV-1 were identical in the telomeric region and agreed with genomic restriction maps as verified by hybridization with probes p495-3ES and p495-3PH. A unique fragment derived from the centromeric region of λHV-1 (pHV-Sac3) and not present in λ495-3 was mapped to human chromosome 4 with a somatic cell hybrid panel (*14*), thereby confirming the identity of the breakpoint clone. The corresponding unrearranged chromosome 4 allele was isolated by screening a human Mbo I partial digest library with pHV-Sac3. A unique fragment, pCH4S-Eco, derived from λCH4-1 correctly maps to chromosome 4 and is located distal to the chromosome 4 breakpoint. As the region between 495 and the chromosome 11 breakpoint is rich in repetitive sequences, the der(11) allele was isolated with probe pCH4S-Eco from a Bgl II digest library of H.V. DNA (*13*).

Comparison of restriction maps for the isolated phage clones showed no differences between rearranged alleles and their normal counterparts outside of the breakpoint region. The breakpoint was localized by restriction mapping within fragments of 250 and 350 bp on chromosome 11 and 4, respectively. There was no indication that the translocation resulted in a concomitant deletion, although loss of a few nucleotides could not be excluded by comparison of the restriction maps. The absence of a significant deletion is substantiated by the fact that p495-3AH, a 350-bp fragment that spans the translocation breakpoint on chromosome 11, detects the der(4) and the der(11) homologs in H.V. DNA (Fig. 3b).

The breakpoint probe p495-3AH and probe p495-3PH, located immediately distal to the breakpoint, hybridized with rodent sequences on the hybrid cell panels used to confirm their chromosome assignment. In fact, both probes identify cross-

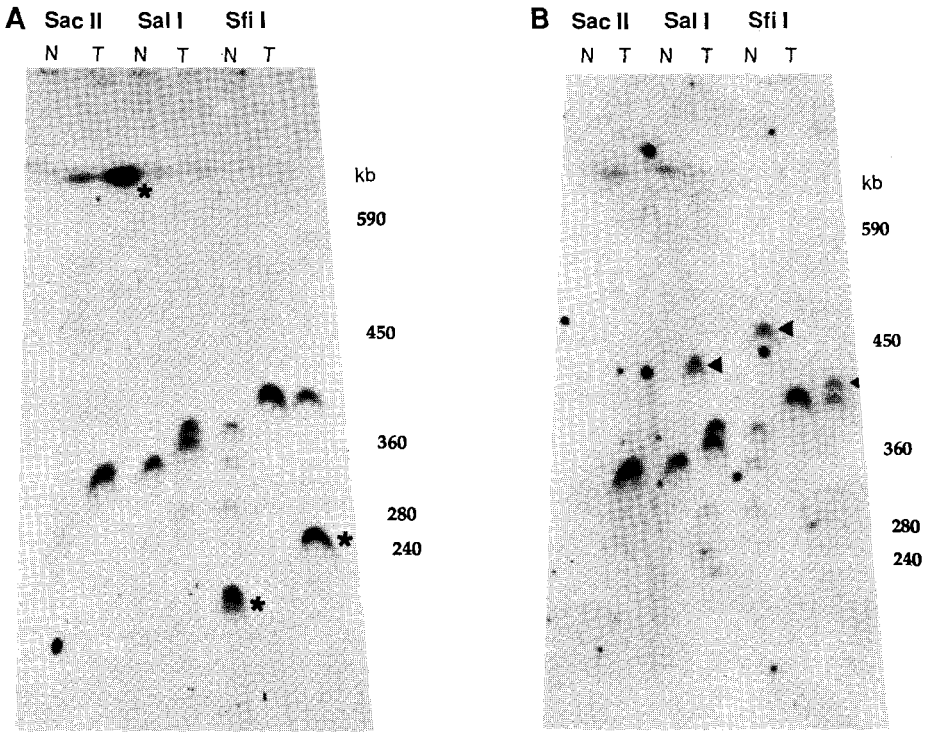
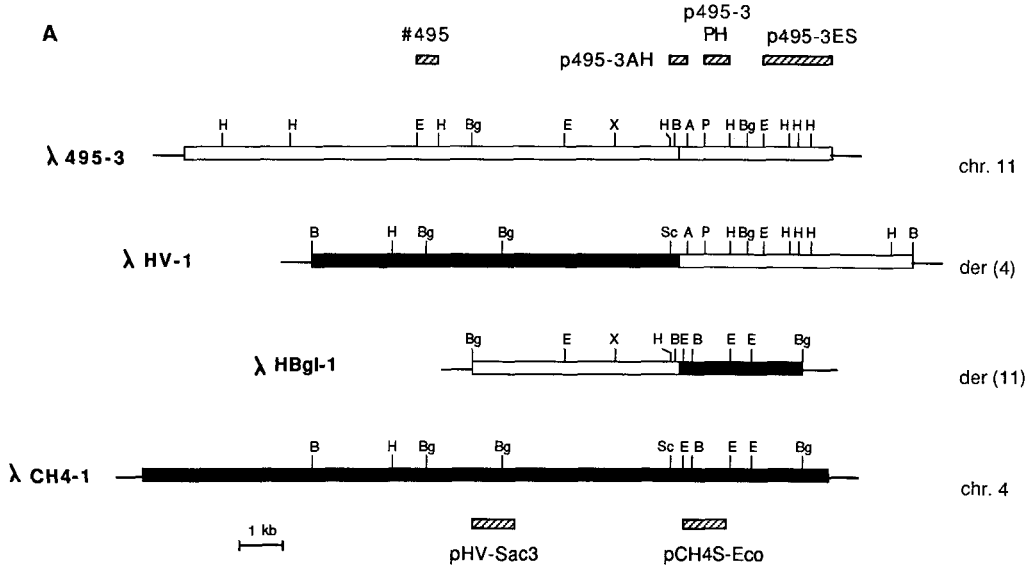


Fig. 2. Detection of the t(4;11)(q22;p13) rearrangement by short-range PFGE. DNA from a normal lymphoblastoid cell line (N) and DNA from H.V. (T) were digested with the enzymes indicated and PFGE blots prepared as described (*17*). Probe 495 (**A**) detects additional fragments in H.V. DNA (*), derived from the der(11) chromosome. With longer switching times, the altered Sac II fragment in H.V. DNA can be resolved and separated from the Sac II partial digest fragment of normal DNA. Probe p495-3PH (**B**) identifies the same normal fragments as probe 495; the altered fragments in H.V. DNA (◀), however, are different and are derived from the der(4) chromosome. Sizes on the right were obtained from *Saccharomyces cerevisiae* chromosomes. Electrophoresis conditions for the gel were 330 V, 11°C, 35-s switching time for 33 hours.



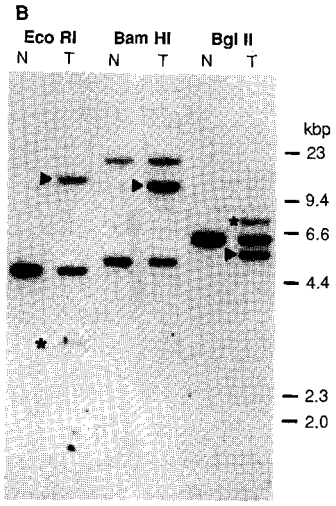


Fig. 3. Restriction maps of phage isolates for both rearranged and normal alleles of the t(4;11)(q22;p13) cell line and localization of the breakpoints. (**A**) Sites for the restriction enzymes Eco RI (E), Hind III (H), Bam HI (B), Bgl II (Bg), Acc I (A), Sac I (Sc), and Xba I (X) are indicated as determined from digests of phage DNA or plasmid subclones. For Bgl II, Acc I, Sac I, and Xba I, only the sites in the vicinity of the breakpoint were mapped precisely. The localization of different plasmid subclones used in this study, representing the chromosome 11 (top) and chromosome 4 (bottom) alleles, is indicated by shaded boxes. (**B**) Probe p495-3AH spans the translocation breakpoint. Normal DNA (N) and DNA from H.V. (T) was digested with the enzymes shown. In H.V. DNA the rearranged fragments corresponding to the der(4) (▶) and the der(11) (*) alleles are identified in addition to the normal fragments, which are seen in the control lane. Since the probe contains an internal Bam HI site, the region of homology with the rearranged der(11) fragment is likely too short to allow visualization of this small fragment [see (A)].

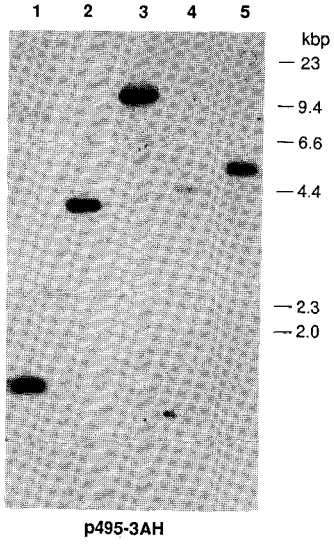


Fig. 4. Conservation of probe p495-3AH sequences. A Southern blot filter containing 4 μg of Hind III–digested DNA from human (1), chimpanzee (2), dog (3), hamster (4), and chicken (5) was hybridized with probe p495-3AH in 0.5*M* $Na(PO_4)$, *p*H 7.2, 1% SDS, 1 m*M* Na_2 EDTA, and salmon sperm DNA (50 μg/ml) at 65°C (*18*). The filter was washed for 1.5 hours in 40 m*M* $Na(PO_4)$, *p*H 7.2, 1% SDS at 65°C, and exposed overnight.

hybridizing DNA sequences in a number of higher vertebrates at high stringency of hybridization (Fig. 4), suggesting that they may contain coding sequences. As the H.V. chromosome translocation cosegregates with the aniridia phenotype in three generations in the Finnish kindred, it represents the likely cause of the disorder. The translocation could disrupt the aniridia gene itself or alter regulatory elements for the gene in the affected individuals. Initial sequence analysis of the breakpoint region showed that the translocation occurred within an open reading frame that is flanked by consensus splice donor and acceptor sites, suggesting that it may represent an exon. An RNA transcript corresponding to the two conserved probes has not yet been detected on Northern blots. This may reflect a very narrow window of expression for this gene during development.

For Duchenne muscular dystrophy and retinoblastoma, the isolation of sequences with extensive evolutionary conservation led directly to the cloning of the genes (*15*). The highly conserved probes at and adjacent to the t(4;11) translocation breakpoint should be similarly important for cloning of the aniridia gene and subsequent elucidation of how dominantly inherited mutations at this locus perturb developmental processes in the eye.

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13. Phage clones λ495-3 and λCH4-1 were isolated by

standard protocols from an Mbo I partial digest human genomic library in EMBL3, provided by S. Orkin. Clone λHV-1 was isolated from a complete Bam HI digest library of H.V. DNA in EMBL3 and packaged with Gigapack plus (Stratagene). The λHBgl-1 clone was isolated from a Bgl II complete digest library of H.V. DNA in EMBL3 prepared without phosphatase treatment or size selection of the insert to ensure clonability of the 8-kbp rearranged fragment.
14. The segregation of pHV-Sac3 was completely concordant with that of chromosome 4 in 15 hybrids. Discordancy fractions for the other chromosomes ranged from 0.21 to 0.66.
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