

Frequent Chromosome Aberrations Revealed by Molecular Cytogenetic Studies in Patients with Aniridia

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Seventy-seven patients with aniridia, referred for cytogenetic analysis predominantly to assess Wilms tumor risk, were studied by fluorescence in situ hybridization (FISH), through use of a panel of cosmids encompassing the aniridia-associated *PAX6* gene, the Wilms tumor predisposition gene *WT1*, and flanking markers, in distal chromosome 11p13. Thirty patients were found to be chromosomally abnormal. Cytogenetically visible interstitial deletions involving 11p13 were found in 13 patients, 11 of which included *WT1*. A further 13 patients had cryptic deletions detectable only by FISH, 3 of which included *WT1*. Six of these, with deletions <500 kb, share a similar proximal breakpoint within a cosmid containing the last 10 exons of *PAX6* and part of the neighboring gene, *ELP4*. Two of these six patients were mosaic for the deletion. The remaining four had chromosomal rearrangements: an unbalanced translocation, t(11;13), with a deletion including the WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) region, and three balanced rearrangements with what appear to be position effect breakpoints 3' of *PAX6*: (a) a t(7;11) with the 11p13 breakpoint ~30 kb downstream of *PAX6*, (b) a dir ins(12;11) with a breakpoint >50 kb from *PAX6*, and (c) an inv(11)(p13q13) with a breakpoint >75 kb downstream of *PAX6*. The proportion and spectrum of chromosome anomalies in familial (4/14, or 28.5%) and sporadic (26/63, or 41%) cases are not significantly different. An unexpectedly high frequency of chromosomal rearrangements is associated with both sporadic and familial aniridia in this cohort.

Introduction

Aniridia (MIM 106200) is a rare developmental defect affecting the anterior segment of the eye, observed in ~1 of 50,000–100,000 newborns (Nelson et al. 1984). Sporadic cases arise de novo but are subsequently inherited as an autosomal dominant phenotype, with virtually complete penetrance but variable expressivity. Aniridia generally occurs in isolation, but it also occurs, more rarely, as part of the WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation [MIM 194072]) deletion syndrome. WAGR deletions are of variable size but always include part or all of chromosome band 11p13. WAGR-associated phenotypes were also found to be variable, even in patients with deletions that are apparently identical cytogenetically (Franke et al. 1979; Turleau et al. 1984). Subsequently, molecular studies were used to define the proximal and distal breakpoints of both visible and submicroscopic deletions in patients with isolated aniridia and WAGR syndrome-associated

aniridia (van Heyningen et al. 1985; Davis et al. 1988; Mannens et al. 1991; Fantès et al. 1992, 1995b; Dreschler et al. 1994; Crolla et al. 1997; Kent et al. 1997).

Early analysis of such deletions led to the positional identification of *PAX6* and *WT1* as candidate genes for aniridia and Wilms tumor predisposition, respectively (Call et al. 1990; Gessler et al. 1990; Ton et al. 1991). *WT1* and *PAX6* map to a 700-kb interval in distal 11p13, which is within a fully sequenced region (GenBank accession number NT_009237). Mutations in *PAX6*, including deletions, have been identified in a high proportion of tested patients with either sporadic or familial aniridia (Fantès et al. 1992, 1995b; Glaser et al. 1992; Jordan et al. 1992; Hanson et al. 1993; Dreschler et al. 1994; Axton et al. 1997; Grønskov et al. 2001). Early studies showed that deletions that include the *WT1* gene are correlated with a significant risk of Wilms tumor, perhaps as high as 60%. More-recent studies have attempted to quantify this empirical risk more accurately, on the basis of larger samples (Grønskov et al. 2001; Muto et al. 2002; authors' unpublished data).

A number of studies have confirmed that human aniridia is a haploinsufficiency disorder resulting from inactivation of one copy of the *PAX6* gene, predominantly through intragenic mutations leading to premature termination of the protein (reviewed by Prosser and van Heyningen 1998). However, chromosomal abnormalities

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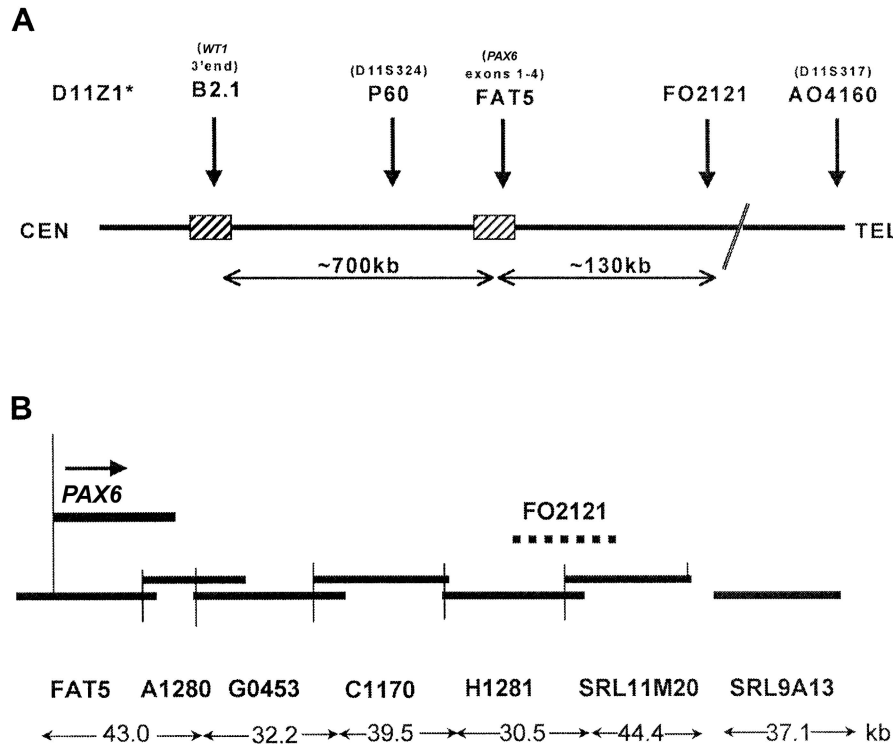


Figure 1 A, Map showing the position of the four cosmids used in the original FISH deletion analysis. D11Z1 is the centromeric alphoid repeat probe used to identify the 11 homologues, and D11S317 is a cosmid A4160, which lies ~400 kb telomeric of FO2121 (see fig. 2). B, Higher-resolution map showing the relative positions of the cosmid contig covering *PAX6* and the ~180-kb region telomeric of the gene.

including deletions, both visible and cryptic (Mannens et al. 1991; Fantes et al. 1992, 1995a; Dreschler et al. 1994; Kent et al. 1997; Crolla et al. 1997; Grønskov et al. 2001; Muto et al. 2002), and inversions, translocations, and insertions (Simola et al. 1982; Moore et al. 1986; Mannens et al. 1991; Fantes et al. 1995b; Crolla et al. 1996, 1997; Llerena et al. 2000), have also been reported in association with both isolated sporadic and familial aniridia, as well as in patients with WAGR syndrome. More recently, cell hybrid studies demonstrated inactivation of two independent, aniridia-associated *PAX6* alleles, each carrying an ~1,000-kb submicroscopic deletion distal to *PAX6*, with the proximal breakpoints at ~11 kb and ~22 kb 3' of the *PAX6* polyA addition site (Lauderdale et al. 2000). Analysis of a region ~150 kb downstream of the *PAX6* transcriptional termination site revealed *PAX6*-specific DNase hypersensitive sites and demonstrated a number of tissue-specific regulatory elements for *PAX6* (Kleinjan et al. 2001; Griffin et al. 2002).

Following the early reports of cryptic 11p13 deletions, a diagnostic FISH service was set up in Salisbury in 1993, to screen patients presenting with aniridia (particularly sporadic cases) and identify those with interstitial deletions, so that the extent of the deletion and

the associated empirical Wilms tumor risk could be assessed and reported clinically. Initially, the FISH analysis used only four cosmids (B2.1, P60, FAT5, and FO2121) (fig. 1A) (Crolla et al. 1997); however, after the identification of a patient with a deletion of FO2121 but not FAT5, an additional six cosmid probes contiguous with FAT5 were added to the panel (fig. 1B). A further seven flanking cosmids were used to give estimates of the overall sizes of the deletions (Fantes et al. 1995a). Patients 1–19 were initially reported by Crolla et al. (1997), and, for these patients, only the additional FISH investigations are presented here.

Subjects and Methods

The Study Population

Seventy-seven patients, falling into four principal categories, were referred for aniridia/WAGR FISH testing (table 1):

1. Sporadic isolated aniridia: 43 patients, of whom 38 (88%) were ≤ 5 years old at referral (mean \pm SD 13.2 ± 13.4 mo). The mean age at referral for all 43 cases was 43.0 ± 117 mo. Patient 94 is an MZ twin

Table 1
Patients by Referral Category and Rates of Abnormality

Referral Category	Total No. (%)	No. (%) Abnormal
Isolated sporadic aniridia	43 (56)	16 (37)
WAGR	12 (15)	8 (66.6)
Aniridia plus other abnormalities (not WAGR)	8 (10.5)	2 (25)
Familial aniridia	<u>14</u> (18.5)	<u>4</u> (28.6)
Total	77	30 (39)

- referred with bilateral isolated aniridia and optic atrophy. His twin brother is phenotypically normal.
- WAGR syndrome: 12 patients had aniridia and one or more of the other features associated with WAGR syndrome (table 2). Eight were ≤ 5 years old at referral (mean 29.9 ± 26.7 mo). The mean age at referral for all 12 patients was 96 ± 151 mo.
 - Aniridia and additional anomalies (not WAGR): Six of these eight patients had eye and/or other abnormalities in addition to aniridia (table 2). All patients were ≤ 2 years old at referral (mean 10.4 ± 9.1 mo).
 - Familial aniridia, all independent pedigrees: the age at referral for the probands in the 14 patients with familial aniridia ranged from a 38-wk-gestation still-born infant, who had inherited aniridia from the mother, to a 57-year-old man. All patients with familial aniridia were from pedigrees showing aniridia in two or more generations.

Clinical outcomes have been collated to determine the empirical risk of developing Wilms tumor in patients with isolated aniridia who are shown by FISH to have cryptic or visible constitutional deletions that include *PAX6* and *WT1*. The results of these studies will be presented as part of a larger study (authors' unpublished data).

Chromosome Preparation, Analysis, and Probe Labeling

Chromosome preparations were made using conventional techniques. All cases referred for FISH were examined by the referring laboratories with G banding at a minimum of 550-band resolution and were reported using the system recommended by the International System for Human Cytogenetic Nomenclature (table 3). Cytogenetic breakpoints for visible deletions and rearrangements are as reported by the referring laboratories.

Whenever possible, spare material from the cytogenetic and/or FISH analyses was stored either at -20°C (cell suspensions in 3:1 methanol:acetic acid fixative) or as lymphoblastoid cell lines. The FISH technique and analyses were performed as described elsewhere (Crolla et al. 1997). In brief, cosmids were labeled by nick translation with digoxigenin and the chromosome 11 centromere probe with biotin. The probe and chromosomal

target DNAs were codenatured at 75°C for 5 min and were incubated overnight at 37°C , and, after two 5-min stringent washes at 42°C in $1 \times \text{SSC}/50\%$ formamide, the sites of hybridization were visualized using antibodies: one layer each of anti-digoxigenin-TRITC (red fluorescence) and avidin-FITC (green fluorescence). Images were captured using a cooled CCD camera and were digitized for analysis through use of PSI Powergene software. A minimum of 10 metaphases were examined after hybridization with each probe; for patients 2 and 94, 30 metaphases were scored.

All cases were initially studied using four cosmids (from centromere to telomere: B2.1, P60, FAT5, and FO2121; see fig 1A) (Fantes et al. 1995a). Additional studies were prompted by the results obtained for patient 64, who was not deleted for FAT5 but was hemizygous for the more telomeric probe, FO2121 (~ 100 kb 3' of *PAX6*). All cases previously reported as normal were subsequently reexamined using the contig of fully sequenced cosmids covering the ~ 180 -kb region 3' of *PAX6* (fig. 1B). The FISH reexamination with this cosmid contig was performed mostly with stored fixed cells. Three cases were examined using fresh chromosome preparations derived from stored lymphoblastoid cell lines. For patient 2, the result was confirmed using chromosome preparations derived from a fresh peripheral blood sample. In four cases, FISH reexamination was not possible because no stored material was available, and these have been excluded from the results.

Anchoring of Cosmids onto the Chromosome 11 Map

Public STSs, gene segments, or sequence data associated with each cosmid used (fig. 2) are as follows: DO8153 (rapsyn, *RAPSN*), c1-11-474 (D11S1103), c1-11-458 (D11S1776), B2.1 (*WT1*), P60 (D11S324), FAT5 (*PAX6*, European Molecular Biology Laboratory [EMBL] accession number Z95332), A1280 (EMBL accession number Z83307), G0453 (EMBL accession number Z83301), C1170 (EMBL accession number Z83306), H1281 (EMBL accession number Z83309), FO2121 (overlaps H1281 and SRL11M20), SRL11M20 (EMBL accession number Z83308), SRL9A13 (EMBL accession number Z86001), AO4160 (D11S317),

Table 2**Clinical Features of Patients with Aniridia and Associated Anomalies**

DISORDER AND PATIENT NUMBER	AGE AT REFERRAL (XX/XY STATUS)	CLINICAL FEATURES				Other Abnormalities	DELETION (V/C) ^a
		W	A	G	R		
WAGR syndrome:							
11 ^b	4 years (XY)	—	+	—	+	Nystagmus, glaucoma	—
12 ^b	1 mo (XY)	—	+	+	—	Falot's tetralogy, 1st and 2nd ribs absent	—
14 ^b	2 mo (XY)	—	+	+	—	Single kidney	V
16 ^b	5 years (XY)	+	+	+	—		C
32	2 mo (XY)	—	+	—	+		—
33	10 mo (XY)	—	+	—	+		V
34	7 years (XY)	—	+	+	+	Bilateral cataracts	V
36	32 years (XY)	+	+	+	+	Nystagmus	V
41	4 years (XX)	—	+	—	+	Immunodeficiency	V
44	5 years (XY)	—	+	—	+	Bilateral colobomata	—
47	37 years (XY)	—	+	+	+		V
96	18 mo (XX)	+	+	—	—		C
Aniridia and other anomalies:							
13 ^b	4 mo (XY)	—	+	—	—	Optic atrophy	V
15 ^b	4 mo (XX)	—	+	—	—	Facial dysmorphism, glaucoma	V
29	2 years (XX)	—	+	—	—	Left microphthalmia, anterior segment dysgenesis	—
45	2 years (XY)	—	+	—	—	Glaucoma, bicuspid valve, mild stenosis	—
50	1 year (XX)	—	+	—	—	Facial dysmorphism	—
68	10 mo (XY)	—	+	—	—	Glaucoma	—
69	4 mo (XY)	—	+	—	—	Coloboma	—
77	1 mo (XY)	—	+	—	—	Exomphalos, glaucoma	—

^a V = visible deletion; C = cryptic deletion; — = no deletion.

^b Previously reported by Crolla et al. (1997).

CO8160 (D11S151, *KCNA4*), F1238 (D11S1446), and EO6182 (D11S1062).

The cosmids B2.1 and P60 and the *PAX6* contig covering the ~180-kb downstream region (fig. 1B) are available on request from J.A.C.

Results

The overall frequency of chromosomal abnormalities found in this cohort of patients with aniridia is summarized in tables 1 and 3 and in figure 2.

Abnormalities by Referral Category

Sporadic isolated aniridia.—Of the 43 patients in this group, 16 (37%) had chromosome abnormalities that accounted for their aniridia. Thirteen of the abnormalities were interstitial deletions—eight cryptic and five cytogenetically visible. Two of the three remaining patients (patients 1 and 85) had apparently balanced chromosome rearrangements; the third was patient 21, the child with a t(11;13) with a deletion at the 11p breakpoint. All five visible deletions involved *WT1*, compared with only one (patient 81) of the eight cryptic deletions. Five of the six cryptic deletions involving a proximal

breakpoint in cosmid A1280 were ascertained in patients with sporadic isolated aniridia.

WAGR.—Eight (66.6%) of the 12 patients with WAGR (tables 2 and 3) had deletions—six visible and two cryptic. Both cryptic deletions included *WT1* (in patients 16 and 96), as did five of the six visible deletions. The one visible deletion not involving *WT1* (in patient 47) had a proximal breakpoint between *WT1* and P60 (D11S324).

Aniridia and other abnormalities.—Two (25%) of the eight patients (tables 2 and 3) were chromosomally abnormal, both with visible deletions; the deletion in one patient included *WT1* (patient 15), whereas the deletion in the other patient did not include *WT1*, with a proximal breakpoint between P60 (D11S324) and *PAX6*.

Familial aniridia.—Four (28.6%) of the 14 patients with familial aniridia were chromosomally abnormal; three cryptic deletions were observed (patients 19, 70, and 75), the last being one of the patients with a proximal breakpoint in cosmid A1280, and a fourth patient had a pericentric inversion of chromosome 11.

Visible Deletions

Thirteen (17%) of the patients, all with sporadic aniridia, had visible deletions, only two of which (in patients 13 and 47) did not include *WT1*. The size of the

Table 3**Conventional Karyotypes of Patients**

Patient Number	Age at Referral	Cytogenetic Result (ISCN)
1	2 years	46,XX,t(7;11)(q31.2;p13)
2	1 year	46,XY
6	6 mo	46,XX
13	4 mo	46,XY,del(11)(p13;p15.1)
14	2 mo	46,XY,del(11)(p13;p14.2)
15	4 mo	46,XX,del(11)(p11.2;p14.1)
16	5 years	46,XY
19 ^a	29 years	46,XX
21	2 years	46,XX,t(11;13)(p?13;q?) de novo
25	4 mo	46,XY
26	3 years	46,XY
33	10 mo	46,XY,del(11)(p13p14.2)
34	7 years	46,XY,del(11)(p13p14.3)
36	32 years	46,XY,del(11)(p11.2p14)
41	4 years	46,XX,del(11)(p12p15.1)
42	1 years	46,XX,del(11)(p11.2p13)
47	37 years	46,XY,del(11)(p13p14.3)
51	2 years	46,XX,del(11)(p12p14)
61 ^a	2 years	46,XX,inv(11)(p13q13)mat
64	6 mo	46,XY
70 ^a	38 wk gestation	46,XX
73	4 mo	46,XX,del(11)(p11.2p13)
74	8 mo	46,XX,del(11)(p13p15.1)
75 ^a	1 years	46,XX
81	23 years	46,XX
83	5 mo	46,XY
85	1 mo	46,XY,dir ins(12;11)(q24.11;p13p15.1) de novo
94	2 mo	46,XY
95	3 mo	46,XX,t(2;11)(q33;q25)del(11)(p?12p?13) de novo
96	18 mo	46,XX

^a Patient with familial aniridia.

deletions varied (fig. 2), but the two patients with *WT1* not deleted had proximal breakpoints between P60 and FAT5 (patient 13), and *WT1* and P60 (patient 47). Seven of the nine deletions tested with the centromeric FISH markers had a breakpoint between c1-11-474 and c1-11-458. Four of these seven shared a similar distal breakpoint between F1238 and EO6182 (patients 15, 34, 51, and 73); two had breakpoints between CO8160 and F1238 (patients 33 and 95), and patient 74 had a more distal breakpoint beyond EO6182.

Cryptic Deletions

Thirteen (17%) of the 70 patients had cryptic 11p deletions that were visible only when FISH was used (fig. 2). Five of these were not detected in the original analysis, because the deletion did not include cosmid FAT5 (*PAX6* exons 1–4). These deletions were subsequently identified using the ~180-kb cosmid contig starting with A1280. Three of the 13 cryptic deletions included *WT1* (patients 16, 81, and 96). Patient 16 presented with Wilms tumor

at age 17 mo, and patient 96 received a diagnosis of Wilms tumor at age 18 mo. Patient 81 was referred at age 23 years and, apart from visual problems associated with her aniridia, she was reported to be well and intellectually normal.

The extent of cryptic deletions was estimated using information from chromosome 11 mapping (Fantès et al. 1995a) and sequencing around the *PAX6* region (Ensembl Genome Browser). Eleven of the 13 cryptic deletions ranged in size from ~75 to <1,500 kb (fig. 2). In patients 16 and 96, the deletion is deduced to cover 900–2,500 kb and 2,500–7,000 kb, respectively. Subsequent conventional reanalysis of these two cases showed that the deletions were not visible through use of light microscopy.

The cryptic deletions can be further subdivided into two groups:

1. Six patients had an apparently recurrent common proximal breakpoint in cosmid A1280, resulting in a diminished signal on one chromosome 11 homologue (fig. 3A). These deletions ranged in size from ~80 kb (patient 25) to ~450 kb (patients 6 and 64).

Centromere														Telomere	
VISIBLE DELETIONS															
Patient Number	DO8153	c1-11-474	c1-11-458	B2.1	P60	FAT5	FO2121	SRL11M20	SRL9A13	AO4160	CO8160	F1238	EO6182	Category	
13§	NT	NT	NT	+/+	+/+	+/-	+/-	NT	NT	NT	NT	NT	NT	p13p15.1	
14*	NT	NT	NT	+/-	+/-	+/-	+/-	NT	NT	NT	NT	NT	NT	p13p14.2	
15	NT	+/+	+/-	+/-	+/-	+/-	+/-	NT	NT	NT	NT	+/-	+/-	+/+	
33	NT	+/+	+/-	+/-	+/-	+/-	+/-	NT	NT	NT	NT	+/+	+/+	p13p14.2	
34	NT	+/+	+/-	+/-	+/-	+/-	+/-	NT	NT	NT	NT	+/-	+/+	p13p14.3	
36*	NT	NT	NT	+/-	+/-	+/-	+/-	NT	NT	NT	NT	NT	NT	p11.2p14	
41§	+/+	+/-	+/-	+/-	+/-	+/-	NT	NT	NT	NT	NT	NT	NT	p12p15.1	
42	+/+	+/-	+/-	+/-	+/-	+/-	+/-	NT	NT	+/-	+/-	+/+	+/+	p11.2p13	
47	NT	NT	NT	+/+	+/-	+/-	+/-	NT	NT	NT	NT	NT	NT	p13p14.3	
51	NT	+/+	+/-	+/-	+/-	+/-	+/-	NT	NT	NT	+/-	+/-	+/+	p12p14	
73	NT	+/+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	p11.2p13	
74§	NT	+/+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	NT	+/-	+/-	+/-	p13p15.1	
95	NT	+/+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	NT	p12p13**	
CRYPTIC DELETIONS															
	c1-11-458	B2.1	P60	FAT5	A1280	GO453	C1170	H1281	FO2121	11M20	9A13	AO4160	CO8160	F1238	Size (kb)
2(m)	NT	+/+	+/+	+/+	+/dim	+/-	NT	+/-	+/-	NT	+/-	+/+	NT	NT	200
6	NT	+/+	+/+	+/+	+/dim	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	NT	450
25	NT	+/+	+/+	+/+	+/dim	+/-	+/-	+/+	+/+	+/+	+/+	NT	NT	NT	80
64	NT	+/+	+/+	+/+	+/dim	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	NT	450
75 (F)	NT	+/+	+/+	+/+	+/dim	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	NT	100
94(m)	NT	NT	NT	+/+	+/dim	+/-	+/-	+/-	+/-	+/-	+/-	+/+	NT	NT	200
16	+/+	+/-	+/-	+/-	NT	NT	NT	NT	+/-	NT	NT	+/-	+/+	NT	>900<2,500
19 (F)	NT	+/+	+/+	+/dim	NT	NT	+/-	NT	+/-	+/-	+/-	+/+	NT	NT	250
26	NT	+/+	+/-	+/-	NT	NT	NT	NT	+/-	+/+	+/+	+/+	+/+	NT	150
70 (F)	NT	+/+	+/+	+/-	NT	NT	NT	NT	+/-	+/-	+/-	+/+	+/+	NT	230
81	+/+	+/-	+/-	+/-	NT	NT	NT	NT	+/-	+/-	+/-	+/+	+/+	NT	>280<1,500
83	NT	+/+	+/+	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	NT	NT	NT	75
96	+/+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	>2,500<7,000
CHROMOSOME REARRANGEMENTS															
	c1-11-458	B2.1	P60	FAT5	A1280	GO453	C1170	H1281	FO2121	SRL11M20	SRL9A13	AO4160	CO8160	F1238	
1	NT	st	st	st	st	st	mv	mv	mv	NT	NT	NT	NT	NT	
21	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	
61(F)	NT	mv	mv	mv	mv	mv	mv	split	st	st	NT	NT	NT	NT	
85	NT	st	st	st	st	st	split	mv	mv	NT	NT	NT	NT	NT	

Breakpoints

Figure 2 Details of the FISH results in the chromosomally abnormal patients. Patient numbers in boldface italic type are those with a proximal breakpoint in A1280. Shaded areas define the approximate size of the deletion. Size (kb) is the deletion size estimated using known mapping and sequence data. m = mosaic for deletion; F = familial; +/+ = signal on both homologues; +/- = one homologue deleted; +/dim = one homologue with diminished signal; mv = cosmid signal moved; st = cosmid signal stays on der(11); split = cosmid signal split; NT = not tested for this probe; § = cytogenetic breakpoint 11p15 (FISH on distal breakpoint not performed); * = no stored material for additional FISH; ** = also had t(2;11)(q33;q25) de novo.

In addition, two unrelated patients, 2 and 94, were mosaics for the deletion, both of which were ~200 kb in size and present in ~50% (patient 2) and 60% (patient 94) of peripheral blood metaphases (fig. 3B). The diminished A1280 signal suggested that the proximal breakpoint had occurred either within exons 5–13 of PAX6, including the ~1 kb 3' UTR (PAX6 3' UTR), or immediately distal to this region. In an attempt to resolve this, FISH with three plasmid probes from the 3' end of the gene and the 3' UTR (fig. 4), was performed on five of the six patients. Use of the plasmids individually did not yield FISH signals, but use of all three as pooled FISH probes gave signal on both 11p13 regions in approximately half of the metaphases examined (fig. 3C and 3D).

Binding of the plasmid pool suggests that the telomeric region of PAX6 is still present, but higher-resolution techniques are required to define these breakpoints with more precision.

Cosmid A1280 is 34,614 bp long, with its centromeric end at nucleotide 1 in the sequence given for EMBL Z83307 and the telomeric end >12 kb into the sequence for G0453 (EMBL Z83001). Exons 5–13 of PAX6 cover a little more than 15 kb at the centromeric end of A1280. The final exon (exon 10) of the ELP4 neighboring gene, transcribed on the opposite strand to PAX6, stretches over ~400 bp at 20 kb from the centromeric end of A1280.

2. Seven patients had a proximal breakpoint centromeric of PAX6. Three of these shared a proximal breakpoint

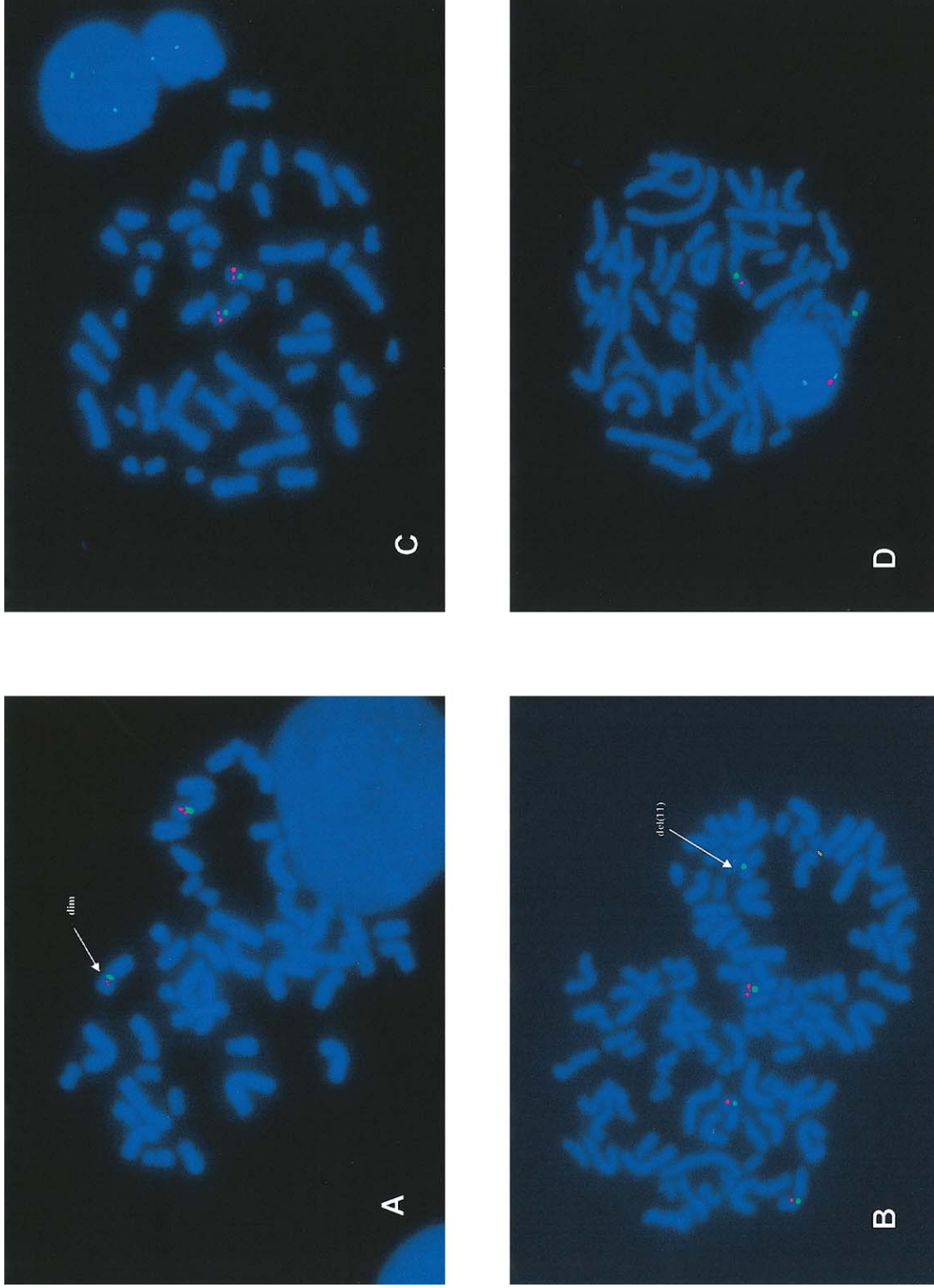


Figure 3 A, Result from use of cosmid A1280 on patient 2. The normal and abnormal homologue (indicated by the arrow) show a different size of hybridization signal, showing that only part of the genomic sequence contained in this cosmid binds to the deleted 11. The green signal represents the D11Z1 centromere probe. B, Result from use of cosmid FO2121 on metaphases from the mosaic patient 2. The metaphase on the left shows FO2121 signal on both 11 homologues, whereas the metaphase on the right has cosmid signal on only one of the 11 homologues. The green signal represents the D11Z1 centromere probe. C, FISH results in patient 64 with the contig of plasmid probes pExδ-11, pEE2250, and pSIP, showing signals on both homologues. D, FISH results in patient 64. In contrast to C, signal is only seen on the normal homologue.

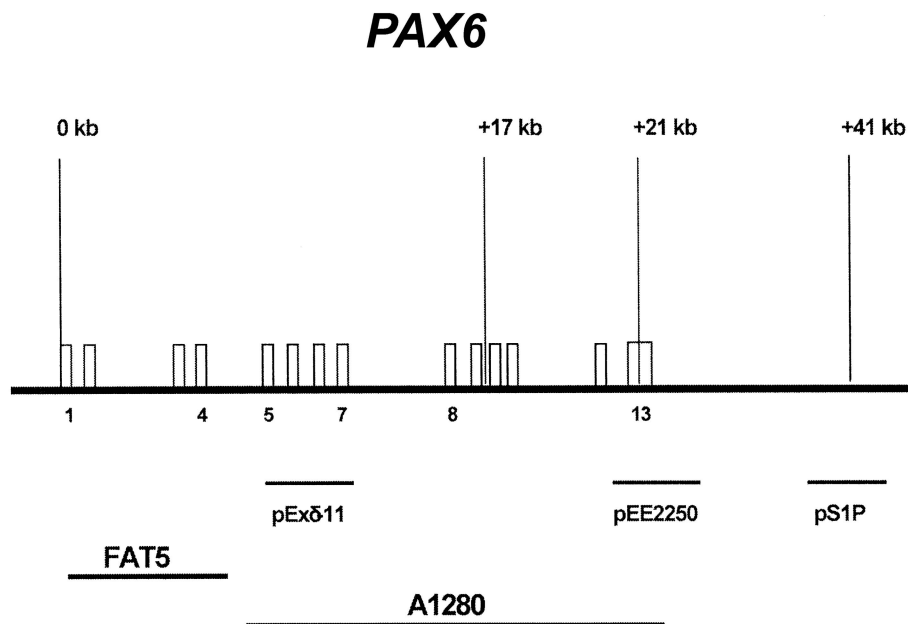


Figure 4 Map showing the position of the plasmid and cosmid probes used for FISH

between c1-11-458 and B2.1 (patients 16, 81, and 96). The proximal and distal breakpoints of the remaining four were heterogeneous (fig. 2).

Chromosomal Rearrangements

Four patients were identified with chromosomal rearrangements involving one breakpoint in distal 11p13 (fig. 2). Patient 1 has an apparently balanced $t(7;11)$ (Crolla et al. 1996), where the 11p breakpoint apparently lies between adjacent cosmids, with the G0453 signal present on the der(11) and C1170 on the der(7), placing the breakpoint ~ 30 kb 3' of *PAX6*. Patient 61, with familial aniridia, has a pericentric inversion of chromosome 11 (p13q13) with the 11p breakpoint identified by a split in cosmid H1281, placing the breakpoint >75 kb downstream of *PAX6*. Patient 85 has an apparently balanced rearrangement defined as a $dir\ ins(12;11)$ where the 11p breakpoint splits cosmid C1170 ~ 50 kb 3' of *PAX6*. These three chromosomal rearrangements almost certainly represent position effects leading to heterozygous loss of *PAX6* function. The overall frequency of position effects in this series of patients with aniridia is 3/77 (3.8%).

The fourth chromosomal rearrangement, an apparently balanced $t(11;13)$, was found by FISH to have an ~ 7.5 -Mb deletion at the breakpoint on the der(11) (Llerena et al. 2000).

Discussion

The Need for Deletion Analysis and Choice of Probes

Since the original identification of *PAX6* as the aniridia candidate gene, intragenic mutations and chromosomal deletions, translocations, and inversions have been associated with a high proportion ($\sim 80\%$) of aniridia cases studied in detail (Axton et al. 1997; Crolla et al. 1997; Grønskov et al. 2001). Most of the intragenic mutations lead to premature protein truncation (Hanson et al. 1993; Axton et al. 1997; Prosser and van Heyningen, 1998; Chao et al. 2000) and haploinsufficiency. FISH analysis is the most efficient way of identifying the extent of aniridia-associated deletions, both visible and cryptic. The aim was to ascertain whether patients with aniridia, particularly children younger than ~ 7 years of age with de novo phenotypes, are deleted for *WT1* and therefore require monitoring for increased risk of Wilms tumor (Fantès et al. 1992; Crolla 1997; Kent et al. 1997). FISH analysis, using cosmids B2.1, P60, FAT5, and FO2121, was also ideal for defining the position of chromosomal breakpoints associated with translocations and inversions leading to aniridia (Fukushima et al. 1993; Fantès et al. 1995b; Crolla et al. 1996). Once it became clear that chromosomal rearrangements with breakpoints outside the *PAX6* transcription unit could also lead to classical aniridia phenotypes, the cosmid contig

used for FISH studies was extended ~180 kb downstream of the *PAX6* polyA addition site (fig. 1B).

Cosmid contigs are ideal probes for use in fine deletion analysis, since the signal is strong enough to be present reliably, but the genomic insert size (~30 kb) is small enough to detect small deletions by loss or diminution of signal. Some smaller structural changes may be detectable only through pulsed field gel or Southern blot analysis.

After the setting up of the molecular cytogenetic service in Salisbury, through use of the four-cosmid set for the identification of aniridia-associated deletions, an affected individual was identified who had an intact *PAX6* cosmid (FAT5) but was deleted for the more distant FO2121 (fig. 1A). Subsequently, the extended cosmid contig (fig. 1B) was used for all new FISH studies and in a retrospective analysis of earlier samples. Subtle rearrangements were defined, with breakpoint positions identified more accurately.

Unexpectedly High Frequency of Rearrangements Seen

The spectrum of patients with aniridia sent by clinicians for FISH studies was broad: the largest category comprised patients with isolated sporadic aniridia (43); patients with syndromic aniridia, including 12 with the WAGR phenotype, accounted for 20 patients in total, and analysis was also requested for 14 patients with familial isolated aniridia (table 1). That 8/12 WAGR phenotypes were found to be associated with deletions is not surprising. In contrast, the identification of cryptic deletions in 8/44 sporadic isolated aniridia cases, (86% of patients were ≤ 5 years old at referral), with no evidence of associated anomalies, was unexpected. A further eight of the submitted patients with sporadic isolated aniridia carried previously identified structural rearrangements or visible deletions. However, one of the structural rearrangements and all eight cryptic deletions were only identified in the course of this analysis. Overall, 9/44 (20%) of patients with sporadic isolated aniridia were found to have unsuspected underlying chromosomal rearrangements.

Perhaps most surprising was the finding of chromosomal rearrangements in four independent pedigrees among the 14 patients with familial aniridia studied, with three cryptic deletions and a previously identified cosegregating pericentric inversion. The frequency of unsuspected aberrations (3/14 [21%]) among patients with familial aniridia is similar to the frequency in the sporadic category. In contrast, Grønskov et al. (2001), using only FAT5 and B2.1 as FISH probes, found no chromosomal anomalies among 19 patients with familial aniridia, but they identified intragenic point mutations in 17 patients. However, some small deletions may not be identifiable when only these two cosmids are used.

Recurrent Breakpoint Region in Cosmid A1280

Six patients were identified (four retrospectively and two prospectively) with a diminished A1280 signal (figs. 2 and 3A) and a deletion involving two or more of the telomeric cosmids in the contig. Cosmid A1280 carries 10 of the 14 *PAX6* exons and >15 kb of downstream region beyond the predicted polyA addition site, which also contains the final exon of the gene neighboring *PAX6*, now known to be the human homologue of transcriptional elongation factor *ELP4*, first defined in yeast (Kleinjan et al. 2002). The latest gene prediction programs also define a predicted additional single-exon 138–amino acid ORF sequence only 2 kb beyond the *PAX6* polyA addition site (like *ELP4*, transcribed on the opposite strand). In addition to the six deletion breakpoints within A1280 among the current cohort, the centromeric end of one of the deletions described by Lauderdale et al. (2000) is ~8 kb from the telomeric end of A1280 (Lauderdale et al. 2000). Sequence comparison reveals no region-specific low-copy repeats (Stankiewicz and Lupski 2002) that might be associated with recurrent breakpoints, although the last 2 kb at the telomeric end of A1280 contain two different 50–60-bp segments that are also found, perfectly repeated, on several other chromosomes, though not on chromosome 11. The second deletion breakpoint described by Lauderdale et al. (2000) is within cosmid G0453. In view of the high frequency of breakpoints within this region, the use of this contig (fig. 1B) may identify aniridia-associated breakpoints in cases in which conventional sequence-based mutation analysis fails to find point mutations.

Mosaicism for Cryptic Deletion in Two Patients with the Aniridia Phenotype

Two of the patients who have a de novo cryptic deletion (patients 2 and 94) with a reduced A1280 signal show the deletion in ~50% of the peripheral blood metaphases (fig. 3B), with the remaining metaphases showing normal signals on both chromosome 11 homologues. In both patients, the telomeric breakpoint of the mosaic deletion is between cosmids SRL9A13 and AO4160 (fig. 2). The results from the use of the pool of the three plasmid markers over *PAX6* exons 10–13 (fig. 4) suggest reduced FISH signals in these patients, implying truncation of the *PAX6* transcription unit by the mosaic deletion. In both patients, chromosomes have been analyzed only from peripheral blood lymphocytes, so that the degree of mosaicism in other tissues is not known. Both of the babies were referred (at ages 12 mo and 2 mo) with bilateral aniridia and optic atrophy, without any other clinical complications. Optic atrophy is a phenotype that has not previously been associated with *PAX6* mutations. Interestingly, the second patient (patient 94) is an MZ twin with an unaffected brother, which suggests that somatic mutation in the affected

twin occurred after the two-blastomere stage, which is the earliest point at which MZ twinning can occur. The presence of the normal cell lines in both patients would have made it very unlikely for the deletions to be detected using conventional DNA mutation-detection techniques, so that overall mosaicism involving cryptic deletions may be underrepresented among patients with aniridia. Furthermore, the fact that we observed mosaicism in 2 of the 26 deletions overall (2/13 of the microdeletions), suggests that mosaicism may be underrepresented as a mutational mechanism in microdeletion syndromes (Pearson 2002).

Somatic mosaicism for structural chromosome abnormalities is a well-recognized but rarely reported phenomenon and has been described for a number of syndromes, including 22q13 deletion (Riegel et al. 2000; Phelan et al. 2001), neurofibromatosis type 1 (Ainsworth et al. 1997; Tinschert et al. 2000), 22q11.3 deletion (Kasprzak et al. 1998), dystrophin (Bunyan et al. 1995), 1p36.33 deletion (Eugster et al. 1997), Angelman syndrome 15q11-13 deletion (Tekin et al. 2000), and 19q13.33 deletion (Mikelsaar et al. 2001). Molecular confirmations of germline mosaicisms for deletions of 22q11.2 (Hatchwell et al. 1998) and Williams-Beuren syndrome (Kara-Mostefa et al. 1999) have also been reported.

The observation of the aniridia phenotype in two mosaic cases is also surprising, in the light of previous findings that cells carrying heterozygous *Pax6* mutations cannot contribute to lens tissue in artificial mouse chimeras (Collinson et al. 2001).

Position Effects as a Mutational Mechanism

Position effects, in which loss or alteration of gene function results from chromosomal rearrangements outside the transcribed region of a gene, have been described for a number of human disease loci (Kleinjan and van Heyningen 1998). On the basis of the cases reported here and those described in previous studies (Lauderdale et al. 2000; Kleinjan et al. 2001, 2002), it is clear that position-effect disruptions of *PAX6* regulatory element(s) affecting the chromosomal domain as distant as 180 kb telomeric of the 3' end of the gene are rare but important causes of human sporadic and familial aniridia. In addition to position-effect breakpoints, we have observed a wide variety of interstitial deletions varying in size from 75 kb to ≥ 7 Mb, which suggests that this region, particularly the ~ 180 kb immediately telomeric to *PAX6*, may be a region of genomic instability.

FISH Analysis Is the Ideal First Choice for Assessing the Molecular Cause of Sporadic Aniridia

The present study illustrates the importance of FISH for the characterization of mutations associated with both familial and sporadic aniridia. The high frequency

of submicroscopic deletions detected suggests that FISH should be used as the primary screening procedure, particularly in the neonatal period, in patients ascertained with sporadic isolated aniridia. Our results, in combination with those of Lauderdale et al. (2000), also show that, in order to detect all deletions, it is necessary to use a contig of FISH probes covering the ~ 180 -kb region 3' of *PAX6*. This is particularly important in young patients with sporadic isolated aniridia who are reported to have a normal karyotype by conventional analysis. In these cases, we recommend that cosmids (listed in order from centromere to telomere) B2.1, P60, FAT5, A1280, GO453, C1170, H1281, and FO2121 should be used as the initial FISH screen. Intragenic mutation-screening protocols can then be used on those patients reported to be normal when tested by conventional cytogenetics and FISH.

Additional Data Reported Since Submission of the Present Article

FISH analysis of the phenotypically normal MZ brother of patient 94 has shown that he is also a mosaic for the same sized (~ 200 kb) cryptic deletion but that in the peripheral blood, the proportion of deleted cells is $\sim 30\%$, compared with $\sim 60\%$ in the affected twin brother. This implies that mosaicism arose before the splitting of the embryo, or that the twins arose from a chimeric embryo.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/Homo_sapiens/
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/>
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for aniridia [MIM 106200] and WAGR syndrome [MIM 194072])

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