The incidence of PAX6 mutation in patients with simple aniridia: an evaluation of mutation detection in 12 cases

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Abstract

Twelve aniridia patients, five with a family history and seven presumed to be sporadic, were exhaustively screened in order to test what proportion of people with aniridia, uncomplicated by associated anomalies, carry mutations in the human PAX6 gene. Mutations were detected in 90% of the cases.

Three mutation detection techniques were used to determine if one method was superior for this gene. The protein truncation test (PTT) was used on RT-PCR products, SSCP on genomic PCR amplifications, and chemical cleavage of mismatch on both RT-PCR and genomic amplifications. For RT-PCR products, only the translated portion of the gene was screened. On genomic products exons 1 to 13 (including 740 bp of the 3' untranslated sequence and all intron/exon boundaries) were screened, as was a neuroretina specific enhancer in intron 4.

Ten of the possible 12 mutations in the five familial cases and five of the sporadic patients were found, all of which conformed to a functional outcome of haploinsufficiency. Five were splice site mutations (one in the donor site of intron 4, two in the donor site of intron 6, one in each of the acceptor sites of introns 8 and 9) and five were nonsense mutations in exons 8, 9, 10, 11, and 12. SSCP analysis of individually amplified exons, with which nine of the 10 mutations were seen, was the most useful detection method for PAX6.

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Aniridia is a rare human congenital malformation of the eye characterised by almost complete absence of the iris. There are associated anomalies of cataracts, corneal vascularisation, and glaucoma, so that affected subjects frequently suffer severe visual impairment. About two thirds of cases are familial with autosomal dominant inheritance and a high degree of penetrance, but considerable phenotypic variability. The remainder of cases are sporadic, either isolated aniridia with no malformations outside the eye, or as part of the WAGR syndrome (Wilms' tumour, aniridia, genitourinary abnormalities, and mental retardation).[1]

Pax6 was originally isolated from mouse[2] and mutations in the gene are responsible for the *Small eye* phenotypes.[3] Subsequently, the human PAX6 homologue was shown to be mutated in aniridia patients.[4] The gene has been studied in a number of organisms, including *Drosophila*,[5][6] showing that its central role in eye development has been conserved throughout the animal kingdom. Evolutionary conservation of the amino acid sequence of the gene is high and between mouse and human there is only a single amino acid difference in the alternatively spliced form.[7][8] Despite great differences between insect and vertebrate eyes, mouse *Pax6* induces ectopic eye development in transgenic *Drosophila*.[9] The protein sequence has recognisable functional domains: a 128 amino acid paired domain and a 61 amino acid paired type homeo domain believed to bind to a bipartite DNA recognition sequence, separated by a 78 amino acid linker region, as well as a 152 amino acid proline, serine, and threonine rich C-terminal region (PST) reminiscent of the activation domain of the Oct-2 transcription factor.[8][10]

Mutations have been published for approximately 40 aniridia cases[1][11–14] and virtually all lead to premature termination of translation of the protein. Since no differences have been observed between the eye phenotypes associated with deletions of the entire PAX6 gene in WAGR patients and intragenic mutations leading to premature protein truncation in familial and sporadic aniridia patients, PAX6 mutations in aniridia are assumed to be caused by haploinsufficiency or the complete loss of function of one gene copy.[1] There is one reported example of a missense mutation in aniridia but the result of the mutation may be a non-functional protein. It affects an invariant arginine (R208) which is part of a four amino acid motif immediately 5' of the homeo domain of certain pax genes and may provide sequence specific contacts between the domain and the DNA target,[15] or act as a nuclear localisation signal.[12]

Peters' anomaly, an anterior segment malformation, and aniridia have been proposed as alternative phenotypes resulting from loss of function of the PAX6 locus[16] based on data which included one patient deleted for PAX6 and affected members of a family with a missense mutation (R26G). The missense

Table 1   Primers for amplifying the 3' untranslated portion of the gene and the neuroretinal enhancer

3' untranslated		
K795	5'GTTCCCGGAAGTGAACCTGATAT	309 bp
G404	5'AAATAATACACCAAAATG	
G647	5'TAGAGCCGCTTCAGTTCTAC	254 bp
G239	5'AGCTTGTTGATCATGGTTTTC	
H830	5'GATCTGTGTTGGTCATGAAGTTG	233 bp
H831	5'ACTTCATCTGTTAACAACCTTTG	
H832	5'TATGTCAGAAATATCTGTTGGT	179 bp
F948	5'CTGAGGATTTCTAGGGAAGAC	
G648	5'GATAGCTAAAAGGTAGAGTGTG	208 bp
H16	5'GAACAATTAACTTTTGCTGGCC	
K796	5'TCATACGTTTCTTTTTTGGCCAGC	247 bp
K794	5'GGTTAAAAGAAACCTGTGTAATAAAGG	
Neuroretinal enhancer		
I132	5'ACGGAAAGGGTCGTTTTATC	185 bp
I174	5'CTGCACGTCAAGTTTGAGCT	

mutation involves another invariant arginine believed to be crucial for DNA binding. A further missense mutation was recently identified in a case of isolated foveal hypoplasia in yet another invariant arginine (R125C) conserved in all identified PAX family members and involved in contact of the paired domain with the DNA target.[17] Foveal hypoplasia is a common feature of recognised ocular disorders such as albinism and aniridia, but isolated foveal hypoplasia is rare and sporadic. The variable phenotypes resulting from these missense mutations at invariant and functionally crucial arginines may be because of the effects of haploinsufficiency on different genetic backgrounds rather than mutation specific differences. The high degree of genetic conservation in PAX6 would indicate that most mutations are deleterious and that missense mutations which result in altered function rather than loss of function must be associated with a phenotype quite distinct from aniridia.

Congenital cataracts and autosomal dominant keratitis are phenotypes which may be caused by altered or reduced activity of mutant PAX6 rather than no activity. Two members of one family with bilateral cataracts and decreased visual acuity but apparently normal iris development carried a stop mutation at amino acid 353 in exon 12. The resultant protein, prematurely terminated in the activation domain, was shown to have reduced activity rather than no activity.[18] In another family, two members carried a PAX6 splice mutation which was shown to alter the ratios of the different splice forms. They, too, suffered from bilateral juvenile cataracts with reduced vision, but had intact irides.[19] Autosomal dominant keratitis has clinical manifestations which overlap with aniridia but iris involvement that is less

severe. In a family with 15 affected subjects of varying severity, an intron 11 splice acceptor mutation was found. While the exact molecular effect of the mutation was not determined, it probably led to excision of exon 12 and a protein prematurely terminated in the activation domain.[20] A mutant protein truncated in this region had some residual transcriptional activity.[18]

Haploinsufficiency of the PAX6 protein is most frequently achieved by premature termination of translation, either through nonsense mutations, frameshifts, or splicing errors, although a mutation leading to the in frame excision of a major part of the homeo domain of the protein has also been reported,[15] as well as certain missense mutations, detailed above, which may produce proteins unable to bind their target DNA sequence. Several functional domains of the protein, including the DNA binding homeo and paired domains, the transcriptional activator region in the C-terminus of the protein, and a highly conserved four amino acids N-terminal to the homeo domain, appear to be equally important functionally since mutation in any one of the domains is apparently equivalent in effect.[1] This argues for a simple or single function for PAX6 which involves DNA binding and transcriptional activation. It is not known what proportion of aniridia patients contain mutations in PAX6 although the gene is now believed to be the major, and probably the only, predisposing locus.[21] This study aims to test exhaustively for mutations in the gene in a selected group of patients.

Materials and methods
MATERIALS
Established lymphoblastoid cell lines were available for all patients and were used to prepare both RNA and DNA.[15] Sequence numbering follows Ton *et al.*[10]

PCR AMPLIFICATION
Genomic PCR
Exons 1-13 were individually amplified for SSCP using oligonucleotides published by Glaser *et al.*[8] The 3' untranslated region was amplified in six overlapping PCR fragments using oligonucleotides from table 1; 185 bp of intron 4 incorporating a neuroretinal enhancer[22] (table 1) was also screened. Standard PCR conditions were used with a "hot start" of five minutes at 94°C before addition of *Taq* polymerase. For genomic HOT analysis exons 3-13 were amplified singly (exons 5, 7, 8, 11) or doubly (exons 3 and 4, 5a and 6, 9 and 10, 12 and 13) using the same primers and conditions.

Table 2   Primers for RT-PCR (numbering according to Ton et al[10])

C139	5'AGAGTGGACAGACATCCGAGA (nt 285-305)
I252	5'GGATCCTAATACGACTCACTATAGGAACAGACCACCATGCAGAA CAGTCACAGCGG (nt 363-382 for the last 20 nts)
I253	5'AGAACTGAAGCGGCTCTAAC (3' untranslated)
I254	5'CTGAATTAACACAATATTTCC (3' untranslated)
I673	5'GGATCCTAATACGACTCACTATAGGAACAGACCACCATGATCGG TGGTAGTAAACCGAG (nt 615-634)
I672	5'GGATCCTAATACGACTCACTATAGGAACAGACCACCATGCGACT TCAGCTGAAGC (nt 1008-1026)
B510	5'TTTACTACCACCGATTGCCCTG (nt 587-566)
B958	5'TGTCCAACGGATGTGTGAGT (nt 505-524)
B508	5'CCTCCTTCCTGTTGCTGGCA (nt 910-891)
B509	5'TGCCAGCAACAGGAAGGAGG (nt 891-910)
D840	5'CGCTGTAGGTGTTTGTGAGG (nt 1344-1325)

RT-PCR
RT-PCR[23] was carried out with nested primers (table 2) on 1 µg of RNA prepared from patient lymphoblastoid cell lines. External primers were C139 to I253 and three products were produced from internal primer pairs: I252-I254 (1357 bp) which includes all the translated sequence; I673-I254 (1104 bp) which initiates in exon 6, 84 amino acids short of the

full length protein; and I672-I254 (711 bp) which initiates in exon 8, 215 amino acids short of the full length protein. Additional primers listed in table 1 were used to amplify selected regions of the transcripts when testing for splicing abnormalities in the RNA.

MUTATION DETECTION
SSCP

PCR products showing a single band of the correct size were used for SSCP screening.[24] Approximately 10 μl of product (depending on DNA concentration) was mixed with 5 μl of denaturing dye (95% formamide/20 mmol/l EDTA), denatured, and run on 15% acrylamide gels (29/1 with bis). All samples were run on 18 cm × 16 cm × 1.0 mm vertical gels for 15 hours at 12°C (water cooled) and constant current (1 milliamp per 10 bp). The gels were silver stained as described by Budowle *et al.*[25]

PTT

Protein truncation test (PTT) was carried out according to Roest *et al*[26] using the TNT-T7 coupled reticulocyte system (Promega). ^{35}S-methionine labelled protein products were resolved on 8, 10, and 14% SDS PAGE gels on a Hoefer "Mighty Small" (SE245) electrophoresis apparatus run at 150 volts until the bromphenol blue reached the base of the gel. A non-radioactive rainbow size marker (Amersham 14 300-200 000) was used. Gels were fixed, dried, and autoradiographed.

HOT

HOT analysis was done according to Condie *et al.*[27]

Sequencing

PCR products were purified from LMagarose by Geneclean TM or Wizard PCR Prep. Sequencing was done according to Condie *et al.*[27]

Restriction site alteration

Restriction sites were altered by the following mutations. In MATJO a *Bst*NI site is destroyed; in HORLA an *Mbo*II site is destroyed; in VAKIN an *Mse*I site is created; in GAWES a *Tsp*45I site is created; and in JOPOT a *Hae*III site is destroyed. In order to verify the double point mutation found in p236113, an amplification which creates two *Bss*HII sites in the mutant but only one in the normal allele was set up. The forward primer (J774 from nt 314-340) introduced two changes (C for A at nt 324 and G for C at nt 327) so that the primer sequence 5' CCCATATTCGCGCGCCGT-GGAATCC creates a *Bss*HII site in both mutant and normal alleles, as shown. The reverse primer (J775 in intron 4) introduced a G for A change in the penultimate nucleotide, and reads 5' GAAGTCCCAGAAAGACCA-GAGGCGC, finishing at +5 of intron 4. In the mutant, the AAGC change together with the introduced change in J775 creates a second *Bss*HII site, not present in the normal allele. Amplification of the 87 bp fragment followed by digestion with *Bss*HII gives a 77 bp fragment in the normal allele and a 77 bp plus a 51 bp fragment in the mutant allele.

Results

The exact mutations in each of the patients are listed in table 3.

Table 3 Mutation detection in 12 aniridia patients

Patient	Mutation	Effect	Inheritance	FISH analysis
MATJO male	G→C intron 9 at −1	?	Sporadic	No deletion
HORLA male	A→T, nt 1161 exon 10	R267X	Sporadic	No deletion
JAMAD male	GT del, nt 1071/2 codon 237, exon 9	Stop 12 codons later	Sporadic	No deletion
VAKIN female	T insertion intron 6, +2	Uses altern splice Deletes 36 aa, ex 6	Sporadic (+talipes)	No deletion
OLGIR female	Not detected	—	Sporadic	No deletion
CUICKLE ?	Not detected	—	Sporadic	ND
MAJMA female	G del, nt 1452 codon 364, exon 12	Stop, immediate	Familial	ND
GAWES male	C→T, nt 969 exon 8	R203X	Familial	ND
MEJOH female	A→C intron 8 at −2	?	Sporadic	No deletion
p236113 female	dbl mut, AA→GC intron 4 at +3, +4	Excludes exon 4	Familial	ND
JOPOT female	C→T, nt 1311 exon 11	R317X	Familial	ND
ANALL male	G→A, intron 6 at +1	Uses altern splice Deletes 36 aa, ex 6	Familial	ND

nt=nucleotide, del=deletion, altern=alternate, ex=exon, aa=amino acid, ND=not done, ?=effect unknown.

RNA

Three RT-PCR products were amplified from each sample with a view to carrying out PTT analysis. The longest product (I252-I254, see methods) included the alternatively spliced exon 5a which, on amplification, would lead to a 42 bp difference in size in the 1.4 kb products, not distinguishable on the 2% agarose gel used to purify the RT-PCR products. In practice, two PCR products were seen, one about 150 bp smaller than expected and presumed to be the result of mispriming. The 5' end of the PAX6 gene is GC rich and suitable priming sites for PCR amplification of the translated 5' portion of the gene frequently terminate with nucleotide runs repeated elsewhere in the region. In order to avoid the complication of alternative splicing and because no ideal priming sequence at the start of PAX6 translation was available, a PCR product was amplified from exon 6 (I673-I254). In order to identify proteins deleted for only a small number of amino acids in the carboxy end of the protein, a product starting in exon 8 was amplified (I672-I254). None of the PCR amplifications of PAX6 from lymphoblastoid cell line RNA was ideal.

Two mutations in these patients could be detected solely on the basis of RT-PCR (VAKIN and ANALL) using primers I673/I254 (fig 1A). These mutations were not visible using I252/I254 because weak amplification of the mutant product was masked by amplification owing to mispriming. The two patients each contain mutations at the donor site of intron 6 (t insertion at +2 in VAKIN, g→a change at +1 in ANALL) and amplification of the region using primers B958/B508 for a shorter product shows the expected wild type size and one 108 nt shorter in both patients (fig 1B). Hanson *et al*[15] reported a subject with a mutation identical to that of ANALL and showed that an alternative splice site within exon 6 (the GT of nts 612 and 613) was used in preference to the defective site, removing 108 nts of RNA from nt 612 to 719. It is interesting that the mutant products in VAKIN and ANALL were considerably under-represented

in comparison to the wild type even though the mutant RNA contains an in frame deletion rather than a premature stop codon. Clearly, however, a shortened RNA is produced.

Two further patients, MATJO and MEJOH, contain splicing mutations at the acceptor site of introns (g→c at -1 of intron 9 and a→c at -2 of intron 8, respectively) but no abnormally sized RT-PCR products were identified in any of the three routine amplifications, nor by using primers B509/D840 to look for differences in a short product encompassing exons 9 and 10 (fig 1C). It is more difficult to predict the effect of 3' splice site alterations since the splicing outcome of such mutations is frequently not reported and, where it is reported, the outcome can be exon skipping, use of cryptic site(s), or no detectable aberrant RNA.[28]

One patient (p236113) contained a 5' splice site mutation in intron 4 leading to the excision of exon 4 from the transcript (fig 1D). The excision was seen on second round amplification using primers C139/B510, but would not be visible in any of the products from the amplifications routinely carried out. The mutation itself is, unusually, a double point mutation and, following sequencing, was validated by mutation specific PCR amplification which generated a restriction site (Methods).

Finally, five patients contained point mutations producing nonsense codons (HORLA, JAMAD, MAJMA, GAWES, and JOPOT) and only normal sized RNA would be expected. No mutations were found in two patients (OLGIR and CUICKLE).

PTT (PROTEIN TRUNCATION TEST)
Because virtually all identified PAX6 mutations result in premature termination of translation of the protein product,[1] PTT should be an ideal method for identification of mutations in this gene. PTT uses PCR amplification of genomic DNA (where exons are large) or RNA, via RT-PCR, and in both cases it incorporates in one primer a T7 polymerase promoter and an in frame eukaroytic translation initiation site. In vitro transcription and translation of the resulting PCR product
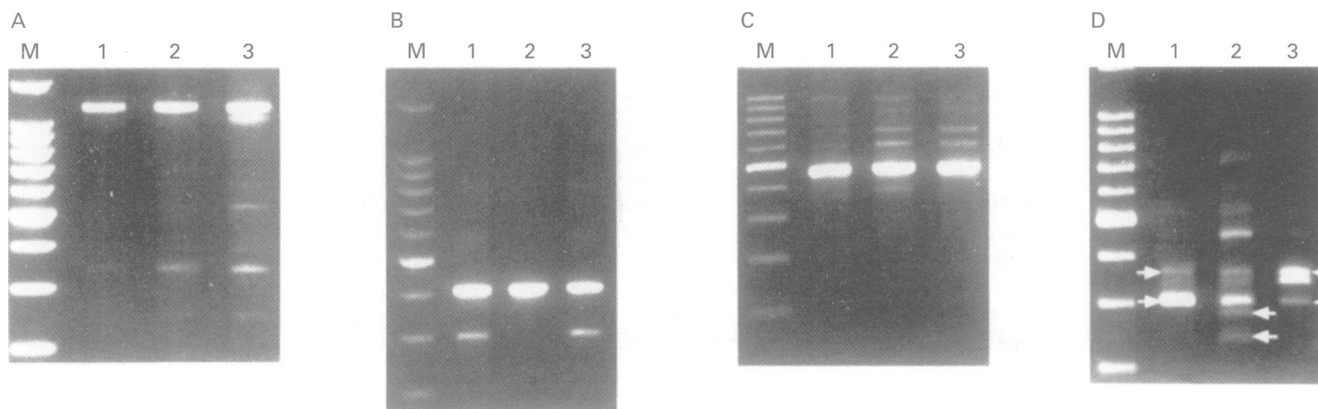

Figure 1   RT-PCR results. In each gel, the unspecified lanes represent wild type results and M refers to the marker, a 100 bp ladder. (A) Primers I672/I254. A full length product of 1104 bp is seen in lanes 1-3 as well as a shortened product of 996 bp in lane 3 (ANALL). (B) Primers B958/B508. A full length product of 406 bp is present in each lane as well as an under-represented mutant band of 298 bp in lane 1 (ANALL) and lane 3 (VAKIN). (C) Primers B509/D840. A full length product of 453 bp is present in each lane. No shortened products are seen because of excision of exon 10 in lane 1 (MATJO) or of exon 9 in lane 3 (MEJOH). (D) Primers C139/B510. The full length sizes of 345 bp (+exon 5a) and 303 bp (-exon 5a) are arrowed in lanes 1 and 3. In lane 2 (p236113) arrowed bands of 284 bp and 242 bp agree with excision of exon 4 from both + and -5a products. Other bands may be the result of mispriming or missplicing.
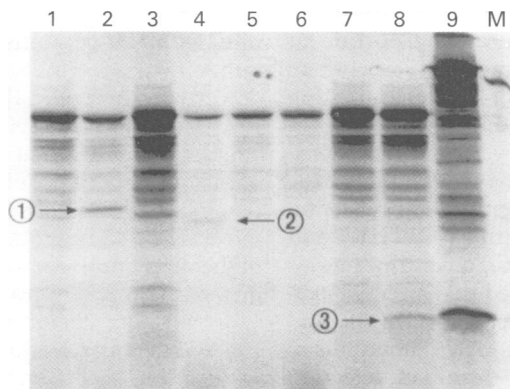
Figure 2   PTT for transcript I673/I254 run on 14% SDS PAGE gel. The lanes are loaded MATJO, HORLA, FATO (normal control), JAMAD, OLGIR, CUICKLE, MAJMA, GAWES, control, and non-isotopic rainbow marker (lanes 1-10 respectively). Numbers 1, 2, and 3 refer to mutant protein bands of 27.5, 26.3, and 17.7 kDa, visible above the background.

followed by SDS-PAGE analysis shows the wild type protein product and any truncated protein fragments from mutant RNAs. Size estimation of the truncated protein indicates the position of the putative nonsense codon caused either by point mutation at the site, or by an insertion, deletion, or out of frame splicing mutation 5' of the stop codon. Direct sequencing identifies the precise mutation. Mutations in three patients were identified in this way.

For PTT on PAX6 we translated three different PCR products in vitro, one incorporating sequence from the ATG which initiates protein synthesis to 40 nucleotides downstream of the termination TAA, one starting in mid exon 6, and one starting in mid exon 8, and the products were run on three densities of acrylamide gel. Proteins differing in size by less than 2 kDa (the -exon 5a product) would not be apparent when analysing the 60 kDa full size protein product.

Truncated proteins indicating mutations in three patients were observed in samples HORLA, JAMAD, and GAWES (fig 2). These truncated proteins, as well as a spurious truncation product (JOPOT), were observed in the I673-I254 products. No mutations were identified on the basis of translation of the full length RT-PCR product, nor by translating the shortest product. Six truncated proteins, which might theoretically have been observed, were not (VAKIN, ANALL, MATJO, MEJOH, MAJMA, and JOPOT). In view of the fact that the abnormally spliced RNA for four of these patients was either noticeably under-represented (VAKIN and ANALL) or not seen (MATJO and MEJOH), inability to see the protein products is not surprising. One mutation was inaccessible to the PTT test (p236113) since no mutant RNA would be amplified. Of the 10 mutations found, three were observed with the PTT test. This is at best a 30% detection rate, at worst 25%. Furthermore, the test was most informative on a template which does not include the initial 70 amino acids of the protein.

PTT itself carries a background of shorter and much less intense protein bands,[29] some-

times attributed to translation from internal methionines in the RNA transcript. Also, it is well known that mRNAs carrying in frame stop codons are under-represented in the cell[30] and when mutant RNA is under-represented the truncated protein translated from it will also be under-represented. The overall effect of under-represented translation products viewed on a background of non-specific truncated proteins, together with an inability adequately to control mispriming in the PCR amplification used for the test, means that PTT is less than ideal as a detection system on breakthrough RNA transcripts from lymphoblastoid cell lines of PAX6 patients.

SSCP

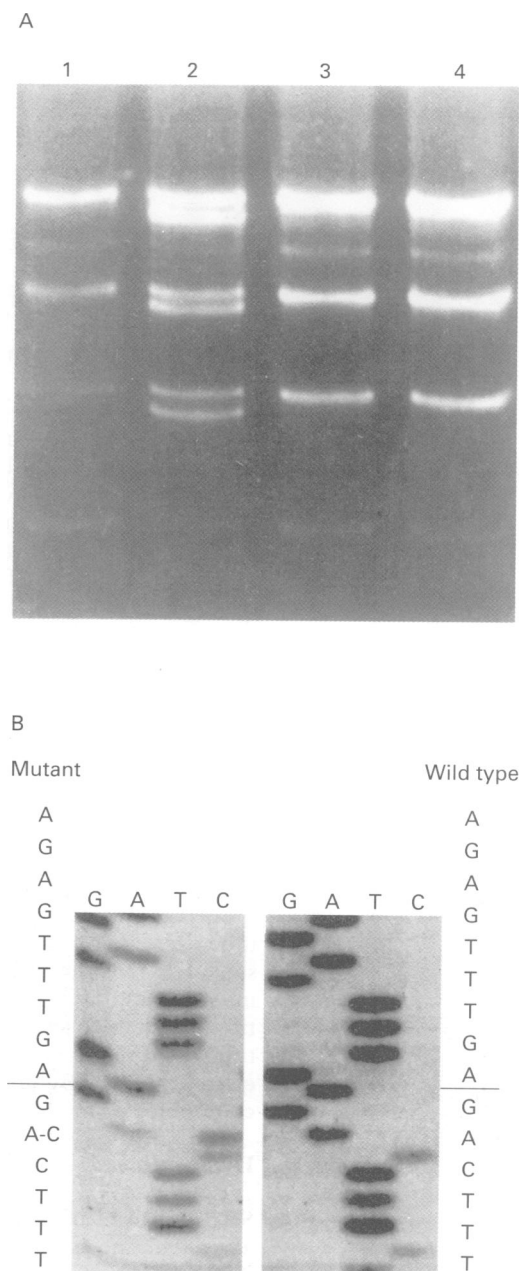All exons of PAX6 in all the patients were screened genomically with individual exons

Figure 3   (A) Silver stained SSCP bands for exon 9 on a non-denaturing 15% acrylamide gel. Lanes 1-4 are loaded MAJMA, MEJOH (mutant), HORLA, and p236113. (B) Sequence change in MEJOH. Horizontal line marks intron/exon boundary.

amplified in fragments ranging in size from 154 to 370 bp. When only the coding portion of exon 13 is considered, all fragments except one (exon 8 at 370 bp) are under 300 bp in size. Fig 3 illustrates a mutant band in exon 9 of MEJOH and verification by DNA sequencing. Nine of the 10 detected mutations were observed in this way (either a 75% detection rate if it is assumed that OLGIR and CUICKLE have as yet undetected mutations or a 90% rate if it is assumed that after exhaustive screening by three methods OLGIR and CUICKLE do not contain PAX6 mutations in the regions screened). A 75% rate of detection is good and 90% would be excellent for a single set of SSCP conditions. Additionally, SSCP is the easiest mutation detection method available. Two polymorphisms were detected in intron 9 (C→T at -12 and A→T at -13, data not shown). A further 740 bp of 3' untranslated sequence in exon 13, much of which is evolutionarily highly conserved,[8] was screened in equivalent sized fragments as well as 185 bp of intron 4 believed to contain a PAX6 neuroretinal enhancer.[22] No polymorphisms were found.

HOT

PAX6 was screened at the genomic level (exons 3 to the translated portion of exon 13) and the RNA level (the intermediate I673-I242 fragment missing the initial 70 amino acids) (fig 4). Although at the genomic level one previously unidentified mutation (VAKIN) was found, it is evident that carrying out eight HOT analyses for each of 12 DNA samples to find one mutation more than could be detected by SSCP is not an efficient use of resources. HOT on RT-PCR products, while theoretically attractive, was practically disappointing, probably for a number of reasons. If RNA transcripts which are misspliced or contain in frame stop codons are severely under-represented they are unavailable for analysis. HOT on RT-PCR products excised from gels precludes analysis of splicing variants, which result in products different in size from the wild type, yet because of the misprimed or misspliced products analysis of non-gel purified samples is not an option. For HOT analysis to approach 100% efficiency of detection, both templates must be used reciprocally as probes. Before labelling the probe DNA, it is an absolute requirement that it be excised and cleaned from gels. In conclusion, HOT at the genomic level is too labour intensive and HOT at the RNA level not practically suitable, since mutant and wild type transcripts are unequally represented, frequently of different sizes, and occasionally contain misprimed products.

Discussion

TECHNIQUES

SSCP analysis of individually amplified exons, by which nine of the 10 mutations (nine mutations in 12 patients) were identifiable, was the easiest and most efficient detection method for PAX6. Because of under-representation of mutant transcripts as well as amplification of misprimed and misspliced products, we found
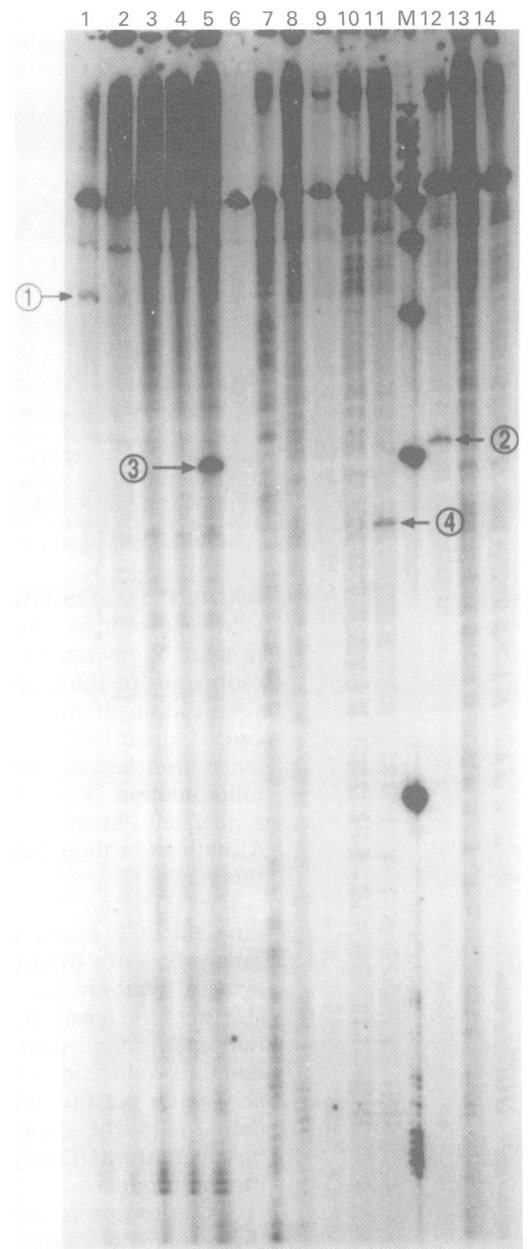

Figure 4   HOT analysis of the genomic exon 9 and 10 fragment (550 bp) in which lanes 1-7 are hydroxlyamine modified, lanes 8-14 osmium tetroxide modified, and lane M is a 100 bp marker. The samples are loaded in the following order: JOPOT, SIMO (normal), CUICKLE, HORLA, MATJO, OLGIR, DEMI (normal). The arrowed changes are the result of the following changes: (1) JOPOT polymorphism C→T at -12 of intron 9; (2) MATJO polymorphism A→T at -13 of intron 9; (3) MATJO mutation G→C at -1 of intron 9; (4) HORLA mutation A→T at 1161 in exon 10.

that RT-PCR on breakthrough transcripts in this gene was not consistently useful. Interestingly, Aoufouchi *et al*[31] discuss depletion of mRNA harbouring in frame nonsense mutations which is independent of protein synthesis, effected at the level of splicing, tissue specific but not species specific, and only loosely overlaps with tissue specific transcription. They also found that nonsense mutations resulted in defective splicing. Certainly much remains to be discovered of the effect of premature nonsense mutations on transcript abundance and splicing products. Our experience of breakthrough transcripts of the PAX6 gene in LCLs is that they are unreliable for mutation

detection. Although PTT would be the theoretical method of choice for a gene in which virtually all detected mutations lead to premature truncation of the protein, we found that because of the unreliability of representation of the RT-PCR products, only three of 10 mutations, or three mutations in 12 patients, were identified. For similar reasons, HOT analysis of breakthrough transcripts was not useful. By using HOT analysis at the genomic level, one mutation not seen with SSCP was identified, but in a gene with 13 short exons this detection route is not practical for screening purposes.

Clearly, for an exhaustive mutation analysis of the PAX6 gene one would choose a DNA based method. Of the techniques tried by us, SSCP was most useful and could be used as a primary screen with genomic HOT as a secondary method for negative cases. However, for concerted analysis at the DNA level and a detection rate approaching 100%, DGGE or CDGE should be considered.[27] This is a DNA based screening techniques with a greater efficiency of detection than SSCP but no more difficult to execute, although it requires a certain effort of input to establish. Before embarking on this study, PTT was the obvious method of choice for mutation detection; retrospectively, however, DGGE might have been a better investment.

MUTATIONS
Ten of the possible 12 mutations in the five familial and five sporadic patients were found so that PAX6 mutations were found in 90% of the aniridia cases (table 3). Of the two patients in whom no mutations were seen, patient OLGIR was thought by the submitting laboratory to be cytogenetically abnormal. However, FISH analysis using a cosmid covering the whole of the PAX6 gene and other cosmids proximal to it showed no rearrangements. Distal markers were not assessed. CUICKLE was received as a transformed cell line from a sporadic aniridia case without further details. It is certainly possible that there are undetected PAX6 mutations in these two patients, either in the regions screened by us, or in regions not screened, including introns and further untranslated regions, but there is no reason to suspect a mechanism which does not involve PAX6. There are many different ways of causing loss of function, not all of which can be easily screened. For instance, mutations outside the exonic regions can lead to loss of expression from one allele. Such mutations may be found deep within introns or in 5' or 3' control elements whose role in PAX6 is suggested by the occurrence of patients with 3' chromosomal rearrangements leading to position effects.[21] Promoter mutations have been described for a number of genes, but the promoter elements of PAX6 are not yet defined. Searching for such long range mutations is currently not feasible.

Five mutations involved splicing errors: two coded for protein products missing 36 amino acids in the paired domain, one led to absence of exon 4 and therefore absence of the initiating ATG, and two had unknown effects on the translated protein but could be presumed to interrupt the protein from exon 9 or 10 onwards, deleting most of the activation domain. Although two splicing mutations were in frame and did not result in a premature stop codon, both the RNA and protein resulting from the event would be shorter. The predicted mutant proteins would be deleted for a helix-turn-helix motif of the paired domain and, as Hanson *et al*[5] pointed out for an identical mutation, it is difficult to imagine that the deletion would not have a deleterious effect on the function of the paired domain. Five mutations created premature stop codons in exons 8, 9, 10, 11, or 12. Three removed or interrupted the homeo domain (exons 8, 9, and 10) and two prematurely terminated the protein after the homeo domain affecting the PST transcriptional activation region (exons 11 and 12). The stop codon in exon 12 (MAJMA) would delete the terminal 58 amino acids of the PST domain and is one of the most C-terminal protein truncations detected in PAX6. The patient, however, was identified in a classical aniridia family and did not manifest a noticeably mild phenotype. As far as is possible to determine from the information given,[12] a mutation at +2 of exon 13, which very likely would delete the terminal 28 amino acids of the PST domain, was found in a patient with aniridia. Clearly, interruption late in the transcriptional activation domain of the protein can be as deleterious as total absence of the copy, but it is worth noting that truncations of PAX6 just N-terminal to MAJMA's mutation in the PST domain were responsible for the milder phenotypes of congenital cataracts[18] and autosomal dominant keratitis.[19] Genetic modifiers play a major role in the final determination of a phenotype and there are now well documented examples, particularly in mouse, of mutant alleles giving rise to different phenotypes on different genetic backgrounds.[32] The segregation of modifier genes that interact with PAX6 would explain phenotypic heterogeneity of mutations which are identical or nearly identical. Non-functional mutant proteins are the presumed outcome of the 10 gene alterations found in this screen and conform to previous data indicating that aniridia is caused by haploinsufficiency of PAX6 protein.

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