Positional cloning in Xp22 : towards the isolation of the gene involved in X-linked retinoschisis

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CHAPTER 5.1

Exclusion of *PPEF* as the gene causing X-linked juvenile retinoschisis

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Abstract X-linked juvenile retinoschisis (RS) is a progressive vitreoretinal degeneration localised in Xp22.1-p22.2. A human homologue of the retinal degeneration gene C (*rdgC*), a gene that in *Drosophila melanogaster* prevents light-induced retinal degeneration, was localised in the RS obligate gene region. We have tested the gene, designated *PPEF* in humans, as a candidate gene in RS patients using RT-PCR and the protein truncation test on RNA and SSCP on DNA. No mutations were identified, making it highly unlikely that *PPEF* is the gene implicated in RS. The data presented facilitate mutation analysis of the *PPEF*-gene in other diseases which have been or will be localised to this region.

Introduction

X-linked juvenile retinoschisis (RS, MIM 312700) is a progressive vitreoretinal degeneration, with a frequency of about 1 in 10 000 (Bergen et al.1995). Symptoms vary from mild loss of visual acuity and peripheral field defects to total blindness due to complete retinal detachment; the age of onset is variable (George et al.1996). The gene causing RS has been localised by extensive linkage analysis to a region on Xp22 between markers DXS418 and DXS999 (George et al.1994; Van de Vosse et al.1996; Huopaniemi et al.1997). Several YAC contigs have been constructed spanning the 1 Mb obligate gene region (Ferrero et al.1995; Alitalo et al.1995; Van de Vosse et al.1997). Gene identification techniques are currently used to isolate candidate genes for RS and to test these in mutation analysis of patients derived samples.

Exon trapping experiments carried out on YAC clone y939H7, covering the RS-gene candidate region, yield several products. Two of the identified exon trapping products corresponded to a novel human transcript (*PPEF*, Montini et al.1997) which was highly homologous to the retinal degeneration gene C (*rdgC*, M89628). In *Drosophila melanogaster*, this gene is required to prevent light-induced retinal degeneration (Steele and O'Tousa 1995; Steele et al.1992). *PPEF* was mapped back to YAC clone y939H7 close to DXS999 and resides therefore in the RS critical region (Fig 1). The localisation in the RS obligate gene region and the phenotype observed in the fly, made *PPEF* an attractive candidate for RS. *PPEF*, Protein Phosphatase with EF hand motifs, encodes a serine/threonine protein phosphatase (Montini et al.1997). The gene consists of 17 exons, with a coding region

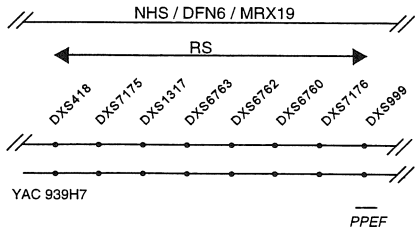

Fig. 1 Localisation of the *PPEF* gene in the retinoschisis (*RS*), Nance-Horan syndrome (*NHS*), DFN6 and MRX19 candidate regions.

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of 1962 bp having a 61.7 % similarity on protein level with *rdgC*. Here we report testing of the *PPEF* gene as a candidate gene for RS.

Materials and methods

Patient samples

Patient DNA was isolated from blood lymphocytes as described (Maniatis et al.1989). Patient RNA was isolated from blood lymphocytes as described (Den Dunnen et al.1996) using RNAzolB (Campro Scientific). All tissue specific RNAs were obtained from Clontech.

Hybridisation

Southern blots containing *Eco*RI, *Msp*I, *Bam*HI, *Eco*RV, *Hind*III digested DNA of RS patients were made using standard techniques (Maniatis et al.1989). A 25-ng aliquot of DNA was labelled with 5 µl [α-^{32}P]-dCTP using the Prime-it II random primer labelling kit (Stratagene). Hybridisation was performed with 10^6 cpm hybridisation mix (0.125 M $NaHPO_4$, 0.25 M NaCl, 1 mM EDTA, 7% SDS, 10% PEG-6.000) at 65°C. Filters were washed once for 5 min at room temperature in 2 x SSC, 0.2% SDS and twice for 30 min at 65°C in 2 x SSC, 0.2% SDS. Autoradiography was done by overnight exposure of Kodak X-Omat AR film.

SSCP analysis

SSCP analysis on patient DNA was performed as described by Orita (Orita et al.1989) and adapted for use of non-radioactive samples (Renieri et al.1994) using the primers and conditions given in Table 1.

RT, PCR and protein truncation test (PTT)

Reverse transcription (using 1-3 µg RNA) and two rounds of PCR and PTT analysis were essentially done according to Den Dunnen et al. (1996). Cycling conditions for the first PCR round: 3 min at 93°C, followed by 30 cycles of 1 min at 93°C, 1 min at 58°C, 4 min at 72°C, and finally one cycle of 7 min at 72°C. Cycling conditions for the second PCR round were 3 min at 93°C, then 32 times 1 min at 93°C, 1 min at 59°C, 3 min at 72°C and finally once for 4 min at 72°C. Approximately 250-500 ng PCR-product was used in a coupled *in vitro* transcription/translation reaction. *PPEF* primers used for RT-PCR (*PPEF-1F, -1R, -2F, -2R*) are indicated in Table 1. Primer *PPEF-2F* has a tail containing a T7 promoter sequence and a eukaryotic translation initiation signal, facilitating subsequent analysis using *in vitro* transcription and translation (Sarkar and Sommer 1989). The primers for the RT-PCR experiments were designed on the sequence available at the time, this sequence did not include the 5' end of the sequence that was revealed later (Montini et al.1997).

Sequence analysis

PCR products were sequenced using an AmpliCycle sequence kit (Perkin Elmer) according to the manufacturers instructions.

Results and Discussion

In most X-linked diseases, DNA deletions of various sizes form a significant fraction of the mutations found. We carried out a scan for the presence of deletions and rearrangements in genomic DNA by hybridisation of the *PPEF* cDNAs (Montini et al.1997) to Southern blots containing DNA of 60 unrelated RS patients. DNA was digested with *Msp*I, *Bam*HI, *Eco*RV (23 samples), *Hind*III (37 samples) and *Eco*RI (all samples). Neither deletions nor aberrant fragments could be detected with the cDNAs, thereby excluding the presence of large deletions in the *PPEF* gene in these RS patients (data not shown).

For a more detailed mutation analysis of the *PPEF*-gene, we performed SSCP analysis on genomic

Table 1 Sequences of the primers and conditions used in RT-PCR and SSCP analysis. Primer *PPEF-2F* has a tail containing a T7 promoter sequence and a eukaryotic translation initiation signal (*= GCTAATACGACTCACTATAGGAACAGACCACCATG)

Primer set	Ta	Product size (bp)	$MgCl_2$ (mM)	Sequence of forward primer	Sequence of reverse primer
1	53	239	1.5	CAGAAGTTGAATTCATGAAC	GTAGTTTCCTATGCTACTC
2	55	238	2.5	GAAGCACCTACTTCTCCTAAC	CCTCGAGGTCGACGGTATC
3	55	182	1.5	TTGTCACAGTAGCTGTTTGG	GCTCTTGATGAAGACAATTG
4	54	318	2.5	AGTGCCTTACATGGGCTAG	GGGCATCTGTTATGTACAAG
5	55	259	1.5	ACGATGTAGGACCAAGAGG	GCTTGCTCCACCTTTACAG
6	56	159	1.5	GGGCATTGCATCTTGTTCTC	TATCTGCCCTAAGACTGCCC
7	55	289	1.5	ACACGGCCTGACTTTAAAAG	CAGCATTTTCCAGAGTGCG
8	55	185	1.5	TGCATGACTCATGGAAGTAG	AATCTGGTCTTTCTTGGCTC
9	51	252	2.5	TTCCCTTCTAAATCCCTGAG	CAATAAACTGAACCTGTCAG
10	55	255	1.5	GAATAAGCAGAGGGTTGGAC	CCCTGTTGTACGTGCGATC
11	55	281	1.5	CTCACTTGTAAGTTACAGCG	TGTGCTTAGGGGAAGGATC
12	52	240	2.5	TTTGAGAACTAATGTTACGTG	GTGATACCGTGATACCAG
13	52	215	2.5	AAATGAAACACAACAGGATG	ATGTAACTTGGTGTGTTAAG
14	57	275	1.5	TCCCAAGAGGTTGCATTC	CACCCTGGCTAGGTTTTAG
15	46	202	2.5	AATATGTTCTAACACTTAG	TCAAAGTGTACTCATTTTG
16	54	312	1.5	ACCCTTGCCTTAGGTGGGTC	TAGCTGTTTCAGGGAGCCTG
PPEF-1	58	2122	6.7	TGAAGGCCAGA CAACACTATG	ACTAGGTCCAACCCAGTTTCT
PPEF-2	59	1960	6.7	*GAATATGCTGATGAACAAGGC	CTTTTCTCTGCTACTGACTATGAA

DNA of 37 patients. Sixteen primersets were designed to amplify 16 of the 17 exons of the *PPEF* gene, excluding exon 1 containing 5'UTR sequences only. No deletions or aberrant fragments could be detected and no polymorphic fragments were found that could be used as RFLPs (Table 1).

To detect mutations either altering the promotor or splice sites of the gene or causing frame shift mutations leading to a premature stop in the open reading frame, we analysed the gene on RNA level using RT-PCR and PTT (Roest et al.1993). Analysis of RT-PCR products made on RNA isolated from lymphocytes of nine unrelated RS patients revealed products ranging in size from 800 to 1900 bp. *In vitro* transcription and translation of the PCR products showed that the size of the translation products corresponded to open reading frames in accordance with the length of all PCR products. Consequently, no mutations affecting transcription of the *PPEF* gene nor mutations causing premature translation termination could be identified.

Based on the extensive mutation analysis in both DNA (hybridisation and SSCP) and RNA (RT-PCR and PTT), we conclude that *PPEF* is not likely to be the gene implicated in RS. Experiments using RNA *in situ* hybridisation of *PPEF* on mouse embryo tissue sections revealed expression of the gene in the brain and basal ganglia but not in the developing eye (Montini et al.1997). The latter suggests a different function for the mammalian homologue of the *Drosophila rdgC* gene. Given the accumulating evidence from SSCP and *in situ* hybridisation experiments, we decided not to complete the PTT analysis, which did not include the 363 bp at the 5' end of the translated region of the gene.

While our data provisionally exclude the *PPEF* gene as the gene involved in RS, its map position (Fig. 1) renders it a candidate gene for other diseases localised in this region, such as Nance-Horan syndrome (Bergen et al.1994), MRX19 (Donnelly et al.1994) and DFN6 (Del Castillo et al.1996). The expression and mutation analysis data presented here, should provide the tools to test the involvement of *PPEF* in these diseases, or others which in the future might be mapped to this region.

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