FEBS Letters 426 (1998) 238–242 FEBS 20121

Genomic organization and chromosomal localization of the human peroxisomal membrane protein-1-like protein (PXMP1-L) gene encoding a peroxisomal ABC transporter

Andreas Holzinger[a,*], Adelbert A. Roscher[a], Pablo Landgraf[a], Peter Lichtner[b], Stefan Kammerer[a]

[a] *Dr. v. Hauner Children's Hospital, Ludwig-Maximilian-University, Department of Pediatrics, Division of Clinical Chemistry and Metabolism, Lindwurmstrasse 4, 80337 Munich, Germany*
[b] *Department of Pediatric Genetics, Ludwig-Maximilian-University, Goethestrasse 29, 80336 Munich, Germany*

Received 28 January 1998; revised version received 17 March 1998

Abstract The cDNA of the peroxisomal membrane protein-1-like protein (PXMP1-L, synonyms: PMP69, P70R), a novel peroxisomal ATP binding cassette transporter of yet unknown function, has recently been cloned. The best known peroxisomal member of this protein family is the adrenoleukodystrophy protein, defects of which are the underlying cause of X-linked adrenoleukodystrophy (X-ALD). Here we describe the complete exon-intron structure (19 exons and 18 introns covering 16.0 kb) of the human PXMP1-L gene, transcript variants, the localization on chromosome 14q24 by cytogenetic analysis and sequencing of the putative promoter region. PXMP1-L has been proposed to play a role as a modifier in determining the phenotypic variations observed in X-ALD. The data presented will enable sequence analysis of the PXMP1-L gene in X-ALD patients and facilitate the analysis of PXMP1-L function.

Key words: ABC transporter; Peroxisome; Very long chain fatty acid

1. Introduction

We and others have recently reported the cloning of the full length cDNA of peroxisomal membrane protein-1-like protein (PXMP1-L, synonyms: PMP69, P70R) [1,2], a novel human membrane protein which belongs to the superfamily of integral membrane transporter proteins containing an ATP binding cassette (ABC transporter). PXMP1-L is localized within the peroxisomal membrane. Members of the ABC transporter superfamily are involved in the transport of a variety of substrates across biological membranes [3]. Mammalian examples include the cystic fibrosis transmembrane conductance regulator (CFTR), the multiple drug resistance proteins (MDR1 and MDR2), the retinal ABC transporter (ABCR) [4] responsible for Stargardt's disease, the transporters of antigen processing TAP1 and TAP2 [5] and many others. Three other members of the family have been located within the peroxisomal membrane: the adrenoleukodystrophy protein (ALDP) [6], the peroxisomal protein 70 (PMP70) [7,8] and the adrenoleukodystrophy-related protein (ALDRP) [9,10]. Defects in the ALD gene are the underlying cause of adrenoleukodystrophy (ALD; McKusick 300100), an X-chromosomal recessively inherited demyelinating disorder of the nervous system with marked heterogeneity of clinical manifestations even between affected individuals carrying the same mutation. ALDP is likely to be involved in the peroxisomal transport or catabolism of very long chain fatty acids (VLCFA; $>$C22) since elevated VLCFA in plasma and tissues are the biochemical hallmark of all forms of X-ALD. It has been proposed that ALDP might be a transporter translocating VLCFA into the peroxisome, the exclusive site of β-oxidation of VLCFA [11]. Mutations in the PMP70 gene have been associated with some forms of Zellweger syndrome, a heterogeneous group of peroxisome assembly disorders [12,13], although recently doubts have been raised whether PMP70 is indeed a Zellweger gene [14]. The function of ALDRP, the protein most highly homologous to ALDP, is also unknown. All known peroxisomal ABC transporters conform to the model of an ABC half-transporter, requiring a partner half-transporter molecule (either as a homodimer or a heterodimer) to form a functional transporter. In *Saccharomyces cerevisiae* two different peroxisomal ABC transporters (Pat1 and Pat2) have been demonstrated to associate with each other and to be involved in the import of fatty acids [15–17]. It is currently not known in which way the four human peroxisomal ABC half-transporters form functional complexes nor which substrates are transported. In the present work we report the detailed genomic organization of the human PXMP1-L gene including the sequence immediately upstream of the transcriptional start, several transcript variants and data on cytogenetic mapping of the PXMP1-L gene.

2. Materials and methods

2.1. Genomic structure analysis

A human genomic DNA library generated with the pCYPAC2 vector and genomic DNA derived from cultured skin fibroblasts with an average insert size of approximately 100 kb [18] was screened with a 793-bp human PXMP1-L cDNA probe. This probe was amplified with primers P1f (5′-GGGCAGCTTCGGGAGACGCTTCTGG) and P1r (5′- GAAAGAACCCAAAGGGTTCAG). The oligonucleotide primer P1r is located in intron 17 which is present on a fraction of PXMP1-L transcripts. The probe was [α-^{32}P]dATP-labeled by random priming and a positive clone (LLNLP704B17234Q13) was isolated (Screening Service of the Resource Center of the German Human Genome Project Heidelberg/Berlin) [19]. Southern blot analysis of *Eco*RI, *Hin*dIII and *Xba*I digests with the same probe confirmed the presence of PXMP1-L-specific fragments. Genomic fragments containing complete introns were PCR-amplified from pCYPAC2-DNA templates using cDNA-derived oligonucleotide primers. Template preparation was performed by ion-

*Corresponding author. Fax: (49) (89) 5160 4192.
E-mail: holzinger@kk-i.med.uni-muenchen.de

Sequence data from this article have been deposited at the EMBL/NCBI-GenBank data bases under the accession numbers AF009746, Y14318, Y14319, Y14320, Y14321, Y14322 and Y14323.

PII S0014-5793(98)00354-8

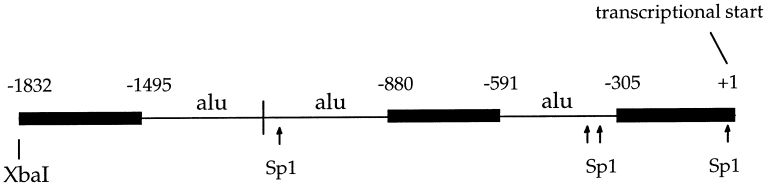

Fig. 1. The putative promoter region of the human PXMP1-L gene. A 1832-bp fragment immediately upstream of the transcriptional start resulting from an *Xba*I restriction digest contains three Alu repetitive elements including a tandem Alu formation at the 5′ end. Non-repetitive sections are indicated by bold lines. Potential Sp1 binding sites are indicated by arrows.

exchange columns (Qiagen midiprep) according to the manufacturer's protocol for P1-derived constructs. PCR amplification was performed using the Expand Long Template system (Boehringer Mannheim). To estimate intron sizes the PCR products were separated on 0.7% agarose gels. These products were directly sequenced without further subcloning from either end by the fluorescent dideoxy dye terminator method on an ABI 377 sequencer using the amplification primers.

A fragment containing the region immediately upstream of the transcriptional start was subcloned into the pcDNA3 vector (Invitrogen) from an *Xba*I restriction endonuclease digest of the pCYPAC2 clone LLNLP704B17234Q13 containing the entire PXMP1-L gene. The fragment size was determined by non-radioactive Southern blot analysis using a digoxigenin-labeled probe derived from exon 1 (DIG Probe Synthesis Kit, Boehringer Mannheim). This probe was amplified from a RACE-PCR product subcloned into the pGemT-easy vector using a T7 vector oligonucleotide primer and an exon 1-specific reverse primer. Transfer of pCYPAC-2 *Xba*I fragments onto nylon membranes was performed by an alkaline capillary method (Turboblotter, Schleicher and Schuell) according to the manufacturer. Hybridization and chemoluminescent detection by an alkaline phosphatase-labeled anti-digoxigenin antibody and the substrate CDP-Star (Boehringer Mannheim) followed the manufacturer's protocol. The presence of PXMP1-L-specific DNA in the resulting subclones was confirmed by dot blot analysis using the same exon 1-specific probe.

2.2. Transcript variant analysis

In order to investigate tissue specific splicing of a transcript lacking exon 3 total RNA was prepared from human tissues using RNAzol (Biozol). RT-PCR amplification from random-primed cDNA from a variety of human tissues was performed with oligonucleotide primers PF (5′-GTTCCTGCAGATACTGAAGG-3′) and PR (5′-GGTGCTCAGTGAGGTCCTTCC-3′) flanking exon 3. Amplitaq Gold (Perkin Elmer) was used for PCR amplification. The PCR products were separated on a 1.6% agarose gel and bands were visualized by ethidium bromide staining.

2.3. Fluorescence in situ hybridization (FISH) analysis

Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes according to standard procedures. FISH was performed using pCYPAC2-DNA from clone LLNLP704B17234Q13 which contained the complete PXMP1-L gene. pCYPAC2-DNA was labeled with biotin-14-dUTP (Life Technologies) by nick-translation and preannealed with Cot-1 DNA (Life Technologies). Detection and visualization was achieved using the avidin-fluorescein isothiocyanate/antiavidin antibody system described elsewhere [20,21] and chromosomes were identified by staining with 4,6-diamino-2-phenylindole dihydrochloride (DAPI).

3. Results

3.1. Characterization of the region upstream from the transcriptional start

A 3.3-kb genomic DNA fragment resulting from an *Xba*I restriction digest was isolated by hybridization with an exon 1-specific cDNA probe and subcloned. This fragment yielded 1832 kb of sequence immediately upstream from the 5′ end of the transcript. The transcriptional start can be expected at or at least very close to the 5′ cDNA end we have previously reported [1] since the sequenced cDNA length corresponds well to the signal size in Northern blot analysis. Fig. 1 shows the putative promoter region. A TATA box was not identified. Three Alu repetitive elements were identified including a tandem Alu formation at the 5′ end of this region. A high GC content (57%) was observed in the region from position −305 to +89 which includes exon 1. The 89 bp of exon 1 including the coding portion of exon 1 demonstrate a GC content of 72%. These findings are consistent with a housekeeping character of PXMP1-L also reflected by its ubiquitous expression [1]. Four putative Sp1 binding sites were identified at positions −16 to −21, −355 to −360, −413 to −418 and −1136 to −1140. Analysis of the putative promoter region did not reveal apparent peroxisome proliferator responsive elements (PPRE) [22–24]. Such an element may, however, theoretically be positioned further upstream [22].

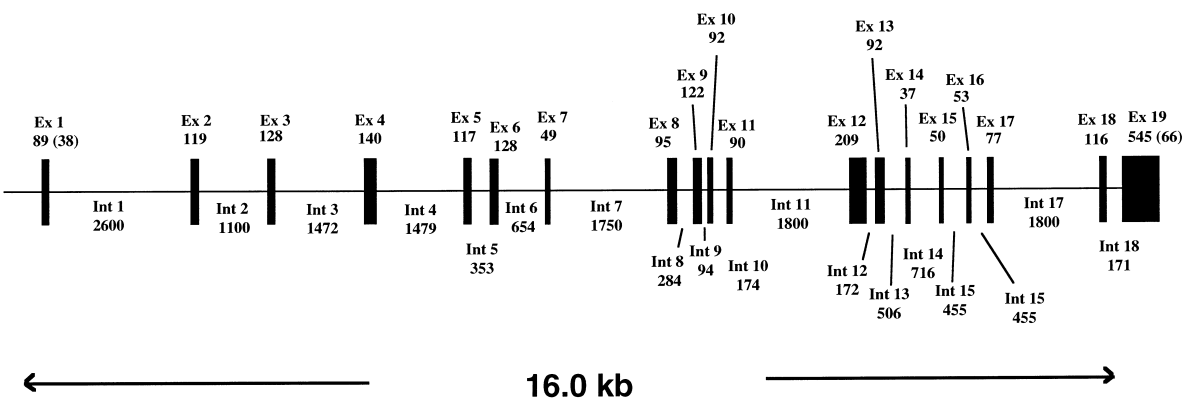

Fig. 2. Genomic organization of the human PXMP1-L gene. Closed boxes represent exons. Intron and exon sizes (in bp) are indicated. Numbers in parentheses indicate the coding sequence of exons that also contain non-coding portions. The human PXMP1-L gene extends over 16.0 kb and consists of 19 exons and 18 introns.

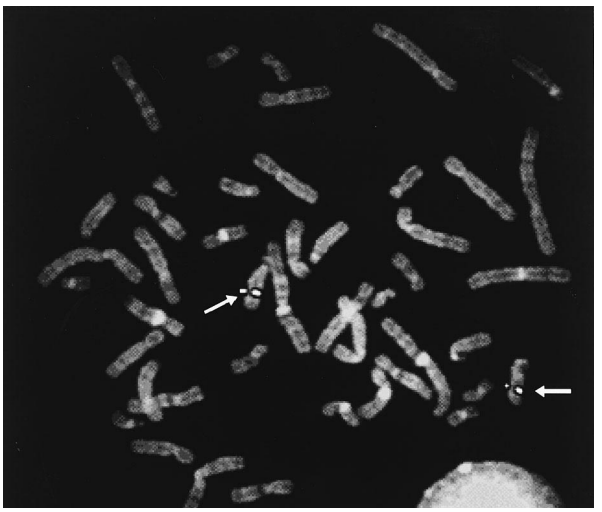

Fig. 3. FISH analysis of the human PXMP1-L gene using PAC LLNLP704B17234Q13 containing the complete PXMP1-L gene. Fluorescent signals on DAPI-stained chromosomes are indicated by arrows. A single signal is detected on chromosome 14q24.

3.2. Determination of the exon-intron structure and Southern blot analysis

The genomic organization was determined by PCR amplification of genomic fragments with cDNA-derived primers using a pCYPAC2 clone containing the entire PXMP1-L gene. Overlapping fragments spanning the complete coding region were generated and sequenced. Comparison of the obtained sequence with the cDNA sequence revealed that the PXMP1-L gene consists of 19 exons and 18 introns (Fig. 2). The last exon is the largest one containing 545 bp, if the predominant transcript derived from utilization of the upstream polyadenylation signal is considered (1083 bp if the more downstream alternative polyadenylation signal is used). All other exons range in length between 39 bp and 210 bp. All exon-intron boundaries conform to the GT-AG rule. We have fully sequenced 11 introns (introns 3–6, 8–10, 12–15, 17) and determined the exon-flanking intronic sequences of all other introns. The approximate sizes of introns not fully sequenced were determined by agarose gel electrophoresis of corresponding PCR products. The total size of the PXMP1-L gene is approximately 16.0 kb (Fig. 2).

Southern blot analysis was performed with the partial cDNA probe described above using *Eco*RI, *Hin*dIII and *Xba*I digests of human genomic DNA isolated from leukocytes. A 12- and a 7.7-kb fragment (*Eco*RI), a 4.3- and a 3.2-kb fragment (*Hin*dIII) and an 11-kb fragment (*Xba*I) were observed. Corresponding restriction digests of the pCYPAC-2 clone containing the entire PXMP1-L gene yielded identical fragments (data not shown).

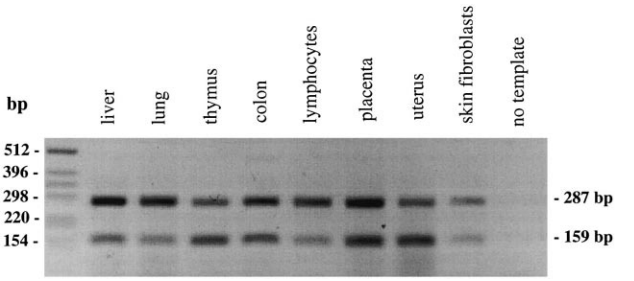

Fig. 4. Tissue analysis of the splice variant Δex-3 lacking exon 3. RT-PCR analysis using cDNA-derived oligonucleotide primers flanking exon 3. The 287-bp product represents the transcript including exon 3, the 159-bp product represents the transcript lacking exon 3. Δex-3 is present in all tissues examined.

3.3. Fluorescence in situ hybridization analysis of the PXMP1-L gene

FISH analysis with a PXMP1-L-specific probe revealed a single signal on chromosome 14q24 (Fig. 3). This finding is in agreement with the previously reported location of a 227-bp PXMP1-L-specific cDNA fragment on a cosmid derived from chromosome 14q24.3 (Sharma et al., unpublished entry in NCBI/GenBank accession number N65934) and is also consistent with recent findings by Shani and colleagues [2]. STS entry A06I12 in the NCBI Human Gene Map which places a PXMP1-L-specific transcript between chromosomal markers D14S71 and D14S76 also confirms this localization. These data correct another report [4] which maps a PXMP1-L-specific cDNA fragment to chromosome 14q12. Our FISH data are consistent with PXMP1-L being a single copy gene which is also supported by data of the Southern blot analysis.

3.4. Transcript variants

In addition to transcript variants we have previously described [1], other transcripts resulting from alternative splicing have been detected. Fig. 4 shows the analysis of RT-PCR products from various tissues for a variant lacking exon 3. This variant is present in all tissues examined. Additionally a variant containing the complete 506-bp intron 13 has been detected. The knowledge of the genomic structure allows assignment of previously described variants to specific splice sites. Table 1 lists all known transcript variants of PXMP1-L including those resulting from alternative polyadenylation.

4. Discussion

PXMP1-L is the fourth known member of the peroxisomal subgroup of ABC half-transporters including ALDP, ALDRP and PMP70. The genomic organization of PXMP1-L differs significantly from other peroxisomal ABC transporters The ALD gene has been reported to contain 10 exons [25], the

Table 1
Transcript variants of the PXMP1-L gene

Variant	Description	Position in cDNA (bp)
Δex-3	missing exon 3	208–336
Δex-4	missing exon 4	337–476
+intron 13	505-bp intron 13 not spliced	1470
+intron 18	171-bp intron 18 not spliced	1803
up polyA	upstream (predominant) poly(A) tail	2351

Splice variants and transcripts arising from alternative polyadenylation as described previously are included. The positions in relation to the cDNA sequence refer to NCBI/GenBank entry AF009746.

PMP70 gene consists of 22 exons (Jutta Gärtner, personal communication). Despite the different total exon number in a comparison between PXMP1-L and PMP70, individual exon-intron boundaries were found in corresponding segments of the aligned cDNA sequences. Exon 10 of PXMP1-L corresponds to exon 14 of PMP70. The exon-intron boundary of exon/intron 2 of PXMP1-L corresponds to the boundary of exon/intron 4 of PMP70. This is in contrast to the relation between the genomic structures of the ALD and ALDR genes which are very similar (Holzinger et al., unpublished). These data may suggest an ancient divergence time of PXMP1-L from ancestor ABC transporters and a more recent duplication of a peroxisomal ABC transporter to result in ALD and ALDR genes.

The genomic organization data allow the assignment of previously reported transcript variants to alternative splicing events of specific exons and introns. The splice variant resulting from the loss of a 140-bp exon [1] was determined to involve exon 4, the variant resulting from retention of an intron to involve intron 18. In this article we describe an additional splice variant resulting from the loss of exon 3 (Fig. 4) as well as a variant containing the complete 506-bp intron 13. A functional role of any of these splice variants is, however, unlikely because they all result in frame shifts leading to premature termination.

The human PXMP1-L gene is localized on chromosome 14q24.3. In contrast to clustering of functionally related ABC half-transporters such as the transporters of antigen processing TAP1 and TAP2 [26], all human peroxisomal ABC transporters cloned to date are localized on different chromosomes (ALD: Xq28, ALDR: 12q12, PMP70: 1p22–p21). Therefore, the chromosomal localization does not allow any speculation on the nature of interaction between peroxisomal ABC transporters. The chromosomal locus 14q24.3 has not been linked to any known peroxisomal disease. This locus has received intense medical interest since it contains the AD3 gene, defects of which are associated with early-onset Alzheimer disease (Alzheimer's Disease Collaborative Group, 1995).

Peroxisomal ABC transporters are of particular clinical interest since defects of the ALD gene are the primary cause of adrenoleukodystrophy [6]. Biochemically, X-linked adrenoleukodystrophy represents a defect of very long chain fatty acid β-oxidation thought to be due to impaired import of the substrate. The function of the other peroxisomal ABC transporters is unknown, a possible involvement in fatty acid β-oxidation is purely speculative. PXMP1-L is a candidate heterodimer partner of one or more of these other peroxisomal membrane proteins. The only two yeast peroxisomal ABC transporters Pat1 and Pat2, which are involved in functions related to fatty acid oxidation, interact with each other [15–17]. Homodimerization as well as tissue-dependent dimerization with various partners in order to fulfil different transport functions are also conceivable [2]. The homology to putative lipid transporters (ALDP, Pat1, Pat2) might be viewed as a support of the speculation that PMP70 and PXMP1-L might also take part in lipid translocation. The four peroxisomal transporters and possible heterodimer combinations might constitute a transport system for various lipids or other substrates. The comparison of temporo-spatial expression and inducibility by hypolipidemic drugs [27] of ALDP, PMP70 and ALDR is – if interaction occurs – best interpreted as that there is no obligatory exclusive dimer formation of any two of these proteins.

A role of PXMP1-L in the biogenesis of peroxisomes cannot be totally excluded. The only link to an involvement of PXMP1-L in peroxisome assembly, however, is weak evidence that a few patients with peroxisomal biogenesis defects (Zellweger syndrome) carry mutations in the PMP70 gene – the closest human homolog of PXMP1-L [12]. This hypothesis is also unlikely in the light of the high conservation of peroxisome assembly between yeast and human [13]. The only peroxisomal ABC transporters in *S. cerevisiae* (Pat1 and Pat2) are apparently not involved in peroxisome biogenesis [15–17].

Functional studies including protein-protein interaction experiments are required to determine whether and in what combinations human peroxisomal ABC transporters form functional complexes. If PXMP1-L plays a role as a modifier gene of adrenoleukodystrophy, sequence analysis of the PXMP1-L gene in affected brothers showing different clinical phenotypes should reveal variants associated with the severity of the disease. The data presented in this article will be useful tools towards clarifying the yet unknown function of PXMP1-L.

Acknowledgements: The authors wish to thank Anja Paulmichl and Heide Hinz for excellent technical assistance and Jutta Gärtner, MD, for sharing unpublished data on the genomic organization of the PMP70 gene and helpful discussions. We thank Dr. Simone Schuffenhauer for her help with the interpretation of FISH data. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (RO-727/2-1).

References

[1] Holzinger, A., Kammerer, S. and Roscher, A.A. (1997) Biochem. Biophys. Res. Commun. 237, 152–157.
[2] Shani, N., Jimenez-Sanchez, G., Steel, G., Dean, M. and Valle, D. (1997) Hum. Mol. Genet. 6, 1925–1931.
[3] Higgins, C.F. (1992) Annu. Rev. Cell Biol. 8, 67–113.
[4] Allikmets, R., Gerrard, B., Hutchinson, A. and Dean, M. (1996) Hum. Mol. Genet. 5, 1649–1655.
[5] Kelly, A., Powis, S.H., Kerr, L.A., Mockridge, I., Elliott, T., Bastin, J., Uchanska-Ziegler, B., Ziegler, A., Trowsdale, J. and Townsend, A. (1992) Nature 355, 641–644.
[6] Mosser, J., Douar, A.M., Sarde, C.O., Kioschis, P., Feil, R., Moser, H., Poustka, A.M., Mandel, J.L. and Aubourg, P. (1993) Nature 361, 726–730.
[7] Kamijo, K., Taketani, S., Yokota, S., Osumi, T. and Hashimoto, T. (1990) J. Biol. Chem. 265, 4534–4540.
[8] Kamijo, K., Kamijo, T., Ueno, I., Osumi, T. and Hashimoto, T. (1992) Biochim. Biophys. Acta 1129, 323–327.
[9] Lombard-Platet, G., Savary, S., Sarde, C.O., Mandel, J.L. and Chimini, G. (1996) Proc. Natl. Acad. Sci. USA 93, 1265–1269.
[10] Holzinger, A., Kammerer, S., Berger, J. and Roscher, A.A. (1997) Biochem. Biophys. Res. Commun. 239, 261–264.
[11] Valle, D. and Gärtner, J. (1993) Nature 361, 682–683.
[12] Gartner, J., Moser, H. and Valle, D. (1992) Nature Genet. 1, 16–23.
[13] Subramani, S. (1997) Nature Genet. 15, 331–333.
[14] Paton, B.C., Sharp, P.C., Crane, D.I. and Poulos, A. (1996) J. Clin. Invest. 97, 681–688.
[15] Shani, N., Watkins, P.A. and Valle, D. (1995) Proc. Natl. Acad. Sci. USA 92, 6012–6016.
[16] Hettema, E.H., Vanroermund, C.W.T., Distel, B., Vandenberg, M., Vilela, C., Rodrigues Pousada, C., Wanders, R.J.A. and Tabak, H.F. (1996) EMBO J. 15, 3813–3822.
[17] Swartzman, E.E., Viswanathan, M.N. and Thorner, J. (1996) J. Cell Biol. 132, 549–563.
[18] Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M.A. and de Jong, P.J. (1994) Nature Genet. 6, 84–89.

[19] Lehrach, H. (1990) in: Genome Analysis (Davies, K.E. and Tilghman, S.M., Eds.), Vol. 1, pp. 39–81, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Lichter, P., Cremer, T., Borden, J., Manuelidis, L. and Ward, D.C. (1988) Hum. Genet. 80, 224–234.
[21] Lichter, P., Tang, C.J., Call, K., Hermanson, G., Evans, G.A., Housman, D. and Ward, D.C. (1990) Science 247, 64–69.
[22] Zhang, B., Marcus, S.L., Sajjadi, F.G., Alvares, K., Reddy, J.K., Subramani, S., Rachubinski, R.A. and Capone, J.P. (1992) Proc. Natl. Acad. Sci. USA 89, 7541–7545.
[23] Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992) Cell 68, 879–887.
[24] Dreyer, C., Keller, H., Mahfoudi, A., Laudet, V., Krey, G. and Wahli, W. (1993) Biol. Cell 77, 67–76.
[25] Sarde, C.O., Mosser, J., Kioschis, P., Kretz, C., Vicaire, S., Aubourg, P., Poustka, A. and Mandel, J.L. (1994) Genomics 22, 13–20.
[26] Powis, S.H., Mockridge, I., Kelly, A., Kerr, L.A., Glynne, R., Gileadi, U., Beck, S. and Trowsdale, J. (1992) Proc. Natl. Acad. Sci. USA 89, 1463–1467.
[27] Albet, S., Causeret, C., Bentejac, M., Mandel, J.L., Aubourg, P. and Bugaut, M. (1997) FEBS Lett. 405, 394–397.