Retroviral-mediated adrenoleukodystrophy-related gene transfer corrects very long chain fatty acid metabolism in adrenoleukodystrophy fibroblasts: implications for therapy

Elise Flavigny, Ahmed Sanhaj, Patrick Aubourg, Nathalie Cartier*

Inserm U342, Hôpital Saint-Vincent de Paul, 82 avenue Denfert Rochereau, 75014 Paris, France

Received 25 February 1999; received in revised form 9 March 1999

Abstract X-linked adrenoleukodystrophy is a demyelinating disorder of the central nervous system with an impaired very long chain fatty acid metabolism. The adrenoleukodystrophy gene encodes a peroxisomal membrane protein that is part of a family of related ATP-binding transporters including the adrenoleukodystrophy-related protein. The adrenoleukodystrophy protein and adrenoleukodystrophy-related protein show 66% identity and have a mirror expression in most mouse tissues. We show that retroviral-mediated adrenoleukodystrophy-related gene transfer corrects very long chain fatty acid accumulation in adrenoleukodystrophy fibroblasts, irrespective of the presence or absence of adrenoleukodystrophy protein. Pharmacological approaches aiming at overexpressing the adrenoleukodystrophy-related gene in the central nervous system of adrenoleukodystrophy patients might thus offer new therapeutic leads.

Key words: Adrenoleukodystrophy; ATP-binding cassette transporter; Peroxisome

1. Introduction

Adrenoleukodystrophy (ALD) is a neurodegenerative disorder characterized by progressive demyelination within the central nervous system (CNS), adrenal insufficiency and accumulation of very long chain fatty acids (VLCFA) due to an impairment of their β-oxidation in peroxisomes [1]. The ALD gene encodes a 75 kDa peroxisomal membrane protein (ALDP) which belongs to a family of ATP-binding cassette (ABC) transporters [2,3]. These ABC transporters are part of a large family that includes the cystic fibrosis transmembrane conductance receptor and the multiple drug resistance glycoproteins [3]. The latter occur as single functional proteins with two related halves. In contrast, ALDP and other peroxisomal transporters are hemi-transporters that need to dimerize to exert an import function. ALD has three homologs that are also located in the peroxisomal membrane: the ALD-related (ALDR) protein (66% identity) [4,5], the more distantly related PMP70 protein (38% identity to ALDP) [6,7] and the PMP70 related (P70R) protein (27% identity to ALDP) [8,9]. Among peroxisomal ABC transporter genes, ALD is the only one known to be associated with a disease. Soon after the ALD gene was discovered, two closely related hemi-transporter genes were cloned in yeast (Pxa1p and Pxa2p) [10,11]. Studies of mutants in these genes have provided significant clues about the function of ALDP. In yeast, long chain fatty acids (LCFA) are first activated into CoA derivatives within the cytosol by a LCFA-CoA synthetase and then imported across the peroxisomal membrane by Pxa1p and Pxa2p heterodimers [11–13]. It is therefore hypothesized that ALDP forms homodimers and/or heterodimers with ALDR, PMP70 or P70R proteins to control the transport of VLCFA-CoA into peroxisomes.

Mice with a targeted mutation in the ALD gene have not yet developed a clinical phenotype and demyelination at 1 year of age, in spite of accumulation of VLCFA in the brain and adrenal gland as well as a decrease in the β-oxidation activity in vitro [14–16]. Gene redundancy could account for these observations if another ABC peroxisomal transporter could partially compensate for the ALD defect in mice. ALDR protein (ALDRP) is an attractive candidate for two reasons: (1) it shows the highest identity (66%) to ALDP [4,5] and (2) ALDRP and ALDP have a mirror expression in most mouse tissues [17], suggesting that both proteins might fulfil similar metabolic functions in different tissues. This prompted us to examine whether the expression of the ALDR gene can correct the metabolic defect of ALD fibroblasts, irrespective of the presence or absence of mutated ALDP.

2. Materials and methods

2.1. Cell culture

Skin fibroblasts obtained from ALD patients and the psi-CRIP packaging cell line were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% inactivated fetal calf serum (Gibco) and 4 mM glutamine.

2.2. Construction and packaging of the retroviral vector MFG-mALDR

Mouse ALDR (mALDR) pTL2 cDNA [18] was inserted into the MFG vector (Somatix Therapy Corporation, Alameda, CA, USA) under the control of the Mo-MLV LTR promoter. To position the initiation ATG codon of the mALDR cDNA at the position normally occupied by the viral env ATG, mouse ALDR cDNA was amplified from the plasmid pTL2 with the 5′-GCAAGCCATGGTA-CACATGCTAAAT-3′ and 5′-CAAGGGATCCTTAGGATGTCTTTTCTGG-3′ primers to create *Nco*I and *Bam*HI sites at the 5′ and 3′ end of the cDNA. The PCR reaction was run in the GeneAmp PCR System 2400 (Perkin Elmer) with 2.5 U Expand High Fidelity PCR System (Boehringer), 0.2 mM of each dNTP, expand HF buffer with 1.5 mM $MgCl_2$, 1 mM of the two primers, 30 ng of the template DNA, in a final volume of 100 μl (25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 3 min). *Nco*I and *Bam*HI sites were then used to insert the PCR product into the MFG vector.

The MFG-mALDR and PGK-Neo vector containing the neomycin-resistance gene [19] were co-transfected into the amphotropic packaging cell line psi-CRIP using DNA/calcium phosphate precipitation as described [19]. Transfected cells were selected in a medium containing 1 mg/ml G418 and clones were isolated by ring cloning and expanded to cell lines. MFG-mALDR producer clones were selected on the basis of a positive immunofluorescence assay with anti-ALDRP antibodies and the capacity to transduce ALD patients fi-

*Corresponding author. Fax: (33) (1) 40 48 83 52.
E-mail: cartier@cochin. inserm.fr

PII: S0014-5793(99)00379-8

broblasts in an infection assay. The M48-ALD-producer psi-CRIP cells have been described elsewhere [19]. The absence of helper virus was assayed by a virus mobilization assay [19].

2.3. Retroviral gene transfer

Supernatant from a confluent monolayer of the highest producer MFG-mALDR and M48-ALD [19] clones were harvested 24 h after a medium change, filtered and used for the infection of ALD skin fibroblasts in the presence of polybrene, 8 μg/ml (Sigma). Infection was performed three times in 24 h. As determined by Southern blotting, infection of ALD skin fibroblasts with MFG-mALDR vector resulted in the integration of approximately one provirus copy per cell genome (not shown).

2.4. Immunocytochemistry

Human skin fibroblasts were cultured for 16 h in the wall of a chamber slide (Nunc), fixed in 2% formaldehyde for 4 min and permeabilized in phophate-buffered saline (PBS)/0.1% Triton X-100. Cells were then incubated at room temperature with mouse monoclonal (1D6) [20] or rabbit polyclonal antibodies (1693) [21] against human ALDP, rabbit polyclonal antibodies against mouse (7373) [17] or human (1872) ALDRP and/or rabbit anti-human catalase polyclonal antibody (Chemicon).

All secondary antibodies were obtained affinity pure from Jackson Immunoresearch as follows: FITC or Cy3 conjugated goat anti-mouse IgG (H+L) and Cy3 or FITC conjugated donkey anti-rabbit IgG (H+L). The double procedures followed the principles of recognition of antibody species and the specificity by the conjugates. Appropriate filters for each fluorochrome and combined filters for fluorescein and Cy3 were used on a light microscope equipped with fluorescence (Nikon Optiphot-2).

2.5. Determination of VLCFA levels

VLCFA concentrations were measured in cultured skin fibroblasts after 2 weeks by gas/liquid chromatography/mass spectrometry [21]. (3,3,5,5-$^2H_4$)-docosanoic (C22:0-d_4) and (3,3,5,5-$^2H_4$) hexacosanoic (C26:0-d_4) acids (gift from Dr H.J. ten Brink, Amsterdam, The Netherlands) were used as internal standard. Ions at m/z 397, m/z 401, m/z 453 and m/z 457 corresponding to the [M-57] ions of 22:0, 22:0-d_4, 26:0 and 26:0-d_4 TBDMS (butyldimethylsilyl) derivatives were selectively monitored using electron impact. The corresponding peaks were integrated and the content of C22:0 and C26:0 fatty acids was calculated according to a calibration curve using C22-d_4 and C26-d_4 as internal standards. Results were expressed as C26:0/C22:0 ratios.

3. Results

3.1. Retroviral transduction of mouse ALDR cDNA corrects the VLCFA metabolism in ALD fibroblasts

To evaluate if the ALDR gene expression could correct the metabolic defect in ALD fibroblasts, we constructed a recombinant retroviral vector, MFG-mALDR, carrying the mouse ALDR cDNA under the control of the LTR promoter of the MFG vector. Protein alignments demonstrate a 93.5% amino acid identity between mouse and human ALDR genes [5]. Skin fibroblasts from three different ALD patients were transduced with supernatant from MFG-mALDR or M48-ALD producer psi-CRIP cells.

The ALD-1 cell line is a SV40-transformed cell line [19] with a R617H mutation leading to the absence of ALDP, as determined by Western-blotting and immunocytofluorescence with anti-ALDP antibody [22]. The ALD-2 cell line is a non-transformed cell line with a R518W mutation that leads to a decreased ALDP immunoreactivity, likely due to an instability of the protein [22]. The ALD-3 cell line is a non-transformed cell line with a large deletion of the ALD gene that leads to no detectable ALD mRNA and absence of ALDP [2,23]. The ALDR gene is expressed at a very low level in normal or ALD skin fibroblasts with no detectable ALDRP, as assayed by Western-blotting or immunocytofluorescence [17] (Fig. 1A).

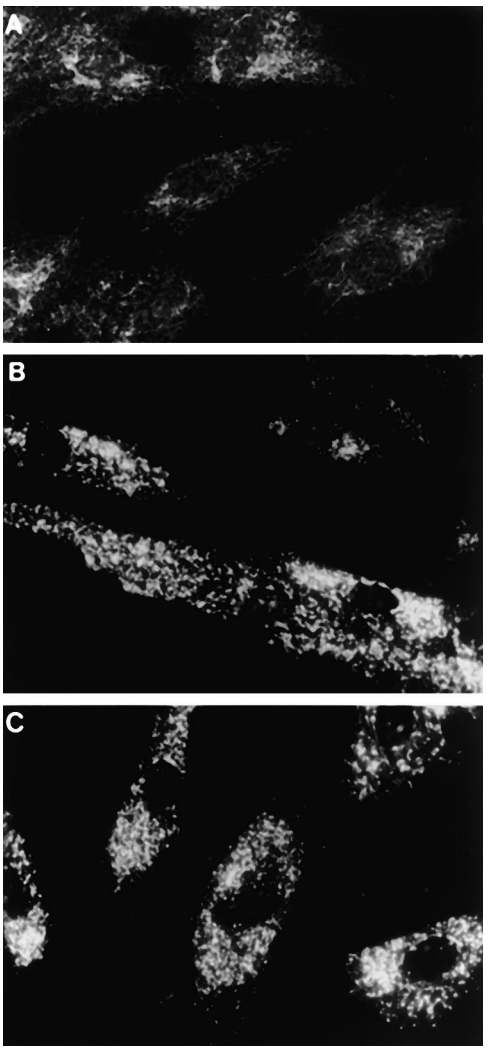

Fig. 1. Expression of ALDRP and ALDP in ALD fibroblasts after transduction with MFG-mALDR and M48-ALD vectors. (A) Prior to transduction, the ALD-3 cell line shows no ALDRP or ALDP immunostaining after incubation with antibodies against human ALDRP (polyclonal 1872) and ALDP (mAb 1D6). The same cells show ALDRP or ALDP expression with peroxisomal localization after transduction with the MFG-mALDR (B) or M48-ALD (C) vectors.

After transduction with the MFG-mALDR vector, a marked punctate fluorescent staining appeared in the ALD-1, ALD-2 and ALD-3 cell lines (Fig. 1B). A similar punctate pattern was observed when these cell lines were transduced with the M48-ALD vector (Fig. 1C). The anti-ALDP and anti-ALDRP staining co-localized with structures immunostained with anti-catalase (not shown), demonstrating the correct peroxisomal targeting of the vector-encoded proteins. Immunocytofluorescence with anti-ALDP antibody of the ALD-1 and ALD-2 cell lines transduced with the MFG-mALDR vector showed no detectable ALDP staining, indicating that overexpression of ALDRP did not stabilize instable mutated ALDP (not shown).

Based on immunocytofluorescence with anti-ALDRP antibodies, 99% of ALD-1, 75% of ALD-2 and 85% of ALD-3 fibroblasts expressed ALDRP after three cycles of infection.

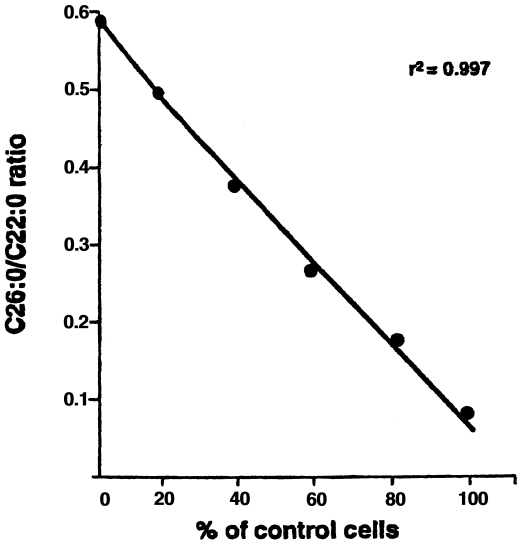

Fig. 2. The C26:0/C22:0 ratio in a mixed cell population. Control and ALD fibroblasts were counted and then mixed in the ratio indicated. The C26:C22:0 ratio per 1×10^5 cells is plotted versus the percentage of control cells in the mixture.

Similarly, using anti-ALDP antibodies, 100% of ALD-1, 96% of ALD-2 and 78% of ALD-3 fibroblasts expressed ALDP after three cycles of infection. We have previously demonstrated that retroviral-mediated transfer of the ALD cDNA with the M48-ALD vector corrects the VLCFA metabolism in ALD fibroblasts [19]. To evaluate the functional complementation of ALD fibroblasts with mouse ALDR cDNA, VLCFA concentrations were measured in ALD fibroblasts before and after transduction with MFG-mALDR and M48-ALD vectors. Table 1 shows that the C26:0/C22:0 ratio of the untransduced transformed ALD-1 cell line is 3-fold of that of normal transformed fibroblasts. Infection with MFG-mALDR or M48-ALD vectors normalized the C26:0/C22:0 ratio in these fibroblasts. Similarly, the non-transformed ALD-2 and ALD-3 fibroblasts showed before transduction a 5–7.5-fold increase of the C26:0/C22:0 ratio that decreased to normal or near normal values after transduction with the MFG-mALDR or M48-ALD vectors.

Next, we wished to compare the efficacy of the MFG-mALDR and M48-ALD vectors to correct the VLCFA accumulation in ALD fibroblasts. Because the percentage of cells expressing ALDRP or ALDP varied between experiments, we designed a mixing experiment to check that a modification of the C26:0/C22:0 ratio in fibroblasts is proportional to the percentage of normal and ALD cells. The C26:0/C22:0 ratio was determined in a pool of ALD fibroblasts with 20, 40, 60 and 80% of control cells. As the proportion of control cells increased, the C26:0/C22:0 ratio decreased proportionally ($r^2 = 0.997$) (Fig. 2). The linearity of the assay allowed thus to calculate by simple linear regression the expected C26:0/C22:0 values of transduced cells with MFG-mALDR or M48-ALD vectors with respect to the number of cells expressing the vector-encoded ALDRP or ALDP, as determined by immunocytofluorescence. The correction of the C26:0/C22:0 ratio was slightly greater than expected after normalization of the C26:0/C22:0 ratio with respect to the number of cells expressing a vector-encoded ALDRP or ALDP (Table 1). Vector-encoded ALDP and ALDRP were expressed at the same level in transduced cells, as determined by Western-blotting (not shown). Overexpression of ALDP or ALDRP most likely increases the import of VLCFA in peroxisomes of transduced cells and leads to an increased degradation of these fatty acids in these cells. No significant difference in the efficacy of correction of the VLCFA metabolism was observed in the three studied ALD cells transduced with the MFG-mALDR or M48-ALD vectors.

4. Discussion

Our study demonstrates that overexpression of ALDR cDNA can correct the accumulation of VLCFA in ALD fibroblasts, confirming recent published data by Kemp et al. [24]. In the latter study however, the correction of the VLCFA metabolism was achieved in a mixed population of ALD cells with different mutations of the ALD gene. One could not therefore discard the possibility that, in these experiments, some ALD cells were corrected while other were not, owing to the presence or absence of mutated ALD protein in the peroxisomal membrane. In our experiments, we did not observe any difference in the correction of VLCFA accumulation after overexpression of ALDRP in ALD cell lines whose ALD gene mutation leads to a complete absence of ALD mRNA and ALDP or to the presence of non-functional but yet normally targeted ALDP in peroxisomes. Our data therefore demonstrate, conclusively, that overexpression of the ALDR gene corrects to the same extent the biochemical defect of

Table 1
Correction of the VLCFA metabolism in ALD fibroblasts with MFG-mALDR and M48-ALD vectors

	n	Observed C26:0/C22:0 ratio[a]	Expected C26:0/C22:0 ratio[b]
Control SV-40 fibroblasts	4	0.178 ± 0.030	0.178
Untransduced ALD-1	3	0.494 ± 0.014	
ALD-1 transduced with MFG-mALDR vector	4	0.159 ± 0.011	0.177 (99%)
ALD-1 transduced with M48-ALD vector	3	0.143 ± 0.015	0.178 (100%)
Control fibroblasts	20	0.082 ± 0.028	0.082
Untransduced ALD-2	3	0.395 ± 0.014	
ALD-2 transduced with MFG-mALDR vector	4	0.136 ± 0.012	0.160 (75%)
ALD-2 transduced with M48-ALD vector	2	0.055 ± 0.017	0.094 (96%)
Untransduced ALD-3	3	0.607 ± 0.022	
ALD-3 transduced with MFG-mALDR vector	4	0.090 ± 0.010	0.161 (85%)
ALD-3 transduced with M48-ALD vector	3	0.113 ± 0.009	0.197 (78%)

[a]The C26:0/C22:0 ratio is given as the mean ± S.D. of three determinations of the number (*n*) of studied samples.
[b]The expected C26:0/C22:0 ratio is calculated by linear regression for the fraction (given in percentage in parentheses) of cells expressing vector-encoded ALDP or ALDRP, as detected by immunocytofluorescence.

ALD, irrespective of the presence or absence of mutant ALD protein. Our results suggest in addition that heterodimers did not form significantly between functional ALDRP or ALDP and non-functional ALDP, arguing against a negative transdominant effect of mutated ALDP over ALDRP or ALDP wild-type proteins. In yeast, Pxa1p and Pxa2p, two orthologs of ALDP and ALDRP involved in the import of long chain fatty acid-CoA, were shown to heterodimerize, the absence of Pxa2p leading to destabilization of Pxa1p [12]. Because ALDP could heterodimerize with ALDRP, it is therefore possible that overexpression of ALDRP could stabilize an instable mutated form of ALDP. However, after infection with the MFG-mALDR vector, ALDP remained undetectable by immunocytofluorescence in the ALD-2 cell line whose ALD gene mutation leads to a decreased amount of ALDP, likely reflecting instability. These results demonstrate that overexpression of the ALDR gene corrects per se the biochemical defect of ALD and that ALD and ALDR proteins did not form significant heterodimers in vitro in fibroblasts.

The ALD mouse shows no clinical phenotype, despite accumulation of VLCFA in the brain and adrenal glands similar to that observed in ALD patients [14–16]. ALDR gene redundancy could account for these observations. ALDRP and ALDP are both markedly expressed in astrocytes, oligodendrocytes and microglia in mouse [25] and a double mutant of ALD and ALDR genes might therefore be required to mimic the human ALD in mice. In contrast, the ALDR gene expression is very low in human [4,5]. We, however, recently found that, in human, the ALDR gene is expressed at a low level in neurons, astrocytes and microglia (unpublished results), raising the possibility that the stimulation of ALDR gene expression in these cells as well as in oligodendrocytes could compensate for the loss of ALDP.

Kemp et al. [24] showed recently that 4-phenyl butyrate treatment of human ALD cells and ALD mice results in decreased levels of VLCFA in cells, brain and adrenal glands. The correction of the VLCFA metabolism was associated with an increased expression of the ALDR gene and a 2.4-fold increase in the number of peroxysomes. This effect was however transitory in ALD mice (K. Smith, personal communication). Fenofibrate, a peroxisome proliferator, stimulates the ALDR gene expression in rat liver [26]. It is however likely that peroxisome proliferators will have little effects in ALD patients for two reasons: (1) although fenofibrate has recently been shown to increase the transaminase gene expression in human cells [27], there is, to our knowledge, no report demonstrating that peroxisome proliferators could induce significantly the expression of peroxisomal genes in human, in contrast to rodents; (2) the effect of peroxisome proliferators on the gene expression is minimal in rodent brain [28], likely due to the inability of these compounds to cross the blood-brain barrier efficiently. The demonstration that overexpression of ALDRP can correct the VLCFA metabolism in ALD cells supports, however, the active screening of drugs that would allow upregulation of the ALDR gene in CNS cells of ALD patients. Such effort would open the possibility to correct pharmacologically the biochemical defect of ALD in the brain of ALD patients.

Acknowledgements: This work was supported by the Association Française contre les Myopathies (AFM), the Association Européenne contre les Leucodystrophies (ELA), the Ministère Français de la Santé (Programme Hospitalier de Recherche Clinique 1994) and the Ministère Français de la Recherche (94C0313).

References

[1] Moser, H.W. (1997) Brain 120, 1485–1508.
[2] 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.
[3] Higgins, C.F. (1992) Annu. Rev. Cell. Biol. 8, 67–113.
[4] Lombard-Platet, G., Savary, S., Sarde, C.O., Mandel, J.L. and Chimini, G. (1996) Proc. Natl. Acad. Sci. USA 93, 1265–1269.
[5] Holzinger, A., Kammerer, S., Berger, J. and Roscher, A.A. (1997) Biochem. Biophys. Res. Commun. 239, 261–264.
[6] Kamijo, K., Taketani, S., Yokota, S., Osumi, T. and Hashimoto, T. (1990) J. Biol. Chem. 265, 4534–4540.
[7] Gärtner, J., Moser, H. and Valle, D. (1992) Nat. Genet. 1, 16–23.
[8] Holzinger, A., Kammerer, S. and Roscher, A.A. (1997) Biochem. Biophys. Res. Commun. 237, 152–157.
[9] Shani, N., Jimenez-Sanchez, G., Steel, G., Dean, M. and Valle, D. (1997) Hum. Mol. Genet. 6, 1925–1931.
[10] Shani, N., Watkins, P.A. and Valle, D. (1995) Proc. Natl. Acad. Sci. USA 92, 6012–6016.
[11] Hettema, E.H., Van Roermund, C.W.T., Distel, B., Van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R.J.A. and Tabak, H.F. (1996) EMBO J. 15, 3813–3822.
[12] Shani, N. and Valle, D. (1996) Proc. Natl. Acad. Sci. USA 93, 11901–11906.
[13] Verleur, N., Hettema, E.H., Van Roermund, C.W.T., Tabak, H.F. and Wanders, R.J.A. (1997) Eur. J. Biochem. 249, 657–661.
[14] Kobayashi, T., Shinnoh, N., Kondo, A. and Yamada, T. (1997) Biochem. Biophys. Res. Commun. 232, 631–636.
[15] Lu, J.F., Lawler, A.M., Watkins, P.A., Powers, J.M., Moser, A.B., Moser, H.W. and Smith, K.D. (1997) Proc. Natl. Acad. Sci. USA 94, 9366–9371.
[16] Forss-Petter, S., Werner, H., Berger, J., Lassmann, H., Molzer, B., Schwab, M.H., Bernheimer, H., Zimmermann, F. and Nave, K.-A. (1997) J. Neurosci. Res. 50, 829–843.
[17] Troffer-Charlier, N., Doerflinger, N., Metzger, E., Fouquet, F., Mandel, J.-L. and Aubourg, P. (1998) Eur. J. Cell. Biol. 75, 254–264.
[18] Sarde, C.O., Thomas, J., Sadoulet, H., Garnier, J.M. and Mandel, J.-L. (1994) Mamm. Genome 5, 810–813.
[19] Cartier, N., Lopez, J., Moullier, P., Rocchiccioli, F., Rolland, M.-O., Jorge, P., Mosser, J., Mandel, J.-L., Bougnères, P.-F., Danos, O. and Aubourg, P. (1995) Proc. Natl. Acad. Sci. USA 92, 1674–1678.
[20] Mosser, J., Lutz, Y., Stoeckel, M.E., Sarde, C.O., Kretz, C., Douar, A.M., Lopez, J., Aubourg, P. and Mandel, J.L. (1994) Hum. Mol. Genet. 3, 265–271.
[21] Doerflinger, N., Miclea, J.M., Lopez, J., Chomienne, C., Bougnères, P., Aubourg, P. and Cartier, N. (1998) Hum. Gene Ther. 9, 1025–1036.
[22] Feigenbaum, V., Lombard-Platet, G., Guidoux, S., Sarde, C.O., Mandel, J.L. and Aubourg, P. (1996) Am. J. Hum. Genet. 58, 1135–1144.
[23] Cartier, N., Sarde, C.-O., Douar, A.-M., Mosser, J., Mandel, J.-L. and Aubourg, P. (1993) Hum. Mol. Genet. 2, 1949–1951.
[24] Kemp, S., Wei, H.M., Lu, J.F., Braiterman, L.T., McGuinness, M.C., Moser, A.B., Watkins, P.A. and Smith, K.D. (1998) Nat. Med. 4, 1261–1268.
[25] Fouquet, F., Zhou, J.M., Ralston, E., Murray, K., Troalen, F., Magal, E., Robain, O., Dubois-Dalcq, M. and Aubourg, P. (1997) Neurobiol. Dis. 3, 271–285.
[26] Albet, S., Causeret, C., Bentejac, M., Mandel, J.L., Aubourg, P. and Maurice, B. (1997) FEBS Lett. 405, 394–397.
[27] Edgar, A.D., Tomkiewicz, C., Costet, P., Legendre, C., Aggerbeck, M., Bouguet, J., Staels, B., Guyomard, C., Pineau, T. and Barouki, R. (1998) Toxicol. Lett. 98, 13–23.
[28] Reddy, J.K. and Mannaerts, G.P. (1994) Ann. Rev. Nutr. 14, 343–370.