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Fenofibrate differently alters expression of genes encoding ATP-binding transporter proteins of the peroxisomal membrane

Sabrina Albet[a], Catherine Causeret[1,a], Marc Bentejac[a], Jean-Louis Mandel[b], Patrick Aubourg[c], Bugaut Maurice[a,*]

[a]*Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences Mirande, BP 400, 21011 Dijon, France*
[b]*Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 rue Laurent Fries, BP 163, 67404 Illkirch, France*
[c]*Unité Inserm U342, Hôpital Saint-Vincent de Paul, Paris, France*

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Abstract The 70-kDa peroxisomal membrane protein (PMP 70), adrenoleukodystrophy protein (ALDP) and adrenoleukodystrophy-related protein (ALDRP) belong to the ATP-binding transporter family, share a structure of half-transporters and are localized in the peroxisomal membrane of mammals. It was suggested that these proteins may heterodimerize to form functional transporters. The expression of the three genes was examined in various tissues of control or fenofibrate (a peroxisome proliferator)-treated rats using Northern and immuno-blotting techniques. The patterns of tissue expression were distinct for the three genes. Upon treatment, expression of the *ALD* gene was not altered while that of the *PMP 70* and *ALDR* genes was strongly increased in intestine and liver, respectively. The absence of coordinated expression excludes that the three transporters function as exclusive and obligatory partners. We also report for the first time that the *ALDR* gene is inducible in rodents and that the corresponding mRNA is different in length in rat (3.0 and 5.5 kb) and in mouse and human (4.2 kb).

Key words: ABC transporter; Adrenoleukodystrophy; Peroxisome

1. Introduction

Several ATP-binding cassette (ABC) transporter proteins are associated with the mammalian peroxisome membrane. These molecules include the peroxisomal membrane protein of 70 kDa (PMP 70) [1], adrenoleukodystrophy protein (ALDP) [2] and adrenoleukodystrophy-related protein (ALDRP) [3]. In Zellweger's syndrome, a genetically heterogeneous disorder of peroxisome biogenesis, some patients have defects in the locus encoding PMP 70 [4]. Similarly, mutations in the *ALD* gene are responsible for a severe X-chromosome linked neurodegenerative disorder, adrenoleukodystrophy [2]. Unlike individuals afflicted with Zellweger's syndrome, patients with ALD possess apparently intact peroxisomes. ALD is biochemically characterized by a decrease in the activity of peroxisomal lignoceroyl-CoA ligase (very-long-chain acyl-CoA synthetase) [5,6]. Although until now the *ALDR* gene has not been found to be involved in peroxisomal disorders, it might be a candidate of the modifier genes predicted to account for the tremendous phenotypic variability of ALD [7] or one of the genes implicated in the complementation groups of Zellweger's syndrome [8].

All functional ABC transporters share an organization with four 'core' domains that can be expressed as separate polypeptides or fused together in larger, multidomain proteins [9]. Because PMP 70, ALDP and ALDRP display a half transporter structure, a homo- or heterodimer of these transporters therefore needs to be formed to constitute a functional transporter. It has been suggested that PMP 70 and ALDP [10] or ALDRP [3] could interact to form an active heterodimer. Recently, the function of two genes encoding peroxisomal membrane ABC proteins, probably ortholog of ALDP, first called YKL 741p [11] and Pxa1p [12] and now called Pat1p and Pat2p [13] or Pxa1p and Pxa2p [14], has been described in *Saccharomyces cerevisiae*. Pat1p and Pat2p heterodimerize to form a functional transporter that imports activated long-chain fatty acids from the cytosolic pool into the peroxisome that is the exclusive site of β-oxidation in yeasts. By inference, the authors suggested that the human ALDP might be involved in the uptake of activated very-long-chain fatty acids into human peroxisomes. Nevertheless, neither the dimeric structure of the PMP 70, ALDP and ALDRP transporters, nor their function or their substrates, are elucidated in mammals.

In this work, expression of the *PMP 70*, *ALD* and *ALDR* genes was studied in a variety of tissues from rats treated with fenofibrate, a potent peroxisomal proliferator in rodents. Comparison of the expression profiles does not support the hypothesis of a heterodimeric form of the three known peroxisomal ABC transporters in mammals.

2. Materials and methods

2.1. Chemicals

Fenofibrate (2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl propanoic acid 1-methylethyl ester) was a gift from Fournier SCA, Dijon, France. Nycodenz and Maxidenz were from Nycomed A/S, Oslo, Norway.

2.2. Animals

Male Wistar rats (250 g b.wt.) were obtained from IFFA-CREDO, L'Arbresle, France. They were kept at a temperature of 22°C with equal periods of darkness and light and had free access to water and food. Treated animals were fed a diet supplemented with 0.3% fenofibrate. The diet was prepared by spraying chow pellets with the hypolipidemic drug dissolved in acetone and, then, by evaporating acetone at room temperature overnight.

2.3. Isolation of peroxisomes

Peroxisomes of liver from control or fenofibrate-treated rats were purified by a discontinuous Nycodenz gradient according to Cher-

*Corresponding author. Fax: (33) 3-80-39-62-50.
E-mail: lbmc@satie.u-bourgogne.fr

[1]*Present address:* Katholieke Universiteit Leuven, Afdeling Farmacology, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

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kaoui-Malki and coworkers [15]. Peroxisomal membranes were prepared using the sodium carbonate procedure [16].

2.4. Northern blot analysis

Total RNA was isolated from fresh or frozen, saline-washed tissues by the method of Chomczynski and Sacchi [17]. Poly(A)$^+$ RNA were prepared as described by Celano and coworkers [18], electrophoresed and transferred onto Amersham Hybond-N membranes. Membranes were prehybridized in Rapid-Hyb buffer (Amersham) for 2 h at the hybridization temperature. Hybridization was carried out for 2 h at 65°C in the same buffer containing freshly-labelled cDNA probe. Probes were full-length cDNAs for human PMP 70, mouse ALDP and ALDRP [3,4,19]. Rat palmitoyl-CoA oxidase (*AOX*) cDNA was a gift of Dr. T. Osumi [20]. Rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA (provided by Dr. W.A. Schulz) [21] was used as a control probe to check the amount of RNA loaded. Probes were labelled by random priming using [α-^{32}P]dATP [22]. The labelled probes were purified on ProbeQuant G-50 Micro Columns (Pharmacia). The blots were washed twice 15 min in 5×SSC, 0.1% SDS at the room temperature, then 2×15 min in 1×SSC, 0.1% SDS at the hybridization temperature and finally once 15 min in 0.1×SSC, 0.1% SDS at the hybridization temperature. The membranes were exposed to Kodak BioMax MS films at −70°C for 1–24 h.

2.5. Isolation of a rat ALDR exonic sequence

PCR primers from the mouse *ALDR* region corresponding to large *ALD* exon 1 [23] were used to amplify a 500 bp segment of the rat *ALDR* gene from rat genomic DNA, as described in [3]. The primers were 5′-TGGACCAGATCCGGTGCTGCT-3′ and 5′-TACTAAGCGAGTTCTAAAGGC-3′. The PCR fragment was extracted using phenol/chloroform and directly sequenced using its own primers and fluorescent dideoxynucleotides.

2.6. Western blot analysis

Proteins of purified peroxisomes and peroxisomal membranes from rat liver were subjected to SDS-PAGE [24]. The proteins were then electrotransferred onto an Immobilon-P membrane (Millipore), incubated with polyclonal rabbit antibody and subsequently with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma).

2.7. Preparation of polyclonal antibodies

Peroxisomal membrane proteins obtained from liver of fenofibrate-treated rats were separated by SDS-PAGE. The polypeptide band corresponding to PMP 70 was cut-out and extracted from the gel by electroelution in 50 mM ammonium bicarbonate containing 1% SDS, using a 422 Electro-eluter (BioRad). PMP 70 was again purified under similar conditions and, then, injected into rabbits to raise serum antibodies. A synthetic peptide was generated that corresponds to the predicted amino acid residues 700–736 in mouse ALDP. The peptide was coupled to bovine serum albumin, and antiserum was raised in rabbits.

2.8. Protein concentrations

Protein contents were determined using the Bio-Rad microassay with bovine serum albumin as the standard.

3. Results

3.1. Expression of the ALD and PMP 70 genes

Northern blot analysis of poly(A)$^+$ RNA prepared from various tissues of adult rats was carried out using full-length cDNAs corresponding to the human *PMP 70*, mouse *ALD* and *ALDR* genes. Fig. 1A shows that *ALD* mRNA was detected more strongly in intestine, heart, lung and spleen than in skeletal muscle and liver, and weakly in brain and kidney. Fenofibrate treatment did not affect the level of *ALD* mRNA. However, as expected the level of peroxisomal acyl-CoA oxidase mRNA used as a marker of peroxisomal proliferation was strongly enhanced in liver and intestine by fenofibrate treatment (Fig. 1D). The *PMP 70* gene was found to be expressed especially in liver, more weakly in kidney and intestine and only slightly in other tissues (Fig. 1B). The level of *PMP 70* mRNA was increased in liver and intestine when rats were treated with fenofibrate.

Expression of the *ALD* and *PMP 70* genes were also examined at the protein level in post-nuclear homogenates from control and fenofibrate-treated rat tissues by using polyclonal anti-mouse ALDP and anti-rat PMP 70 antibodies. Only a fine band at about 75 kDa consistent with the molecular mass

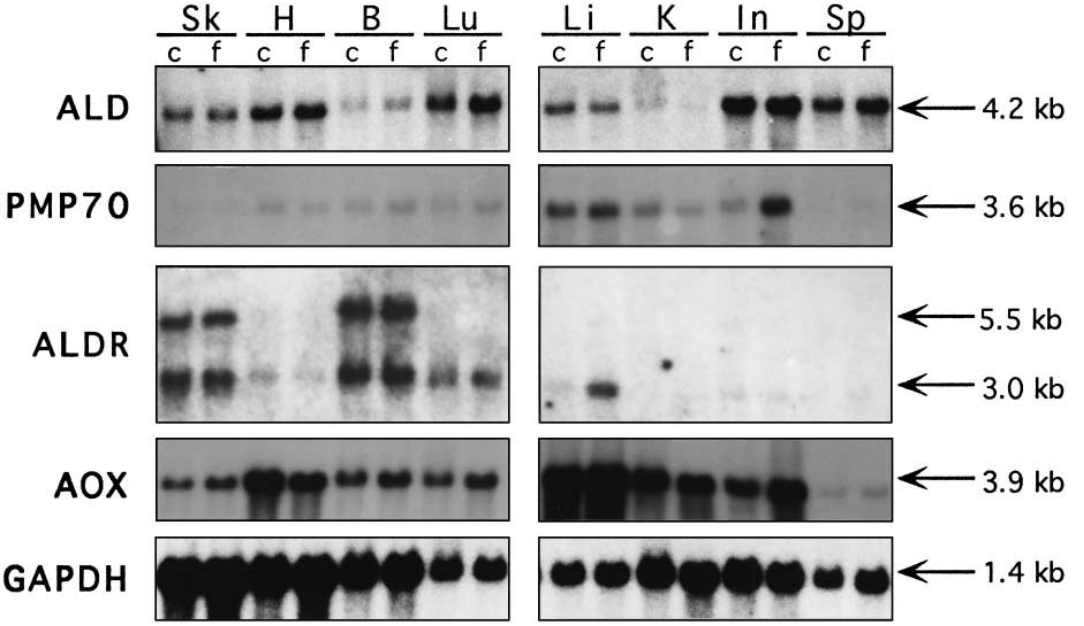

Fig. 1. mRNA expression of the *ALD*, *PMP 70* and *ALDR* genes in rat tissues. The Northern blot containing 5 μg of poly(A)$^+$ RNA isolated from tissues of control (lane c) and fenofibrate-treated (lane f) rats (skeletal muscle (Sk), heart (H), brain (B), lung (Lu), liver (Li), kidney (K), intestine (In) and spleen (Sp)) was hybridized as described in Section 2. Probes were mouse *ALD* cDNA, human *PMP 70* cDNA, mouse *ALDR* cDNA, rat acyl-CoA oxidase (*AOX*) cDNA. Rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe was used as a control for the loaded amount of RNA.

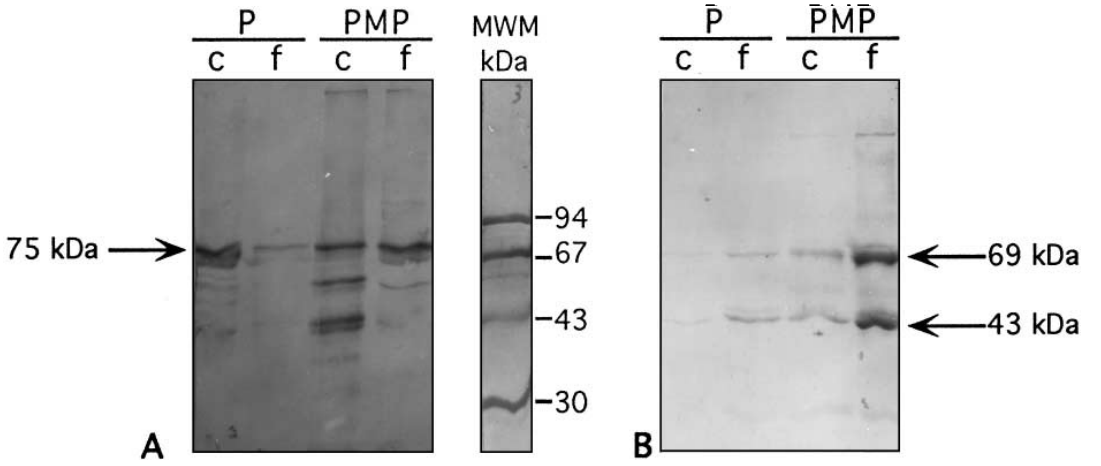

Fig. 2. Western blot analysis of proteins in purified peroxisomes and peroxisomal membranes. Proteins of purified peroxisomes (8 μg) (lanes P) and proteins of peroxisomal membranes (14 μg) (lanes PMP) from liver of control (c) and fenofibrate-treated (f) rats. The 100-fold diluted serum against ALDP (A) or PMP 70 (B) was used as the first antibody, and the second antibody was 1000-fold diluted goat alkaline phosphatase-conjugated anti-rabbit IgG. Visualization was carried out using 5-bromo-3-chloro-indolyl phosphate/nitroblue tetrazolium as the substrate. The 43-kDa band detected in (B) was probably a result of proteolysis of PMP 70 [1].

of ALDP could be detected in most tissues, and the fenofibrate treatment had apparently no effect (results not shown). PMP 70 was detected especially in liver and kidney and was enhanced by the fenofibrate treatment (results not shown). When purified peroxisomes and peroxisomal membrane proteins from liver were examined, Fig. 2 shows clearly that fenofibrate did not alter the ALDP level whereas it strongly increased the PMP 70 level.

3.2. Expression of the ALDR gene

As far as the *ALDR* gene is concerned, the 4.2-kb mRNA normally found in mouse and human tissues was not detected in rat tissues using mouse *ALDR* cDNA as a probe (Fig. 1C). However, two other transcripts at about 3.0 and 5.5 kb were strongly detected in skeletal muscle and brain. The 3.0-kb mRNA alone was detected in lung and heart. In liver, the 3.0-kb mRNA level was strongly enhanced by fenofibrate. When mice were treated with fenofibrate, a strong increase in the 4.2 kb *ALDR* mRNA was observed in liver but not in intestine and kidney (data not shown). Interestingly, the 3.0- and 5.5-kb mRNA species were also observed in the mouse brain besides the predominant signal at 4.2 kb corresponding to *ALDR* mRNA (Fig. 3A).

Using primers within putative mouse *ALDR* exon 1, a fragment of 500 bp was amplified by PCR from rat genomic DNA. The rat amplicon was very similar in nucleic sequence (95% identity) to the corresponding sequence of the mouse *ALDR* cDNA (data not shown). It was used as a probe in Northern blot analysis of rat and mouse poly(A)$^+$ RNA under the same conditions as those described for Fig. 1 Whereas the 4.2-kb *ALDR* mRNA was strongly expressed in the mouse brain, it was not detected in all the rat tissues examined (results not shown) including the brain (Fig. 3B). On the other hand, the 3.0- and 5.5-kb mRNA were again detected in the rat tissues in a similar pattern as already observed in Fig. 1C, except in the lung in which the 5.5-kb transcript was also detected (results not shown). However, using the rat *ALDR* probe, the intensity of the mRNA detected at 3.0 and 5.5 kb in some rat tissues was comparable with that of the 4.2-kb *ALDR* mRNA in mouse as shown in Fig. 3A,B for the brain. Futhermore, fenofibrate induction was again observed only in the liver (results not shown). Southern blot experiments performed using either mouse *ALDR* cDNA or a rat *ALDR* PCR fragment as the probe and mouse and rat genomic DNA confirmed the presence of the *ALDR* gene in both rodents (results not shown).

4. Discussion

ALDP and PMP 70 are 'half transporter' versions with one hydrophobic and one hydrophilic domains, share a 38.5% amino-acid identity, and both have a peroxisomal membrane location. It was suggested that ALDP and PMP 70 heterodimerize to form a functional transporter [10]. However, Northern blot analysis of the *ALD* and *PMP 70* genes had shown distinct expression patterns in

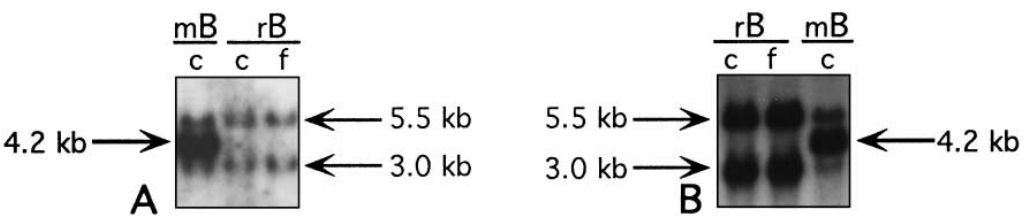

Fig. 3. Northern blot analysis of *ALDR* mRNA in rat and mouse tissues. The Northern blots of poly(A)$^+$ RNA (5 μg per each lane) were prepared from brain of control (c) mice (mB) and control (c) and fenofibrate-treated (f) rats (rB). A: The membrane was hybridized using the full-length mouse *ALDR* cDNA as the probe. B: The membrane was hybridized using the 500-pb PCR fragment amplified from rat genomic DNA, as the probe.

mouse [3]. These observations were confirmed by the present results obtained in the rat by Northern and Western blot analyses. Moreover, we observed no effects of a fenofibrate treatment on the *ALD* mRNA level in the eight rat tissues examined whereas fenofibrate induction of the *PMP 70* gene was found in liver and intestine. An increase in the *PMP 70* mRNA had already been pointed out in the liver of rats treated with fenofibrate [25], ciprofibrate [25] or diethylhexylphtalate [1]. Finally, different spatial and temporal expression of *ALD* and *PMP 70* mRNA during post-natal development has been demonstrated using in situ hybridization histochemistry in rat brain [26]. Absence of coordinated expression of the *PMP 70* and *ALD* genes in different tissues and in response to peroxisome proliferators indicates that these two genes are regulated by different mechanisms. Our results and those from other authors taken together suggest therefore that ALDP and PMP 70 are unlikely to function as exclusive and obligatory partners in the peroxisomal membrane.

Because the *ALDR* gene is presently the closest known homologue to the *ALD* gene (66% amino-acid identity), it was suggested that ALDRP may also interact with ALDP or PMP 70 to form an active heterodimer [3]. Recently, Lombart-Platet and coll. [3] showed that the expression patterns of the *ALDR*, *ALD* and *PMP 70* genes were distinct in some mouse tissues and cell lines, as revealed by Northern blotting. Similar conclusions can be drawn from the present results obtained in the rat. For example in the brain of rat, and of mouse as well [3], the *ALDR* gene is strongly expressed whereas the *ALD* and *PMP 70* genes are weakly expressed. Furthermore, fenofibrate appears to alter the expression of the *ALDR* gene in the liver, but not in the intestine, of the rat and mouse. All together, the data are not in favor of functional heterodimerization of the members of the peroxisomal ABC protein subfamily but rather suggest that ALDP, ALDRP and PMP 70 may be specialized for function in specific tissue types.

Interestingly, the 4.2-kb *ALDR* mRNA strongly expressed in mouse brain [3] was not expressed in all of the rat tissues tested. Instead, two other transcripts (3.0 and 5.5 kb) were found to be expressed in the rat. These two transcripts were also expressed in mouse brain, but weakly. The hybridization conditions used (high stringency) suggest that these two *ALDR* homologous mRNA are closely related to the 4.2-kb *ALDR* mRNA. The *ALDR* gene actually appears to be present in rat as shown by PCR amplification of putative *ALDR* exon 1 and Southern blot hybridization. One of the two additional transcripts at least (the 3.0-kb mRNA more probably) corresponds to the *ALDR* mRNA in rat and could arise from use of multiple transcription or polyadenylation sites. Heterogeneity in *ALDR* mRNA size may be relevant to the regulation of this gene. Indeed, it should be noticed that in rat liver, only the 3.0-kb *ALDR* transcript is markedly increased following fenofibrate treatment. The biological significance of this phenomenon and its possible relevance to the control of expression of this gene are the subject of further investigations.

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References

[1] Kamijo, K., Taketani, S., Yokota, S., Osumi, T. and Hashimoto, T. (1990) J. Biol. Chem. 265, 4534–4540.
[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] Lombard-Platet, G., Savary, S., Sarde, C.-O., Mandel, J.-L. and Giovanna, C. (1996) Proc. Natl. Acad. Sci. USA 93, 1265–1269.
[4] Gärtner, J., Moser, H. and Valle, D. (1992) Nature Genet. 1, 16–23.
[5] Wanders, R.J.A., van Roermund, C.W.T., van Wijland, M.J.A., Schutgens, R.B.H., van den Bosch, H., Schram, A.W. and Tager, J.M. (1988) Biochim. Biophys. Res. Commun. 153, 618–624.
[6] Lazo, O., Contreras, M., Bhushan, A., Stanley, W. and Singh, I. (1989) Arch. Biochem. Biophys. 702, 722–728.
[7] Moser, H.W. and Moser, A.B. (1989) in: The Metabolic Basis of Inherited Disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle D. eds.), pp. 1511–1532, Mc Graw-Hill, New York.
[8] Shimozawa, N., Suzuki, Y., Orii, T., Moser, A.B., Moser, H.W. and Wanders, R.J.A. (1993) Am. J. Hum. Genet. 52, 843–844.
[9] Higgins, C.F. (1995) Cell 82, 693–696.
[10] Valle, D. and Gärtner, J. (1993) Nature 361, 682–683.
[11] Bossier, P., Fernandes, L., Vilela, C. and Rodrigues-Pousada, C. (1994) Yeast 10, 681–686.
[12] Shani, N., Watkins, P.A. and Valle, D. (1995) Proc. Natl. Acad. Sci. USA 92, 6012–6016.
[13] 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.
[14] Shani, N. and Valle, D. (1996) Proc. Natl. Acad. Sci. USA 93, 11901–11906.
[15] Cherkaoui-Malki, M., Bardot, O., Lhuguenot, J.C. and Latruffe, N. (1990) Biol. Cell 69, 83–92.
[16] Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) J. Cell Biol. 93, 97–102.
[17] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[18] Celano, P., Vertino, P.M., and Casero, Jr. R.A. (1993) Biotechniques 15, 27–28.
[19] Sarde, C.-O., Thomas, J., Sadoulet, H., Garnier, J.-M. and Mandel, J.-L. (1994) Mam. Genome 5, 810–813.
[20] Miyazawa, S., Hayashi, H., Hijikata, M., Ishii, N., Furuta, H., Kagamiyata, T., Osumi, T. and Hashimoto, T. (1987) J. Biol. Chem. 262, 8131–8137.
[21] Tso, Y.J., Sun, X.-H., Kao, T.-H., Reece, K.S. and Wu, R. (1985) Nucl. Acid Res. 13, 2485–2502.
[22] Feineberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[23] Sarde, C.-O., Mosser, J., Kioschis, P., Kretz, C., Vicaire, S., Aubourg, P., Poustka, A.-M. and Mandel, J.-L. (1994) Genomics 22, 13–20.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Causeret, C., Bentéjac, M. and Bugaut, M. (1993) Biol. Cell 77, 89–104.
[26] Pollard, H., Moreau, J. and Aubourg, P. (1995) J. Neurosci. Res. 42, 433–437.