Activity of human Δ5 and Δ6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids

Roberto J. de Antueno*, Leah C. Knickle, Heidi Smith, Michele L. Elliot, Stephen J. Allen, Solomon Nwaka, Michael D. Winther[1]

QuantaNova Canada Ltd., P.O. Box 818, Kentville, NS, Canada B4N 4H8

Received 5 October 2001; revised 29 October 2001; accepted 2 November 2001

First published online 13 November 2001

Edited by Pierre Jolles

Abstract **Yeast co-expressing human elongase and desaturase genes were used to investigate whether the same desaturase gene encodes an enzyme able to desaturate n-3 and n-6 fatty acids with the same or different carbon chain length. The results clearly demonstrated that a single human Δ5 desaturase is active on 20:3n-6 and 20:4n-3. Endogenous Δ6 desaturase substrates were generated by providing to the yeast radiolabelled 20:4n-6 or 20:5n-3 which, through two sequential elongations, produced 24:4n-6 and 24:5n-3, respectively. Overall, our data suggest that a single human Δ6 desaturase is active on 18:2n-6, 18:3n-3, 24:4n-6 and 24:5n-3.** © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Desaturase; Elongase; Polyunsaturated fatty acid; Essential fatty acid biosynthesis

1. Introduction

In human and other mammalian cells, microsomal Δ6 desaturation of essential fatty acids linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) is the first and rate limiting step in the biosynthesis of n-6 and n-3 polyunsaturated fatty acids (PUFAs). The products of these reactions, γ-linolenic (18:3n-6) and stearidonic acids (18:4n-3), are elongated to dihomo-γ-linolenic acid (20:3n-6) and 20:4n-3, respectively (Fig. 1). These fatty acids are then substrates of a Δ5 desaturase that generates arachidonic acid (20:4n-6) and 20:5n-3 which are further elongated to 22:4n-6 and 22:5n-3 and finally to their respective products 24:4n-6 and 24:5n-3. A second microsomal Δ6 desaturation takes place on these PUFAs. The products of this desaturation, 24:5n-6 and 24:6n-3, are converted to 22:5n-6 and 22:6n-3, respectively, by peroxisomal β-oxidation [1]. In addition, two sequential elongations of 20:4n-3 (i.e. 22:4n-3 and 24:4n-3) were described by Sauerwald et al. as part of putative alternative steps in the synthesis of 22:6n-3 [2]. These authors proposed that 24:4n-3 could undergo Δ9 desaturation to 24:5n-3 which can act as substrate of the Δ6 desaturase. Direct elongations on 18:2n-6, 18:3n-3 and 20:3n-6 are also reported.

To date, there is no conclusive evidence that the Δ6 desaturase, which recognizes 18-carbon unsaturated fatty acids (18:2n-6 or 18:3n-3), is the same enzyme that desaturates 24-carbon substrates [3]. In this regard, Marquardt and coworkers have cloned three human fatty acid desaturase genes [4]. Two of these genes encode either a Δ5 or a Δ6 desaturase, however the third gene, to which a function has not been ascribed yet, is unlikely to represent a second human Δ5 or Δ6 desaturase. We have recently demonstrated that, in certain malignant human cells, the Δ6 desaturase impairment is not specific to any particular step of the PUFA biosynthetic pathways [5]. Similar results were obtained with skin fibroblasts from a patient with an inherited abnormality in lipid metabolism [6]. All these findings from different laboratories suggest that the hypothesis of the existence of at least two distinct enzymes still remains unsubstantiated.

Recent reports have shown that the levels of 20:4n-6, 22:4n-6, 20:5n-3 and 22:5n-3 significantly increased when a human Δ5 desaturase was expressed in mouse fibroblasts [7]. However, direct activity on both substrates of this human Δ5 desaturase, 20:3n-6 and 20:4n-3, has not been reported yet.

A heterologous host, *Saccharomyces cerevisiae*, known for its absence of PUFA biosynthesis, was transformed with a human fatty acid elongase and either a Δ6 or Δ5 desaturase gene in an attempt to elucidate whether the same human desaturase is active on n-3 and n-6 fatty acids with the same or different chain length. We here describe the functional analysis of these fatty acid biosynthetic enzymes using different radiolabelled PUFAs.

2. Materials and methods

2.1. Chemicals

Fatty acid free bovine serum albumin (BSA), tergitol, Tris–HCl, carbohydrates, amino acids and fatty acids were obtained from Sigma-Aldrich Canada (ON, Canada). Yeast nitrogen base without amino acids was purchased from Difco-Becton Dickinson (Kansas City, MO, USA). All organic solvents (high performance liquid chromatography (HPLC) grade) were obtained from Fisher-Scientific (Fair Lawn, NJ, USA).

[1-^{14}C]18:3n-3, [1-^{14}C]20:4n-6, [1-^{14}C]20:5n-3, [1-^{14}C]20:3n-6 and [1-^{14}C]18:2n-6 (99% radiochemical purity; specific activity: 51–56

*Corresponding author. 1520 Ashlee Drive, Coldbrook, NS, Canada B4R 1A1.
E-mail address: r.deantueno@mailexcite.com (R.J. de Antueno).

[1] Present address: Xenon Genetics Inc., 2386 East Mall, Vancouver, BC, Canada V6T 1Z6.

Abbreviations: SC-Leu and SC-U-Leu, synthetic minimal medium lacking either leucine or uracil and leucine, respectively; PUFAs, polyunsaturated fatty acids; fatty acids are designated with a number that refers to the number of carbons in the chain; the number after the colon indicates the number of double bonds and the n refers to the location of the nearest double bond from the methyl end

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)03135-0

μCi/μmol) were purchased from NEN (Boston, MA, USA). [1-^{14}C]20:4n-3, [1-^{14}C]24:4n-6 and [1-^{14}C]24:5n-3 (99% radiochemical purity; specific activity: 55 μCi/μmol) were provided by ARC (St. Louis, MO, USA). Fatty acids were saponified with 0.1 M LiOH and dissolved in synthetic minimal medium for yeast lacking either leucine (SC-Leu) or uracil and leucine (SC-U-Leu), containing 1% tergitol.

2.2. Cloning

Human Δ5 and Δ6 desaturases (GenBank accession numbers AF226273 and AF126799, respectively) were cloned into the pYES2/CT yeast expression vector (Invitrogen, Carlsbad, CA, USA) for production of these desaturases as fusion proteins with a C-terminal peptide containing the V-5 epitope and polyhistidine tag. The desaturases were amplified by PCR from Chang Liver cell (America Type Culture Collection (ATCC), Rockville, MD, USA) cDNA using Advantage HF polymerase (Clontech, Palo Alto, CA, USA). The cDNA was prepared by reverse transcribing RNA, isolated with Trizol reagent (Gibco BRL, Rockville, MD, USA), using MuLV reverse transcriptase and random hexamers. The forward primers, 5′-CACGCGAAGCTTAAAA**ATG**GCCCCCGACCCGG-3′ for Δ5 desaturase and 5′-CACGCGAAGCTT**ATG**GGGAAGGGAGGG-AAC-3′ for Δ6 desaturase, contained the translation initiation codon (bold-faced) as well as a *Hin*dIII site (underlined). The reverse primers, 5′-CACGCGTCTAGATTGGTGAAGATAGGCATCTAGCC-AGAGCTG-3′ for Δ5 desaturase and 5′-CACGCGTCTAGATTT-GTGAAGGTAGGCGTCCAG-3′ for Δ6 desaturase, contained an *Xba*I site (underlined). PCR products were gel-purified, digested with *Hin*dIII and *Xba*I, and ligated into pYES2/CT cut with the same enzymes. The resulting plasmids encoding Δ5 and Δ6 desaturase were designated pTh5009.1 and pTh5002.1, respectively. In these plasmids the desaturase is under the control of the inducible *GAL1* promoter.

BLASTP of the GenBank NR database with yeast ELO1 identified a protein with unknown function (GenBank accession number BAA91096), as a potential elongase. This protein was deduced from cDNA (GenBank accession number AK000341) and is termed ELG3 herein. ELG3 was amplified by PCR from ZR-75-1 cell (ATCC) cDNA, prepared as described above, using Advantage HF polymerase. The forward primer, 5′-CACGCGGGATCCATC**ATG**GAACA-TCTAAAGGCC-3′, contained the translation initiation codon (bold-faced) as well as a *Bam*HI site (underlined). The reverse primer, 5′-ATATCACGATGCGGCCGC**TTA**TTGTGCTTTCTTGTTCATCA-CTCC-3′, contained the translation stop codon (bold-faced) as well as the *Not*I site. The PCR product was gel-purified, digested with *Bam*HI and *Not*I, and ligated into pYES2/CT cut with the same enzymes. ELG3 was transferred from pYES2/CT into the *Bam*HI and *Xba*I sites of the pBEVY-L yeast expression vector [8]. The resulting plasmid was designated pLh5015.1. In this plasmid ELG3 is under the control of the constitutive glyceraldehyde 3-phosphate dehydrogenase promoter. (In comparison to GenBank accession number BAA91096, the protein encoded by the ELG3 gene contains two amino acid substitutions, T31M and V179I.)

2.3. Yeast transformation

S. cerevisiae strain INVSc1 (Invitrogen) was transformed using the lithium acetate method. Double recombinant yeast cells containing both pTh5002.1 and pLh5015.1, or pTh5009.1 and pLh5015.1, were grown on SC-U-Leu medium. Yeast cells transformed with pBEVY-L alone, the cloning vector for ELG3, were grown on SC-Leu medium.

2.4. Incubation with radiolabelled fatty acids

Transformed yeast cultures (approximately 3.2×10^6 cells/ml; OD_{600} 0.4) were divided in two experimental groups. The first group was incubated in a 125 ml Erlenmeyer flask containing 10 ml of SC-U-Leu medium with 2% raffinose and 1% tergitol. Yeasts of the second group were incubated in 10 ml of SC-U-Leu medium containing 1% raffinose, 2% galactose (to induce the expression of the Δ6 or Δ5 desaturase which are under the control of the *GAL1* promoter supplied by the pYES2/CT yeast expression vector) and 1% tergitol. Lithium salts (1 μCi) of either [1-^{14}C]18:3n-3, [1-^{14}C]20:4n-6, [1-^{14}C]20:5n-3, [1-^{14}C]18:2n-6, [1-^{14}C]20:3n-6, [1-^{14}C]20:4n-3, [1-^{14}C]24:4n-6 or [1-^{14}C]24:5n-3 were added to both experimental groups at a final concentration of 25 μM. The general incubation conditions in this study are a modification of those described in the literature for the rat Δ6 desaturase expressed in yeast [9]. Briefly, after 24 h incubation in an orbital incubator at 270 rpm and 30°C, cells were harvested by centrifugation at $5000\times g$ for 10 min at 4°C. The cell pellet was washed with Tris–HCl buffer (100 mM, pH 8.0) containing 0.1% BSA. Total lipids were extracted and radiolabelled fatty acids analyzed as described below. Triplicate incubations with each fatty acid were performed from at least two yeast cultures.

The host yeast transformed with pBEVY-L and grown in SC-Leu medium was used as negative control.

2.5. Lipid extraction

Cells were resuspended in 1.5 ml of water and total lipids were saponified with 2 ml of 10% KOH in ethanol. The free fatty acids from the saponified samples were methylated using 14% boron trifluoride in methanol at 90°C for 30 min. The resultant methyl esters (FAME) were analyzed as described below.

2.6. Reverse phase-HPLC (RP-HPLC) analysis

Analyses of radiolabelled FAME were carried out on a Hewlett Packard 1090, series II chromatograph equipped with a diode array detector set at 205 nm, a radioisotope detector (model 171, Beckman, CA, USA) with a solid scintillation cartridge (97% efficiency for ^{14}C detection) and a reverse phase ODS (C-18) Beckman column (250 mm×4.6 mm i.d.; 5 μm particle size) attached to a pre-column with a microBondapak C-18 (Beckman) insert. FAME were separated isocratically with acetonitrile/water (95:5 v/v) at a flow rate of 1 ml/min and were identified by comparison with authentic standards obtained from ARC and NuChekPrep (Elysian, MN, USA).

3. Results and discussion

In the presence of galactose, the cotransformed *S. cerevisiae* cells, containing the human Δ6 desaturase and elongase (ELG3) genes, were able to desaturate 18:2n-6 to 18:3n-6 and 18:3n-3 to 18:4n-3 (Fig. 2). These products were then substrates of the ELG3 that elongated them to 20:3n-6 and 20:4n-3, respectively (Fig. 2B,D). The constitutively expressed ELG3 was slightly active on 18:3n-3 as per the low levels of 20:3n-3 produced within the 24 h incubation (Fig. 2C,D). ELG3 did not appear to elongate 18:2n-6 (Fig. 2A). This

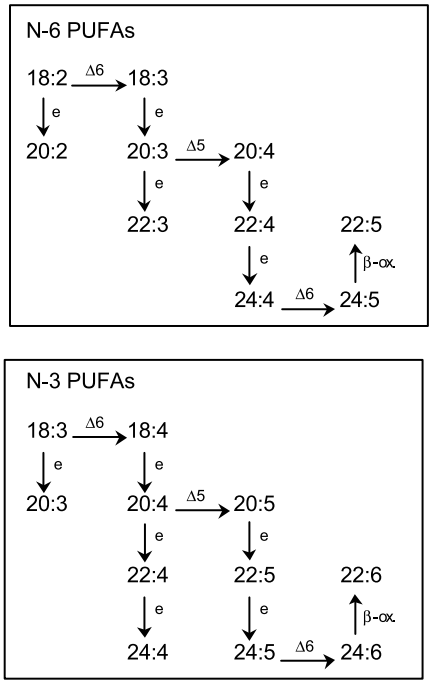

Fig. 1. n-6 and n-3 PUFA biosynthetic pathways.

observation might suggest the possible existence of multiple human elongases with various substrate specificities.

When the cotransformed yeast was incubated with [1-^{14}C]-24:4n-6 or [1-^{14}C]24:5n-3 in the presence of galactose the activity of the Δ6 desaturase was not detected, probably due to the low uptake and thus low intracellular concentrations of these long chain PUFAs. Indeed, under the experimental conditions of this study, we have found that not all PUFAs were taken up by *S. cerevisiae* to the same degree. Only 14% of the total radioactivity provided as [1-^{14}C]24:4n-6 or [1-^{14}C]24:5n-3 was recovered in yeast cells, whereas the remaining 86% of that radioactivity was detected in the medium after a 24 h incubation. Conversely, more than 79% of [1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-6 and 40% of [1-^{14}C]20:4n-6 was detected in yeast at the same incubation time (data not shown). Therefore, radiolabelled 20:4n-6 and 20:5n-3 were added to the culture medium in order to test the capability of the cotransformed yeast to desaturate the 24-carbon substrates as shown in Fig. 1. The ELG3 elongase was highly active on these two PUFAs which, through two sequential elongations, produced endogenous substrates for the desaturase, 24:4n-6 and 24:5n-3 (Fig. 3A,C). Upon induction of the Δ6 desaturase with galactose, the 24-carbon PUFAs were converted into 24:5n-6 and 24:6n-3, respectively (Fig. 3B,D). No activity on any of the substrates was detected in yeast transformed with pYES2/CT and pBEVY-L alone (data not shown).

These data expand the results of Cho and co-workers [10] who were the first to clone this human Δ6 desaturase and test its activity on the generally used 18-carbon PUFA substrate. In addition, our findings in this yeast model support those

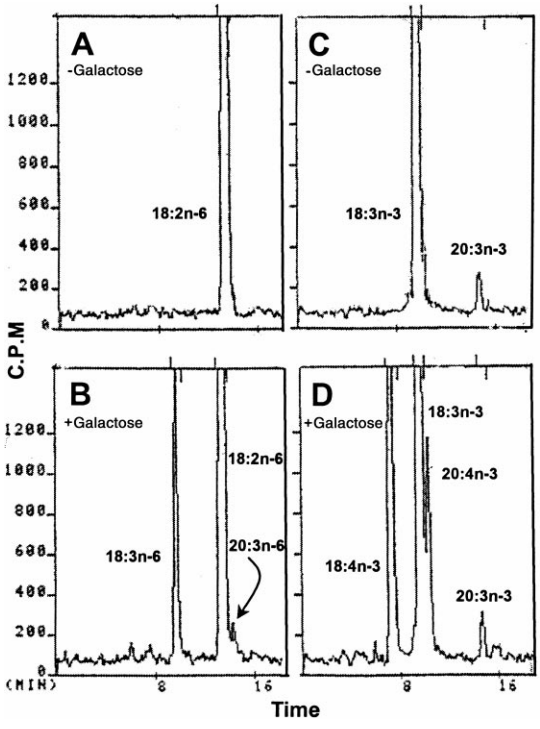

Fig. 2. RP-HPLC analysis of radiolabelled methyl esters of fatty acids from yeast co-expressing human Δ6 desaturase and elongase, incubated with [1-^{14}C]18:2n-6 (A and B) or [1-^{14}C]18:3n-3 (C and D). Galactose was used to induce the expression of the desaturase gene.

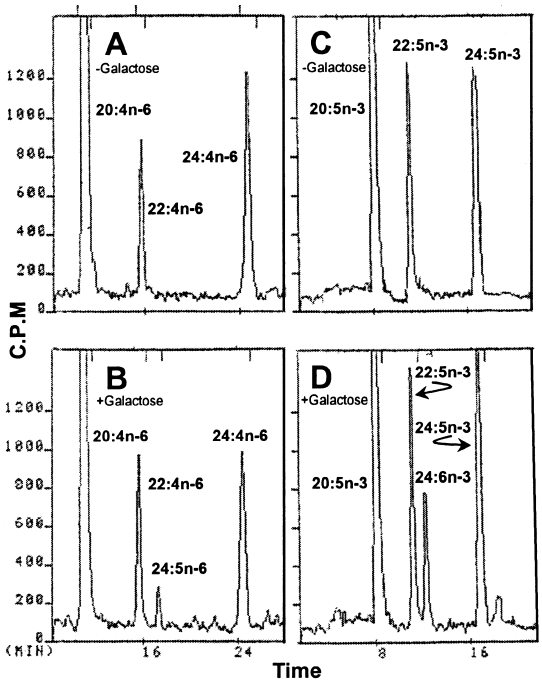

Fig. 3. RP-HPLC analysis of radiolabelled methyl esters of fatty acids from yeast co-expressing human Δ6 desaturase and elongase, incubated with [1-^{14}C]20:4n-6 (A and B) or [1-^{14}C]20:5n-3 (C and D). Galactose was used to induce the expression of the desaturase gene.

from Sauerwald and co-workers [2] who have suggested that two Δ6 desaturation steps active on 18:3n-3 and 18:2n-6 would compete not only with each other but also with 24:5n-3 and 24:4n-6. Sprecher, in a recent review addressing this subject, has emphasized that the control of the Δ6 desaturase would be of considerable interest in animals or human studies if a single enzyme is active on four different fatty acids from both n-6 and n-3 families [1].

Further studies, this time with yeast co-expressing human Δ5 desaturase and ELG3 genes, showed significant conversion of the classical substrate, 20:3n-6 to 20:4n-6 by the desaturase (Fig. 4). As expected, this cotransformed yeast as well as the yeast transformed with the vectors alone (pYES2/CT and pBEVY-L) were not capable of desaturating substrates of the Δ6 desaturase (data not shown). ELG3 was able to elongate 20:4n-6 sequentially to 22:4n-6 and 24:4n-6 (Fig. 4B). A slight elongation of 20:3n-6 was only detected when the desaturase was not induced (Fig. 4A). Similarly, when the yeast was incubated with 20:4n-3 without galactose, two higher PUFA homologs were produced by the constitutively expressed ELG3 (Fig. 4C). However, 24:4n-3 was not detected when the desaturase was induced. In such cases, 20:5n-3 was produced from 20:4n-3 (Fig. 4D). These results confirm indirect observations from Leonard et al. [7] performed in mouse fibroblasts transfected with a human Δ5 desaturase. Additionally, our results show that ELG3 is active on both 20:5n-3 and 22:5n-3.

Kinetic experiments for any of the desaturases with the elongase in the cotransformed yeast model were not within the scope of this study. Therefore, we were not able to determine if a competition between these enzymes for the same substrates (e.g. 20:3n-6) could explain the lack of elongation

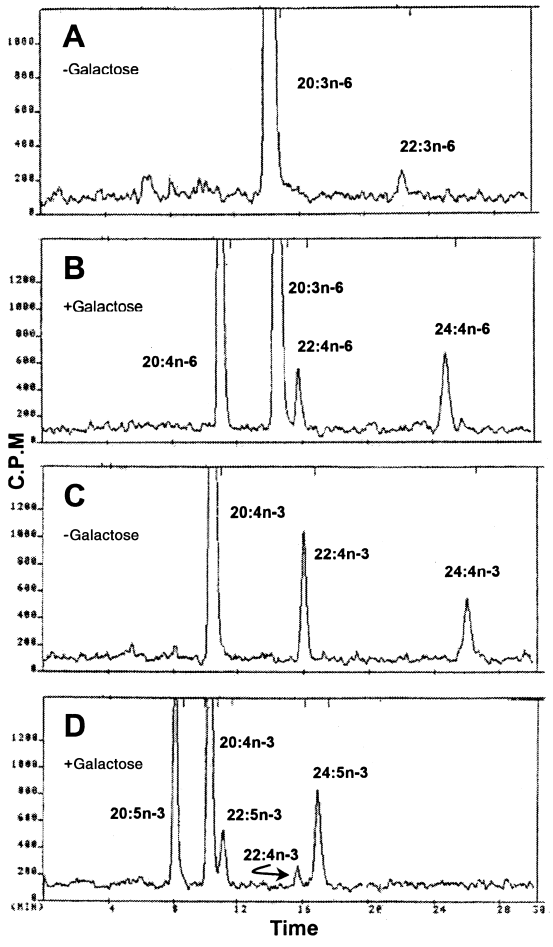

Fig. 4. RP-HPLC analysis of radiolabelled methyl esters of fatty acids from yeast co-expressing human Δ5 desaturase and elongase, incubated with [1-^{14}C]20:3n-6 (A and B) or [1-^{14}C]20:4n-3 (C and D). Galactose was used to induce the expression of the desaturase gene.

of 20- and 22-carbon PUFAs upon the induction of the Δ5 desaturase.

Our data support the existence of putative alternative steps of the PUFA biosynthetic routes depicted in Fig. 1. In this regard, the presence of 22:4n-3 and 24:4n-3 has already been shown in the plasma phospholipid fraction of infants that received a dose of [U-^{13}C]18:3n-3 [2]. In our experiment, the cotransformed yeast was capable of producing 22- and 24-carbon n-3 PUFAs from 20:4n-3 (Fig. 4). However, we could not detect any activity of the endogenous Δ9 desaturase on 24:4n-3 which would have produced 24:5n-3, the substrate for the Δ6 desaturase as proposed by Sauerwald et al. [2]. We have previously confirmed that this yeast model has a Δ9 desaturase which is active on its typical substrate, 16:0, palmitic acid (data not shown).

In short, our results suggest that the human Δ6 desaturase gene cloned by Cho et al. [10] encodes an enzyme which is able of generating a Δ6 double bond in both 18- and 24-carbon PUFAs and that a single Δ5 desaturase is capable of introducing a Δ5 double bond on n-3 and n-6 20-carbon PUFAs. We may hypothesize that these findings can be extrapolated to other mammalian desaturases based on the similarities between the human, mouse and rat desaturases. Indeed, an analysis of the amino acid sequences for the mouse and human Δ6 desaturases revealed an 87% identity (96% similarity) for both mammalian homologs [10]. In particular, we may speculate that the rat Δ6 desaturase described by Aki et al. [9] has a similar enzymatic capability to that ascribed to the human gene herein. Similarly, it has recently been reported that the 3413-bp full-length Δ5 desaturase cDNA cloned from rat liver contains an open reading frame of 447 amino acid residues sharing 92% similarity with its human counterpart [11]. Further investigations are needed to confirm this hypothesis and to determine the substrate specificity of these enzymes. Such experiments may require the cloning, isolation and purification of these enzymes from different mammals in an attempt to reconstitute them in artificial membranes and perform kinetic analyses with n-3 and n-6 PUFAs. Overall, our findings will contribute to the understanding of the biochemical and physiological roles of fatty acid desaturases in normal tissues and to the design of strategies for the modulation of their activities in disease.

Acknowledgements: pBEVY plasmid was a generous gift from Dr. C.A. Miller (University of Tulane Medical Center, New Orleans, LA, USA). We thank K.A. Chisholm, M. Sweeney and K. Reeves for their skillful technical assistance.

References

[1] Sprecher, H. (2000) Biochim. Biophys. Acta 1486, 219–231.
[2] Sauerwald, T.U., Hachey, D.L., Jensen, C.L., Chen, H., Anderson, R.E. and Heird, W.C. (1997) Pediatr. Res. 41, 183–187.
[3] Innis, S.M., Sprecher, H., Hachey, D., Edmond, J. and Anderson, R.E. (1999) Lipids 34, 139–149.
[4] Marquardt, A., Stohr, H., White, K. and Weber, B.H.F. (2000) Genomics 66, 175–183.
[5] De Antueno, R.J., Allen, S.J., Ponton, A. and Winther, M.D. (2001) FEBS Lett. 491, 247–251.
[6] Williard, D.E., Nwankwo, J.O., Kaduce, T.L., Harmon, S.D., Irons, M., Moser, H.W., Raymond, G.V. and Spector, A.A. (2001) J. Lipid Res. 42, 501–508.
[7] Leonard, A.E., Kelder, B., Bobik, E.G., Chuang, L.-T., Parker-Barnes, J.M., Thurmond, J.M., Kroeger, P.E., Kopchick, J.J., Huang, Y.-S. and Mukerji, P. (2000) Biochem. J. 347, 719–724.
[8] Miller, C.A., Martinat, M.A. and Hyman, L.E. (1998) Nucleic Acids Res. 26, 3577–3583.
[9] Aki, T., Shimada, Y., Inagaki, K., Higashimoto, H., Kawamoto, S., Shigeta, S., Ono, K. and Suzuki, O. (1999) Biochem. Biophys. Res. Commun. 255, 575–579.
[10] Cho, H.P., Nakamura, M.T. and Clarke, S.D. (1999) J. Biol. Chem. 274, 471–477.
[11] Zolfaghari, R., Cifelli, C.J., Banta, M.D. and Ross, A.C. (2001) Arch. Biochem. Biophys. 391, 8–15.