Transfection of L6 myoblasts with adipocyte fatty acid-binding protein cDNA does not affect fatty acid uptake but disturbs lipid metabolism and fusion

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We studied the involvement of fatty acid-binding protein (FABP) in growth, differentiation and fatty acid metabolism of muscle cells by lipofection of rat L6 myoblasts with rat heart (H) FABP cDNA or with rat adipocyte (A) FABP cDNA in a eukaryotic expression vector which contained a puromycin acetyltransferase cassette. Stable transfectants showed integration into the genome for all constructs and type-specific overexpression at the mRNA and protein level for the clones with H-FABP and A-FABP cDNA constructs. The rate of proliferation of myoblasts transfected with rat A-FABP cDNA was 2-fold higher compared with all other transfected cells. In addition, these myoblasts showed disturbed fusion and differentiation, as assessed by morphological examination and creatine kinase activity. Uptake rates of palmitate were equal for all clone types, in spite of different FABP content and composition. Palmitate oxidation over a 3 h period was similar in all clones from growth medium. After being cultured in differentiation medium, mock- and H-FABP-cDNA-transfected cells showed a lower fatty acid-oxidation rate, in contrast with A-FABP-cDNA-transfected clones. The ratio of [^{14}C]palmitic acid incorporation into phosphatidylcholine and phosphatidylethanolamine of A-FABP-cDNA-transfected clones changed in the opposite direction in differentiation medium from that of mock- and H-FABP-cDNA-transfected clones. In conclusion, transfection of L6 myoblasts with A-FABP cDNA does not affect H-FABP content and fatty acid uptake, but changes fatty acid metabolism. The latter changes may be related to the observed fusion defect.

INTRODUCTION

Fatty acid-binding proteins (FABPs) belong to a conserved family of intracellular lipid-binding proteins with low molecular mass (14–15 kDa) [1–3]. Eight different FABP types have been described: heart, liver, adipose, myelin, intestinal, epidermal, brain and ileal. Many FABP cDNAs have been isolated, cloned and the proteins expressed in bacterial systems. In this way, large amounts of proteins have become available for three-dimensional analysis by crystallography and/or NMR (reviewed in [1,3]). All FABPs studied thus far contain a folding motif designated an up-and-down β-barrel [3].

The main function of FABP is thought to be intracellular binding and targeting of fatty acids. FABPs may also modulate the effect of fatty acids on various metabolic enzymes and receptors and cellular processes such as signal transduction and gene expression [1,4]. The function significance of the different FABP types is not clear and may vary with the tissues and the physiological conditions. Adaptation may be related to metabolic requirements of the cell, ligand trafficking and/or modulatory functions.

A relation has been observed between fatty acid-oxidation capacity and FABP content of various rat tissues and various human and rat muscles [5–7]. FABP content of muscle showed no change under various physiological conditions, except an increase in fast-twitch muscles on chronic electrostimulation and endurance training [7]. Heart FABP (H-FABP) content and fatty acid-oxidation capacity increase in skeletal and heart muscle during postnatal development [7,8]. Some indications of a role for H-FABP in regulation of growth and differentiation have been obtained. Mammary-derived growth inhibitor, which appeared to be identical with a mixture of H-FABP and adipocyte FABP (A-FABP) [9], caused specific growth inhibition and terminal differentiation of mammary epithelial cells [10,11] and modest anti-proliferative activity in human breast cancer cells [12]. The protein also appeared to be an inducer of hypertrophy of cardiac myocytes [13]. Bovine H-FABP expression in yeast transformants retarded growth [14].

Much of our previous work was related to the *in vitro* characteristics of FABPs in general and H-FABP in particular [1,4,15]. In studies with cultured human primary skeletal-muscle cells, we observed that the FABP content increases with the degree of maturation of the cells [16]. This increase was, however, not accompanied by a rise in palmitate-oxidation capacity. In a mouse skeletal muscle cell line, H-FABP expression increased 60-fold on differentiation [17]. A 5–7-fold increase in fatty acid oxidation was found on L6 myoblast differentiation [18]. In an abstract, Claffey et al. [19] reported a larger fatty acid uptake and esterification in these cells containing overexpressed H-FABP. Fatty acid oxidation was not studied in these transfected cells.

Transfection studies of liver FABP (L-FABP), intestinal FABP (I-FABP) and A-FABP have been performed in fibroblasts [20–25] and Chinese hamster ovary (CHO) cells [26], which express no or very little FABP. We used the L6 rat skeletal-muscle cell line to study FABP function in a cell culture model marked by changes in endogenous FABP content. Myoblasts of this cell line are able to fuse and differentiate if the culture conditions are changed. We studied the effect of transfection

Abbreviations used: A-FABP, adipocyte fatty acid-binding protein; CHO, Chinese hamster ovary; CK, creatine kinase; CS, citrate synthase; D-PBS, Dulbecco's modified PBS; H-FABP, heart fatty acid-binding protein; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver fatty acid-binding protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RT, reverse transcriptase; DMEM, Dulbecco's modified Eagle's medium; SV40, simian virus 40; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pac, puromycin acetyltransferase.

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with rat H-FABP cDNA on cell growth and differentiation and fatty acid uptake and metabolism. Furthermore we studied the effect of the presence of a second non-muscle FABP by introducing rat A-FABP cDNA in this cell line.

EXPERIMENTAL

Materials

Oligonucleotide primers were obtained from Eurogentec, Seraing, Belgium; *Taq* DNA polymerase and RNasin were from Promega, Madison, WI, U.S.A.; Sequenase version 2.0 DNA sequencing kit was from United States Biochemical, Cleveland, OH, U.S.A.; Superscript RNase H^- reverse transcriptase (RT), restriction endonucleases, agar, bacto yeast extract, casein hydrolysate, isopropyl β-D-thiogalactopyranoside, Ultroser G and guanidinium isothiocyanate were from Life Technologies Inc., Gaithersburg, MD, U.S.A.; TA Cloning system was from Invitrogen Corp., San Diego, CA, U.S.A.; Lipidex 1000 was from Canberra-Packard, Groningen, The Netherlands; transfection reagent (DOTAP), creatine kinase (CK) *N*-acetylcysteine-activated monotest kit and the random-primed DNA labelling kit were from Boehringer-Mannheim GmbH, Mannheim, Germany; puromycin dihydrochloride and BSA were from Sigma, St. Louis, MO, U.S.A.; Dulbecco's modified Eagle's medium (DMEM) was from Gibco, Hoofddorp, The Netherlands; fetal calf serum was from Integro, Zaandam, The Netherlands; Hybond-N^+, ECL Western-blotting analysis system, [α-[^{35}S]thio]dATP (1000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [1-^{14}C]oleic acid (55 mCi/mmol), [1-^{14}C]-palmitic acid (55 mCi/mmol) and [6-^{3}H]thymidine (5 mCi/mmol) were from Amersham International, Little Chalfont, Bucks, U.K.; Aqualuma Plus was from Lumac LSC, Groningen, The Netherlands; trypsin was from Difco Laboratories, Detroit, MI, U.S.A.; TLC aluminium sheets of silica-gel 60 F_{254} were from Merck. All other reagents were of analytical grade.

Culture media and buffers

Dulbecco's modified PBS (D-PBS) contained 145 mM NaCl/5.4 mM KCl/5 mM Na_2HPO_4/25 mM glucose/25 mM sucrose, pH 7.3. Growth medium was composed of DMEM with 10% fetal calf serum. Differentiation medium contained 0.4% Ultroser G and 10% rat brain extract in DMEM [16].

Cell culture

L6 myoblasts were proliferated in growth medium. Before confluence was reached the cells were washed with D-PBS, treated with 2 mM EDTA (in D-PBS) for 1 min, and then with trypsin (0.05% trypsin in D-PBS for 7–10 min at 21 °C), collected by centrifugation and seeded again in fresh growth medium. Medium was changed every 2–3 days. To induce fusion, the medium was changed to differentiation medium at confluence. Cells were grown in this medium for 3–9 days. Routinely, a mycoplasma control was performed using fluorochrome Hoechst 33258 staining of air-dried cultures. Cultures of cells grown on differentiation medium were photographed (Zeiss Pan-Neofluar, 10 × lens) after staining with Haematoxylin solution. Primary rat muscle cells from 10-day-old rats were cultured in the same medium as the L6 cells.

Cloning and sequencing of rat H-FABP and A-FABP cDNA

Rat H- and A-FABP mRNAs were reverse-transcribed and amplified using rat poly(A) RNA isolated from adult rat quadriceps muscle as a template. The primer sets used were for the rat H-FABP cDNA sequence: forward primer, 5′-TCTCATTGCACCATGGCGGACGCCTTT-3′; reverse primer, 5′-AGTGACGGGGGATCCAGGTCACGCCTCCTT-3′. For rat A-FABP cDNA the primers were derived from the mouse and human A-FABP cDNA sequences: forward primer, 5′-GGCCATATGTGTGATGCYTTTGTRGG-3′; reverse primer, 5′-CATGGATCCGGCTYATGCYCTYTCATAAAC-3′. Reverse primers (20 pmol) were used to reverse-transcribe 1 μg of rat muscle mRNA with 20 units of Superscript RNase H^- RT in 50 mM Tris/HCl, pH 8.3, containing 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM each dNTP and 40 units of RNasin in 1 h at 42 °C. PCR amplification was carried out in a total volume of 50 μl containing 20 pmol of each primer, 1 unit of *Taq* DNA polymerase and 1.5 mM $MgCl_2$ in buffer comprising 50 mM KCl, 10 mM Tris/HCl, pH 9.0 and 0.1% Triton X-100. After 5 min denaturation at 94 °C, 30 cycles of amplification were carried out: 94 °C for 1.5 min; 55 °C for 2 min; 74 °C for 2 min. The amplified cDNAs were cloned using the TA Cloning kit. DNA sequencing was performed using Sanger sequencing on either double-stranded or single-stranded pCRII plasmids.

Expression and purification of rat H-FABP and A-FABP

Coding parts of rat A-FABP and H-FABP cDNA were cloned into bacterial expression vectors pET-11a and pET-3d, using the *Nde*I and *Nco*I restriction sites respectively as translation-start sites. The recombinant plasmids were transformed to *Escherichia coli* BL21(DE3) cells, and FABP expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside [27,28]. The procedure for purification of rat H-FABP and A-FABP was essentially the same as for human H-FABP [15,28]. The binding affinity for [1-^{14}C]oleic acid was analysed by the Lipidex method [29]. K_d values were derived from Scatchard plots of binding assays at 0.1–2.0 μM oleic acid and 0.5 μM protein. Polyclonal rabbit anti-(rat H-FABP) and anti-rat A-FABP) sera were prepared by standard procedures [6].

Construction of eukaryotic expression constructs

The constructs were based on the pSG5 expression plasmid [30]. Transcription of the cDNAs was under the control of a simian virus 40 (SV40) early promoter and the construct contained a rabbit β-globin intron II and an SV40 poly(A) signal [30]. A modified pSG5 plasmid, with an extended multiple cloning site and a 1.7 kb pac (puromycin acetyltransferase) cassette ligated in the *Sal*I site of the multiple cloning site was used as expression construct. Rat H-FABP and rat A-FABP cDNAs in the pCRII vectors were cut out by *Eco*RI digestion and inserted into the *Eco*RI site of the multiple cloning site of the pSG5-pac expression plasmid. The orientation and size of the cloned cDNAs were verified by PCR using a T7 promoter (forward) primer and a cDNA-specific (reverse) primer or an SV40 poly(A) addition signal (reverse) primer and a cDNA-specific (forward) primer. The mock construct was the pSG5-pac plasmid without an FABP cDNA.

Introduction of plasmids into rat L6 myoblasts by lipofection; selection of transfected cells

L6 myoblasts were seeded at a density of 5×10^5 cells per 60 mm dish, and cultured for 18 h. The medium was refreshed and lipofection was started after 6 h. Linearized plasmid DNA (5 μg) was mixed with 37.5 μl of transfection reagent DOTAP, added to the cells and incubated for 18 h at 37 °C/5% CO_2. Selection with 5 μg puromycin/ml of growth medium started 24 h after lipo-

fection [31]. One week after transfection, separate colonies were picked and grown to check for clonal variation.

Southern-blot analysis

Genomic DNA of non-transfected and transfected L6 myoblasts was digested over 18 h with *Eco*RI and *Hin*dIII. The digestion products were separated on a 0.8 % agarose gel and blotted on to a Hybond-N^+ filter. Southern-blot hybridization was performed using a ^{32}P-labelled pac cassette (1.7 kb) as a probe in a mixture of 0.5 M sodium phosphate, pH 7.2, containing 1 mM EDTA, 7 % SDS and 100 μg/ml denatured herring sperm DNA. Hybridization with the labelled probe was performed in the latter mixture for 16 h at 65 °C. Blots were washed with high stringency in a solution containing 40 mM sodium phosphate (pH 7.2)/0.1 % SDS at 65 °C.

Northern-blot hybridization

Total RNA was isolated by the RNAzol method. Total RNA (15 μg) was separated on agarose/formaldehyde gels and blotted on to Hybond-N^+ filters. Northern-blot hybridization was performed using ^{32}P-labelled cDNA probes for rat H-FABP cDNA, rat A-FABP cDNA or murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The hybridization and wash conditions were as described for Southern-blot analysis. After exposure (1–3 days), the nylon membrane was dehybridized and reused for subsequent hybridizations.

SDS/PAGE and immunoblotting

Cell extracts were analysed for FABP expression by separation of 20 μg of protein on an SDS/15 % polyacrylamide gel and blotting. RAT H- and A-FABP were detected with polyclonal rabbit antisera and the ECL detection system (Amersham).

Cell proliferation

Cell proliferation was assayed by measuring [^{3}H]thymidine incorporation. Myoblasts were seeded at a density of 10000 cells per 35 mm dish (five dishes were assayed for each clone) and proliferated in growth medium for 36 h. After a medium change, cells were pulsed for 24 h with 3 μCi of [6-^{3}H]thymidine per dish. Cells were washed three times with PBS, fixed with methanol and air-dried. They were then solubilized by the addition of 1.0 ml of 0.5 M NaOH, neutralized and radioactivity was determined.

Assay of palmitic acid uptake

L6 myoblasts were plated on 35 mm dishes in growth medium. After 36 h of growth, subconfluent myoblasts were washed twice with 2 ml of D-PBS, and 1.75 ml of DMEM was added. After a 1 h equilibration period, 0.25 ml of a solution containing 600 μM [1-^{14}C]palmitic acid (1.4 μCi/μmol) bound to 120 μM albumin in 0.9 % NaCl was added and incubation was prolonged. After 1–30 min, this solution was removed and the cells were washed three times with ice-cold PBS containing 0.5 % fatty acid-free BSA. Cells were lysed with 1 ml of 0.5 M NaOH, neutralized with 6 M HCl and radioactivity was determined in 10 ml of Aqualuma by liquid-scintillation counting. At each time point three plates were assayed. Values were corrected for a blank which was determined by aspirating the fatty acid-containing solution immediately after addition. The data were normalized for protein content, determined on four identical plates. Cells were washed three times with PBS, lysed with 0.5 M NaOH, neutralized with 6 M HCl and used for protein determination by the method of Lowry et al. [32].

Assay of palmitate oxidation

For this assay, we used a procedure previously described for the measurement of fatty acid oxidation of human myotubes [33]. Cells were cultured in 24-well plates on growth and differentiation medium. The medium was removed and the cells were washed three times with PBS. Wells with cells in 0.5 ml buffer containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 10 mM Hepes, pH 7.4, were placed separately in 40 ml incubation vials, containing 5 ml of water. Incubation at 37 °C was started by the addition of 120 μM [1-^{14}C]palmitate (1.6 μCi/μmol) bound to 24 μM fatty-acid free albumin (final concentrations) to wells. Incubation at 37 °C proceeded for 180 min. Palmitate-oxidation rate was calculated from the production of $^{14}CO_2$ and ^{14}C-labelled acid-soluble products [34]. For protein assay, intact L6 cells were washed with PBS and dissolved in 500 μl of 2 M NaOH per well. Contents of six wells were pooled and neutralized with HCl before protein determination.

Assay of palmitic acid incorporation

Cells were grown on 35 mm dishes in culture medium for 24 h in the presence of 120 μM [1-^{14}C]palmitic acid bound to 24 μM albumin. The cells were washed three times with 0.15 M NaCl, and sonicated in the same medium (0.3–1.0 and 3–7 mg of protein/ml for cells on growth and differentiation medium respectively). Extracts were analysed for lipids by TLC as described by Mazière et al. [35]. Neutral lipids were separated in hexane/diethyl ether/acetic acid (70:30:2, by vol.), and phospholipids in chloroform/methanol/water/acetic acid (50:30:4:8, by vol.). Incorporation of [1-^{14}C]palmitic acid was determined by liquid-scintillation counting of separated spots.

Biochemical assays

Cells were homogenized in a buffer containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris/HCl, pH 7.4. Citrate synthase (CS) activity was measured in sonicated culture homogenates as described by Shepherd and Garland [36]. Units of activity of this enzyme are μmol of CoA formed at 25 °C/min. CK activity was determined by the CK *N*-acetylcysteine-activated monotest at 37 °C. Units of activity are μmol of NADPH formed/min. CK isoenzymes were separated by agarose-gel electrophoresis (procedure no. 715-EP; Sigma, St. Louis, MO, U.S.A.). The gels were scanned using a Bio-Rad GS-670 densitometer and analysed with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The content of rat H-FABP was assayed by a modified ELISA [6]. Briefly, cellular protein (25 ng) was adsorbed on 96-well microtitre plates by incubation for 16 h at 4 °C. The FABP was probed with affinity-purified anti-(rat H-FABP) antibodies, and peroxidase-conjugated swine anti-rabbit IgG was used as secondary antibody. Results were determined by reference to a standard curve (0–0.2 ng of rat H-FABP). Protein content was determined by the method of Lowry et al. [32] with BSA as standard.

RESULTS

Cloning and sequencing of rat A-FABP and H-FABP cDNAs and expression of the proteins

Rat A-FABP cDNA was isolated and sequenced (GenBank/EMBL accession no. U75581). Comparison with mouse and human A-FABP cDNA sequences [37,38] showed similarities of 93 and 85 % respectively. Conserved amino acid substitutions are the most common substitutions between the three A-FABP sequences (10 of 17). Isolated rat H-FABP cDNA showed no

differences in nucleotide sequence from previously reported sequences [8,39], except one conserved nucleotide substitution on the codon of Val-32 (GTG to GTC). Purified recombinant rat A-FABP and H-FABP showed dissociation constants for the binding of oleic acid of 2.19 ± 0.51 μM (mean $\pm$ S.D. for five experiments) and 0.30 μM respectively. These values are in good agreement with the data for tissue-derived or recombinant A-FABP [38,40] and H-FABP [41]. The FABP preparations were applied in subsequent assays as standards.

Rat L6 cell line as a model

Rat L6 myoblasts show a low H-FABP content on growth medium (about 30 pmol/mg of protein) which increases to 50 pmol/mg of protein in myotubes. No additional induction of H-FABP was detected at mRNA or protein level on addition of 20 or 200 μM palmitate or oleate, 0.3 μM insulin or 6–25 mM glucose to the culture medium of L6 myoblasts and myotubes for 24 h (results not shown). Primary rat muscle cells show a gradual increase in H-FABP content from myoblasts (45 ± 2 pmol/mg of protein; $n = 2$) on culturing for 7 days on differentiation medium to a maximal content of 76 ± 21 pmol/mg of protein ($n = 7$).

Transfection of L6 myoblasts and clonal variation

The isolated rat A- and H-FABP cDNAs were used for preparation of constructs that contained both a FABP cDNA and a pac cassette. In this way, no co-transfection is necessary and all puromycin-resistant clones automatically include an FABP cDNA (except the mock-transfectants). After lipofection of L6 myoblasts, separate colonies were picked and grown to check for clonal variation.

Clonal variation was monitored by SDS/PAGE and subsequent immunoblotting of transfected myoblast homogenates and by ELISA. Of the seven isolated clonal cell lines transfected with rat H-FABP cDNA, only one showed a six to eight times higher expression of H-FABP than mock-transfected myoblasts; the other clones showed 1–3-fold overexpression. Clonal cell lines isolated after transfection with rat H-FABP antisense cDNA (nine) and with mock construct (nine) all showed an expression of H-FABP comparable with that of non-transfected myoblasts. The clonal variation between the latter two types of clone was similar. From this, we concluded that the antisense approach, i.e. inhibition of H-FABP expression, had not worked.

All three isolated clones transfected with rat A-FABP cDNA showed H-FABP and A-FABP expression, whereas A-FABP was not detectable in non-transfected cells and all other transfected clone types by Western blotting (Figure 1). A strong signal at about 30 kDa points to the existence of dimers of A-FABP in transfected myoblasts. A protein of approx. 20 kDa also reacted strongly with the rabbit anti-(rat A-FABP) polyclonal serum (Figure 1) and with affinity-purified antibodies (not shown). This phenomenon was also observed with antisera raised against human A-FABP in mouse and chicken as well as against rat A-FABP in another rabbit. Pre-adsorbing the antibodies with a homogenate of non-transfected L6 myoblasts also did not work (results not shown).

One representative clone of mock- and A-FABP-cDNA-transfected clone types was used in most of the following experiments, but major observations were checked in at least three clones of each type. For H-FABP cDNA, the highest overexpressing clone was generally used to investigate the maximal effect.

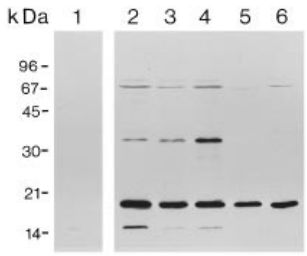

Figure 1 Immunodetection of A-FABP expression

Total cell extracts of transfected L6 clones (20 μg) were analysed by SDS/PAGE (15% gel). Immunodetection was performed with rabbit anti-(rat A-FABP) serum. Lane 1, purified rat A-FABP (50 ng); lanes 2–4, A-FABP-cDNA-transfected clones 1–3; lane 5, mock-transfected clone; lane 6, non-transfected L6 cell line.

Genomic analysis of transfected L6 clones

Selection for resistance to puromycin was an indirect test to check the stable integration of the constructs in the genome. A direct test for integration is Southern-blot hybridization of genomic DNA isolated from the clones (Figure 2). Indeed only transfected clones tested showed a positive hybridization signal with the isolated pac cassette. Different patterns occurred as the result of concatemer formation of linear plasmids and subsequent integration in the genome. A positive signal meant that clones were stably transfected, but did not give information about the extent of transfection.

Northern-blot hybridization

Total RNA of representative transfected clones was used in Northern-blot hybridization. Clones transfected with the rat H-FABP cDNA and rat A-FABP cDNA showed a clear overexpression at the mRNA level (Figures 3a and 3b). Changes in the levels of mRNAs are not due to differences in the quantity of

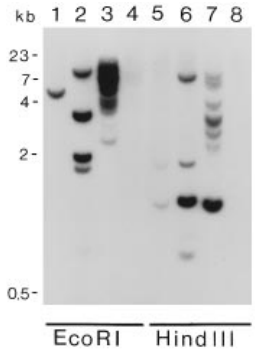

Figure 2 Genomic analysis of transfected and non-transfected rat L6 clones

*Eco*RI- or *Hin*dIII-digested genomic DNAs were separated on a 0.8% agarose gel. Southern-blot analysis was performed using the ^{32}P-labelled pac cassette as a probe. Lanes 1 and 5, mock-transfected clone; lanes 2 and 6, H-FABP antisense cDNA-transfected clone; lanes 3 and 7, H-FABP cDNA-transfected clone; lanes 4 and 8, non-transfected L6 cell line.

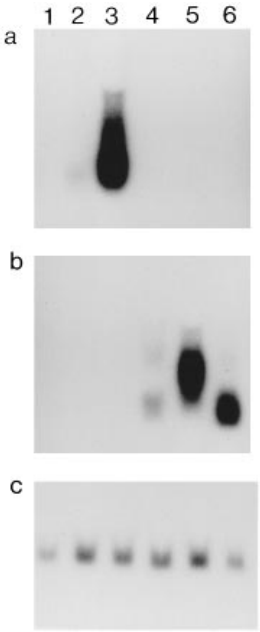

Figure 3 mRNA expression in non-transfected and transfected L6 clones

Total RNA (15 μg) was separated on a 1 % formaldehyde/agarose gel. Northern-blot hybridization was performed using ^{32}P-labelled rat H-FABP cDNA (**a**), rat A-FABP cDNA (**b**) or murine GAPDH cDNA (**c**) as probe. Lane 1, non-transfected L6; lane 2, mock-transfected clone; lane 3, H-FABP-cDNA-transfected clone; lanes 4–6, A-FABP-cDNA-transfected clones 1–3.

Table 1 [^{3}H]Thymidine incorporation of L6 myoblasts transfected with four different constructs

[^{3}H]Thymidine was added (3 μCi/dish) and incorporation measured after 24 h of incubation. Values are given as percentages of those in control mock-transfected L6 myoblasts. The incorporation in these cells varied between 1061 and 3353 d.p.m./dish (eight independent experiments). Data are means $\pm$ S.D. for four to five experiments. * $P < 0.01$. Three clones of A-FABP-cDNA-transfected cells all showed the higher incorporation.

Clone type	Thymidine incorporation (%)
Mock transfected	100
H-FABP cDNA	104 $\pm$ 28
A-FABP cDNA	189 $\pm$ 45*

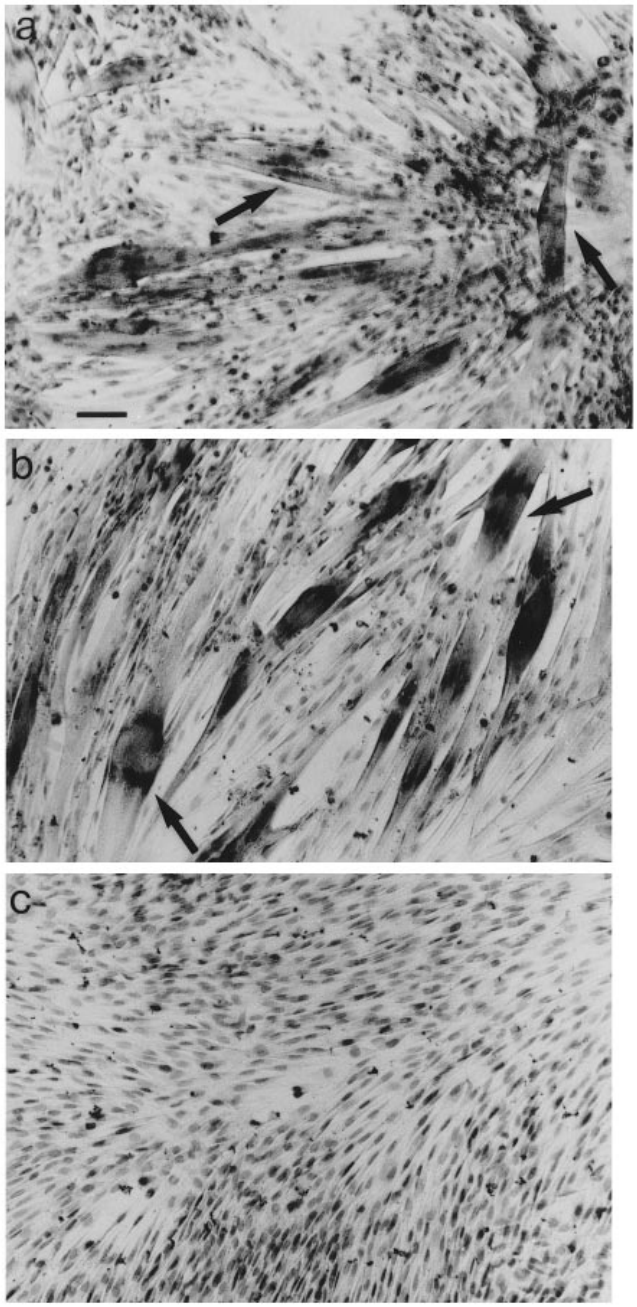

Figure 4 Micrographs of transfected L6 cell lines

Confluent L6 myoblasts were cultured for 6 days on differentiation medium. (**a**) Mock-transfected; (**b**) H-FABP-cDNA-transfected; (**c**) A-FABP-cDNA-transfected. Cultures were stained with Haematoxylin solution for 15 min. Arrows indicate myotubes. The bar represents 70 μm.

RNA loaded on to the gel (Figure 3c). The clones transfected with rat H-FABP antisense cDNA showed no change in H-FABP mRNA signal (results not shown), compared with the mock-transfected clones, again indicating that the antisense approach did not work. In the three clones derived from transfection with A-FABP cDNA, differences in length of the A-FABP transcripts were observed. This phenomenon is detected more often in systems for heterologous expression and is probably due to differences in the length of the poly(A) tail.

Morphological and growth characteristics

In the proliferative stage, all transfected clones were morphologically similar to non-transfected L6 myoblasts. [^{3}H]Thymidine-incorporation studies revealed a significant increase in the proliferation rate of A-FABP-cDNA-transfected myoblasts compared with mock-transfected L6 myoblasts (Table 1). Rat H-FABP cDNA transfectants showed no significant differences in growth rate compared with mock-transfected myoblasts. Transfected clones were mycoplasma-negative during all the culturing and assay procedures.

After the growth medium had been changed to differentiation medium, morphological differences became visible. Both mock-transfected and H-FABP-cDNA-transfected L6 cells showed normal myotube formation after 3–6 days on differentiation medium (Figures 4a and 4b). The very dark regions in the largest myotubes were due to the typical clustering of nuclei and their

Table 2 CK and CS activities and H-FABP content of transfected L6 cells on growth and differentiation media

Cells were cultured for 2–3 days on growth medium or 3–5 days on differentiation medium. CK and CS activity are given in units/mg of protein and m-units/mg of protein, and H-FABP in pmol/mg of protein (means ± S.D. for the number of cultures indicated in parentheses). Statistically different from mock-transfected cells: * $P < 0.001$. No significant differences were observed between different clones of one clone type except for H-FABP DNA for H-FABP content.

	Growth medium (6–18)			Differentiation medium (10–12)		
Clone type	CK	CS	H-FABP	CK	CS	H-FABP
Mock	0.05 ± 0.02	42 ± 23	46 ± 14	1.35 ± 0.61	76 ± 16	56 ± 25
H-FABP cDNA	0.08 ± 0.02	82 ± 36	177 ± 67*	1.18 ± 0.66	84 ± 24	127 ± 38*
A-FABP cDNA	0.19 ± 0.07	55 ± 32	54 ± 56	0.05 ± 0.03*	77 ± 15	62 ± 28

staining by Haematoxylin. Rat A-FABP-cDNA-transfected myoblasts cultured on differentiation medium remained mononuclear and were arrested in the alignment process (Figure 4c). These cells were unable to fuse and form myotubes even after 9 days on differentiation medium (observed with three different clones). After 5–7 days the mock- and H-FABP-cDNA-transfected myotubes detached from the dishes, but the A-FABP-cDNA-transfected cells remained stuck for at least 9 days.

Determination of CK activity established the difference in differentiation of the three clone types (Table 2). After 3–5 days on differentiation medium, the fusion-incapable A-FABP-cDNA-transfected clones showed very low CK activities, comparable with myoblast values, whereas the myotube-forming clones (mock- and H-FABP-transfected) showed the usual CK activities. The proportion of the muscle-specific isoenzyme CK-MM, as a measure of myogenic differentiation, was already over 90% of total content of CK isoenzymes in proliferating myoblasts. Therefore this is not a useful marker for the degree of maturation of cultured L6 muscle cells, in contrast with the mouse muscle cell line C2C12 and primary rat and human skeletal-muscle cells [16]. Spontaneous contractions of L6 myotubes were never observed in our cultures, nor reported in the literature, and therefore could not be used as a marker of differentiation. CS activities did not differ significantly between the different clone types on differentiation medium (Table 2) and were only about twice the values of myoblasts. These activities appeared also to vary between the different clone cultures, leading to relatively high S.D. values.

FABP content of transfected cells

H-FABP content was estimated by ELISA. Proliferating myoblasts of the H-FABP-cDNA-transfected clone showed a fourfold overexpression of H-FABP (0.26% of total protein; Table 2). H-FABP contents of mock- and A-FABP-cDNA-transfected myoblasts were all in the same range, similar to those in non-transfected L6 myoblasts. In the H-FABP-cDNA-transfected myotubes, H-FABP expression was also significantly increased compared with mock-transfected myotubes (0.19 and 0.08% of total protein respectively). H-FABP contents of mock- and A-FABP-cDNA-transfected cells were slightly higher on differentiation medium than on growth medium.

The A-FABP content in three A-FABP-cDNA-transfected clones was estimated as 240–910 pmol/mg of protein (0.3–1.3% of total cell protein) as means of two densitometric determinations of Western blots. The A-FABP content could not be measured by ELISA because of the strong reaction of a 20 kDa L6 cell protein with the anti-(rat A-FABP) antibodies.

Table 3 Uptake of [1-^{14}C]palmitic acid in non-transfected and transfected rat L6 myoblasts

Cells were used as subconfluent cultures on growth medium, and equilibrated for 1 h at 37 °C and 5% CO_2 on DMEM without serum. The assay was started by the addition of 75 μM [1-^{14}C]palmitic acid bound to 15 μM albumin. Values (in nmol of palmitic acid/mg of protein) are the means ± S.D. for four to six experiments. No significant differences were detected between the different clones of one clone type and between different clone types.

	Uptake of palmitic acid			
Clone type Time (min) ...	1	5	15	30
Non-transfected	2.7 ± 1.0	14.4 ± 8.0	24.5 ± 5.8	39.3 ± 8.4
Mock-transfected	4.5 ± 1.3	12.1 ± 5.2	20.2 ± 5.1	31.7 ± 8.0
H-FABP-cDNA	3.2 ± 0.3	11.5 ± 2.4	20.6 ± 2.3	35.0 ± 6.0
A-FABP-cDNA	3.6 ± 1.4	11.5 ± 1.1	24.5 ± 2.3	41.9 ± 3.4

Table 4 Palmitate oxidation of transfected and non-transfected L6 cells on growth and differentiation media

L6 myoblasts were grown for 2–3 days in growth medium and used before confluence. Confluent L6 myoblasts were cultured for 3 days on differentiation medium. Oxidation was measured for 3 h with 120 μM [1-^{14}C]palmitate bound to 24 μM albumin as substrate. Values (in nmol per h mg of protein) are means ± S.D. for three to five experiments. Different clones of one clone type did not show differences. Significantly different from mock-transfected cells: * $P < 0.05$. The $^{14}C_2$ contribution of the total palmitate oxidation was 59 ± 6% and 67 ± 7% for cells on growth medium and differentiation medium respectively.

Clone type	Growth medium (2–3 days)	Differentiation medium (3 days)
Non-transfected	13.2 ± 0.5	3.4 ± 0.9
Transfected		
Mock	11.6 ± 2.2	4.6 ± 0.5
H-FABP cDNA	11.0 ± 3.4	4.7 ± 0.8
A-FABP cDNA	12.1 ± 3.4	8.4 ± 2.6*

Palmitic acid uptake

We were unable to detect significant differences in palmitic acid uptake in the transfected myoblast cultures (Table 3). In the first 5 min the uptake showed a high initial rate (about 2.6 nmol of palmitic acid/min per mg of protein), but it slowed down after 5 min incubation (about 1.3 nmol/min per mg of protein measured over 30 min). Overexpression of H-FABP and expression of A-FABP did not have an effect on palmitic acid uptake.

Table 5 Distribution of [1-^{14}C]palmitic acid in neutral lipid species of transfected L6 cells cultured on differentiation medium

Cells were cultured for 4 days on differentiation medium, the last 24 h with addition of 120 μM [1-^{14}C]palmitic acid bound to 24 μM albumin. DG, diacylglycerols; TAG, triacylglycerols; C, cholesterol; CE, cholesterol esters; NEFA, non-esterified fatty acids. Cholesterol and 1,2- and 1,3-diacylglycerol were taken together. Values (in % of radioactivity) are given as means ± S.D. for the number of cultures (*n*). Different clones of one clone type did not show differences. Significantly different from mock-transfected cells: * $P < 0.05$; ** $P < 0.01$.

Clone type	*n*	CE	TAG	NEFA	C + DG
Mock	7	11.1 ± 0.7	53.1 ± 3.8	14.3 ± 3.1	21.7 ± 1.6
H-FABP	5	14.2 ± 0.8**	47.0 ± 2.7	13.0 ± 3.9	26.0 ± 1.2**
A-FABP	9	11.9 ± 2.7	43.6 ± 10.6*	19.4 ± 5.7*	25.2 ± 4.7

Fatty acid oxidation

Fatty acid-oxidation assays were performed with transfected L6 cells on growth and differentiation medium. The assay used for palmitate oxidation has previously been shown to be linear over 300 min in intact myotubes [33].

At subconfluence, all transfected myoblast clones showed the same rate of palmitate oxidation (Table 4). Differences in palmitate oxidation became visible when the transfected clones were cultured on differentiation medium for 3 days. The palmitate-oxidation rate decreased except when fusion was disturbed by transfection with A-FABP cDNA. These latter clone types showed rates comparable with myoblasts. The lower palmitate oxidation in our non-transfected myotubes compared with myoblasts is in contrast with the data of Sauro and Strickland [18], who observed a 3–5-fold increase in fatty acid oxidation on L6 myoblast differentiation. This may be related to differences in assay conditions and medium.

Distribution of [^{14}C]palmitic acid in neutral and phospholipid species

We studied by TLC the distribution of [^{14}C]palmitic acid in the lipid species of cells cultured for 24 h with [1-^{14}C]palmitic acid on growth or differentiation medium. No marked differences were detected in total incorporation among the four different types of clone on both media. The extent of uptake varied for different experiments between 811 and 1808 pmol/μg of protein or 31–37 % of added radioactivity for myoblasts and 215–346 pmol/μg of protein or 58–81 % of added radioactivity for myotubes. The ratio of incorporation of radioactivity into phospholipids compared with neutral lipids was also similar for the four clone types, and was 2.0 ± 0.1 in cells on growth medium compared with 3.4 ± 0.4 in cells on differentiation medium.

Proliferating myoblasts of the four clones did not show any differences in distribution of [^{14}C]palmitic acid among the different neutral lipid species (results not shown). Incorporation into cholesterol esters (about 24 %) was higher and into triacylglycerols (about 33 %) lower than in cells grown on differentiation medium (Table 5). Slight changes were observed in the relative distribution of palmitic acid incorporation into neutral lipids in H-FABP- and A-FABP-cDNA-transfected cells after culture on differentiation medium (Table 5).

Mock transfectants showed a significant decrease in [^{14}C]palmitic acid incorporation into PC after culture on differentiation medium compared with growth medium (Table 6). On growth medium, all transfected clones exhibited no differences in [^{14}C]palmitic acid distribution over the phospholipid species, except for a higher PE and a lower PC percentage in all three A-FABP-cDNA-transfected clones. In cells cultured on differentiation medium, a significantly lower incorporation into PE is observed in A-FABP-cDNA-transfected cells and a significantly higher incorporation into PC in both FABP-cDNA transfectants compared with mock transfectants. Incorporation into sphingomyelin was lower in transfectants of H-FABP cDNA on differentiation medium. The PC/PE ratio of A-FABP- and H-FABP-cDNA-transfected cells on differentiation medium was significantly higher than that of mock-transfected myotubes (Table 6). Furthermore palmitic acid incorporation into phospholipids of A-FABP-cDNA-transfected cells on differentiation medium differed from that of the same cells on growth medium, but was very similar to the mock-transfected clones on growth medium. The PC/PE ratio of mock-transfected cells and H-FABP-cDNA-transfected cells decreased, whereas that of A-FABP-cDNA-transfected cells increased on culture on differentiation medium compared with growth medium.

DISCUSSION

To study the function of H-FABP in cells that show low endogenous H-FABP expression, we introduced H-FABP cDNA into rat L6 myoblasts, in both the sense and antisense orientations.

Table 6 Distribution of [1-^{14}C]palmitic acid in phospholipids of transferred L6 cells cultured on growth and differentiation media

Cells were cultured on growth (for 2–3 days) or differentiation (for 4 days) medium, the last 24 h with the addition of 120 μM [1-^{14}C]palmitic acid bound to 24 μM albumin. PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin. Values (in % of radioactivity) are given as means ± S.D. for the number of cultures (*n*). Different clones of one clone type did not show differences. [a] $P < 0.05$ compared with corresponding cells on growth medium; [b] $P < 0.01$ compared with corresponding cells on growth medium; [c] $P < 0.05$ compared with mock-transfected cells on growth medium; [d] $P < 0.01$ compared with mock-transfected cells on growth medium; [e] $P < 0.01$ compared with mock-transfected cells on differentiation medium.

		Distribution (%)				
Clone type	*n*	PE	PS + PI	PC	SM	PC/PE ratio
Growth medium						
Mock	4	19.7 ± 6.3	12.3 ± 1.1	57.5 ± 4.8	10.5 ± 2.7	3.2 ± 1.1
H-FABP cDNA	2	17.7; 18.1	13.6; 14.2	56.2; 56.6	11.7; 11.8	3.1; 3.2
A-FABP cDNA	4	31.9 ± 1.9[d]	12.2 ± 0.5	48.8 ± 0.9[c]	7.1 ± 0.8	1.5 ± 0.1[c]
Differentiation medium						
Mock	7	23.8 ± 1.4	13.8 ± 1.2	49.5 ± 1.2[b]	12.8 ± 1.0	2.1 ± 0.1[a]
H-FABP cDNA	6	22.6 ± 1.0	13.4 ± 1.0	54.8 ± 1.6[e]	9.2 ± 1.3[e]	2.4 ± 0.1[e]
A-FABP cDNA	10	18.8 ± 2.4[b,e]	12.5 ± 1.4	53.5 ± 1.1[b,e]	13.9 ± 2.5[b]	2.9 ± 0.4[b,e]

Another approach followed by us was to introduce a second non-muscle FABP into cells already expressing an FABP, as proposed by Matarese et al. [42]. Expression of A-FABP in muscle cells may compromise fatty acid oxidation, because it may be more involved in fatty acid utilization for triacylglycerol synthesis and lipolysis. The results presented here show a clear effect of transfection with A-FABP cDNA on cell growth and differentiation by biochemical assays and morphological observations. The introduction of A-FABP cDNA into L6 myoblasts caused a marked increase in their rate of proliferation. The fusion-incompetence of these cells on culturing for 4–9 days on differentiation medium is also striking. The mononuclear myoblasts clearly enter the first stages of myogenesis, i.e. alignment and adhesion [43], but the cells arrest in this stage instead of fusing to multinucleated myotubes. Transfection of L-cell fibroblasts with L-FABP or I-FABP cDNA did not change their growth characteristics [21,24].

The H-FABP-cDNA-transfected clone did not show a different proliferation rate from that of the mock-transfected clone, in contrast with yeast, mouse mammary epithelial cells and human breast cancer cell lines, which show a specific inhibition of growth and exhibit a differentiated morphology on H-FABP cDNA transfection [11,12,14]. The mammary-derived growth inhibitor from bovine lactating mammary glands, now identified as a mixture of A- and H-FABP [9], reversibly suppresses cell multiplication in a variety of cultured epithelial cells [10–12]. In cultured cardiac myocytes, extracellularly added H-FABP seems to induce hypertrophy [13]. The mechanism by which H-FABP leaves or enters these cells is not known.

Members of the FABP family appear to be associated with modulation of cell growth. L-FABP cDNA transfection increases the efficacy of utilization of unsaturated fatty acids, especially linoleic acid, leading to proliferation of cultured hepatoma cells [44,45]. L-FABP specifically mediates the induction of cell multiplication of transfected hepatoma cells by two classes of carcinogenic peroxisome proliferators [46]. Moreover, the binding of eight prostaglandins to L-FABP correlates with their growth-inhibitory activity [47]. Porcine ileal lipid-binding protein (ileal FABP) stimulates the growth of gastrointestinal epithelial cells in culture [48]. Cellular retinol-binding protein I and cellular retinoic acid-binding proteins are associated with the cessation of cell multiplication that accompanies differentiation [49]. In our transfected L6 myoblasts, A-FABP expression has the same effect as L-FABP expression in hepatoma cells, i.e. the promotion of cell proliferation. The underlying mechanism is, however, not understood. It has been reported that A-FABP is able to bind retinoic acid, in contrast with H-FABP [38,40]. This difference could underly the increased proliferation rate of the A-FABP-cDNA-transfected cells since retinoic acid is a potent inducer of myogenesis [50,51]. Recent data on mice with a null mutation in the A-FABP gene indicate that A-FABP is possibly involved in the expression of the gene of tumour necrosis factor α [52]. Exposure of C2C12 mouse myoblasts and primary mouse myoblasts to thiazolidinediones or fatty acids prevented their differentiation to myotubes and led to the expression of a typical adipose differentiation programme [53]. Our A-FABP-expressing clones did not exhibit lipid droplets on morphological observation.

The original L6 cell line used in our studies shows a low content of endogenous H-FABP. In myotubes, the amount of H-FABP increases nearly twofold, as in cultured primary rat muscle cells, but is still 30-fold lower than in quadriceps muscle [5,6]. A larger differentiation-dependence of expression of H-FABP was found in the mouse C2C12 cell line, which shows a much lower FABP content in myoblasts [17]. A low content of endogenous H-FABP should favour the use of the antisense approach, because the number of endogenous messengers may not titrate out the number of messengers encoded by the H-FABP antisense cDNA construct. Nonetheless, H-FABP could still be detected by immunoblotting and ELISA in myoblasts of these clones in a similar concentration to that in mock- and non-transfected clones. Antisense L-FABP cDNA transfection caused only a decrease in L-FABP content in some transfected HepG2 cell clones [54]. Our H-FABP-overexpressing transfected L6 myoblasts showed the same level of expression (0.26 % of total protein) as the L- and I-FABP-overexpressing fibroblasts used by others [23–25]. Our A-FABP-cDNA-transfected L6 clones showed a higher A-FABP content (0.3–1.3 %) than A-FABP-cDNA-transfected CHO cells (0.01 %) [26].

To establish the role of FABP in the cellular uptake of fatty acids, FABP cDNAs have been transfected in cell lines, most of which do not express endogenous FABP. The results were variable. In mouse fibroblasts, overexpression of L-FABP increased the initial rate and extent of *cis*-parinaric acid uptake by 50 and 29 % respectively [23]. Expression of I-FABP did not influence fatty acid uptake [23,24]. In addition, high L-FABP expression caused a larger initial rate of fatty acid uptake compared with low expression [25]. In an abstract, Rolf et al. [54] reported a reduction of fatty acid uptake into HepG2 liver cells transfected with antisense L-FABP cDNA and a decreased L-FABP content. Furthermore the total amount of L-FABP and I-FABP in I-FABP-cDNA-transfected Caco-2 cells was not proportional to fatty acid uptake [55]. CHO cell transfectants expressing A-FABP demonstrated a 1.5–2-fold increase in the rate of [^{3}H]oleate uptake [26]. We did not observe any effect of H-FABP overexpression or A-FABP expression on the rate and extent of fatty acid uptake by L6 myoblasts. In contrast, Claffey et al. [19] reported a larger uptake and esterification of fatty acids by L6 myoblasts, which were stably transfected with rat H-FABP cDNA and producing the protein. No quantitative results of these studies were published. Transfection of 3T3 pre-adipocytes and COS-7 cells with H-FABP cDNA did not alter fatty acid uptake [56]. We conclude that the effect of FABP on fatty acid uptake may be related to the FABP type and cell type studied.

The higher uptake of fatty acid into total lipids and the higher incorporation into triacylglycerols of L6 myoblasts compared with that into myotubes were also observed previously after 6–18 h of incorporation [18]. Esterification of [^{3}H]oleic acid was increased in both L-FABP- and I-FABP-cDNA-transfected fibroblasts [23–25]. The type of FABP cDNA transfected influenced the targeting of fatty acids to triacylglycerols or phospholipids and to PC or PE [23–25]. A higher esterification of fatty acids was also found in CHO cells transfected with A-FABP [26]. Expression of bovine H-FABP in yeast did not affect either the phospholipid composition or the fatty acid pattern of neutral lipids or total phospholipids [14]. Fusion-incompetence of the myoblasts expressing A-FABP could be related to alterations in plasma-membrane structure, as found in transfected fibroblasts expressing L-FABP [20–22]. Their PC/PE ratio increased at (higher) expression of L-FABP [20,25]. The PC/PE ratio of A-FABP-cDNA-transfected clones increased on differentiation medium, whereas the PC/PE ratio of the mock- and H-FABP-cDNA-transfected clones decreased with normal fusion and differentiation. Future studies are necessary to reveal the stage(s) of myogenesis at which the former clones are disturbed and which factors are involved.

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