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F1Fo-ATPASE SUBUNIT e GENE ISOLATED IN A SCREEN FOR DIET REGULATED GENES

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Described is a protocol for isolating genes differentially regulated by the level of an essential nutrient in the diet. One gene, designated LFM-1 (*L*ow *F*at *M*ammary), was identified by subtractive hybridization and plus/minus screening in a λ-cDNA library constructed with mRNA from mammary glands of virgin, tumor-free Balb/c mice fed a low fat diet; a second gene, stearoyl CoA desaturase, known to be regulated by the level of fat intake, was also isolated. The procedure can be modified for use with other constituents. The differential expression of LFM-1 was noted in mammary glands, liver, and kidney but not other tissues of mice fed low fat compared to high fat. LFM-1 is 86% identical to the gene encoding the e subunit of bovine F1-Fo ATPase and 89% identical to an UV-light induced gene (DDIU4) from Chinese hamster fibroblasts. Differential hybridization analyses indicated that LFM-1 may belong to a gene family.

Epidemiological data suggest that increased fluxes through fat metabolic pathways correlates with incidence and severity of cancer, cardiovascular diseases, and obesity (rev. in [1,2]). Dietary conditions are known to change short term transcriptional responsiveness (3 - 6) and/or post-transcriptional regulation (7,8). Long term intakes of dietary fat may change gene expression to facilitate the development and progression of these diseases. Certain lipids or peroxisomal proliferators are known (9) to activate a nuclear orphan - glucocorticoid chimeric receptor in transactivation assays in CHO cells suggesting that they, or their metabolic products are transcriptional activation ligands. This communication

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Abbreviations: SCD, *s*tearoyl *C*oA *d*esaturase; LFM, *L*ow *F*at *M*ammary; DDIU4, *D*NA *d*amage *i*nducible - *U*V; RBF, *r*eceptor *b*inding *f*actor; AIN, *A*merican *I*nstitute of *N*utrition.

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describes the isolation and identification of diet-responsive genes which will allow analyses of their regulation and possible role in disease.

METHODS AND MATERIALS

Reagents: Sucrose was purchased locally, corn oil from Anderson Clayton Foods (Dallas, TX), maltodextrin from Teklad (Madison, WI), with all other dietary constituents from Research Diets (New Brunswick, NJ). Other chemicals were reagent or molecular biology grade.

Animal Management and Diets: Six week old Balb/c virgin female mice (Harlan Sprague-Dawley, Inc. Indianapolis, Indiana) fed a fat free semipurified diet for one week were then randomly assigned to either a low fat (3% corn oil) or a high fat (20% corn oil) diet for two weeks formulated according to AIN-76 guidelines (10,11). One hundred g of the low fat diet contained: 20g vitamin-free casein, 0.3g DL-methionine, 34.5g maltodextrin, 18g cornstrach, 14.5g sucrose, 5g cellulose, 3g corn oil, 3.5g AIN-76 mineral mix, 1g AIN-76 vitamin mix, 0.2g choline bitartrate. Corresponding amounts in the high fat diet were: 23.5, 0.35, 22.9, 11.8, 10.0, 5.88, 20.0, 4.12, 1.18, 0.24. Nutrient/calorie ratios were equal in the two diets. Daily individual food consumption averaged from 15 to 15.4 kcals daily for both groups. Animal care satisfied guidelines of the University of Illinois and the National Institutes of Health. After consuming experimental diets for 14 days, the mice were killed by cervical dislocation in random order between groups from 0900 to 1300 hours. Tissues were immediately excised and frozen in liquid nitrogen and stored at -80°C.

Nucleic Acid Manipulation: Total RNA was isolated from pooled and pulverized tissues according to Chirgwin (12). Poly A+ RNA was isolated by oligo(dt) cellulose chromatography (13). DNA was isolated from livers of retired male Balb/c breeders by protease/phenol extraction methods (14) followed by isopycnic centrifugation in CsCl gradients.

cDNAs were synthesized using standard procedures (13) and DNA modifying enzymes (Bethesda Research Laboratories, Gaithersburg, MD). cDNA was ligated to λ ZAP II cDNA cloning vector (Stratagene, La Jolla, CA), packaged, and transfected into XL1-Blue cells according to manufacturer's protocols. Each of the λ ZAPII cDNA libraries constructed from mammary gland mRNA of mice fed high fat and low fat diets contained approximately 1 x 10^6 transformants with more than 90% of phages containing cDNA inserts. Approximately 3 x 10^5 primary recombinants from the low-fat cDNA library were analyzed (13). High fat-subtracted, low fat enriched probes were prepared with the Invitrogen (San Diego, CA) Subtraction kit. After phagemid rescue with R408 f1 helper phage, ampicillin resistant colonies were plated and re-screened by 4 rounds of plus/minus colony hybridization of duplicate filters. The high fat mRNA and low fat cDNA probes were made by first strand synthesis (13,14).

PolyA+ RNA concentrations were determined by UV spectroscopy ($E_{1mg/ml}$ = 40). Identical amounts of mRNA (usually 2 μg) from each dietary treatment were analyzed on 1% or 1.5% agarose gels containing 1% formaldehyde. β-Actin was used as an internal control for the amount of RNA added to each lane. Probes were synthesized from isolated inserts using the Prime-It kit (Promega Biotec, Madison, WI).

Northern and Southern Analyses: Asymmetric probes were constructed with α-^{32}P-dCTP (New England Nuclear, Boston, MA) as described in BRL protocols with minor modifications. Hybridization conditions were 50% formamide, 10% dextran sulfate, 25mM $KHPO_4$ (pH 7.4), with 100 μg/ml salmon sperm DNA. Following hybridization, the nylon membranes (Magnagraph, MSI, Westborom, MA) were washed in 2X SSC-0.1% SDS at room temperature for 30 minutes, then twice for 15 minutes at 60°C in 0.25X SSC - 0.1% SDS. Some filters were washed at 65°C in 0.1X SSC-0.1% SDS. Hybridization was analyzed by densitometric scans of autoradiographs. For Southern blot analyses, 7.5 μg of total genomic DNA were digested overnight with 15 units of either *Eco*RI, *Hind*III, *Bam*HI, *Bgl*II, *Pst*I, or *Pvu*II in 25 μl. Following overnight incubation at 37°C, the digested DNAs were separated by electrophoresis in 1% agarose gels, and blotted to nylon membranes. Probes and hybridization conditions were as above.

DNA Sequencing: Double stranded DNA sequencing was done with Sequenase (US Biochemicals, Cleveland, OH) as per the manufacturer's protocol. The *r*apid *a*mplification of *c*DNA *e*nds (16) was used to produce clones containing the 5' noncoding and coding sequences. An oligonucleotide complementary to nucleotides 81 through 106 (National Biosciences, Plymouth, MN) of the above sequence was the primer for reverse transcription of liver poly A+ RNA from animals fed 3% dietary fat using the Stratagene first strand synthesis kit. The resulting cDNA was tailed with dATP and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) following manufacturer's protocols. The dA-tailed cDNA was amplified with the Promega (Madison, WI) PCR kit using the $(GA)_{10}$-*Xho*I-Oligo-dT oligonucleotide (Stratagene) and one complementary to nucleotides 36 to 61 (Genetic Engineering Facility, Univeristy of Illinois) of LFM-1 as primers. The amplified product was ligated to *Eco*RV digested, dT-tailed pcDNAII (Dr. Hugh Robertson, University of Illinois), and the ligated products were transformed into LE392 cells (13,14). Sequences were compared to the GENBANK data base (Genetic Engineering Facility, University of Illinois).

RESULTS AND DISCUSSION

Two diet regulated genes, designated LFM-1 and LFM-2, were isolated by screening a low fat, mammary gland cDNA library (see Methods) once with low fat enriched-cDNA probes followed by four successive rounds of low (3%) *vs* high (20%) fat (plus/minus) cDNA screening (13,14). The high fat library has not been screened. Figure 1 shows a representative analyses of LFM-1 mRNA levels in 2 grams of tissues pooled from 150 mice fed low or high dietary fat. The radiolabeled LFM-1 cDNA probe detected four fold more transcript (~350 bp) in the mammary glands and liver, 2 fold more in kidney, but similar levels in heart, spleen, brain, and lung of mice fed low fat *vs* high fat. The differences in LFM-1 mRNA levels between dietary conditions also was found in tissues from a second feeding study.

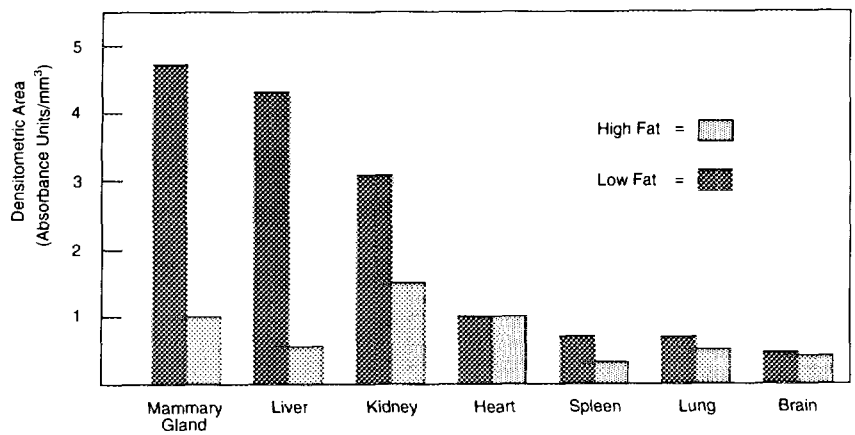

FIGURE 1. **Northern Analyses of LFM-1 mRNA in Mouse Tissues.** mRNA was isolated from frozen, pooled tissues of mice fed low or high levels of dietary fat. Two micrograms of each mRNA was analyzed as described in Methods. RNA molecular weight markers indicated that LFM-1 was about 350 nucleotides in length.

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                10v        20v        30v        40v        50v        60v        70v
LFM1        ATGGTgCCCCCGGTTCAGGTCTCTCCaCTCATCAAGtTCGGCCGgTACTCCGCtCTGaTCaTCGGCATGGCaTA
DDIU4       ATGGTaCCCCCGGTTCAGGTCTCTCCGCTgATCAAGCTCGGCCGaTACTCaGCCCTGgTCCTCGGCATGGCCTA
ATPase e    ATGGTtCCgCCGGTgCAGGTCTCTCCGCTCATCAAGCTCGGCCGtTACTCCGCCCTGtTCCTCGGCATGGCCTA

                80v        90v       100v       110v       120v       130v       140v       150v
LFM1        CGGCGCCAAGCGCTACAGTTACCTAAAACCCCGGGCAGAGGAGGAGAGGAGaaTaGCAGCGGAGGAAAAGAAGAG
DDIU4       CGGCGCtAAaCGCTACAGTTACCTAAAACCCCGGGCAGAAGAGGAGAGGAGGgTgGCAGCGGAGGAAAAGAAGAG
ATPase e    CGGCGCCAAGCGCTACAaTTACCTgAAACCtCGGGCAGAAGAGGAGAGGAGGCTtGCAGCcGAGGAgAAGAAGAa

                160v       170v       180v       190v       200v       210v
LFM1        ACTAGATGAGtTGAAACGGATtGAGAGAGAACTGGCGGAAGCtCAAGATGACAgCATtCTCAAGTGA
DDIU4       ACTAGATGAGCTGAAACGGATCGAGAGAGAACTGGCaGAAGg------TGACACCATACTCAAGTGA
ATPase e    gCggGATGAGCaGAAgCGcATCGAGcGgGAgCTGGCGGAAGCcCAAGAgGAtACCATAtTgAAGTGA
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FIGURE 2. DNA Sequence Comparisons of LFM-1, F1Fo-ATPase e Subunit, and DDIU4. The ALIGN program from the *DNASTAR* package was used to determine the homology between genes. Differences between sequences are denoted by lower case letter. Data are summarized in Table 1.

DNA sequences of the 5 isolated LFM-1 clones were identical with 249 nucleotides but lacked an initiating region with an ATG codon. The remaining LFM-1 sequences were obtained with the RACE (rapid amplification of cDNA ends) protocol (16). LFM-1 was 82% identical to a gene encoding the e subunit of the F1Fo-ATPase complex isolated from bovine hearts (17); and 89% identical to DDIU4, a gene induced by UV light (Figure 2) isolated by subtractive screening of a cDNA library constructed with mRNAs from UV-irradiated Chinese hamster cells (18,19). The predicted protein sequences are shown in Figure 3 and their similarities are summarized in Table 1. With conserved amino acid substitutions considered, the predicted sequence of LFM-1 was 97% similar to the e subunit suggesting that these proteins are similar if not identical.

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LFM1        M V P P V Q V S P L I K F G R Y S A L I I - G M A Y G A
DDIU4       M V P P V Q V S P L I K L G R Y S A L V L - G M A Y G A
ATPase e    M V P P V Q V S P L I K L G R Y S A L F L - G M A Y G A
RBF1        M I P P V Q V S P L I K F T X Y S A L L L V G M I Y G K

LFM1        K R Y S Y L K P R A E E E R R I A A E E K K R L D E L K
DDIU4       K R Y S Y L K P R A E E E R R V A A E E K K R L D E L K
ATPase e    K R Y N Y L K P R A E E E R R L A A E E K K K R D E Q K
RBF1        K X Y D Y L K P I A V

LFM1        R I E R E L A E A Q D D S I L K
DDIU4       R I E R E L A E G - - D T I L K
ATPase e    R I E R E L A E A Q E D T I L K
```

FIGURE 3. Comparisons of Predicted Protein Sequences. The AALIGN program from the *DNASTAR* determined the similarity between the 38 known residues of RBF-1, the predicted protein sequences of LFM-1 and DDIU4, and the e subunit of F1Fo-ATPase. The identities are summarized in Table 1.

TABLE 1
DNA AND PROTEIN SEQUENCE IDENTITIES
(Percent)

	DDIU4	e SUBUNIT	RBF1[b]
LFM-1[a]	89 (89)	82 (86)	(72)
e SUBUNIT	84 (87)	-	(75)
DDIU4	-	-	(72)

[a] Percent identities of DNA and protein (in parentheses) sequences of mouse LFM-1, Syrian hamster DDIU4, bovine F1Fo ATPase e subunit, and chicken oviduct RBF-1 using ALIGN (DNA) and AALIGN (protein) programs from DNASTAR.

[b] The gene and complete protein sequences of RBF-1 have not been reported. Comparisons are with 38 residues of the amino terminus.

The e subunit co-purifies in a 1:1 stoichiometry with other bovine F_1F_o-ATPase subunits. Although its function is unknown, the e subunit may be involved in regulating ATPase activity since the highly similar LFM-1 is regulated by the level of dietary fat, a macronutrient needed for metabolic energy. UV-induced stress also requires energy for cellular repair processes which may explain the isolation of a similar, if not identical gene from UV-light treated cells (18,19).

Hybridization analyses of Balb/c genomic DNA (Figure 4) showed that LFM-1 hybridized to multiple fragments at moderate (M) stringencies but a higher (H) stringency wash eliminated hybridization to several fragments (compare M vs H with each restriction enzyme). These differences indicate the presence of nonidentical but similar sequences in Balb/c DNA suggesting that LFM-1 may belong to a gene family. Similar LFM-1 - like sequences exist since the partial sequence of the progesterone receptor binding factor (RBF-1) is 70 - 75% identical to LFM-1, DDIU4, and the e subunit (Figure 3 and Table 1). RBF-1 apparently provides high affinity binding sites for the progesterone receptor (20,21).

Nuclear membranes contain ATP-dependent calcium pump consisting of a single polypeptide chain regulated by phosphorylation (22). Since RBF-1 is a chromatin acceptor protein, it is presumably not involved in regulating this ion pump. At least two possibilities may explan the unexpected finding that two functionally different proteins have such high sequence similarities. The e subunit/LFM-1/RBF-1 protein may be involved in nuclear-mitochondrial regulatory processes or RBF-1 is not functionally identical to the e subunit, but these proteins share sequence similarities. Further experiments are required to test these possibilities.

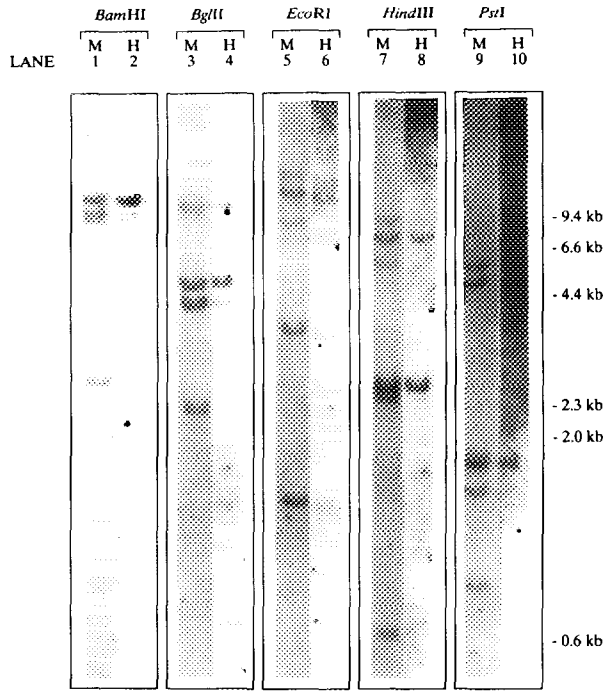

FIGURE 4. Differential Hybridization Analyses of LFM-1 in Mouse Genomic DNA. Balb/c genomic DNA was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, or *Pst*I, electrophoresed, blotted, and hybridized as described in Methods. M refers to moderate stringency wash (2X 15 minutes at 0.25X SSC-0.1% SDS at 60°C). After exposure for 3 days with a screen, the filter was washed at high stringency (H - 2X 15 minute at 0.1X SSC-0.1% SDS at 65°C). The filter was exposed for 7 days with a screen following the high stringency wash.

The LFM-2 transcript was found to be about 5 fold more abundant in liver mRNA of mice fed 3% *vs* 20% dietary fat. The nucleotide sequence of the two LFM-2 clones were identical and encoded nucleotides 3325 to 3659 of *s*tearoyl *C*oA *d*esaturase (SCD-1 [23]). Its transcription is induced by insulin and certain fatty acids in rodents (24) and by insulin in 3T3-L1 adipocytes (23,25). The effectiveness of our protocol was confirmed by the isolation of SCD-1 since the low fat/high carbohydrate diets fed in this study mimic conditions that stimulate SCD-1 transcription.

CONCLUSION

Two diet-regulated genes were isolated by the protocol described in this report. A similar approach identified lithocholic acid 6β-hydroxylase, an enzyme involved in cholic acid metabolism (26). The high and low fat groups consumed equivalent total calories but had a 5 fold difference in calories from fat but only a 1.7 fold difference in carbohydrate

calories; the major dietary variable was fat level. To represent long term dietary conditions in the lifespan of mice, a two week feeding period was chosen to simulate conditions associated with the promotion of disease states (1,2). Changes in gene expression by other variables were minimized by using normal, Balb/c virgin female mice that do not produce an active mammary tumor virus. Assessment of the role of diet-regulated genes in lipid metabolism and possibly in disease promotion will require further studies.

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