The e Subunit Gene of Murine F₁F₀-ATP Synthase

GENOMIC SEQUENCE, CHROMOSOMAL MAPPING, AND DIET REGULATION*

(Received for publication, January 16, 1996, and in revised form, June 3, 1996)

Deborah A. Swartz‡§, Eric I. Park‡¶, Willard J. Visek‡, and Jim Kaput**

From the Departments of Internal Medicine, Division of Nutrition, University of Illinois College of Medicine, University of Illinois, Urbana, Illinois 61801 and Molecular Genetics in Nutrition Program, The Sapient’s Institute, Dallas, Texas 75209

Genomic sequences encoding murine Lfm1, whose predicted protein sequence is 96% and 98% similar to bovine and rat F₁F₀-ATP synthase e subunits (respectively), have been amplified from BALB/cByJ DNA, cloned, and sequenced. The 1.1-kilobase gene has 3 introns and 4 exons, and its coding sequence differs by two nucleotides compared to the previously published BALB/cHnn Lfm1 cDNA sequence. A PstI restriction site polymorphism in intron 2 between C57BL/6J and Mus spretus was used to map this gene to Chromosome 5 near D5Mit9. Related sequences were identified on Chromosomes 8, 11, and 2 and unlinked loci on Chromosome 2 using Southern blot analyses with the 1.1-kilobase gene as a probe. Previous studies from this laboratory indicated that the Lfm1/e subunit was regulated by the level of dietary fat and carbohydrate. Northern hybridization analyses demonstrated that e subunit mRNA abundance showed statistically significant differences (p < 0.025) between hearts of BALB/c mice fed 3% and those fed 20% corn oil for 2 weeks and in liver (p < 0.05) from the same animals. Significant differences were also observed in hepatic and heart mRNA expression at different times after eating in animals subjected to a fast/refeed regimen. The implications of the high degree of sequence similarity to the e subunit for rat and bovine F₁F₀-ATP synthase and its regulation by diet are discussed.†

One of the ultimate outcomes of the metabolism of nutrients is the establishment of a proton gradient across the inner mitochondrial membrane. F₁F₀-ATP synthase, located on the inner surface of the inner membrane, converts energy derived from the gradient into ATP (reviewed in References 1 and 2). Its activity is partly regulated by the availability of substrates and the proton flux through the membrane (1, 2). The synthase complex consists of a soluble F₁ catalytic unit with the F₀ portion composed of a joining stalk and inner membrane proteins (1–7). Proton flux through F₀ membrane proteins has been postulated to cause conformational changes, which may pass to the catalytic F₁ subcomplex through the stalk (2). Three proteins, designated e, f, and g, co-purify in a 1:1 stoichiometry with F₁F₀ subunits (4–7) but are peripherally associated with the membrane or stalk (2). Their functions are unknown. In a screen for genes regulated by the level of dietary lipids and carbohydrates (8), we isolated and characterized low fat mammary 1 (Lfm1), whose predicted protein sequence is 96% and 98% similar to e subunit sequences in bovine heart (4) and rat liver (7). Since the latter two proteins are 94% similar, we suggested that Lfm1 is the murine F₁F₀-ATP synthase e subunit gene (8). Northern hybridization analyses revealed higher Lfm1/e-actin mRNA ratios in livers, kidneys, and mammary glands of BALB/cHnn mice fed semipurified diets containing 3% instead of 20% corn oil. The association of the e subunit to a region of F₀ postulated to transmit conformational energy to the F₁ catalytic subunits (2) and the regulation of its mRNA abundance by concentrations of dietary constituents (see Ref. 8 and this report) suggest that the e subunit may play a role in regulating F₁F₀-ATP synthase activity.

We report here the sequence of a 1.1-kb e subunit gene, amplified from BALB/cByJ genomic DNA with primers derived from the Lfm1 cDNA sequence (8). The genomic clone encodes 3 introns and 4 exons with two nucleotide differences in the coding sequence compared to the published BALB/cHnn Lfm1 cDNA sequence (8). A 370-bp “spliced” pseudogene was also amplified from genomic DNA. The 1.1-kb e subunit gene was mapped to Chromosome 5 (Chr 5) near D5Mit9 by scoring a PstI RFLP in PCR-amplified (C57BL/6J × Mus spretus)F₁ × C57BL/6J (BSB) segregants of The Jackson Laboratory Backcross DNA panel (9). Four related sequences mapped to loci on Chromosomes 11 and 8, and at two unlinked positions on Chromosome 2 by Southern hybridization analyses of TaqI-digested (C57BL/6J × SPRET/Ei)F₁ × SPRET/Ei (BSS) segregants (9). We have also analyzed the relative abundance and temporal regulation of e subunit/glycer aldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in heart tissue of BALB/cHnn mice during a fast/refeed regimen following their 2-week ad libitum consumption of diets containing 3%, 10%, and 20% corn oil. Statistically significant differences in main

---

*This work was supported in part by Grant 91-01631 from the United States Department of Agriculture, and by grants from the United States Department of Agriculture, and by grants from the USDA, Ross Laboratories (Columbus, OH), and the University of Illinois Research Board. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by a predoctoral fellowship from the American Institute of Nutrition and National Institutes of Health Traineeship PHS ST32-AM07497. Current address: Division of Gastroenterology, Washington University School of Medicine, St. Louis, MO 63110.

‡ The contributions of these two authors were similar.

§ Supported by a predoctoral fellowship from the American Institute of Nutrition and National Institutes of Health Traineeship PHS ST32-AM07497. Current address: Division of Gastroenterology, Washington University School of Medicine, St. Louis, MO 63110.

**To whom correspondence should be addressed: The Sapient’s Institute, c/o University of Illinois College of Medicine, 506 S. Mathews, Urbana, IL.

¶ The abbreviations used are: Lfm1, low fat mammary (refers to primers, cDNA, or the gene sequence, but not to the gene locus); e subunit, the genomic clone or mRNA; Atpob, Chr5 e subunit locus; rs, related sequence; B or B6, C57BL/6J; Spret, M. spretus; C, BALB/c; H C3H; BSB is (C57BL/6J × Spret/Ei)F₁ × C57BL/6J (backcross); BSS is (C57BL/6J × M. spretus)F₁ × Spret/Ei backcross DNA; bp, base pair(s); Chr, chromosome; cm, centimorgan(s); GAPDH, glycer aldehyde-3-phosphate dehydrogenase; kb, kilobase(s); PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; UTR, untranslated region.
effects were observed between e subunit mRNA levels when there were differences in dietary corn oil concentrations and in times after eating. Similar results were obtained with liver.

MATERIALS AND METHODS

Mapping the Initiation of the e Subunit Gene—To determine the transcription start sites for the e subunit gene, total RNA samples were isolated from hearts of BALB/c mice (8 weeks of age), and Northern blots of total heart RNA were hybridized with 32P-labeled e subunit probes. Northern blots were washed at 65°C in 0.1× standard sodium phosphate buffer (SSC) containing 1% SDS, and autoradiograms were obtained by placing blots next to an Imager (Fuji, Stamford, CT) according to manufacturer’s specifications. The radiolabeled probes emitted at least 106 dpm/μg.

DNAs and RNA were isolated as described above. The e subunit gene probe was a 200-bp region spanning the entire cDNA probe and a 100-bp region extending into the 5′-untranslated region (UTR).

DATA ANALYSIS

Effects of sacrifice time and linear trends with dietary fat level were evaluated in two-way analyses of variance, followed by examination of trends with fat level at individual sacrifice times. The SAS GLM procedure (12) was employed for statistical computing, with Tukey’s Studentized range test used to adjust for testing of fat trend at five separate sacrifice times. The standard of statistical significance for fat trend at any single sacrifice time was 0.05.

RESULTS

Animal Feeding—Animals, diet composition, 2 and liver RNA isolation for the experiments reported briefly here are described by Paisley et al. 3 BALB/c mice (Harlan-Sprague-Dawley, Indianapolis, IN) were fed a semipurified diet containing 3% corn oil for 1 week and then randomly assigned for 2 weeks to the same 3% corn oil diets or otherwise identical control diets, with 10% of the corn oil replaced isocalorically. Group of animals were killed at predesignated times at the end of the 2-week feeding period as follows; at the end of a 12-h light cycle (ad libitum fed), after 12 h without food (−2 h, fasted), and at the end of a 12-h fasting phase followed by a 2-h period with food provided (0 h). All untreated food was removed from the cages at 0 h, and three other groups were killed at 1, 2, and 6 h after the end of the 2-h period with food available. The quantity of food placed in the cages at the beginning of the 2-h feeding period was 80% of the average daily food consumption during the preceding 2 weeks. 3

RNA Hyridization Analyses—Total RNA was extracted from ground livers and hearts by the Ultra-Speed RNA isolation system (BioTeck, Houston, TX). Twenty μg of total heart RNA were resolved by electrophoresis in gels containing 1.2% agarose and 2.2 M formaldehyde at 110 V for 2.5 h. Sizes were estimated by comparison to a 0.24–9.5-kb ladder of synthetic RNA (Life Technologies, Inc.). Gels were stained with 0.5 mg/liter ethidium bromide, photographed, and blotted onto nylon membranes (U. S. Biochemical Corp.) following standard protocols (10). Hybridizations were conducted at 7% SDS, 0.5 × NaPO4 (pH 7.2), 1 × EDTA, and 1% SDS at 65°C with 4% SDS, 40 μg/ml NaPO4 (pH 7.2), 1 × EDTA wash at 65°C for 15 min and three 15-min washes with 1% SDS, 40 μg/ml NaPO4 (pH 7.2), 1 × EDTA at 65°C (11). Hybridization signals were quantified using a Molecular Dynamics (Sunnyvale, CA) 4250 PhosphorImager, and autoradiograms were obtained by placing blots next to a phosphorimager screen and scanning through a diffuse plate to give A (Fig. 1, lanes 1 and 5).

The e subunit mRNAs were normalized to GAPDH mRNA levels because two actin mRNAs are expressed in heart tissues. The e subunit/GAPDH mRNA ratios from individual hearts were normalized to the ratio of e subunit/GAPDH in identical aliquots (approximately 20 μg) of a control RNA analyzed on each of the five blots. The control RNA, isolated from 5 pooled hearts of BALB/c mice fed 10% corn oil diets ad libitum, was used to control for blot to blot variation. Based upon the specific activity of the probes, PhosphorImager counts and efficiency, and the duration of exposure, there were approximately 1010 molecules of a subunit mRNA/20 μg of total RNA. Liver RNA hybridizations and analyses were as described previously (8). Hepatic e subunit mRNA was normalized to β-actin mRNA as a control for loading and expressed as a ratio of e subunit/β-actin mRNAs. The data were normalized to e subunit/β-actin mRNAs in hepatic total RNA from mice fed 3% corn oil. Effects of sacrifice time and linear trends with dietary fat level were evaluated in two-way analyses of variance, followed by examination of trends with fat level at individual sacrifice times. The SAS GLM program (12) was employed for statistical computing, with p < 0.05 employed as the nominal criterion of statistical significance. The method (13) was used to adjust for testing of fat trend at five separate times, requiring that an observed p < (0.05/5 = 0.01) be the standard of statistical significance for fat trend at any single sacrifice time.

RESULTS

e Subunit Gene Amplification—Four prominent DNA fragments A (900 bp), B (800 bp), C (350 bp), and D (200 bp) from BALB/c (not shown), C57BL/6J, and M. spretrus genomic DNA were amplified by PCR using the internal Lfm1-specific primers, +36 → +101 and +222 → +198 (Fig. 1, lanes 1 and 5). Fragment A encoded an e subunit gene beginning at nucleotide +36 of the cDNA sequence (with +1 the A of the initiating ATG) and ending at +216. This gene contained 3 exons and 2 introns and the sequence of the exons differed by 1 base pair (Fig. 2, nucleotide 611) from the previously reported Lfm1 cDNA sequence (8) but lacked the expected coding nucleotides +1 → +8. A second PCR reaction was performed with oligonucleotide primers +29 → +48, the untranslated region of the cDNA sequence, and +233 → +208, which overlaps the TAG termination codon (+214 → +216). Three prominent DNA fragments of 1.1 kb (A′), 900 bp (B′), and 250 bp (D′) were produced (data not shown).

2 All diets contained a base of 20 g of alcohol-extracted casein, 5 g cellulose, 1 g of AIN-76A vitamin mix (25, 26), 0.2 g of choline bitartrate, and 0.1 g of dye. In addition, the 3% corn oil diet contained 3 g of corn oil, 40 g of corn starch, 14.5 g of sucrose, 0.3 g of α-methionine, 2.5 g of AIN-76A vitamin mix (25, 26) for a total of 100 g. The 10% corn oil diet contained the base plus 9.8 g of corn oil, 28.1 g of corn starch, 11.2 g of sucrose, 0.3 g of α-methionine, and 3.5 g of mineral mix for a total of 91.7 g. The 20% corn oil diet contained the base plus 16.6 g of corn oil, 16.1 g of corn starch, 7.9 g of sucrose, 0.4 g of α-methionine, and 4.5 g of mineral mix for a total of 83.1 g.

3 Paisley, E. A., Park, E. I., Swartz, D. A., Mangian, H. J., Visek, W. J., and Kaput, J. (1996) J. Nutr., in press.
The 1.1-kb fragment (A') encoded the 900-bp A fragment obtained with the internal primers plus a 5' exon and an additional intron. The sequences of the 4 exons match the cDNA sequence exactly (Fig. 2) except for two substitutions. Cytosine at 310 of the genomic clone is replaced by thymidine in the cDNA. This base overlapped the 36 bp amplified with primer sequences were found within 390 and 320 basespair of e subunit cDNA (8) as substrate for amplification.

Additional or different primers producing the 650-bp fragment are identified in Fig. 2. The 1.1-kb fragment (A) amplified by the internal set of primers followed by B fragment C (370 bp) amplified by the internal set of primers encoded nucleotides +36 to +218 of the e subunit cDNA sequence joined by 4 nucleotides of unknown origin to nucleotides +38 to +222 of a second e subunit cDNA sequence (data not shown). Some of the nucleotide substitutions (5 total) in the first half of this gene created translation termination codons. The second half had seven substitutions with several termination codons and a dinucleotide deletion. This pseudogene was not found in over 200 clones of BALB/cJ, C57BL/6J, and M. spretus genomic DNAs amplified with oligonucleotides hybridizing to noncoding sequences (external primers) of the e subunit gene.

Introns are numbered, and the numbers refer to the Lfm1 cDNA sequence (8). Internal primers yielded an 800-bp fragment that started at +36 of the cDNA sequence and ended at +233. Nucleotide sequence was determined by sequencing both strands. The second amplification with external primers yielded a 1094-bp fragment encoding 3 introns and 4 exons. Exons are in uppercase letters and the introns in lowercase. The PstI site at 419 bp is not found in over 200 clones of BALB/cJ, C57BL/6J, and M. spretus genomic DNAs amplified with oligonucleotides hybridizing to noncoding sequences (external primers) of the e subunit gene.

Fig. 2. Sequence of 1.1-kb e subunit gene. A, simplified restriction map and genomic organization of 1.1-kb e subunit gene amplified from BALB/cJ genomic DNA. Restriction enzymes shown above the map were used for producing subclones for DNA sequencing. Arrows below the map show sequencing strategy. Fifty nucleotides (nucleotides 476 through 426) of the second intron were sequenced on one strand. Introns are open boxes, and exons are filled. Numbers below the introns refer to their amino acid sequence. B, nucleotide sequence of the 1.1-kb gene. Introns are in lowercase and exons in uppercase. BALB/cJ genomic DNA was used as a substrate for two amplifications with oligonucleotides complementary to +36 to +61 and +222 to +198 (referred to as internal primers) and -29 to -4 and +233 to +208 (external primers) of the e subunit sequence. Primer oligonucleotides are underlined, and the numbers refer to the Lfm1 cDNA sequence (8). Internal primers yielded an 800-bp fragment that started at +36 of the cDNA sequence and ended at +233. Nucleotide sequence was determined by sequencing both strands. The second amplification with external primers yielded a 1094-bp fragment encoding 3 introns and 4 exons. Exons are in uppercase letters and the introns in lowercase. The PstI site at 426 bp is not found in C57BL/6J but a different or additional site(s) is present in M. spretus.

Fig. 1. C57BL/6J and M. spretus genomic DNA amplification of e subunit and PstI RFLP. C57BL/6J by M. spretus genomic DNAs (middle panel) were amplified with +36 to +61 and +222 to +198 primers (lanes 1 and 3, respectively), purified by Qiagen columns (lanes 2 and 6), and digested with PstI (lanes 3 and 7) or XhoI (lanes 4 and 8). + and − refer to the addition of enzyme. PstI and XhoI sites produce a 450-bp fragment. Lane 10 is Lfm1 cDNA minus Taq polymerase, and lane 11 is minus DNA plus Taq polymerase.
Data base and Nomenclature Committees (Jackson Laboratory, Bar Harbor, ME).

Radiolabeled e subunit sequences were also hybridized to Southern blots of TaqI-digested Jackson Laboratory BSS Backcross Panel DNAs (Fig. 4). The 1.1-kb e subunit probe detected sequences on Chr 5 (5.1- and 0.9-kb bands, equivalent to the locus mapped in the BSB cross if the 13 missing noncrossover animals were inferred). Bars between the two maps join loci mapped in both backcrosses.

FIG. 3. Map position of Chromosome 5 e subunit locus, typed in Jackson BSS and BSB backcrossed. On the left is a map figure for the BSB backcross data for the region surrounding the e subunit gene, Atp5k. This BSB mapping of Atp5k was done by PCR/RFLP, and all 94 segregants were scored. On the right side of the figure is a partial map of the data from the Jackson BSS backcross, where an equivalent locus was mapped by Southern hybridization (see Fig. 4). In this analysis, 82 animals were typed for Atp5k and the phenotypes of the remaining 12 noncrossover animals were inferred. Bars between the two maps join loci mapped in both backcrosses.

rs1 Chromosome 2 (loci D2Mit61, D2Bir9, Atp5k-rs1) is MGD-CREX-505; Atp5k-rs2, Chromosome 2 (loci D2Mit52, D2Mit25, Atp5k-rs2, Gnas, D2Mit74) is MGD-CREX-497; Atp5k-rs3, Chromosome 8 (loci D8Mit29, Gcdh, Atp5k-rs3, D8Bir23, D8Mit15) is MGD-CREX-498; Atp5k-rs4, Chromosome 11 (loci Scy11, D11Mit36, Atp5k-rs4, D11Bir11, D11Mit14) is MGD-CREX-499.

designated Atp5k-rs1, for related sequence-1. A 5.8-kb band was scored in 85 animals, inferred from the remaining 9 animals, and co-segregated with Gnas (17) on Chr 2 but unlinked to Atp5k-rs1. This distal Chr 2 locus has been named Atp5k-rs2. From these same Southern blots, 82 animals were also typed for a 2.9-kb C57BL/6j band, which cosegregated with Gcdh (18) on Chr 8. This locus is designated Atp5k-rs3. Since all crossover animals were typed, the remaining phenotypes can be inferred to give 0 crossovers/94 animals between Gcdh and Atp5k-rs3. This locus had previously been identified in mapping studies with mice from CBX, CXH, BXA, and AXB recombinant inbred (R1) strains (19). PhosphorImager analyses indicated that the Chr 8 locus had about 1.5 times as many counts as other e subunit-related sequences, suggesting that it might encode the 370-bp pseudogene previously described. A 7.0-kb C57BL/6j band, scored in 72 of the animals cosegregated with D11Mit36 and D11Bir11 (16). Since none of the untyped animals were crossovers, typings in the remaining animals can be inferred from calculating the map position of this locus, designated Atp5k-rs4. Two of the three related loci are likely to encode another e subunit pseudogene and a cDNA-like gene which we have isolated from genomic libraries (not shown). Establishing the sequences at each locus will require additional studies.

Regulation of e Subunit mRNA Abundance—Mice fed 3%, 10%, and 20% corn oil containing diets consumed the equivalent of approximately 5 kcal during the 2-h feeding period. The autoradiogram in Fig. 6 shows heart e subunit mRNA hybridization signals at 6 h after eating for mice fed 3% (lanes 2–6), 10% (lanes 7–11), and 20% corn oil (lanes 12–16). Similar analyses were done for mice that had been fed 3%, 10%, and 20% corn oil and killed at −2 h (fasted) and 0, 1, and 2 h after having food available for a 2-h period (i.e., fed mice). ANOVA showed that the e subunit/GAPDH mRNA ratios were greater in all mice fed 20% corn oil compared to those fed 3% corn oil (p < 0.05). There was also a main effect (p < 0.0001) for time, showing that the e subunit/GAPDH ratio was significantly

designated Atp5k-rs1, for related sequence-1. A 5.8-kb band was scored in 85 animals, inferred from the remaining 9 animals, and co-segregated with Gnas (17) on Chr 2 but unlinked to Atp5k-rs1. This distal Chr 2 locus has been named Atp5k-rs2. From these same Southern blots, 82 animals were also typed for a 2.9-kb C57BL/6j band, which cosegregated with Gcdh (18) on Chr 8. This locus is designated Atp5k-rs3. Since all crossover animals were typed, the remaining phenotypes can be inferred to give 0 crossovers/94 animals between Gcdh and Atp5k-rs3. This locus had previously been identified in mapping studies with mice from CBX, CXH, BXA, and AXB recombinant inbred (R1) strains (19). PhosphorImager analyses indicated that the Chr 8 locus had about 1.5 times as many counts as other e subunit-related sequences, suggesting that it might encode the 370-bp pseudogene previously described. A 7.0-kb C57BL/6j band, scored in 72 of the animals cosegregated with D11Mit36 and D11Bir11 (16). Since none of the untyped animals were crossovers, typings in the remaining animals can be inferred from calculating the map position of this locus, designated Atp5k-rs4. Two of the three related loci are likely to encode another e subunit pseudogene and a cDNA-like gene which we have isolated from genomic libraries (not shown). Establishing the sequences at each locus will require additional studies.

Regulation of e Subunit mRNA Abundance—Mice fed 3%, 10%, and 20% corn oil containing diets consumed the equivalent of approximately 5 kcal during the 2-h feeding period. The autoradiogram in Fig. 6 shows heart e subunit mRNA hybridization signals at 6 h after eating for mice fed 3% (lanes 2–6), 10% (lanes 7–11), and 20% corn oil (lanes 12–16). Similar analyses were done for mice that had been fed 3%, 10%, and 20% corn oil and killed at −2 h (fasted) and 0, 1, and 2 h after having food available for a 2-h period (i.e., fed mice). ANOVA showed that the e subunit/GAPDH mRNA ratios were greater in all mice fed 20% corn oil compared to those fed 3% corn oil (p < 0.05). There was also a main effect (p < 0.0001) for time, showing that the e subunit/GAPDH ratio was significantly
FIG. 6. Autoradiogram of heart e subunit mRNA at 6 h after eating. Representative Northern hybridization analyses of e subunit/glycerolaldehyde-3-phosphate dehydrogenase mRNA in total RNA from hearts of individual BALB/c mice. Twenty μg of total RNA from heart tissue of individual mice 6 h after eating. Lane 1, pooled RNA from five BALB/c mice fed 10% corn oil diet ad libitum was analyzed on each blot as a standard; lanes 2–6, total RNA from mice fed 3% corn oil diets; lanes 7–11, total RNA from mice fed 10% corn oil diets; lanes 12–16, total RNA from mice fed 20% corn oil diets. Differences in intensity between lanes from the same dietary treatment are due to differences in total RNA loaded. GAPDH was used to control for the loading differences between the lanes. Sizes were determined from standards on each blot.

FIG. 7. Analyses of e subunit/GAPDH ratios in heart as a function of time and level of dietary oil. Fasted mice had no food available for 12 h (−2 h) until −2 h and were then provided a weighed quantity of food for 2 h. The end of this eating period is designated 0 h. Postprandial hours after the 2-h feeding period are 1, 2, and 6 h. All values are the means ± S.E. (n = 4–5) for each experimental group. Analyses of the effects of fat by nested time periods adjusted for multiple comparisons using Bonferroni’s method show no statistically significant differences (p < 0.05) for fat level at each time point. However, differences between e subunit/GAPDH mRNA ratios approached statistical significance at the 6-h time point (p < 0.075).

lower in fasted (2 h) relative to fed mice at 0, 1, and 6 h. The data were also analyzed at each time point for fat effects using Bonferroni’s method (13) for multiple comparisons (Fig. 7). Differences approached significance 6 h after eating (p < 0.075, after Bonferroni’s correction).

Liver tissues from mice in this fast/refeed regimen that were fed 3% and 20% corn oil were analyzed for e subunit abundance in fed mice ad libitum (−14 h), fasted mice (−2 h), and fed mice (2 h). A statistically significant (p < 0.05) difference in hepatic e subunit/β-actin mRNA ratios was observed between different times during the fast/refeed regimen (Fig. 8). There was also a highly significant interaction (p < 0.0003) between time and fat level. Analyses by Bonferroni’s method (13) showed highly significant (p < 0.0007, after Bonferroni’s correction) differences in e subunit/β-actin mRNA ratios as a function of fat levels following the 12-h fast (−2 h) and differences approaching statistical significance (p < 0.08, after Bonferroni’s correction) at the beginning of a 12-h fast (Ad lib, Fig. 8).

DISCUSSION

Sequence analyses of a 1.1-kb genomic BALB/cByJ F1F0-ATP synthase e subunit gene with 3 introns and 4 exons (fragments A and A’) showed two differences in the coding sequence when compared to the BALB/cHnh cDNA reported previously (8). The C → T difference at position 251 produces a Phe → Ser substitution, which would be expected to alter protein structure, while the other change at 611 (A → G) would cause a conservative change (Lys → Arg). These differences in coding sequence may be due to substrains of mice (Harlan Sprague-Dawley versus Jackson Laboratory) because at least two reverse transcriptase-PCR amplifications of RNA from liver, heart, and kidneys of BALB/cHnh mice consistently yielded only the BALB/cHnh cDNA sequence in multiple isolates. The structure of the gene, which codes for the same 5’ UTR as the cDNA, suggests that it would be transcriptionally active. We also isolated a 370 bp (Fragment C) pseudogene from genomic PCR products which apparently arose from an abortive splicing event. The 1.1-kb gene was mapped to Chr 5 in The Jackson BSS and BSS interspecific backcrosses. Related sequences were mapped to Chrs 11, 8, and 2. Preliminary evidence indicated that the 370-bp pseudogene may be the Chr 8 locus for Atp5k-rs3. Further characterizations of e subunit sequences at this and other loci, including analyses of transcriptional activity, await additional studies.

Heart e subunit/GAPDH mRNA ratios were increased by the level of dietary fat and were also affected by time of eating. The trends observed in e subunit mRNA levels in fasting and 1 and 6 h postprandial, which apparently are due to different levels of

6 D. A. Swartz, unpublished data.
corn oil, were not statistically significant, although Bonferroni's correction method of multiple comparisons showed values approaching significance at 6 h. An experiment of greater statistical power would be required to achieve definitive results. The available data suggest that heart e subunit mRNA expression, as measured by its ratio to the housekeeping gene GAPDH, increases with increasing dietary fat (and concomitantly decreases in carbohydrate) and the nutritional state of the animals (fasting versus fed). The trend in e subunit/GAPDH mRNA ratios as a function of level of dietary oil was not observed immediately following eating (0 h, refed) and 2 h after eating (+2 h, postprandial). These results indicate that e subunit expression is regulated by dietary constituents, their levels and availability, and by other factors. Explanation of these relationships will require additional observations.

Since we had shown previously that hepatic e subunit/β-actin ratios are decreased by high fat diets (8), we also analyzed e subunit mRNA abundance in livers of BALB/c mice fed 3% or 20% ad libitum for 2 weeks before fasting (in light phase), after eating their assigned diets following fasting (in the dark phase), and then 2 h after being allowed feed for 2 h. The 3 versus 20% corn oil and fasting versus refed states were the most extreme nutritional conditions imposed. The ad libitum fed mice confirmed our previous results (8) showing that the e subunit/β-actin mRNA ratios increased significantly with the 3% corn oil diet compared to the 20% corn oil diet. Fasting and eating altered the response to the level of dietary corn oil, since e subunit/β-actin ratios were increased in mice fed 20% corn oil compared to those fed 3% corn oil following a fast/refeed regimen similar to that observed in heart tissue. Differences in e subunit/β-actin ratios between ad libitum feeding (mice killed during the light phase) and the fasting/refeeding regimen (mice killed during the dark phase) may reflect diurnal variations and/or differences in nutritional status, which were not addressed in our previously published studies (8). Others have shown similar dependence of transcript abundance on time of feeding for certain diet-regulated genes (20, 21). The responses of hepatic and heart e subunit mRNA are likely caused by differences in nutrient supply and metabolic changes induced by the 3%, 10%, and 20% corn oil-containing diets. The role of insulin, glucagon, fatty acids (22), carbohydrates (23), and other metabolic factors (24) in regulating e subunit expression by diets differing in fat and carbohydrate content remain to be clarified.

Implications of Diet Regulation and Sequence Similarities—Since F1F0-ATP synthase has a central role in producing cellular energy, it is likely that multiple factors such as substrate concentrations, reducing equivalents, and cellular energy balance will regulate its activity (1, 2). Many of these factors will differ if the primary nutrient source changes from lipids to carbohydrates. In addition, other agents may modify its activity. Higuti et al. (7) have proposed that the e subunit contains a putative Ca2+ binding site, suggesting that a Ca2+-e subunit complex participates in regulating e subunit binding or activity (7). Regulating F1F0-ATP synthase activity by altering transcriptional responses may seem less probable because the required enzyme machinery must be constantly responsive to changes in energy balance and substrate concentration. However, one or more regulatory subunits may be transiently regulated at the transcriptional level by different concentrations of the same or different nutrients. For example, certain carbohydrates and lipids may regulate gene transcription directly as ligands for transcription factors (22, 23). Based upon its presumed interaction with the F0 portion of the complex and the data presented here showing modulation of e subunit expression by the dietary concentration of nutrients and by the time of their consumption, we suggest that the e subunit participates in regulating ATP production. Since our studies did not directly address the function of the e subunit protein or analyze the promoter of the gene, further in vitro and in vivo studies will be required clarify these relationships.

Acknowledgments—We acknowledge the support and assistance of Drs. Beverly Paigen, Jian-long Mu, and other members of Dr. Paigen’s laboratory; Lucy Rowe and Mary Barter of the Genome Center at the Jackson Laboratory (Bar Harbor, ME); and Lois Maltais of The Jackson Laboratory (Bar Harbor, ME) Informatics Group. Figs. 3 and 5 were prepared by Lucy Rowe and Mary Barter, and we are indebted to them for assistance in preparing this manuscript. We also thank Dr. Peter Imrey of the University of Illinois College of Medicine for advice on statistical analyses of the data and for reading the manuscript.

Note Added in Proof—After the submission of this manuscript, Matchinsky reviewed evidence that the glucose-dependent increase of the ATP mass action ratio in certain cell types is governed by F1F0-ATP synthase (see Matchinsky, F. M. (1996) Diabetes 45, 223–241, and references therein).
11. Church, G., and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1991–1995
12. SAS Institute, Inc. (1987) SAS/STAT® Guide for Personal Computers, Version 6, pp. 549–640, SAS Institute, Cary, NC
13. Glantz, S. A. (1992) Primer of Biostatistics, 3rd Ed., McGraw Hill, New York
14. Fornace, A. J., Jr., Alamo, I. Jr., and Hollander, M. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8800–8804
15. Fornace, A. J., Jr., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasiou, M., Fargnoli, J., and Holbrook, N. J. (1989) Mol. Cell. Biol. 9, 4196–4203
16. Jackson Laboratory (Bar Harbor, ME) (1996) BSS and BSB Backcross Data Base, http://www.jax.org/resources/documents/cmdata
17. Pilz, A., Letissier, P., Moseley, H., Peters, J., and Abbott, C. (1992) Mammalian Genome 3, 633–636
18. Koeller, D. M., Digiulio, K. A., Andeloni, S. V., Dowler, L. L., Freman, R. E., White, R. A., and Goodman, S. I. (1995) Genomics 28, 508–512
19. Kaput, J., Swartz, D., Patisley, H., Mangian, H., Daniel, W. L., and Visek, W. J. (1994) J. Nutr. 124, 12965-13055
20. Goldman, M. J., Back, D. W., and Goodridge, A. G. (1985) J. Biol. Chem. 260, 4404–4408
21. Leducq, R. C., Caldwell, M., and Kirk, E. (1994) J. Lipid Res. 35, 121–133
22. Auwerx, J. (1992) Horm. Res. 38, 269–277
23. Shi, S., and Towle, H. C. (1994) J. Biol. Chem. 269, 9380–9387
24. J. Jumpy, D. B., Clarke, S. D., Theilen, A., and Limatta, M. (1994) J. Lipid Res. 35, 1076–1084
25. American Institute of Nutrition (1977) J. Nutr. 107, 1340–1348
26. American Institute of Nutrition (1980) J. Nutr. 110, 1726–1734