Developmental, Nutritional, and Hormonal Regulation of Tissue-specific Expression of the Genes Encoding Various Acyl-CoA Dehydrogenases and α-Subunit of Electron Transfer Flavoprotein in Rat*

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Short chain (SCAD), medium chain (MCAD), and long chain acyl-CoA dehydrogenases (LCAD) catalyze the first step of fatty acid oxidation, while isovaleryl-CoA dehydrogenase (IVD) is involved in leucine oxidation. They are homologous flavoproteins belonging to the acyl-CoA dehydrogenase (ACD) family. Electron transfer flavoprotein (ETF) serves as an obligatory electron acceptor for these reactions. We demonstrated that the expression of SCAD, MCAD, and LCAD and the α-subunit of ETF (α-ETF) showed a similar developmental pattern, while that of IVD was distinctly different from others. The ontogenic pattern of each enzyme in the liver differed distinctly from that in the heart. The degree of glucagon-enhanced ACD expression in vivo and in vitro in both the liver and heart was especially high in fasted rats. Dexamethasone induced all ACD mRNAs in the heart. In contrast, it strongly suppressed mRNAs of all ACDs and α-ETF mRNA in the liver, except IVD mRNA. Dexamethasone induced IVD mRNA in both the liver and heart. Starvation strongly stimulated expression of all five genes in various tissues, with the highest in the heart, except the IVD gene which was down-regulated. The degree of induction by 3-day starvation differed in different age groups of rats. Feeding the rats a fat-free diet for 7 days caused a marked increase of IVD mRNA in the heart, whereas the high fat diet for the same period resulted in a severe decrease of the same degree, suggesting a protein-sparing mechanism. However, these manipulations of dietary fat content had little effect on the expression of other ACD genes.

Five mitochondrial acyl-CoA dehydrogenases have been well characterized (1-4). Three of them, short chain (SCAD), medium chain (MCAD), and long chain acyl-CoA dehydrogenases (LCAD), catalyze the first step of β-oxidation of fatty acids with varying chain length, whereas two others, isovaleryl-CoA dehydrogenase (IVD) and 2-methyl-branched chain acyl-CoA dehydrogenase, catalyze the third step in the leucine and valine/isoleucine oxidation pathways, respectively. These five acyl-CoA dehydrogenases (ACDs) are homologous in their reaction mechanisms, protein size, and in molecular structure. Each of these acyl-CoA dehydrogenases catalyzes the β-dehydrogenation of acyl-CoA and transfers electrons to electron transfer flavoprotein (ETF). They differ from each other mainly in the chain length and configuration of their acyl-CoA substrates. All ACDs are homotetramers of a subunit with a similar size. Comparison of the amino acid sequence of four acyl-CoA dehydrogenases (SCAD, MCAD, LCAD, and IVD) revealed a distinct homology, indicating that these enzymes belong to a gene family, the acyl-CoA dehydrogenase family (5, 6).

In contrast to the mechanistic and structural aspects of ACDs, relatively little is known about the regulation of expression of the acyl-CoA dehydrogenase genes at present. The available biochemical evidence has suggested, however, the importance of the regulatory mechanisms of these genes. Foster and Bailey (7) demonstrated that the activities of several enzymes involved in mitochondrial fatty acid oxidation, such as acyl-CoA synthetase, carnitine acyltransferase, 3-hydroxyacyl-CoA dehydrogenase, general 3-oxoacyl-CoA thiolase and acetoacetyl-CoA thiolase, markedly increased in rat liver immediately after birth, but decreased after weaning. They speculated that these developmental changes were caused by the increased fatty acid supply to the liver from drinking milk and also by the hormonal changes occurring during perinatal development. Carroll et al. (8) recently observed that SCAD, MCAD, and LCAD activities dramatically increased immediately after birth in such tissues as skeletal muscle, heart, and liver, which heavily utilize fatty acids. Since all three genes are involved in fatty acid oxidation, these previous enzymatic data were suggestive that expression of the SCAD, MCAD, and LCAD genes exhibit similar responses to physiological stimuli.

In spite of the homologous structure and identical reaction mechanisms, there has been evidence suggesting that the regulation of the IVD gene along the development, or in response to hormones and diet, may differ from the other ACD genes, since the metabolic roles of IVD differ from those of others. Previously, Van Veen et al. (9) showed that there was rapid oxidation of leucine in fetal lamb and that the oxidation rate increased in response to maternal fasting. Denne and Kalhan (10) demonstrated that the rate of in vivo leucine oxidation was significantly greater in human newborns than in adults. Since the leucine oxidation rate probably reflect the turnover rate of the whole-body protein, the data...
by Van Veen et al. and that by Denne and Kalhan were suggestive that proteins were used as a major energy source in the fetal and newborn period. At the cellular level, it has been reported that during the differentiation of 3T3-L1 fibroblasts, the activity of IVD was induced together with other enzymes in the leucine oxidative pathway (11). Therefore, the developmental expression of the IVD gene could take a pattern differing from those of the other three acyl-CoA dehydrogenase genes. It was not possible, however, to draw further insights on the developmental and regulatory aspects of ACDs from the physiological and biochemical data. The mechanisms on how the ACD genes are regulated needs to be studied at the molecular level.

In this paper, we report our study of developmental, nutritional, and hormonal regulation of tissue specific expression of various acyl-CoA dehydrogenases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glyoxal and dexamethasone were obtained from Sigma. Fat-free (less than 0.2%) and high fat (cottonseed oil 45%) diets were purchased from ICN Pharmaceuticals.

**Animals**—Pregnant Wistar rats were obtained from Charles River (Wilmington, MA), and were housed in individual cages. Fetal age was calculated from the day of mating. Birth usually occurred on day 22 of gestation. The first 24 h after birth was called day 0 and the period from 24 to 48 h after birth was called day 1. Subsequent days were likewise numbered. Pups were kept with their mothers until the weaning period. For mRNA determination at each time point, tissues were pooled from 12-40 pups, representing 4-6 litters. The adult male laboratory rat diet (Wilmington, MA), and were housed in individual cages. Fetal age was calculated from the day of mating. Birth usually occurred on day 22 of gestation. The first 24 h after birth was called day 0 and the period from 24 to 48 h after birth was called day 1. Subsequent days were likewise numbered. Pups were kept with their mothers until the weaning period. For mRNA determination at each time point, tissues were pooled from 12-40 pups, representing 4-6 litters. The adult male Wistar rats (5-6 weeks in age), previously stabilized on a standard laboratory rat diet (libitum and weighed 120-160 g at the time of experiment.

**Protocol for In Vivo Hormonal Regulation Studies**—Groups of three male Wistar rats (5-6 weeks in age), previously stabilized on a standard diet, were injected subcutaneously with glyoxal (150 μg/100 g of body weight) or sterile water (control) and were sacrificed 3 h later. Another group of three male Wistar rats, previously fasted for 24 h, received glyoxal injection in a similar manner. In a separate experiment, a group of three male Wistar rats were administered dexamethasone by intraperitoneal injection (10 mg/kg body weight) and sacrificed 12-14 h later. A second group of three rats were treated with dexamethasone similarly, but further received glyoxal injection (150 μg/100 g of body weight) 3 h before sacrifice.

**Dietary Experiments**—In experiments in which effect of specific diet was tested, young adult rats were first maintained on a standard rat diet ad libitum and then fed a specific diet for a week. On the 7th day on the special diet, total RNA and mitochondria were isolated from liver and heart.

**RNA Preparation and Isolation of Mitochondria**—Rats were sacrificed by a blow to the head, immediately followed by decapitation. Tissues were rapidly removed, weighed, and used for further experiments. Total RNA was prepared from each tissue by the guanidine isothiocyanate method (16). Mitochondria was isolated form rat liver and heart by the method of Schnaitman and Greenwalt (17).

**Dot-blot Hybridization**—Total RNA was denatured with 1 M glyoxal in 10 mM sodium phosphate buffer (pH 6.8) at 50 °C for 1 h. Dot-blotts were prepared by applying the glyoxylated RNAs to a nylon membrane and a microsample filtration manifold (Schleicher & Schuell, Inc.). The Nylon membrane was then baked at 80 °C and was boiled in 20 mM Tris-HCl (pH 8) for 10 min. The membrane was then prehybridized with sonicated and denatured salmon sperm DNA (250 μg/ml) for 24 h at 42 °C in prehybridization buffer consisting of 50% formamide, 5 × SSC (1 × SSC, 150 mM sodium chloride and 15 mM sodium citrate), 50 mM sodium phosphate buffer (pH 6.5), and 1 × Denhardt’s solution. The Nylon membrane thus prepared was hybridized with an appropriate radiolabeled cDNA. After hybridization, the membrane was washed at room temperature with four changes of 2 × SSC, 0.1% SDS for 15 min each and then at 64 °C with two changes of 0.2 × SSC, 0.1% SDS for 30 min each, and was exposed to an x-ray film at −70 °C for 4-6 h. 

**Isolation and Use of Rat Liver Nuclei for Transcription and Analysis of Specific RNA Transcripts**—Nuclei were isolated from rat liver according to the method of Marzluff and Huang (18). The prepared nuclei were suspended in a concentration of 1-2 × 10⁷ nuclei per 0.1 ml of storage buffer (50 mM Tris-HCl (pH 8.0) containing 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5.0 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and stored at −70 °C. The yield of this procedure was approximately 5 × 10⁷ nuclei, 1 g of liver.

For transcription, a suspension of 1 × 10⁴ nuclei in 10 μl was incubated in 100 μl of reaction mixture containing 100 μCi of [α-32P]UTP (800 μCi/mmol), 1 mM each of ATP, CTP, and GTP, 240 mM KCl, and 5 mM magnesium acetate at 25 °C for 30 min. The reaction was stopped by adding 2 ml of 1% SDS, 10 mM EDTA (pH 7.0) solution (18). Radiolabeled RNA was then purified by using QuickSpin column (Sephadex G-50, Boehringer Mannheim). Individual 32P-labeled RNA was then quantitated via hybridization to a respective cDNA probe (5 μg) that had been bound to Nylon membrane as follows (19). After prehybridization was first performed as described above under "Dot-blot Analysis," 32P-RNA was added in the same buffer (final amount 0.5 ml), and hybridization was carried out at 42 °C for 36 h. The membrane was then washed at 64 °C four times with gentle shaking in 2 × SSC, 0.1% SDS for 15 min and then twice with 0.1 × SSC, 0.1% SDS for 30 min. The amount of hybridized RNA was determined via autoradiography, by punching out the spot and counting its radioactivity in a liquid scintillation counter.

**Immunoblot Analysis**—Mitochondria were solubilized by boiling for 3 min with 10-fold volume of buffer containing 4% SDS, 125 mM Tris-HCl (pH 6.8), 20% glycerol, 0.01% bromphenol blue, and 10% 2-mercaptoethanol. Samples were electrophoresed in a 12.5% SDS-polyacrylamide gel with a 4% stacking gel. After electrophoresis, the gel was electrobotted onto an Immobilon-F membrane (Millipore), according to the method of Towitz et al. (20). Immunoreactive protein bands, were densitometrically quantitated using a Bioimage system (Millipore) and is presented in Fig. 2.

**FIG. 1.** Dot-blot analysis of SCAD, MCAD, IVD, and OTC mRNAs. A, preliminary dot-blot of SCAD and MCAD mRNAs in liver and heart. Adult Wistar rats were the source of tissues. For each tissue, samples that contained 4, 2, 1, and 0.5 μg of total RNA were hybridized with the respective cDNA probe as described under "Experimental Procedures." Yeast RNA was used as a negative control. Hybridized samples were exposed to a film 6 h. B, dot-blot hybridization of liver MCAD, IVD, and OTC mRNAs at various stages of development. For each gene at each stage, 4 μg of total RNA was used. The samples hybridized with a labeled cDNA probe were exposed to an x-ray film for 6 h. Other experimental conditions were described under "Experimental Procedures." The intensity of these and other ACD mRNA dots, together with those of respective protein bands, were densitometrically quantitated using a Bioimage system (Millipore) and is presented in Fig. 2.
Results

Developmental Regulation of Various Acyl-CoA Dehydrogenase and α-ETF Genes in Rat Liver—We first carried out a preliminary dot-blot hybridization of cellular SCAD and MCAD mRNAs in liver and heart of adult Wistar rats. For each tissue, samples contained 4, 2, 1, and 0.5 μg of total RNA. Each sample was hybridized with the respective cDNA probe as described under "Experimental Procedures." Yeast tRNA was used as a negative control. The results are shown in Fig. 1A. After only 4 h exposure of the film, dot-bLOTS of SCAD and MCAD mRNAs in the heart were intensely labeled even at the level of 0.5 μg of total RNA. Although the dot-bLOTS in the liver were not as intense as in the heart, they were intensely labeled at 4 and 2 μg of RNA. Hybridization with 1 and 0.5 μg of RNA was not as intense, but still can be clearly seen. In contrast, there was no hybridization to 4 μg of yeast tRNA at all. These data demonstrate that the dot-bLOTS of SCAD and MCAD mRNAs were specific, and the cellular levels of SCAD and MCAD mRNAs are well in the range which allows accurate quantitation.

We then determined the concentrations of cellular mRNAs and intramitochondrial proteins of four acyl-CoA dehydrogenases, including SCAD, MCAD, LCAD, and IVD, and α-ETF during development using RNA dot-blot hybridization and immunoblot analyses, respectively. The intensity of mRNA dots and protein bands were densitometrically quantitated using a Bioimage system (Millipore) (Fig. 2). The original dot-bLOTS of MCAD, IVD, and OTC mRNAs in the liver are shown in Fig. 1B. Densitometric measurements of mRNA and protein of all four ACDs and α-ETF along the course of development are summarized in Fig. 2. In the liver, the levels of mRNAs involved in fatty oxidation, including SCAD, MCAD, LCAD (these three ACDs are grouped as fatty ACDs hereafter), and α-ETF, followed a similar pattern from the fetal stage to adult age: these four mRNAs were already present in the late fetal period, but their concentrations were low. Immediately after birth, the levels of these four mRNAs rapidly rose, reaching a maximum at day 10 to day 14. After the peak was reached, the amount decreased until day 35, and then slightly restored back to the adult level (Fig. 2, A, B, C, and E). The changes in the respective immunoreactive proteins (Fig. 2, A, B, C, & E) and the changes in enzyme activities (data not shown) paralleled the changes in the amount of mRNA.

The developmental pattern of IVD mRNA in the liver was markedly different from the four enzymes involved in fatty acid oxidation (Fig. 2D). Unlike the other enzymes, the level of IVD mRNA was considerably higher than others at day -5, but precipitously decreased for several days until birth. Immediately after birth, there was a short period of increase. The amount of IVD mRNA decreased again around day 10, but increased gradually thereafter. The highest level of expression of IVD mRNA was observed at 60 days of age. This pattern of IVD mRNA was reproducible in three independent experiments. In general, the trend of the changes in the amount of immunoreactive IVD protein followed that of IVD mRNA.

The relative amount of protein bands in the blot was determined densitometrically using a Bioimage system (Millipore).
mRNA, except at day -5 when a marked discrepancy in the amount of IVD mRNA and that of IVD protein was observed. In repeated experiments, the amount of IVD mRNA was high while that of IVD protein was low at day -5. Also, unlike in the case of IVD mRNA, there was no temporary decrease in the IVD protein level in the 7 day period after day 3.

In order to test whether the pattern of expression of the genes involved in the fatty acid oxidation, such as the SCAD, MCAD, LCAD, and α-ETF genes, in the neonatal period was unique or common for all nuclear-coded mitochondrial enzymes, we examined the developmental expression of OTC, another nuclear-coded mitochondrial enzyme that is involved in the urea cycle (Fig. 2F). It has been shown that OTC is expressed only in the liver, and that its expression increased significantly in the postnatal period (21). Our data indicate that the level of OTC mRNA was very low in the fetal period, but rapidly increased after birth, reaching the peak at days 17-24, and then declined approximately 60%, settling at the adult level by day 35. Thus, the overall pattern of OTC expression is similar to those of ACDs and α-ETF in its rapid increase in the early neonatal period. However, it distinctly differed from those related to fatty acid oxidation in that its maximum level was reached at the weaning period rather than late suckling period when straight chain ACDs and α-ETF reached the peak.

Developmental Regulation of Various Acyl-CoA Dehydrogenase and α-ETF Genes in Rat Heart—In the heart, the expression of three fatty ACDs and α-ETF exhibited a similar developmental pattern (Fig. 3A, B, C, and E), but the pattern in the heart was quite different from that of the respective enzyme in the liver. The levels of mRNAs of these enzymes in fetal heart were extremely low or absent at day -5. The amount of these mRNAs increased toward birth, reaching to 30-50% of the adult level, but remained at this level during the sucking period until 12 days of age. The fatty ACDs and α-ETF mRNAs then rapidly increased around 14-17 days of age reaching the level of 2.5-4 times over that at birth, and retained the high level thereafter.

In contrast, the level of IVD mRNA at day -5 was almost as high as those of neonatal and suckling rats. IVD mRNA started to increase later than other mRNAs (20 days of age), and the magnitude of the increase was not as great as those of other acyl-CoA dehydrogenases (Fig. 3D).

The changes in the amount of all immunoreactive ACDs and α-ETF proteins were consistent with the changes in the amount of respective mRNAs as observed in the liver.

Hormonal Regulation of Acyl-CoA Dehydrogenases and α-ETF mRNAs in Rat Liver and Heart in Vivo—It has been previously shown that fatty acid oxidation is regulated by the changes of hormonal and nutritional status. For instance, carnitine palmitoyltransferase activity, the rate-limiting enzyme in β-oxidation of fatty acids, increased in starvation and diabetes; the physiological states in which the insulin/glucagon ratio is low (22). Glucagon administration to intact rats enhanced the amount and transcription of carnitine palmitoyltransferase mRNA (23).

In the present study, we first tested the effects of glucagon (150 μg/100 g of body weight) on the levels of various ACD and α-ETF mRNAs in the liver and heart of both fed and fasted rats (Table 1). The rats in the fed group were fed on a standard laboratory diet for 7 days before glucagon injection. When glucagon was administered to fed animals, there was 1.7-2.3-fold increase in the amount of MCAD, LCAD, IVD, and α-ETF mRNAs in the liver, but the amount of SCAD mRNA remained essentially unchanged. We also tested the behavior of OTC mRNA as a positive control for glucagon.

![Fig. 3. Developmental changes in the amount of mRNAs and immunoreactive proteins of various acyl-CoA dehydrogenases and α-ETF in rat heart. A: SCAD, B: MCAD, C: LCAD, D: IVD, E: α-ETF. Symbols are: ●, mRNA; ○, immunoreactive protein.](image-url)
Effects of glucagon on the amount of mRNAs for various acyl-CoA dehydrogenases, α-ETF and OTC in fed and starved rats

Adult male Wistar rats (6 weeks of age) were fed with a standard chow for 7 days, and injected with glucagon solution (150 μg/100 g of body weight), or sterile water. Liver and heart were isolated 3 h after injection, and total RNA was prepared from both organs. Another group of animals, which were starved for 24 h, were also treated in the same way and total RNA was prepared. The amount of each mRNA was determined by dot blot hybridization with 32P-labeled cDNAs. Serial 2-fold dilutions of total RNA (4, 2, 1, and 0.5 μg) were blotted and results were quantitated using a densitometric scanner (Bioimage System, Millipore). Values are obtained in at least duplicate or multiple experiments, and expressed in the mean of relative amounts ± S.D., taking the value of fed rats without glucagon as 1.0. Each experiment was performed using three rats. Standard deviations less than ± 0.05 are not listed.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Amount of mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>SCAD MCAD LCAD IVD α-ETF OTC</td>
</tr>
<tr>
<td>None (fed)</td>
<td>1.0 1.0 1.0 1.0 1.0 1.0 1.0</td>
</tr>
<tr>
<td>Glucagon (fed)</td>
<td>0.9 ± 0.1 2.0 ± 0.2 2.3 ± 0.2 1.8 ± 0.1 1.7 ± 0.1 2.4 ± 0.2 1.0</td>
</tr>
<tr>
<td>None (fasted)</td>
<td>1.2 ± 0.1 1.0 ± 0.1 1.1 ± 0.1 0.6 ± 0.1 1.0 0.9 ± 0.1</td>
</tr>
<tr>
<td>Glucagon (fasted)</td>
<td>3.0 ± 0.2 2.5 ± 0.3 3.7 ± 0.2 0.9 ± 0.1 2.6 ± 0.1 2.8 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>SCAD MCAD LCAD IVD α-ETF OTC</td>
</tr>
<tr>
<td>None (fed)</td>
<td>1.0 1.0 1.0 1.0 1.0</td>
</tr>
<tr>
<td>Glucagon (fed)</td>
<td>0.9 ± 0.1 1.1 ± 0.1 1.1 ± 0.1 1.1 ± 0.1 1.0</td>
</tr>
<tr>
<td>None (fasted)</td>
<td>1.1 ± 0.1 1.1 ± 0.1 1.4 ± 0.1 0.7 1.2 ± 0.1</td>
</tr>
<tr>
<td>Glucagon (fasted)</td>
<td>2.6 ± 0.2 3.3 ± 0.2 2.4 ± 0.1 1.9 ± 0.1 3.1 ± 0.3</td>
</tr>
</tbody>
</table>

In another experiment, rats were fasted for 24 h after 6 days at the standard laboratory diet. One-half of the rats in the fasted group were sacrificed without further intervention. The remaining rats were treated with glucagon (150 μg/100 g of body weight). In the 24-h fasting alone group, there was no significant changes in the amount of SCAD, MCAD, and LCAD mRNAs in both liver and heart, whereas that of IVD mRNA markedly decreased. With glucagon administration, the degrees of induction SCAD, MCAD, and LCAD mRNAs in the fasted rats were much more prominent than those in the fed rats. Glucagon caused a marked increase in the amount of IVD mRNA as well. Reaching the level of 1.9 times without intervention control in the heart. In the liver, glucagon administration restore the amount of IVD mRNA to the control level.

It has been previously shown that glucocorticoids enhanced the expression of various enzymes involved in gluconeogenesis. These include phosphoenolpyruvate carboxykinase (24) and tyrosine aminotransferase (25). However, little is known about the effect of glucocorticoids on the expression of enzymes involved in fatty acid oxidation. In the present study, we studied the effects of dexamethasone on the expression of ACD and α-ETF mRNA with and without the combined use of glucagon. Typical dot-blot hybridization data of MCAD and IVD mRNAs are also shown in Fig. 4. All results are summarized in Table II. In the liver, IVD mRNA behaved very differently from fatty ACD and α-ETF mRNAs. The expression of fatty ACD and α-ETF mRNAs in the liver were suppressed by the administration of dexamethasone to less than 50% of the control level, and the concurrent use of glucagon did not reverse this downward effect. In contrast, IVD mRNA in the liver was slightly induced by dexamethasone alone, and was further enhanced by the simultaneous use of glucagon.

In the heart, all ACD and α-ETF mRNAs, including IVD mRNA, were induced to the level of 1.5–2.1 times the control level by dexamethasone. The combined use of glucagon further induced MCAD and IVD mRNA, but exhibited no further effects on SCAD, LCAD, and α-ETF mRNAs. The effects of these hormones on the amount of immunodetectable proteins generally paralleled those of the respective mRNAs, although the magnitude of their changes were generally smaller than those of the corresponding mRNAs, except SCAD protein which exhibited a similar degree of the changes in the amount of its protein and mRNA (data not shown).

Effects of Hormones on in Vitro Transcription of the MCAD and IVD Genes—In view of the marked effects of glucagon and dexamethasone on the level of ACD mRNAs, we then studied the effects of glucagon and dexamethasone on in vitro transcription of the MCAD and IVD genes using isolated liver nuclei. Seven experimental groups, each consisting of 3 rats, were studied in two sets of experiments (Table III). Liver from three rats in each experimental group were combined, and nuclei were isolated. In the first experiment, fed rats were used in all three groups. With dexamethasone administration alone, the transcription rate of the MCAD mRNA decreased.
Effects of dexamethasone and glucagon on the amount of mRNAs for various acyl-CoA dehydrogenases and \( \alpha \)-ETF

Adult male rats (6 weeks of age), which were kept on normal rat diet, injected with dexamethasone (Dex, 10 mg/kg of body weight) and sacrificed 12-14 h later. Some of the rats were injected, in addition, with glucagon (Glc, 150 \( \mu \)g/100 g of body weight) 3 h before sacrifice. Total RNA was isolated from both liver and heart, and the amount of individual mRNA was analyzed using dot blot hybridization, followed by densitometric measurement of the blots. Experiments were performed in multiple or at least duplicate experiments, and each individual experiment was carried out using pooled RNA from three rats. Values are expressed in mean of relative amounts \( \pm \) S.D., taking the value of control as 1.0.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>SCAD</th>
<th>MCAD</th>
<th>LCAD</th>
<th>IVD</th>
<th>( \alpha )-ETF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Dex</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Dex + Glc</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>None</td>
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<td>1.0</td>
</tr>
<tr>
<td>Dex</td>
<td>2.1 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Dex + Glc</td>
<td>1.9 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

### Table III

Nuclei were isolated from liver of rats, which received dexamethasone or dexamethasone plus glucagon as described for Experiment 1 in Table II. Nuclei were also isolated from fed and starved animals, which received glucagon injection as described in Table I. Transcription in the isolated nuclei was assayed as described under “Experimental Procedures.” Each experiment was performed using pooled nuclei from three rats. The amount of \( ^3P \)-RNA, that hybridized to the respective cDNA probe, was used to express the relative transcription activity of the respective gene. The unit is ppm of the total \( ^3P \)-RNA that was used for hybridization. The average efficiency of hybridization was 25%. The radioactive count of RNA, hybridized to pBR322 (ranging from 60 to 100 cpm), was subtracted as background from the individual values.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>MCAD mRNA</th>
<th>IVD mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \times 10^5 ) cpm</td>
<td>ppm of total</td>
<td>cpm ppm of total</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>None</td>
<td>3.25</td>
<td>377</td>
<td>11.6</td>
</tr>
<tr>
<td>Dex</td>
<td>4.15</td>
<td>182</td>
<td>4.4</td>
</tr>
<tr>
<td>Dex + Glc</td>
<td>3.72</td>
<td>168</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (fed)</td>
<td>3.00</td>
<td>317</td>
<td>10.5</td>
</tr>
<tr>
<td>Glc (fed)</td>
<td>3.05</td>
<td>540</td>
<td>17.7</td>
</tr>
<tr>
<td>None (starved)</td>
<td>2.74</td>
<td>401</td>
<td>14.6</td>
</tr>
<tr>
<td>Glc (fasted)</td>
<td>3.16</td>
<td>2972</td>
<td>94.0</td>
</tr>
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</table>

60%, whereas the transcription rate of IVD mRNA increased 80%. Concomitant administration of dexamethasone and glucagon essentially did not change the MCAD mRNA transcription rate, but it further enhanced the IVD mRNA transcription rate to the level of 200% of control.

In the second experiment, the effects of 24-h fasting and glucagon administration were tested. In fed rats, glucagon markedly enhanced the transcription of the MCAD gene (69%) but only slightly enhanced the IVD gene (21%). Fasting alone without the hormone significantly enhanced the transcription of the MCAD gene (38%), but inhibited the IVD gene transcription 37%. When glucagon was administered in fasted rats, the degree of enhancement of transcription of both the MCAD and IVD genes, particularly the former, was markedly greater than in fed rats (895 and 170%, respectively, of fed control). These changes in the \( \text{in vitro} \) transcription rate were consistent with the changes in the amount of the respective mRNA species \( \text{in vivo} \) shown in Tables I and II.

In Vivo Effects of Hormones and Age of Rats—The results shown in Figs. 2 and 3 and Tables I and II suggest that glucocorticoid may be an important regulator of ACD expression during the development of rats (see “Discussion”). Therefore, we investigated the changes of ACD mRNA levels in the liver and heart after a single dose of dexamethasone in rats of various ages. The typical results for MCAD and IVD are summarized in Fig. 5. In the liver, dexamethasone treatment
caused a severe decrease in the amount of MCAD mRNA at all stages of development, whereas in the heart MCAD mRNA was substantially induced after 7 days of age. The magnitude of induction was particularly high at the 7th day, bringing the level of MCAD mRNA to nearly as high as that in adult rats. Dexamethasone treatment caused similar changes in the level of SCAD and LCAD mRNAs (data not shown). The IVD mRNA was induced by dexamethasone in both the heart and the liver, but the degree of induction was much greater in the heart than in the liver during the entire developing stage.

Effects of Starvation on Expression of Acyl-CoA Dehydrogenases and α-ETF—It has been previously shown that starvation leads to increased mobilization and utilization of fatty acids. Starvation caused an increase of palmitoyl-CoA oxidation and enhanced short chain acyl-CoA dehydrogenase activity in rats (26). Therefore, we examined the effects of starvation on the expression of ACDs and α-ETF. In this experiment, adult rats were starved for 3 days before sacrificing. The amounts of four ACD and α-ETF mRNAs in various organs in both the fed and the starved rat groups were determined using dot-blot analysis followed by densitometric scanning. The dot-blot data of MCAD and IVD mRNAs are shown as typical examples in Fig. 6. The results on five proteins are summarized in a graphic form in Fig. 7. In the starved rats, the amount of fatty ACD mRNAs, including SCAD, MCAD, and LCAD mRNA, all increased in the heart, liver, and small intestine, while their behavior in the skeletal muscles and kidneys did not take the same pattern (Fig. 7, A–C). The increases of fatty ACD mRNAs in the heart was particularly large, reaching the levels of approximately twice the level in the fed controls. IVD mRNA responded to starvation in a completely different manner, with decreases of varying degrees in all tissues (Figs. 6 and 7D). The amount of α-ETF mRNA decreased in the heart and skeletal muscles, but increased in the liver, kidneys, and small intestines (Fig. 7E).

We then examined the effects of 3-day starvation on the amount of various mRNAs in the heart and liver in 3 different age groups, including 3-, 6-, and 10-week-old groups. Data for MCAD and IVD are shown in Fig. 8. In the heart, the degree of induction of MCAD mRNA expression was the highest in 10-week-old rats, with its level reaching the level of more than 4 times the control after 3 days of starvation. Induction in 3- and 6-week-old rats were not as prominent as in 10-week-old rats. In the liver, in contrast, MCAD mRNA was induced only slightly in 3-week-old rats. There were essentially no changes in 7-week-old rats, and the expression was slightly suppressed to below control levels in 10-week-old rats. The effects of starvation on SCAD and LCAD mRNAs in both organs took patterns that were similar to that of MCAD mRNA (data not shown).

The effects on the expression of the IVD gene in 3-day starved young rats considerably differed from those on the fatty ACD genes. IVD mRNAs were slightly enhanced in 3-week-old rats, but suppressed more than 50% by starvation in 7- and 10-week-old groups in both heart and liver.

Effect of Fat-free and High Fat Diets on the Expression of ACD Genes—There are two major nutritional changes in the life of rats. The first change occurs at the perinatal period, that is transition from transplacental alimentation providing mainly carbohydrate to milk containing a high concentration of fat. The second change occurs at weaning period when young rats start eating solid food. The exposure to the high fat content of milk has been considered to be one of the major factors causing the changes in the activities of the enzymes involved in fatty acid oxidation during development (27). Hence, we tested the effects of fat content in the diet on ACD expression.

In the heart, after 7 days on a fat-free diet, IVD mRNA increased to the level of 170% of the control, whereas fatty ACD mRNAs remained near normal level or slightly decreased (MCAD) (Fig. 9A). In the liver, the amounts of all ACD mRNAs, including IVD mRNA, remained near-normal (IVD and LCAD) or slightly decreased (SCAD and MCAD).

In the heart, 7 days after the high fat diet was instituted, fatty ACD mRNA, including SCAD, MCAD, and LCAD mRNAs, remained within 75–125% of control levels, whereas IVD mRNA declined 55% (Fig. 9B). While rats were on the high fat diet, the levels of all ACD mRNAs in the liver decreased 40–67%.

The amounts of immunodetectable protein encoded in each gene generally showed changes which are similar to those of respective mRNA (data not shown).

Effect of Protein-free and High-protein Diets on the Expression of ACD Genes—It has been previously reported that the branched-chain α-ketoacid dehydrogenase is a rate-limiting enzyme in the branched-chain amino acid pathways, and that it is subject to metabolic regulations by such inputs as the amount of branched-chain amino acid intake. The activity of branched-chain α-ketoacid dehydrogenase increased linearly in the rat liver, when dietary protein was increased from 0 to 30% (28). It is important to know whether IVD is also regulated in the same manner by the dietary manipulation or not. We then examined the effects of dietary protein content on ACD expression. After 7 days on a protein-free diet, IVD mRNA increased 39% over the control in the heart, whereas fatty ACD mRNAs remained near normal level or slightly decreased (MCAD) (Fig. 10A). In the liver, the amounts of all ACD mRNAs, except IVD mRNA, decreased 44–53%, except LCAD, which decreased only marginally.

After 7 days on a high-protein diet, all ACD mRNAs, except IVD mRNA, decreased both in heart and liver (Fig. 10B). The amount of IVD mRNA slightly (8%) increased in the heart, but somewhat (10%) decreased in the liver.

**DISCUSSION**

We have shown here at the molecular level that the expression of various ACD genes are regulated by at least several
FIG. 7. Tissue specific changes in the amount of mRNAs for various acyl-CoA dehydrogenases and α-ETF caused by starvation. Adult male Wistar rats (6 weeks of age; \( n = 5 \)) were fasted for 3 days and total RNA was prepared from psoas muscle, kidney, small intestine, heart, and liver, and serial 2-fold dilutions of total RNA (4.0, 2.0, 1.0, and 0.5 \( \mu g \)) were blotted onto a nylon membrane and hybridized with the radiolabeled cDNA probes for MCAD and IVD. Total RNA was also prepared from control rats (\( n = 4 \)), which were fed with a standard chow for 3 days, and analyzed in the same way. Total RNA was isolated from various organs from both fed control (\( n = 4 \)) and starved rats (\( n = 5 \)). The results were quantitatively analyzed using a Bioimage System. A, SCAD; B, MCAD; C, LCAD; D, IVD; E, α-ETF.

FIG. 8. Time course of the changes in the amount of MCAD and IVD mRNAs during the 3-day starvation in rats at various ages. Twelve male Wistar rats were used for each of three age groups (3, 6, and 10 weeks). Total RNA was prepared from livers and hearts from three rats at each time point. Dot-blot hybridization analysis was performed as described in the legend to Fig. 6, and the results were quantitatively analyzed using a densitometer. A, MCAD; B, IVD. The symbols are: ○, 3 week rats; ▲, 6 week rats; □, 10 week rats.
of high fat diet. They found that the expression of these genes was tissue-specific and were developmentally regulated. Brady and associates (30) studied the regulation of enzymes of fatty acid oxidation, by various factors. They found that the developmental changes in the expression of these two mitochondrial enzymes mainly paralleled those in energy metabolism.

Kelly and associates (29) studied the level of MCAD mRNA, along with that of mitochondrial malate dehydrogenase mRNA, in a variety of tissues and in various developing stages of rats. They concluded that the expression of these genes were tissue-specific and were developmentally regulated. Since the changes in the levels of MCAD and malate dehydrogenase mRNAs were similar in their time course and magnitude, they attributed the peripartum and early postnatal rises of mRNAs of these two mitochondrial enzymes mainly to the maturation of mitochondria during this period. They considered that the developmental changes in the expression of these two enzymes paralleled those in energy metabolism.

Brady et al. (30–32) studied the regulation in vivo of carnitine palmitoyltransferase, an enzyme involved in mitochondrial fatty acid activation, by various factors. They found that the amount of carnitine palmitoyltransferase mRNA and its enzyme activity increased in riboflavin deficiency (30) and after administration of hypolipidemic agents, such as clofibrate and 2-(diethylhexyl)phthalate (31). In BB Wistar spontaneously diabetic rats, hepatic carnitine palmitoyltransferase mRNA increased 2–6-fold, but returned to control value upon insulin treatment (32). We have previously reported that the pattern of tissue-specific distribution of SCAD, MCAD, LCAD, and IVD, and α-ETF was essentially similar, with the highest in the heart for all of them, followed by liver, skeletal muscle, or kidney (6). We have also demonstrated that transcription of all ACD genes, except IVD, an enzyme involved in leucine oxidation, was markedly stimulated in riboflavin deficiency in vitro and in vivo, elevating the levels of respective mRNAs severalfold over controls. However, the increased mRNA levels failed to maintain the amounts of respective proteins because of the instability of the apoenzymes (33). The lack of stimulation of IVD transcription alone in riboflavin deficiency provided clues that the IVD gene expression is regulated separately from the three fatty ACD genes.

In the present study, we have shown that the expression of the SCAD, MCAD, LCAD, and α-ETF followed similar developmental patterns both in liver and heart during development, whereas the developmental expression of IVD mRNA in both the liver and heart took patterns that are distinctly different from three fatty ACD and α-ETF mRNAs. These and other results presented here gave further support for the notion that the expression of the IVD gene is regulated separately from the three fatty ACD genes. The overall hepatic expression pattern of OTC, a mitochondrial enzyme involved in the urea cycle, was similar to those of fatty ACDs and α-ETF in its rapid increase in the early neonatal period, but distinctly differed from those related to fatty acid oxidation in that its maximum level was reached in weaning period.

Fig. 9. The influence of fat-free and high fat diets on the level of various acyl-CoA dehydrogenases mRNAs liver and heart. Adult male Wistar rats were divided into two groups. One group of rats (n = 6) was fed on fat-free diet for 7 days, and the other group (n = 6) was fed on high fat diet for 7 days. Three rats for each group were sacrificed on day 0 and day 7. Total RNA was prepared from liver and heart from three rats at each time point. Dot-blot hybridization analysis was performed as described in the legend to Fig. 6, and the results were quantitatively analyzed using a densitometer. The results on the amount of each mRNA at day 7 was expressed as a shift from that at day 0 in percentage. Each value represents the mean of two experiments. A, the effects of fat free diet; B, the effects of high fat diet.

Factors including tissue specific, developmental, hormonal, and nutritional factors, providing the molecular basis for the hypotheses that have been forwarded from previous biochemical/nutritional studies. There had been only a few molecular studies on the regulation of enzymes of fatty acid oxidation.

Fig. 10. The effects of protein-free and high-protein diets on the expression of mRNAs for various acyl-CoA dehydrogenases. Male Wistar 12 rats, weighing 71 g in average, were divided into two groups. One group of rats (n = 3) was fed a protein-free diet for 7 days, and the other group (n = 3) was fed a high-protein diet for 7 days. Rats were sacrificed on day 0 and day 7. Total RNA was prepared from liver and heart. Dot-blot hybridization analysis was performed as described in the legend to Fig. 6, and the results were quantitatively analyzed using a densitometer. The results on the amount of each mRNA at day 7 was expressed as a change from that at day 0 in percentage. A, the effects of protein-free diet; B, the effects of high-protein diet.
rather than in late suckling period. The expression of the fatty ACD genes in the liver was most active during the suckling period. We also observed that in contrast to the observation in the liver, the expression of the fatty ACD genes in the heart was most active at the weaning period. These data indicate that during development, the expression of an individual mitochondrial enzyme, or a group of mitochondrial enzymes in a specific metabolic pathway, is under regulation of specific factors, and these factors for a given enzyme may differ from organ to organ. Thus, the maturation of mitochondria and the changes in energy metabolism are not the only factors determining the developmental patterns of mitochondrial enzymes as previously suggested by Kelly and associates (29).

The results of developmental changes in the level of fatty ACD mRNAs in the liver presented in Fig. 2, A-C, correlate well to the previous biochemical findings on perinatal and postnatal development of enzymes. These previous observations include the surge in ['C]palmitate oxidation by liver mitochondria immediately after birth, the almost exclusive reliance on fatty acid oxidation energy in suckling rats (34), and the maintenance of the high level of its activity until weaning before dropping to the adult value (34, 35). In the heart, in contrast, the rate of palmitoyl-CoA oxidation by rat heart homogenates increased steadily from late fetal life to maturity as shown by Warshaw (36), again correlating well with our finding on the peripartum and postnatal levels of the three fatty ACD mRNAs in the rat heart shown in Fig. 3, A-C.

It had previously been shown that glucocorticoid affects the expression of many genes such as tryptophan oxygenase (37) and tyrosine aminotransferase (38) in the course of development. The results from the present study confirm that glucocorticoid is indeed an important developmental regulator. The expression of three fatty ACD genes was stimulated by dexamethasone treatment in the heart, but was inhibited in the liver over the entire course of development. Furthermore, it is important to note that in the course of development, the rapid increases of fatty ACD mRNAs in the heart (Fig. 3) and their reciprocal decrease in the liver (Fig. 2) both start around day 14. This date coincides well with the known second rise of adrenal activity, that begins around day 10 (39, 40). Thus, these data suggest that steroid plays an important role in the developmental regulation of expression of the three fatty ACD genes.

We found that glucagon is another hormonal regulator of ACD expression, causing the elevation of the levels of all four ACD mRNAs levels, including that of IVD mRNA, in both liver and heart. The degree of induction was particularly high when glucagon was administered to fasted rats. It has been reported that glucagon enhances carnitine palmitoyltransferase activity (41) and induces the expression of carnitine palmitoyltransferase at the transcriptional level (23). However, the nature of glucagon effects are not limited to the genes in the fatty acid oxidation pathways, and appear to be rather broad, since the genes in different pathways, such as the IVD and OTC genes were also induced by glucagon. It has been previously shown that insulin antagonizes the glucagon action. During peripartum period, marked changes occur in the insulin and glucagon concentrations in opposite directions, with insulin decreasing and glucagon rising (42). Therefore, insulin may also be playing a role in the regulation of ACD expression, and the ratio of glucagon and insulin may be an important factor in determining the level of expression of ACDs and other enzymes.

We have shown here that the expression of ACD genes are also regulated at the transcriptional level by various nutritional interventions, particularly by starvation. The levels of SCAD, MCAD, and LCAD mRNAs were high in the heart of starved rats, whereas only slight increases or no significant changes were observed in the liver. Thus, it is possible that when rats are starved, fatty acid oxidation in the heart is enhanced by increased expression of ACD genes for use of fatty acids as the major energy source, in response to the decreased energy supply from other nutrients. It is important to note that expression of IVD is suppressed by starvation in both heart and liver.

In contrast to the starvation experiment, results from feeding fat-free or high fat were not as clear. For instance, we could not observe a clear cut correlation between the expression of ACD genes and dietary fat content. It is interesting to note, however, that the IVD mRNA level in rats which were fed a high fat diet was suppressed both in liver and heart. This and the decrease in IVD mRNA level during starvation may represent a protein-sparing effect of fatty acids. The marked increase of IVD mRNA in the heart of rats fed a fat-free diet is consistent with this hypothesis. Conservation of body protein is an important metabolic response during fasting (43, 44). However, the mechanism of protein-sparing has yet to be elucidated. Previous studies showed that during starvation, plasma glucose and insulin concentrations were decreased, whereas those of free fatty acids, ketone bodies, and glucagon were increased (45–47). Krebs (48) suggested that glucose and fatty acids, or their partial oxidation products, such as ketone bodies, could spare the essential amino acids for ongoing protein synthesis by decreasing their oxidation. Tessari et al. (49) demonstrated that the inverse relationship of leucine and long-chain fatty acid in their flux and oxidation in vivo. Thus, the induction of SCAD, MCAD, and LCAD expression, and the down-regulation of IVD expression during starvation as presented in this paper provide a likely molecular mechanism for protein sparing.

The significance of the effects of dietary protein manipulation on ACD expression was less clear, but nonetheless two clear tendencies were observed in the results. One was that the regulation of ACD expression in the heart was completely different from that in the liver, as in the case of other stimuli. The other tendency was that the IVD gene was regulated differently from fatty ACD genes in the heart. However, the metabolic basis for the increased amount of IVD mRNA in the heart during protein-free diet is not clear as were the uniformly suppressed expression of all ACDs following the feeding of high-protein or protein-free diet.

In this paper, we presented several lines of evidence indicating that the expression of the three fatty ACD genes were similarly regulated by various factors including the developmental pattern, the responses to hormones, such as dexamethasone and glucagon, and those to starvation and high fat diet. The marked enhancement of transcription of the three fatty ACD genes in riboflavin deficiency as previously observed (33) was also in line with the above observations. In riboflavin-deficient rats, transcription of the three fatty ACD genes was induced 4–8-fold, while no effects on the IVD and OTC genes were observed. These present and previous data together suggest the possibility that the three fatty ACD genes are coordinately regulated. In contrast, the expression of the IVD gene was subject to factors that are not shared by fatty ACD genes. It is unknown at present, however, whether or not the three fatty ACD genes mechanistically share common factors or regulatory motifs in their sequences. The mechanism of regulation of eukaryotic genes by these factors involves specific interactions of cis-acting DNA segments with
promoter elements such as TATA and CAAT boxes. It con-

sequences homologous to triiodothyronine/retinoic acidles-

and developmental stage-specific expression, as well to the

tains a number of sequences for the binding of transc~ption

increase the tran~riptional activity in response to members

binding site for GF1, as did the MCAD gene.  Unlike in the

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