Transgenic studies of fatty acid oxidation gene expression in nonobese diabetic mice

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Abstract **Type 1 diabetes mellitus is a devastating disorder affecting both glucose and lipid metabolism. Using the nonobese diabetic (NOD) mouse model, we found that diabetic mice had a liver-specific increase in steady state mRNA levels for enzymes involved in oxidation of fatty acids. Increased mRNA abundance was observed in very long-chain acyl-CoA dehydrogenase, long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), carnitine palmitoyltransferase I (CPT-1a), and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, whereas short-chain acyl-CoA dehydrogenase mRNA remained unchanged. In contrast, minimal elevations in LCAD and CPT-1a mRNA were observed in hearts of diabetic mice with no significant differences found for the other enzymes. We developed NOD mice with transgenes containing regulatory elements of human MCAD gene controlling a reporter gene to determine if the increase in MCAD gene expression occurred via the well-characterized nuclear receptor response element (NRRE-1). These results demonstrated that the transgene containing the NRRE-1 and adjacent 5′ sequences had elevated liver expression in diabetic mice compared with prediabetic or normal control mice. Surprisingly, the transgene that contains NRRE-1 with adjacent 3′ sequences and the transgene with the NRRE-1 deleted showed minimal response to the fulminant diabetic condition. Collectively, these results indicate that in type 1 diabetes there exists an excessive and liver-specific activation of fatty acid oxidation gene expression. Using human MCAD as a prototype gene, we have shown that this increased expression is mediated at the transcriptional level but does not occur via the well-characterized NRRE-1 site responsible for baseline expression in normal mice.**—Kurtz, D. M., L. Tian, B. A. Gower, T. R. Nagy, C. A. Pinkert, and P. A. Wood. **Transgenic studies of fatty acid oxidation gene expression in nonobese diabetic mice.** ***J. Lipid Res.*** **2000.** 41: **2063–2070.**

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Prominent aspects of type 1 diabetes mellitus include insufficient circulating insulin, resulting in an increased glucagon-to-insulin ratio associated with hyperglycemia and ketoacidosis (1). Although type 1 diabetes has been the focus of intense studies for many years, a detailed understanding of the pathophysiology of the disease has yet to be reached (2). Insulin replacement therapy and strict diet regulation have reduced the acute morbidity and mortality of human patients with type 1 diabetes, but the abnormalities in both glucose and lipid metabolism often lead to chronic problems including cardiovascular, neurological, and renal disease (1). These problems arise from prolonged aberrant metabolism. A better understanding of the metabolic derangements could help in finding ways to reduce the associated morbidity and mortality (1, 2).

It is recognized that hepatic gluconeogenesis and ketogenesis are excessive in patients with type 1 diabetes (1, 2). Currently, there is major interest in the roles of elevated free fatty acids (FFAs) and the peroxisome proliferator-activated receptor (PPAR) in regulating expression of the enzymes of fatty acid oxidation (FAO) and gluconeogenesis with particular relevance in diabetes (3, 4). For example, regulation of a prototype enzyme of mitochondrial FAO, human medium-chain acyl-CoA dehydrogenase (hMCAD; encoded by gene *ACADM*) occurs via the steroid/thyroid family of nuclear receptors (5–8), including PPAR (9), by interacting at a well-characterized response element known as the nuclear receptor response element-1 (NRRE-1). We demonstrated previously in normal transgenic mice that the NRRE-1 was pivotal in regulating MCAD expression in vivo, especially in heart and brown adipose, and to a lesser extent liver (10). Therefore, we hypothesized that in type 1 diabetes, when insulin becomes deficient for repressing li-

Abbreviations: CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CPT-1a, carnitine palmitoyltransferase I (liver isoform); CPT-1b, carnitine palmitoyltransferase I (muscle isoform); FAO, fatty acid oxidation; FFA, free fatty acid; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; MD, metabolic domain; NOD, nonobese diabetic; NRRE, nuclear receptor response element; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SCAD, short-chain acyl-CoA dehydrogenase; VLCAD, very long-chain acyl-CoA dehydrogenase.

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polysis, the resultant elevated FFAs would stimulate expression of the MCAD gene via PPAR and the NRRE-1 site, as well as other genes encoding enzymes of FAO and gluconeogenesis, particularly phosphoenolpyruvate carboxykinase (PEPCK, encoded by gene *Pepck*) (11). Many genes in these two pathways have been shown to be possibly regulated by elevated FFAs acting via PPAR (3, 4, 12). Therefore, stimulated expression of these enzymes would promote undesired ketogenesis with excessive acetyl-CoA supplied by increased FAO and hyperglycemia supplied by excessive gluconeogenesis. This would provide a mechanism independent of direct insulin action on the regulation of these genes in insulin-dependent diabetes mellitus.

We report here the use of the nonobese diabetic (NOD) mouse as an in vivo model of type 1 diabetes (13) to study altered gene expression of enzymes involved in FAO and gluconeogenesis. We measured steady state mRNA levels of the four mouse acyl-CoA dehydrogenases, very long-chain acyl-CoA dehydrogenase (VLCAD; encoded by *Acadvl*), long-chain acyl-CoA dehydrogenase (LCAD; encoded by *Acadl*), medium-chain acyl-CoA dehydrogenase (MCAD; encoded by *Acadm*), and short-chain acyl-CoA dehydrogenase (SCAD; encoded by *Acads*), as well as liver carnitine palmitoyltransferase I (CPT-1a; encoded by *Cpt-1a*) and PEPCK. In addition, we created transgenic NOD mouse lines that contained a chloramphenicol acetyltransferase (CAT) reporter gene under the control of human MCAD gene regulatory sequences. We report here that FAO gene expression is elevated in a liver-specific manner during the development of type 1 diabetes. The stimulated expression represented by the prototype MCAD gene occurs at the transcriptional level independent of the NRRE-1. The steriod/thyroid family of nuclear receptors including PPAR acts via the NRRE-1 as the predominant control mechanism in normal mice (10), but had virtually no additional effect in type 1 diabetes. Our sequence analyses of the acyl-CoA dehydrogenase genes indicate that there are several other possible target response element mechanisms involved (14–16).

MATERIALS AND METHODS

Mice

NOD/LtJ (NOD) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and further propagated at the University of Alabama at Birmingham. All mice were maintained in a specific pathogen-free barrier facility and housed in Microisolator™ cages (Lab Products, Maywood, NJ). They were tested by a regular health surveillance program and found to be negative for commonly tested murine pathogens. Mice were maintained on a standard autoclavable rodent diet [Teklad-LM485 (5.0% crude fat, 19% crude protein); Harlan Teklad, Madison, WI] and water ad libitum. All mice were monitored for development of diabetes by daily observation for polyuria. If polyuria was noted, urine was tested for the presence of glucose (Keto-Diastix; Bayer, Elkhart, IN). Positive glucosuric mice were retested 3 to 5 days later to confirm glucosuria and overt diabetes was diagnosed when serum glucose was above 17 mM. The incidence rate of diabetes as determined by serum glucose assays was monitored in all mice in the colony over a period of 12 months. All mice used for studies were between 12 to 24 weeks of age. Prediabetic, age-matched NOD mice were used as a control group in all studies. In the steady state mRNA study, adult mice of the ICR strain, the predecessor to the NOD strain, also were used as nondiabetic controls. In the transgenic studies, MCAD-CAT.1197 transgenic mice on a C57BL/6J × SJL/J-F_2 (B6SJLF_2) background were used as unrelated, nondiabetic controls. Animal care and euthanasia protocols were in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham.

Serum glucose, insulin, glucagon, and free fatty acid analyses

All samples for biochemical analysis were collected from experimental animals between 4 and 6 h after the beginning of the light cycle (between 10 AM and 12 PM). All experimental groups were fed ad libitum; prolonged fasting of diabetic NOD mice resulted in increased morbidity/mortality and was avoided. Serum glucose was measured in all mice used experimentally by the glucose oxidase method (Sigma, St. Louis, MO). Serum insulin was measured by double-antibody radioimmunoassay in 30 μl of serum in duplicate (sensitive rat insulin reagents; Linco Research, St. Charles, MO). Glucagon was assayed in duplicate 25-μl aliquots of serum, using a double-antibody kit (Linco Research). Serum free fatty acids were measured by an enzymatic, colorimetric method (NEFA-C reagents; Wako Diagnostics, Richmond, VA). The assay was modified to accommodate a reduced sample volume (10 μl), and use of a microplate reader for measurement of optical density at 550 nm.

RNA isolation and slot-blot analysis

Total RNA from liver and heart was isolated by the guanidium-acid phenol method as described (17). RNA slot-blot analysis was done with a Bio-Rad (Hercules, CA) 48-well slot-blot apparatus. The concentrations of total RNA applied to the slots ranged from 0.8 to 0.025 μg and were applied to Duralon (Stratagene, La Jolla, CA) membranes. All samples were evaluated within a linear range of signal.

Replicate filters of total RNA from liver and heart were made and probed separately with antisense RNA probes. Probes were synthesized as described (18) with incorporation of [α-^{32}P]CTP by T7, T3, or SP6 RNA polymerase (Promega, Madison, WI). The cDNA templates for probe synthesis were cloned into pGEM-11zf(+) or -3zf(+) (Promega), pPCR-II (InVitrogen, San Diego, CA), or pBluescript (Stratagene) vectors. The cloned cDNA probes included mouse VLCAD (19), LCAD (20), MCAD (21), and SCAD (22), as well as rat PEPCK (23) and CPT-1a (24). Hybridizations occurred at 65°C overnight in 50% formamide, 5× Denhart's solution, 5× saline-sodium citate (SSC), 1% sodium dodecyl sulfate (SDS), yeast tRNA (200 μg/ml), and salmon sperm DNA (200 μg/ml). All filters were washed in 1× SSC and 0.1% SDS at 65°C for 1–2 h. After washing, filters were exposed for autoradiography, using intensifying screens at −80°C.

After the inital probing and decay, the filters were reprobed with an antisense, human 18S ribosomal RNA probe (Ambion, Austin, TX) to control for RNA loading and normalization of densitometric readings. Densitometry was done with a Bio-Rad GS-670 densitometer. Gene-specific mRNA readings were corrected by subtracting background signal prior to normalization based on 18S rRNA values, and reported as average normalized optical density.

Production and characterization of the MCAD-CAT.1197, MCAD-CAT.372, and MCAD-CAT.318 transgenic mouse lines

The MCAD-CAT.1197 [−1197 to +189 of the MCAD regulatory sequence relative to the transcription start site (+1)], MCAD-CAT.372 (−372 to +189), and MCAD-CAT.318 (−318 to +189) transgene constructs (**Fig. 1**) were previously described (8, 10).

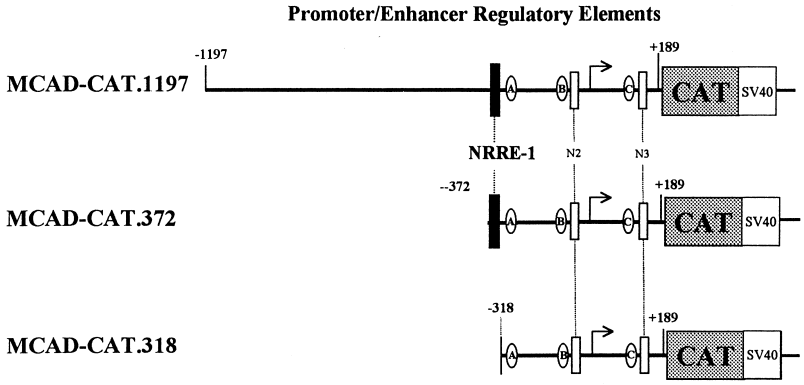

Fig. 1. Human MCAD transgenes. MCAD-CAT.1197 contains human MCAD gene regulatory sequences from −1197 to +189, in relation to the transcription start site (arrow), fused to a CAT reporter gene and the simian virus 40 (SV40) intron-polyadenylation signal. MCAD-CAT.372 contains the functional NRRE-1 regulatory element. The NRRE-1 site located at −345 to −306 contains hexamer-binding sites of the nuclear hormone receptors and is required for CAT reporter expression comparable to the endogenous MCAD gene in vivo (10). Additional MCAD regulatory elements defined previously (8) are represented by the symbols labeled A, B, and C (SP1-binding sites) and N2 and N3 (nuclear receptor response elements 2 and 3). MCAD-CAT.318 contains MCAD regulatory elements from −318 to +189. The hexamer-binding sites for the nuclear hormone receptors at the NRRE-1 have been deleted.

The gene constructs were microinjected into pronuclear one-cell, NOD mouse zygotes and implanted into pseudopregnant ICR mice. B6SJLF$_2$ nondiabetic transgenic mice were produced as described previously (10). Transgenic mice were identified by polymerase chain reaction analysis of DNA isolated from tail biopsies, using CAT gene-specific primers. Transgenic founders and offspring were bred to nontransgenic NOD mice to generate hemizygous offspring. If transgenic founders were unable to produce offspring because of the development of diabetes, only the founder animals were analyzed. Transgene copy number was determined by DNA slot-blot analysis. Five micrograms of genomic DNA from each transgenic line was applied to Hybond-N+ membrane (Amersham, Arlington Heights, IL) as described (25). A standard curve was generated by adding known amounts of a 600-bp *Nco*I−*Xba*I fragment of the CAT gene (from 0 to 100 copies per haploid genome) into 5 μg of genomic DNA from a nontransgenic NOD mouse. Membranes were probed with an [α-^{32}P]-dCTP-labeled (25), 600-bp *Nco*I−*Xba*I fragment of the CAT gene. All resulting images were analyzed by densitometry.

Tissue CAT analysis

Liver, heart, and skeletal muscle samples were isolated from transgenic diabetic and nondiabetic mice, snap frozen in liquid nitrogen, and stored at −80°C until analyzed. Protein extracts were prepared by sonication in 0.25 M Tris buffer, pH 7.4. The suspension was cleared by centrifugation. Protein concentrations were determined on supernatants by the Bradford method (26). CAT assays were performed on 10 μg (heart and skeletal muscle) or 25 μg (liver) of total protein by CAT enzyme-linked immunosorbent assay (Boehringer Mannheim, Indianapolis, IN) in duplicate and reported as picograms of CAT per milligram of total protein per transgene copy.

Statistical analysis

All values were reported as means ± standard deviation (SD) and analyzed by a one-way analysis of variance (ANOVA) for significant differences, using the Statistix 4.0 program (Analytical Software, Orlando, FL). Paired mean comparison was done according to the Tukey method with $P < 0.05$ accepted as significant.

RESULTS

Diabetic mice

NOD mice were classified as diabetic when their serum glucose exceeded 17 mM. Incidence rate and serum glucose results are summarized in **Table 1**. We found significantly ($P < 0.05$) elevated FFA concentrations in the diabetic mice with values almost double those of the unrelated B6SJLF$_2$ normal control transgenic mice; values were intermediate for the prediabetic NOD mice (**Fig. 2A**). We also found a markedly elevated glucagon-to-insulin ratio in the diabetic NOD mice as compared with both the unrelated normal control mice and the prediabetic NOD mice (Fig. 2B), although because of extreme variability associated with the disease process, the differences were not significant.

Steady state mRNA expression in type 1 diabetes

Steady state mRNA expression for enzymes involved in FAO and gluconeogenesis was measured in liver and heart. A total of 12 diabetic NOD mice, 4 prediabetic NOD mice, and 4 ICR mice were evaluated. RNA signals of the slot blots are reported in **Fig. 3** as average normalized optical density (means and SD) after normalization of signal with 18S rRNA expression. Differences in measured densitometry values between the prediabetic NOD and ICR mice were not observed and values were combined and reported as nondiabetic controls. In liver, significant elevations ($P < 0.05$) were observed in steady state mRNA levels for genes encoding MCAD (2.5-fold), LCAD (3.0-fold), VLCAD (1.9-fold), PEPCK (5.7-fold), and CPT-1a (3.1-fold) in the diabetic as compared with

TABLE 1. Diabetes incidence rate and serum glucose concentrations in NOD mouse colony

	Prediabetic	Diabetic[a]	Mean Age at Onset
			Weeks (range)
Females, n = 92	23 (24%)	69 (76%)	21 (13–30)
Males, n = 108	38 (36%)	70 (64%)	24 (16–44)
Glucose, mM[b]	11.3 ± 1.94 (n = 43)	48.0 ± 16.1 (n = 61)	

[a] Diabetes in NOD mice determined by presence of glycosuria (as indicated by Keto-Diastix).

[b] Determined by application of glucose oxidase method to 104 experimental of 200 total NOD mice (52%).

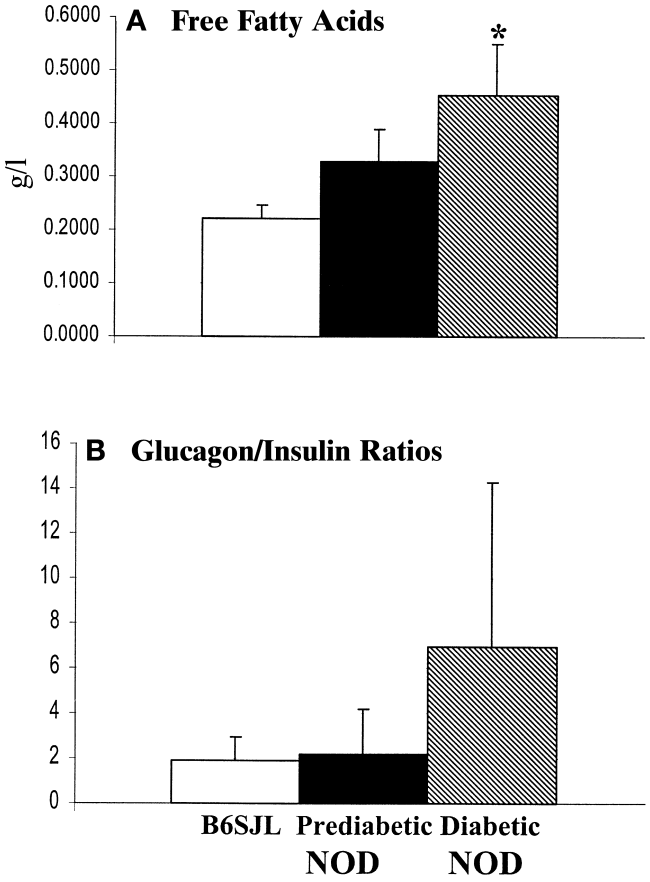

Fig. 2. Serum FFA concentrations and glucagon-to-insulin ratios. (A) Serum FFA concentrations (mean and SD) in nondiabetic controls (n = 3), prediabetic (n = 10), and diabetic (n = 12) NOD mice. The diabetic NOD group (hatched column) is significantly different ($P < 0.05$) from both the $B6SJLF_2$ group (open column) and the prediabetic NOD group (solid column). (B) Serum glucagon (ng/l)-to-insulin (pM) ratios from nondiabetic controls (n = 3), prediabetic (n = 10), and diabetic (n = 12) NOD mice. $B6SJLF_2$ nondiabetic controls, open column; prediabetic NOD mice, solid column; diabetic NOD mice, hatched column.

the nondiabetic controls. Significant ($P < 0.05$) but less pronounced elevations were observed in the heart for LCAD (1.3-fold) and CPT-1a (1.6-fold), while there were no significant differences found for the other enzyme RNAs evaluated in heart.

Tissue CAT expression of the MCAD-CAT.1197 (852 bp 5′/NRRE-1/+189) transgenic mice

Independent lines of transgenic NOD mice were produced by microinjection of the MCAD-CAT.1197 construct containing the NRRE-1, -2, and -3 sites (Fig. 1). One of the founders (line L1, integrating 14 transgene copies per haploid genome) was able to generate offspring for analysis. The founder of a second line (L3, integrating 10 transgene copies per haploid genome) was also analyzed. A total of 20 diabetic and 13 prediabetic mice that contained the MCAD-CAT.1197 construct was analyzed for CAT protein in liver, heart, and skeletal muscle. A significant elevation ($P < 0.05$) was observed in liver CAT protein levels (2.7-fold) in the diabetic as compared with the prediabetic mice (**Fig. 4**). Although much higher expression of CAT protein was observed in both the heart and skeletal muscle as compared with liver in all groups, there were no significant differences in CAT levels between diabetic and prediabetic NOD transgenics. Because all but one of the mice analyzed were derived from the same founder, integration site effects on transgene expression were controlled for between the diabetic and prediabetic controls. The CAT expression results observed in the prediabetic NOD mice were significantly higher ($P < 0.05$) than those found in normal $B6SJLF_2$ mice with the same transgene. Therefore, we saw significantly increased expression of the endogenous genes for FAO and PEPCK in liver from diabetic mice, which occurred in parallel with the increased expression of MCAD-CAT.1197 transgene.

Tissue CAT expression in the MCAD-CAT.372 (NRRE-1/+189) transgenic mice

Six independent transgenic lines were produced with the MCAD-CAT.372 construct, which includes an intact NRRE-1 site and also the NRRE-2 and -3 sites. None of these founder mice produced offspring prior to analysis. Three transgenic NOD mice became diabetic while three remained prediabetic. Surprisingly, we found low liver expression in all six lines analyzed, whether diabetic or prediabetic (Fig. 4). These liver expression results were indistinguishable from those of the transgenic mice with the entire NRRE-1 sequence deleted (MCAD-CAT.318). In skeletal muscle and heart there were no differences in CAT values among all six lines analyzed, whether diabetic or prediabetic.

Tissue CAT expression in the MCAD-CAT.318 (no NRRE-1; −318 to +189) transgenic mice

Ten independent lines were produced that contained the MCAD-CAT.318 construct, in which the NRRE-1 site had been deleted while retaining the NRRE-2 and -3 sites. Copy number ranged from 3 to 52 copies per haploid genome as analyzed by DNA slot-blot analysis (data not shown). Three of these lines (S1, S2, and S4) produced offspring for analysis. Analysis of all remaining lines consisted of the founder mice only.

A total of 29 diabetic and 21 prediabetic mice with this construct were analyzed. CAT protein levels in the liver, heart, and skeletal muscle were reduced to little or no activity in both diabetic and nondiabetic animals. Nine of the 10 lines produced detectable levels of CAT protein, showing that the transgene itself was functional. CAT expression levels are summarized in Fig. 4.

DISCUSSION

Two major features of acute type 1 diabetes include hyperglycemia and ketoacidosis (1, 2). Therefore, we were searching for an underlying gene regulation mechanism, independent of insulin deficiency only, which may affect both metabolic pathways, using members of the steroid/

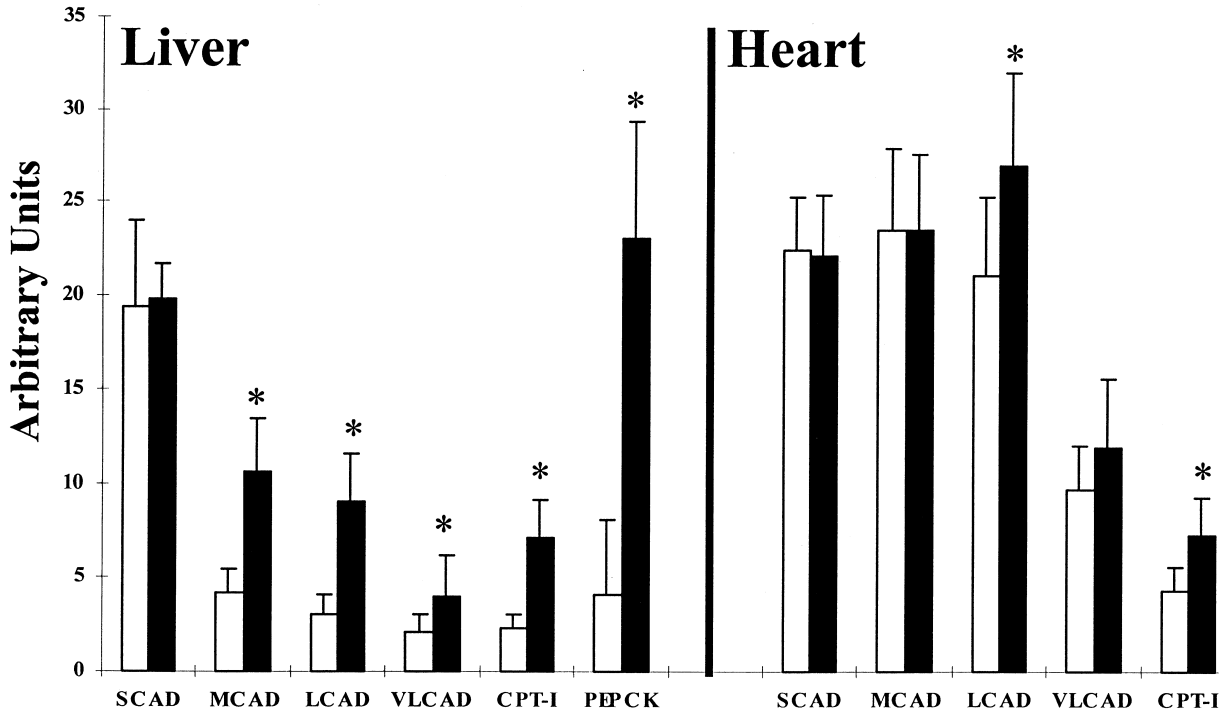

Fig. 3. mRNA expression of genes encoding enzymes of fatty acid oxidation and gluconeogenic enzymes. Total RNA was isolated from liver and heart from diabetic (n = 12) and nondiabetic NOD (n = 4) and ICR (n = 4) control mice. Nondiabetic controls, open columns; diabetic NOD mice, solid columns. An asterisk (*) indicates significant ($P < 0.05$) differences between diabetic and nondiabetic mice.

thyroid family of nuclear receptors (27) such as PPARα. Hepatic glucose production is increased in type 1 diabetes as a result of overproduction and excessive activity of PEPCK, the rate-controlling step of the pathway (28, 29). PEPCK expression has been well studied and shown to have a complex regulatory mechanism that not only includes the influence of insulin and glucagon, but several other ligand/nuclear receptor mechanisms including PPAR (29–39). Ketogenesis is dependent on a supply of acetyl-CoA produced by mitochondrial FAO. Fatty acid oxidation is dependent first on the carnitine-mediated transport of long-chain fatty acids across the mitochondrial membrane, in part requiring CPT-I- and CPT-II-regulated activity (2). Subsequent flow of acyl-CoA through the enzymatic steps of β-oxidation ultimately produces acetyl-CoA for ketogenesis. In addition, mitochondrial β-oxidation of fatty acids includes a dehydrogenation step, which is considered to be rate limiting. This step is catalyzed by a family of chain length-specific acyl-CoA dehydrogenases, VLCAD, LCAD, MCAD, and SCAD. Of the four genes, MCAD is the best characterized at the molecular level.

MCAD expression is regulated in part by several members of the steroid/thyroid family of nuclear receptors, including retinoic acid receptors (RAR/RXR) (5), hepatic nuclear factor 4 (HNF-4) (7), chicken ovalbumin upstream promoter transcription factor (COUP-TF) (6), and PPAR (9). These nuclear receptors have all been found to act via a response element of the MCAD gene designated the NRRE-1. We found striking sequence similarities among human and mouse NRRE-1 sequences and the corresponding response element of rat PEPCK gene (32) (**Fig. 5**), the metabolic domain (MD) or glucocorticoid response element. This MD region contains response element sequences for interaction with RAR/RXR (35, 36), HNF-4 (39), COUP-TF (39), and glucocorticoid receptor (29, 34). The transcription factors HNF-4, RAR/RXR, and COUP-TF in this region are the same transcription factors described above for MCAD expression. Because PEPCK expression was increased in diabetes via mechanisms that included the MD response element, we hypothesized that FAO enzymes, particularly the acyl-CoA dehydrogenases, would also have increased expression and this would be mediated by a fatty acid-activated PPAR signal via the NRRE-1 response element modeled transgenically with MCAD transgenes (10). In addition, there has been major interest in not only the role of PPAR in mechanisms of diabetes (3, 4), but specifically the role of PPARα in expression of *CPT-1b* and the acyl-CoA dehydrogenases, because fatty acids appear to be an important ligand for PPAR activation of transcription of the genes encoding these enzymes (3, 4, 12). Thus, this establishes a direct substrate link to regulated expression of these genes and potential regulation of the FAO pathway. This led to our hypothesis that elevated FAO enzymes, needed to provide acetyl-CoA for excessive ketogenesis in type 1 diabetes, may share a mechanism shown to play a role in elevated expression of PEPCK in type 1 diabetes, that is, PPAR. We hypothesized, on the basis of the remarkable sequence homology, that the MD element of PEPCK may respond to PPARα in the case of FFA-mediated increased expression (11) of PEPCK in liver. Thus, this approach may demonstrate a mechanism independent of insulin by which these genes and re-

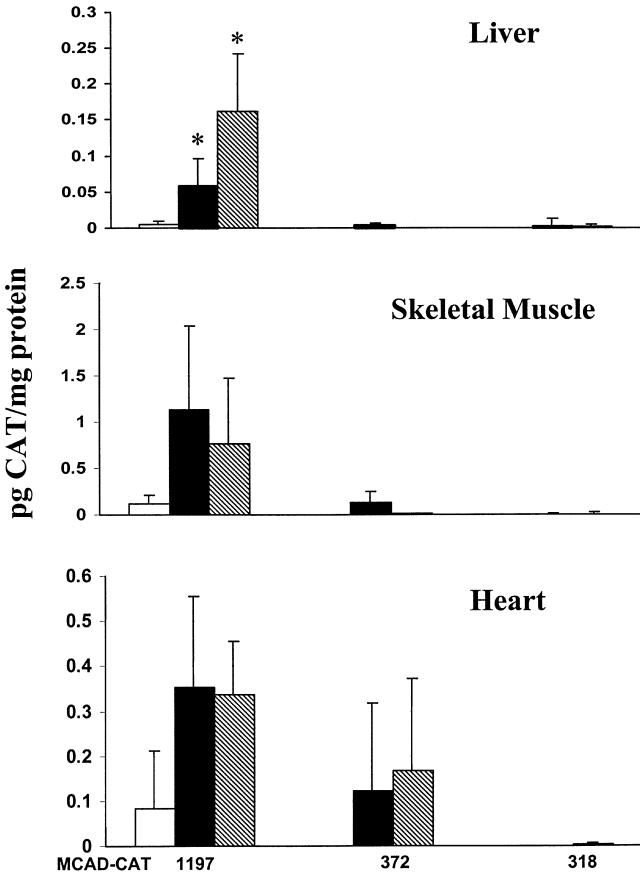

Fig. 4. CAT expression results for the human MCAD transgenes. We find a significant elevation (* $P < 0.05$) in CAT expression from the longest construct, MCAD-CAT.1197 (835 bp 5′/NRRE-1/+189), in the liver of the diabetic NOD mice as compared with the prediabetic NOD and nondiabetic, B6SJLF$_2$ groups. In addition, there exists a trend for an increase in CAT expression in both skeletal muscle and heart in the NOD mice (both diabetic and prediabetic) as compared with the B6SJLF$_2$ mice; however, these differences were not significant. Expression of MCAD-CAT.372 construct (containing the intact NRRE-1 site) in the diabetic and prediabetic NOD mice was similar to that seen in the B6SJLF$_2$ mice possessing the MCAD-CAT.1197 construct. We previously have reported similar CAT expression in B6SJLF2 mice for the MCAD-CAT.1197 and MCAD-CAT.372 transgenes (10). In contrast, the markedly elevated CAT expression in liver from the MCAD-CAT.1197 transgene in the NOD mice shows that the NRRE-1 site is not responsible for the elevated liver expression. Elimination of the NRRE-1 site (MCAD-CAT.318) virtually abolishes CAT expression in all tissues. B6SJLF$_2$ nondiabetic controls, open columns; prediabetic NOD mice, solid columns; diabetic NOD mice, hatched columns.

spective metabolic pathways may be influenced by new therapeutic agents directed at disrupting the PPAR-mediated events that occur to an excessive level, specifically gluconeogenesis and ketogensis.

We have demonstrated here that steady state mRNA levels of genes encoding enzymes of FAO and PEPCK are all markedly increased in liver with the exception of the SCAD gene in acutely diabetic NOD mice (Fig. 3). This included not only the acyl-CoA dehydrogenases, including MCAD, but also CPT-1a and PEPCK. In contrast, there were only relatively minor increases in LCAD and CPT-1a in heart tissue from the same diabetic NOD mice (Fig. 3). These FAO genes are at a relatively high expression level in normal mice because of the high reliance of the normal heart on FAO. That is, they may be at maximal expression, thus not responding further to the diabetic condition.

To test the hypothesis of fatty acid-activated/PPAR-stimulated expression of FAO enzymes, we used transgenes for the prototype FAO enzyme MCAD to evaluate the role of the NRRE-1 in the increased expression of the endogenous MCAD gene observed in acute type 1 diabetes. We demonstrated previously in normal mice that the NRRE-1 was crucial for regulated, high level expression of MCAD, particularly in heart and brown fat, and that the overall expression of the endogenous MCAD gene and the MCAD transgenes is much lower in liver than heart and brown fat, although the NRRE-1 was still required for at least one-half of the expression observed (10). Therefore, we expected liver MCAD expression in type 1 diabetes to be increased to levels similar to those found in the normal heart and that this would be mediated via the NRRE-1 coincident with rising FFA concentration (Fig. 2). We find, as shown in Fig. 4, that there is markedly increased CAT expression in liver with the longest MCAD transgene (MCAD-CAT.1197), which contains not only the NRRE-1 but approximately an additional 800 bp of upstream adjacent 5′ regulatory sequence. As shown in Fig. 4, when the adjacent sequences 5′ to the NRRE-1 are deleted, the excessive expression is eliminated, even though the NRRE-1 remains intact. Surprisingly, this shows that the NRRE-1 site of MCAD, found to be critical in regulated expression in normal mice via PPAR and other nuclear receptors, plays no role in the excessive liver expression found in the diabetic NOD mice. At this point, we cannot rule out the presence of a cryptic nuclear receptor responsive region in this adjacent 5′ sequence that is critical to MCAD regulation during periods of extreme metabolic derangement. In addition, there are possible regulatory elements based on sequence consensus such as an E-box responding to USF, a CArG box responding to insulin, and CRE that responds to cyclic AMP response element-binding protein, that would likely respond to elevated glucagon in an insulin-deficient state such as type 1 diabetes in NOD mice. As noted in Fig. 4, the level of expression for the MCAD-CAT.1197 transgene in nondiabetic mice (B6SJLF$_2$) was similar to what we found previously (10), but well below the level we found in prediabetic NOD mice with the same transgene. This may result from undetermined strain differences between B6SJLF$_2$ and NOD mice. In addition, early "diabetogenic" programs may already be at work in prediabetic NOD mice that cannot be measured biochemically, yet result in the differences in gene expression compared with normal mice (B6SJLF$_2$). Glucagon has been shown to be increased in the NOD mice prior to absolute insulin deficiency (40). Although we did not observe a notable increase in glucagon/insulin ration in the prediabetic NOD compared with the normal mice (B6SJLF$_2$), the trend for an increase in serum FFA (Fig. 2) may indicate an increase in substrate availability

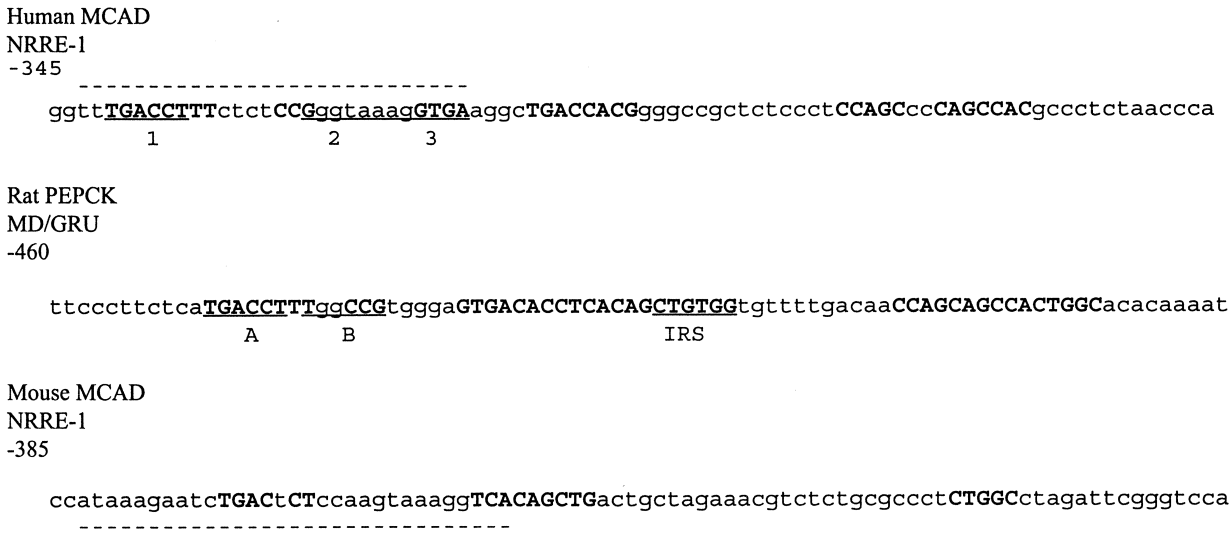

Fig. 5. Comparison of the MCAD gene and PEPCK gene regulatory regions. Sequences of the human and mouse MCAD gene regulatory regions designated as the nuclear receptor response element-1 (NRRE-1) are compared with the rat PEPCK gene regulatory region known as the MD or glucocorticoid response unit (GRU). The capital letters indicate exact sequence homology when the PEPCK sequence is compared with either the human or mouse MCAD gene sequences. There are 45 of the 89 nucleotides of the PEPCK sequence displayed that have the exact sequences in segments when compared with one or both species of the MCAD gene sequences. The dashed lines above and below the MCAD gene sequences indicate regions examined previously by electromobility shift assays (EMSA) to bind several nucle ar hormone receptors. The glucocorticoid response sequence (GRS) of the rat PEPCK regulatory region has been mapped to a region between −455 to −402 [relative to transcription start site (+1)] and is responsible for regulated PEPCK expression in diabetes. The underlined regions indicate specifically mapped nuclear hormone receptor-binding sites. The human MCAD hexamer-binding sites designated 1, 2, and 3 interact with RAR/RXR, HNF-4, PPAR, and COUP-TF. The PEPCK regions designated A and B interact with RAR/RXR, HNF-4, as well as COUP-TF.

and an increase in transgene expression. Because one of the MCAD-CAT.1197 NOD transgenic lines was passed through the germline, we were able to make the comparison between the prediabetic and diabetic lines using the same NOD transgenic line, thus controlling for any transgene integration site effects. The overall MCAD transgenic results for muscle and heart were not significantly different between the prediabetic and diabetic states, and they had a pattern of expression similar to those we saw previously in nondiabetic mice (10), indicating that type 1 diabetes had little effect.

In summary, we find that the spontaneous model of type 1 diabetes in the NOD mouse appears to have many metabolic features similar to those described in human type 1 diabetes, although there are few reports describing the aberrant metabolic characteristics of this model. These mice develop an autoimmune-mediated insulitis resulting in a total insulin deficiency (13) and acute diabetes usually by 20 weeks of age as shown in Table 1. We establish that FAO enzyme gene expression in liver is stimulated as reflected in steady state mRNA analyses of CPT-1a, and the acyl-CoA dehydrogenases, except SCAD, and this occurred in parallel with elevated PEPCK expression. This would fit with a pattern of stimulated expression of this pathway to supply acetyl-CoA substrate for excessive ketogenesis found in type 1 diabetes. We also showed minor to no increased expression of the endogenous genes of FAO in heart from diabetic and control mice. Next, we demonstrated that the increased expression seen using MCAD transgenes is apparently mediated through mechanisms other than PPAR activation via the NRRE-1 of MCAD. These results demonstrate that the increased activity of a pathway important in the pathogenesis of disease may not necessarily be simply an exaggeration of a normal mechanism.

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