Characterisation of the rodent genes for arylacetamide deacetylase, a putative microsomal lipase, and evidence for transcriptional regulation.

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ABSTRACT

In the current study, we have determined the cDNA and the genomic sequences of the arylacetamide deacetylase (AADA) gene in mice and rats. The AADA genes in the rat and mouse consist of 5 exons and have 2.4 kb of homologous promoter sequence upstream of the initiating ATG codon. AADA mRNA is expressed in hepatocytes, intestinal mucosal cells (probably enterocytes), the pancreas and also the adrenal gland. In mice, there is a diurnal rhythm in hepatic AADA mRNA concentration, with a maximum 10 hours into the light (post-absorptive) phase. This diurnal regulation is attenuated in peroxisome proliferator-activated receptor α knockout mice. Intestinal but not hepatic AADA mRNA was increased following oral administration of the fibrate, Wy-14,643. The homology of AADA with hormone-sensitive lipase and the tissue distribution of AADA are consistent with the view that AADA plays a role in promoting the mobilisation of lipids from intracellular stores, and in the liver for assembling VLDL. This hypothesis is supported by parallel changes in AADA gene expression in animals with insulin deficient diabetes and following treatment with orotic acid.
Running title: Regulation of the arylacetamide deacetylase gene.

Abbreviations: AADA, arylacetamide deacetylase; COUP-TF/HNF4, chicken ovalbumin upstream promoter transcription factor/hepatocyte nuclear factor 4; EST, expressed sequence tag; HSL, hormone-sensitive lipase; GRE, glucocorticoid response element; PAC, phagemid artificial chromosome; PPAR, peroxisome proliferator-activated receptor; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; TAG, triacylglycerol; TGH, triglyceride hydrolase; VLDL, very low density lipoprotein.

Keywords: Lipase, lipid stores, liver, PPARα, secretion, transcription.

The nucleotide sequences in this paper have been submitted to GenBank with accession numbers AF182426 (rat cDNA), AF264017 (rat genomic DNA) and AF306788 (mouse genomic DNA).

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INTRODUCTION

It is now clear that one or more lipases must play a role in the secretion of very low density lipoprotein triacylglycerol (VLDL-TAG) from the liver. A large proportion of the VLDL-TAG secreted is derived from the cytosolic TAG storage pool in hepatocytes (1). This pool enables short term storage of TAG in the immediate post prandial period, and its subsequent secretion as VLDL-TAG when the level of circulating chylomicrons falls. Thus the store may act as a buffer to prevent post prandial hyperlipidemia (for review see (2)). For VLDL assembly, the stored TAG undergoes intracellular lipolysis either to glycerol plus fatty acids (3) or to mono- plus diacylglycerol (4) followed by reesterification to TAG (3, 4). In addition to the mobilisation of stored TAG, there is also evidence that the lipids associated with nascent VLDL in the ER lumen undergo remodelling prior to secretion (5) and this may also involve the action of the same or of a distinct lipase. The identities of the relevant lipases have not been established. Lehner et al have proposed that both triglyceride hydrolase (TGH - an ER luminal carboxylesterase) and other lipases together play a role in the mobilisation of stored lipid for secretion (6-8). Of particular interest, they have shown that TGH is not expressed during the suckling period of life, and have suggested that this results in decreased VLDL secretion during this phase (7).

Arylacetamide deacetylase (AADA) is a 45 kDa esterase with an uncleaved amino terminal signal anchor sequence. The enzyme was purified from human liver (9) and the human cDNA cloned by Probst et al (10) during studies of carcinogen metabolism. Immunoblotting of human tissues led Probst et al to conclude that the enzyme is expressed in liver and small intestine, but not in the bladder, which is particularly susceptible to arylamine induced carcinogenesis (10). The presumed active site domain of AADA shows considerable homology to that present in hormone-sensitive lipase (HSL), so that the enzyme has been classified as a lipase (10), and see Fig. 1. We have proposed that AADA,
also, may function as a lipase during the process of lipoprotein secretion, and preliminary transfection evidence has supported the possibility that AADA promotes TAG secretion from hepatoma cells (2).

In the current study, we have determined the cDNA and the genomic sequences of the AADA gene in mice and rats. This has enabled the development of an RT-PCR assay, and has indicated that transcription varies throughout the diurnal cycle in a manner consistent with its proposed role, and also changes under certain regimes associated with altered hepatic lipid secretion.
MATERIALS and METHODS

Identification and characterisation of cDNA and genomic clones. Mouse AADA EST clone AA419661 was identified by homology with the published human cDNA sequence (10) and was obtained from the Medical Research Council Human Genome Mapping Project, Cambridge U.K. A mouse cDNA probe was obtained by PCR amplification of AA419661 with Taq polymerase of a 405bp fragment using the forward oligonucleotide ATCTCTGTGGTCTTGTGA and reverse oligonucleotide GCCTCCATCATGAATGAAACA. The product was labelled with $^{32}$P-dCTP (Rediprime II, Amersham Pharmacia Biotech, Little Chalfont, U.K.), purified on a Sephadex G50 spin column (Amersham Pharmacia Biotech), and then used to screen a rat liver λ triplex cDNA library, (BD Clontech, Basingstoke, U.K.), plated at 25 000 plaques per dish. Positive plaques were identified by hybridisation at medium stringency (3 sequential 0.4x SSC washes at 62°C) to duplicate Hybond N filters (Amersham Pharmacia Biotech), and were purified by secondary and tertiary plating. To analyse the cDNA inserts, bacteriophage were converted to ampicillin-resistance conferring plasmids using the cre-lox excision sites present in λ triplex. Plasmid DNA was purified on silica-based columns (Qiagen, Crawley, U.K.) and then sequenced using the ABI Prism (Applied Biosystems, Foster City, CA, U.S.A.) Big Dye cycle sequencing kit and the products analysed on an ABI model 377 automated sequencer.

5'RACE was carried out using Marathon RACE - ready rat liver cDNA exactly as described by the manufacturers (BD Clontech). Total cDNA was PCR- amplified with the Advantage 2 polymerase mix using a primer to the 5’ tag sequence and a gene specific primer (CAGCACTCCCAACACCAGCCAC) derived from the cDNA sequence. Bands approximating to the predicted size of the desired product were excised from the gel and reamplified using Taq DNA polymerase. Southern blotting confirmed the presence of AADA-related sequences among the
reamplified products. The reamplified fragments were gel purified and subcloned into T-vector (Promega, Southampton, U.K.), and positive subclones detected by colony hybridisation and the recombinant plasmids sequenced.

Rat cDNA inserts and the mouse 405 bp PCR product (above) were next used to screen gridded filters representing mouse and rat PAC genomic libraries, (constructed by Kazutoya Osoegawa and Pieter de Jong and supplied by the Medical Research Council Human Genome Mapping Project). Hybridising clones were grown to an optical density of 0.15U in 400ml of 2xYT medium, (bacto-tryptone; 16g/L, bacto-yeast extract; 10g/L, NaCl; 5g/L) and induced with 0.15mM isothiopropyl β galactoside to promote P element replication. Plasmid DNA was purified on silica-based columns (Qiagen), and elution was performed at 65°C to enhance recovery of large DNA molecules. The large insert size of the PAC clones (130-150kB) precluded direct analysis, therefore restriction fragments were shot-gun subcloned into *Bam*HI, *Eco*RI or *Hind*III digested and dephosphorylated pUC 18 (Amersham Pharmacia Biotech). Subclones containing AADA exonic sequences were identified by hybridisation of ^32^P labelled cDNA to denatured bacterial colonies immobilised on Hybond N filters. To obtain internal sequence within subclones, either HPLC-purified gene specific oligonucleotides (Amersham Pharmacia Biotech) were used as primers or deletions were made between suitably positioned internal restriction sites and polylinker sites, where necessary generating blunt ends with T4 DNA polymerase. The pUC subclones mH4 and mE1 which do not span exonic sequences, were obtained by screening with fragments of intronic sequence derived from the subclones mB1 (see Fig. 3), (following amplification with oligonucleotides GATTGGATTGGGTAGGCGCTG and AGTGCCCTTTGAAACAGTG) and mH1 (following isolation of a 646 bp *Hind*II- *Eco*RI fragment).
Promoter function

Sequences (2180, 1679, 941 and 66bp) spanning the putative mouse promoter region and extending into the 5’UTR were PCR amplified with Taq polymerase using the following forward gene specific oligonucleotides: 5’-GCAGTAAGTGGTACC

GTAGTTCT-3’,
5’-GACTCTCATTTTCTTTTCATAG-3’, 5’-AGATCTAAATTCAACATCCAA-3’, and a common reverse oligonucleotide 5’-TAACCTGCCAAAAGCAGATCTAAGCTTAGG-3’. Products were isolated from agarose gels and were subcloned into T-vector (Promega). Fragments were excised by digestion in each case at the introduced HindIII (underlined) site, together with either the introduced KpnI (underlined) site or the natural BamHI or SacI sites and transferred to suitably digested pGL3 basic vector (Promega). The recombinant plasmids (1ug) were cotransfected with pSV-βgal (0.5ug, Promega) into subconfluent HepG2 cells (0.5x10^6 per well) using the lipofectin procedure as described by the manufacturer (Life Technologies, Paisley, UK). Cells were harvested after 48h, and were assayed for β-galactosidase and luciferase activity as previously described (11).

Northern blotting

For Northern blotting, total RNA was purified using a column binding protocol (RNeasy), as described by the manufacturer (Qiagen). Approximately 30mg of rat male adult liver (Sprague Dawley 200g), foetal liver (Sprague Dawley, embryonic day 19), neonate liver (Sprague Dawley, 14 days post natal) or 30mg of mouse liver (ex breeder female C57/black 6) was ground to a fine powder with a mortar and pestle under liquid N2. RNA was also prepared from 2x10^7 HepG2 cells (a gift from Dr Philippa Talmud, the Rayne Institute, University College London) which were cultured in Dulbecco’s modified Eagle’s medium containing Glutamax (Life Technologies, Paisley, U.K.) supplemented with 10% foetal calf serum. Total RNA was eluted in a volume of 50µl of RNase free water. The integrity
of the total RNA preparation was checked by electrophoresis of 5µl of eluate (prestained with 10ng of ethidium bromide) onto a 1 % MOPS-formaldehyde agarose gel (12) and visualising the 28S and 18S rRNA bands. Resolved RNA was transferred to Hybond N+ membranes (Amersham Pharmacia Biotech) by capillary transfer in 10x SSC (1.5M NaCl, 0.15M sodium citrate) and the membranes probed with 32P cDNA as described. Additionally these blots were probed with a 203 nucleotide fragment of mouse triglyceride hydrolase (EST AA245304) amplified with the forward oligonucleotide AGTCCTGGGGAAGTACGTC and the reverse oligonucleotide ATCTTGGGAGCACATAGG, or with a full-length human lysosomal lipase cDNA (13), a kind gift from Dr Richard Anderson, (Wake Forest University School of Medicine, North Carolina, U.S.A.) or with β actin cDNA (BD Clontech).

A human multiple tissue RNA dot blot and human multiple tissue Northern blots were obtained from BD Clontech and were probed initially with a human AADA full-length cDNA probe derived by ligating fragments from EST clones N76660, H71389, T83264, H71337, H65969. Hybridisation and washing conditions were exactly as described by the manufacturers. Subsequently the blots were stripped and reprobed with the HSL cDNA (14) a kind gift from Dr Cecilia Holm (Lund University, Sweden) and β actin cDNA.

Quantitation of mRNA in animal tissues and in isolated cells.

i) Animals Male Sprague Dawley rats (220g starting weight) were rendered diabetic by subcutaneous injection of streptozotocin (80mg/kg body weight of a freshly prepared 80 mg/ml solution in 25mM sodium citrate, 150mM NaCl pH4.5). A control group was injected with citrate-buffered saline alone. Both groups were maintained on a standard laboratory chow diet ad libitum, and additionally were given access to a 10% D-glucose solution overnight following injection to offset acute hypoglycaemia. In some cases, diabetic animals were treated with 3 units of soluble insulin and
4 units of Zn\(^{2+}\) insulin complex 24h before killing as described (15). Animals from the control and diabetic groups were killed after 7 days and tissues immediately frozen in liquid N\(_2\). Glycosuria (>10mM) was verified in each of the diabetic animals but was excluded in the controls by withdrawal of urine from the bladder for a Clinistix test (Bayer PLC, Newbury, U.K.). The establishment of diabetes was also apparent from changes in body weight. An average 38 ± 3.44g (SEM) increase was apparent in the control animals while a 28 ± 6.7g (SEM) decrease in body weight was observed in the treated animals.

In other experiments male Sprague Dawley rats were fed with fish oil, orotic acid or fructose for 14 days essentially as previously described (16-18). Powdered low fat laboratory chow (breeding diet no3, Special Diet Service, Witham, Essex, U.K) was reconstituted after mixing with either with 8g/800g orotic acid (mixed as an aqueous solution) or with 18% (w/w) fish oil (Max EPA, containing 18% C20:5 and 12% C22:6 Seven Seas Ltd, Kingston–upon-Hull). The modified diets were reformed into pellets and then dried with appropriate antioxidants described in (16, 17). For fructose administration 10% (w/v) fructose was present in the drinking water throughout (18).

The diurnal regulation of AADA transcription was investigated in male PPAR\(\alpha\) knockout mice bred onto a SV/129 genetic background, and using wild type SV/129 mice as a control. Mice were maintained in temperature controlled rooms (22-24°C) on a 12 h light/12 h dark cycle. Food (low fat pellets as above) was available ad libitum. Mice were used between the ages of 14 and 20 weeks as described (19). Food consumption for both genotypes was similar between the groups and in each case was approx 4 fold higher in the dark than the light phase (\(P<0.001\) in each case). The livers were removed, frozen and ground to a powder under liquid N\(_2\) and then used to prepare total RNA by the acid guanidium thiocyanate method (20). In other experiments control and PPAR\(\alpha\) knockout mice were fed ad libitum for 14 days a diet reconstituted with Wy-14,643 (Chemsin laboratories, Lenexa,
KS, U.S.A.) (0.1%) (21). Animals were killed either at the midpoint of the dark (D6) or light (L6) phases.

ii) Isolated cells Adipocytes were obtained from rat epidydimal fat pads essentially as described in (22), by digesting finely chopped pieces in Krebs Ringer bicarbonate buffer containing 20mg/ml bovine serum albumin, 1mg/ml collagenase (type II, 210U/mg, Worthington Biochemical Corporation, Lakewood, NJ, U.S.A.) and 5mM glucose with vigorous shaking and aeration (O2/CO2 (95:5)) at 37°C. Following filtration through nylon gauze the cell mass was centrifuged for 20s at low speed and the floating adipocyte layer was aspirated and rewashed in fresh buffer plus albumin and glucose. Total RNA was prepared by the RNeasy protocol (Qiagen) from approximately 4ml of packed adipocytes.

Intestinal cells enriched in enterocytes were obtained by immersing and flushing rat small intestine in ice cold Krebs Ringer bicarbonate solution, everting the intestine over a glass rod and scraping the mucosal cells into plastic tubes precooled with liquid N2. Total RNA was prepared by the Qiagen protocol from approx 30mg of tissue. Samples of whole intestine tissue were also flash frozen in liquid N2 and ground to a fine powder prior to isolation of total RNA. J774 cultured mouse macrophages were a kind gift from Dr Lisa O’Rourke (Department of Biochemistry and Molecular Biology, University College London). Total RNA was prepared by the Qiagen protocol from approximately 5x 10^7 cells.

iii) RT-PCR AADA mRNA was assayed by reverse transcription (12) followed by real-time PCR using an ABI PRISM Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA, U.S.A.) essentially as described by the manufacturers. PCR oligonucleotides and dually modified probe oligonucleotides containing TAMRA (N’N’N’N’ tetramethyl-6-rhodamine) and either FAM (carboxy fluorescein) or VIC were manufactured by Perkin Elmer Biosystems, and are shown in Table I.
AADA mRNA assays were carried out in 30ul of TaqMan Universal PCR master mix containing 300nM of each primer and 200nM probe under the standard conditions recommended by the manufacturers. Primers (67nM) and probe (85nM) for β-actin were included in each sample to act as an internal standard to correct for assay variation. Amplifications of 18S rRNA were carried out with the primers supplied as a standard kit by ABI in triplicate reactions. All values were related to a curve generated by a standard liver preparation.

(iv) Nuclear run on transcription

Nuclei were prepared as previously described (23) from diabetic, orotic acid treated and diabetic rats treated with insulin (as above). Nuclear run on was performed essentially as described in (24, 25). 32P-labelled RNA was hybridised to AADA or GAPDH cDNA, which had been spotted onto Hybond-N membranes. After extensive washing the spots were excised and the radioactivity assayed by scintillation counting.

Nucleotide and peptide alignments and searches were carried out using the programs multalin (26), GCG (27) and blast (28).
RESULTS

cDNA and genomic sequences

Fig. 1 shows alignments of the predicted peptide sequences of human (10), rat and mouse arylacetamide deacetylase. The rat cDNA sequence was determined by comparison of 21 independent cDNA clones derived from a liver cDNA library. The mouse peptide sequence was deduced from the sequences of the relevant EST clones (AI182380 and AI574013) and was subsequently confirmed by determining exonic sequences from the mouse gene (see below). The predicted rat and mouse sequences are each one amino acid residue shorter than the human sequence within the amino terminal signal anchor domain. As previously described, (10), homology is apparent with HSL particularly over a region spanning an upstream HGGG box characteristic of lipases and the active site motif, GXSXG. In addition, a further segment of homology between AADA and HSL includes an aspartic acid and histidine residue known to comprise the catalytic triad of HSL (29).

Fig. 2A shows alignments of the 3’ region of the AADA cDNAs. The 3’UTR of the available mouse EST clones is shorter than the human 3’ region, owing to the presence of a polyadenylation signal 47 nucleotides from the termination codon. The rat 3’UTR of three independent rat clones was similar in length to the human 3’ UTR. Although a cryptic polyadenylation signal was present in the rat sequence at a similar point to the polyadenylation signal in the mouse, (underlined in Fig. 2A), the 3’UTR extended instead to a downstream polyadenylation signal. Preferential use of the second polyadenylation signal in the rat is apparent on Northern blots of mouse and rat liver RNA (Fig. 2B) in which the rat transcript appears approximately 300bp longer. Sequencing the cDNA clones did not define the extent of the 5’UTR in the rat, nor were the available mouse EST clones fully informative. It was anticipated that the identification of potential promoter elements in the rat and mouse genomic sequences would clarify the situation.
The genomic sequencing strategy, (Fig. 3), revealed that the rodent AADA gene is comprised of 5 exons with intron-exon boundaries which were clearly identifiable by consensus splice donor and acceptor sequences (Table II). The active site motif is encoded in exon 4. There was extensive homology between the rat and mouse sequences, which extended across both introns and exons (not shown). In addition a 6.4kb retroposon (LINE1 element) had integrated into intron 2 of the mouse gene, while a partial retroposon sequence (1.1kb) was present in intron 4 of the rat gene.

The 5’ flanking regions of the rodent and human AADA genes were homologous, and were 64% (A+T) rich in the rat and mouse and 56% (A+T) rich in the human for 2.4 kb upstream of the initiating ATG codon, at which point the sequences diverged into two distinct simple repeats, (Fig. 4). Many, but not all eukaryotic genes have a TATA box, approximately 25 nucleotides upstream of the transcriptional start (30). Searching the TRANSFAC database with the mouse and rat genomic sequences using the Matinspector program (31) detected a strongly predicted TATA box (32) (TATAAACAGAACA) approximately 500 nucleotides upstream of the translational start codon (Fig. 4). No TATA boxes were identified closer to the initiation codon. An alternative initiator sequence (Inr) is present in certain TATA-less genes, which causes initiation at an adenine nucleotide (A+1) through the sequence (pyr pyr (A+1) N (T/A) pyr pyr) (33). A single potential Inr sequence was detected at position –79 in the rat (shown in bold, Fig. 4), which was not present in the mouse or human. Certain genes, lacking either TATA boxes or Inr sites, have multiple transcriptional start sites, causing heterogeneous 5’ ends to be present on the transcripts (33). In the case of the AADA gene, the longest 5’UTR in rat clones obtained by λ triplex library screening was 50 nucleotides, with two additional groups of clones of about 25 nucleotides and some of about 6 nucleotides (Fig. 4). A similar distribution of 5’ UTR sizes was apparent by analysing 16 independent rat 5’ clones obtained by RACE. The longest reported mouse EST clones have 5’UTRs of about 44 nucleotides with others of
about 25 nucleotides. In contrast the human 5’UTR reported in (10) was 85 nucleotides. An EST search using the 5’UTR of human AADA revealed a clone (AV705031) with a similar sized 5’UTR to that in (10) and a longer clone (AV686493) that was 128 nucleotides in length. Human 5’UTRs of about 19 nucleotides were also found in two clones (AA377126 and AW951425). Although artefacts of cDNA cloning may contribute to the heterogeneity observed, the distribution of 5’UTR sequences may also indicate that transcription of the AADA gene begins at multiple start sites, and that different sites of initiation predominate in the different species.

The ability of the mouse promoter to drive luciferase expression was assessed following transfection of HepG2 cells. As shown in Fig. 5 pronounced promoter activity was observed with a construct spanning 1679 bp of the mouse promoter, with somewhat lower activity associated with the 2180 and 941bp constructs. As expected no activity was associated with the 66bp construct spanning the 5’UTR. Promoter activity was also observed with a rat construct similar to the mouse 941bp construct (not shown).

Sites of AADA gene expression

To determine the tissues in which AADA is expressed a human multiple tissue dot blot was probed. Strong signals were apparent in liver, foetal liver and adrenal glands, with weak signals detected in other tissues including small intestine, stomach, kidney and pancreas (Fig. 6A). Northern blotting confirmed that a hybridising band of the same size as the predominant liver transcript was present in both the cortex and medulla of the adrenal gland (Fig. 6B). Reprobing the adrenal blot with the cDNA for HSL revealed a similar distribution of HSL mRNA between the cortex and the medullary fractions, suggesting that the medullary fraction did not contain exclusively chromaffin cells. Stomach and pancreas showed similar signals by dot blots, but Northern blotting could only confirm expression
in the pancreas. AADA mRNA was not detected in total RNA fractions from HepG2 cells (Fig. 7A), which had previously been shown to be deficient in lipid mobilisation (34). AADA mRNA could be detected in livers from both foetal and neonate (suckling) rats, (Fig. 7B). This contrasts with the expression of TGH mRNA, which was absent in utero and expressed at very low levels in suckling animals (Fig. 7B) exactly in agreement with Lehner et al. (7).

An RT-PCR assay was developed using oligonucleotides to amplify the cDNA between exons 1 and 2. The length of intron 1 (3.8kb in both the rat and mouse), coupled with DNase I treatment of the samples, ensured that genomic contamination did not compromise the analysis. Further use of exon 1 conferred specificity, because the signal anchor sequence to which the primer was designed is not present in other (currently known) lipase family members. For quantitation, RT-PCR was carried out under real time conditions, but to confirm the fidelity of the PCR reactions it was also shown that amplification of liver RNA generated products of the expected size (approx 85bp). Highly reproducible quantitation of AADA mRNA in 9 adult rat livers was achieved from animals killed at a similar body weight and time of day: (SEM<5.5% of mean value, data not shown). AADA mRNA levels in various tissues are shown relative to liver levels in Table III. Similar average levels of expression were observed in livers from 19 day foetal rats and from 14 day old neonate rats, but in contrast to adult livers, mRNA levels in immature animals were somewhat variable. However, expression was apparent in all samples assayed by RT-PCR and in the Northern blot shown above. Low levels of AADA mRNA were detected by RT-PCR in two separate preparations of rat kidney (Table III) and also on Northern blots of rat kidney total RNA (data not shown). This agrees with previous reports that the protein was not detected in human kidney (10). It was also shown that AADA mRNA was not present at significant levels in rat epidydimal adipocytes. Some expression was detected in cultured J774 mouse macrophages (0.165 units relative to mouse liver = 1). Comparable
levels of AADA mRNA to that in adult liver were detected in rat small intestine and a small enrichment was apparent in scraped mucosal cells. The levels of AADA mRNA in small intestine are further confirmed by experiments on the mouse described below.

Regulation of hepatic gene expression in vivo

The expression of several genes, including various of those involved in fuel homeostasis, is under diurnal regulation in rodents. As shown in Fig. 8, AADA mRNA is also diurnally regulated in wild type mice with steady state levels being maximal towards the end of the light phase (L10) in the postabsorbtive period. Each mRNA assay point has been adjusted for the concentration of internally coamplified β actin mRNA (which does not undergo diurnal fluctuations), although the diurnal rhythm in AADA mRNA was also apparent in the nonadjusted data (not shown). The diurnal regulation of AADA mRNA was suppressed in PPARα knockout mice, such that there were no statistically significant differences in AADA mRNA concentration at any of the time points examined. To test the hypothesis that the diurnal regulation of AADA mRNA might reflect direct activation of transcription by PPARα, control and PPARα knockout mice were treated with the PPARα ligand Wy-14,643 which has been shown to strongly induce acyl CoA oxidase (21). Liver wet weight increased approximately 3 fold in the treated wild type animals but there was no change in the liver weights of treated knockout mice. Hepatic acyl CoA oxidase mRNA levels were increased at least 7-fold in the control animals when killed at the L6 point of the diurnal cycle but were not increased in the knockout animals (Table IV). However, there was no comparable increase in AADA mRNA levels in the livers of either the wild type or the knockout animals. In contrast, a 2-fold increase in AADA mRNA was apparent in the intestine of the wild type, but not the knockout mice (Table IV). Similar results (not shown) were obtained at the D6 point in the cycle. Steady state hepatic AADA mRNA levels were also measured
under conditions associated with altered VLDL-TAG secretion. Significant decreases in AADA mRNA steady state levels, (corrected for 18S rRNA), were apparent in both acutely diabetic and in orotic acid-treated rats, (Table V), two conditions which markedly inhibit VLDL-TAG secretion (17, 35). AADA mRNA levels were not significantly altered by simple dietary manipulations such as fructose or fish-oil feeding. To confirm that changes in steady state mRNA levels in diabetes and orotic acid treatment resulted from altered transcriptional rates, nuclear run-on assays were carried out. The rate of production of AADA transcripts normalised to GAPDH transcripts was decreased by 50% in diabetes and 70% following orotic acid treatment. Administration of insulin to diabetic animals reversed the effects of diabetes, (Fig. 9).

Probst et al (10) suggested that AADA is the predominant activity in liver capable of deacetylating 2-acetoaminofluorene (2-AAF). We therefore assayed 2-AAF deacetylation by microsomes (9) from control and treated animals to determine whether this mirrored the observed changes in AADA mRNA concentration. A 50% decrease in microsomal 2-AAF deacetylase was apparent in streptozotocin diabetes (38 ± 5.0 SEM, nmol h⁻¹ mg⁻¹ protein n=3, p <0.01) relative to control animals (77±1.2 nmol h⁻¹ mg⁻¹), and the effect of diabetes was partially reversed by insulin administration (51± 4.2 nmol h⁻¹ mg⁻¹ p=0.06). In contrast, orotic acid administration increased 2-AAF deacetylase activity (98± 8.6 nmol h⁻¹ mg⁻¹ p<0.05), in contrast to its effect on AADA mRNA levels. However in our hands (Dr Nigel Turner, EDS and RJP unpublished observations) purification of microsomal 2-AAF deacetylases has shown that other esterases contribute significantly to total activity, therefore the above values may not accurately reflect the behaviour of AADA itself. In support of this, hydrolysis by each of the microsome preparations of an alternative amide substrate for esterases (36), (proline β naphthylamide), paralleled their relative activities using 2-AAF (r=0.846, p=0.0005).
DISCUSSION

Abnormal regulation of hepatic VLDL secretion is a major cause of hyperlipidemia in humans (37). The enzymology involved in the incorporation of triacylglycerols into VLDL is complex. It is known that a significant proportion of the hepatic TAG secreted as VLDL undergoes a cycle of lipolysis and reesterification prior to or during VLDL assembly (3, 4). However, neither the identity of the lipase(s) involved nor the intracellular location(s) of the lipolytic event have been established. The number of molecules of TAG lipolysed by isolated hepatocytes greatly exceeds that secreted as VLDL, the majority of reesterified TAG being redeposited in the storage pool, (a process termed recycling (3). Under some conditions, changes in the rate of TAG lipolysis correlate with changes in the rate of VLDL-TAG secretion suggesting that the lipase is subject to physiological regulation (16-18). Two candidates, TGH (6-8) and AADA (2) have been proposed as the physiological lipase. AADA was first described in studies of carcinogen metabolism (9, 10). In this respect it is interesting that a potential aryl hydrocarbon response element was detected in the promoter of the human gene (Fig. 4), which could cause the AADA gene to be induced in response to xenobiotics (38). However, similar elements were not detected in either the mouse or rat. Irrespective of any role it may play in xenobiotic metabolism, AADA also has properties that might be predicted for the TAG mobilisation lipase. Firstly, AADA is the only currently described mammalian protein that shows strong active site homology with HSL (10), the enzyme that mobilises TAG and cholesterol ester stores in extrahepatic mammalian tissues (39, 40). Secondly, AADA has an unusual tyrosine-rich signal anchor sequence (similar to that of steroid 11 β hydroxylase (41)), which directs AADA to the ER, and which, judged by the high mannose content of the isolated enzyme, (41) may cause it to be retained there. A lipase targeted to the ER would ensure efficient delivery of lipolytic products to diacylglycerol acyl transferase and prevent equilibration with other intracellular fatty acid pools (2). Thirdly, AADA was
found to show a limited tissue distribution, but to be present in liver and small intestine, two tissues whose major common function is lipoprotein secretion. Finally, deacetylation of the model substrate for AADA, 2-acetylaminofluorene, (9) by partially purified protein fractions from detergent-solubilised rat liver microsomes is strongly inhibited by tetrahydrolipstatin (RJP and Nigel A Turner unpublished results), which is a specific inhibitor of lipases (42).

Hepatoma cells are deficient in lipid mobilisation (34), and the present work indicates that they under-express AADA as was previously shown for TGH (8). Transfection of the cDNA for either AADA or TGH into hepatoma cells has resulted in a modest increase in lipid secretion (2, 7), although full restoration of lipid mobilisation to levels comparable with primary hepatocytes has not so far been reported. Because hepatoma cells may also lack other factors required for efficient VLDL assembly, we have examined physiological regulation in animal models. The absence of a specific assay to determine AADA enzyme activity has meant that we have examined mRNA expression by real-time PCR. To this end, the rat and mouse genomic sequences were cloned. A 6kb retroviral insertion was detected in intron 2 of the mouse. Although unusual, this need not impair gene function since in at least one other instance (a mouse annexin gene), a complete retroposon sequence is efficiently spliced out of an intron (43). The mouse and rat AADA genes reported here appear in all other respects to be functional since mutations have not accumulated in the coding sequences, the untranslated regions or the splice sites. Although, common core promoter elements could not be identified, transfection assays showed that the mouse promoter sequence acted efficiently in transfected hepatoma cells, with lower but clear activity associated with the rat promoter.
Tissue distribution of AADA gene expression.

AADA gene expression is limited to a few tissue types. A high level of expression in adult liver was expected from the previous reports (9, 10). Lehner et al (7) have suggested that any lipase which mobilises hepatic TAG for VLDL assembly may be down-regulated in suckling rats. In particular, they showed that TGH is absent in utero and is only strongly induced as the animals reach the adult stage (7). While we observe a similar pattern of TGH expression, it is clear from our results that AADA is expressed both in utero and during the suckling period. In fact, while circulating VLDL levels may be low in rats in utero, VLDL are nevertheless produced from day 21 of gestation and rise sharply over the first 5-10 days of suckling (44, 45). A similar induction of VLDL secretion occurs during the first week of suckling in humans (46). Moreover, isolated foetal rat hepatocytes are competent to secrete triacylglycerols at a significant rate (40% the rate of that in adult hepatocytes) if supplied with exogenous fatty acids (47). Assuming that a process of lipolysis and reesterification is involved in VLDL secretion by foetal and immature animals, as in adults, this confirms the view of Lehner and colleagues (8) that TGH cannot be the only lipase responsible for lipid mobilisation in the liver and suggests that a perinatally expressed lipase such as AADA may also be involved.

In the current study AADA was also found to be strongly expressed in the adrenal cortex and in the medulla. However, since HSL was also detected in both fractions the possibility of mixed tissue types cannot be excluded. Assuming that AADA is present within cortical tissues, it may contribute to the mobilisation of cholesterol esters for glucocorticoid production. While HSL has been previously presumed to mediate this process (39, 40), it may not be the only lipase involved, since transgenic mice deficient in HSL continue to produce glucocorticoids (48). The current results also suggest that AADA is expressed in the pancreas. While the significance of this is unclear, it is notable that HSL is expressed in β cells and may play a role in regulating insulin secretion (49).
Immunoreactive AADA protein was previously detected in human small intestine (10), and in the current study it was shown by RT-PCR that AADA mRNA levels in rat small intestine were comparable to those in liver in from animals fed *ad libitum*. Immunoreactive protein was not detected in human colon (10), which might imply that the gene is expressed in the enterocytes of the small intestine rather than the smooth muscle common to both organs. The enrichment of AADA mRNA in mucosal cells relative to total small intestine observed here is also consistent with a localisation to enterocytes. Lipolysis within enterocytes yields 2-monoacylglycerol which is reesterified to TAG prior to chylomicron secretion (50, 51). The poor detection of AADA mRNA in blots of human small intestinal polyA (+) RNA (Fig. 4) was surprising, in view of the above. The precise circumstances of the human organ donor may have determined whether detectable amounts of mRNA were present in particular post-mortem samples of small intestine. Moreover, the normalisation of the polyA (+) RNA to β-actin mRNA content, which is high in intestine, may have caused AADA mRNA to appear underrepresented.

**In vivo regulation of AADA gene expression**

The expression of several genes involved in lipid metabolism in rodents and in man is diurnally regulated according to the pattern of food intake over the 24 h cycle. As shown in Fig. 7, AADA mRNA is also diurnally regulated in normal mice with a peak during the later part of the light phase, which corresponds to the post-absorptive period in rodents. Hepatic VLDL secretion shows an identical pattern *in vivo* (52-54). Although further work will be required to relate AADA mRNA levels to enzyme activity, diurnal regulation of AADA transcription in rodents is consistent with the view that AADA plays a role in the integration of hepatic fuel homeostasis.
The transcription of some genes related to the feeding cycle in rodents is dependent on the function of the peroxisomal proliferator-activated receptor α (PPARα), (55, 56). In certain instances (including apolipoproteins and enzymes of fatty acid oxidation) transcriptional control by PPARα is directly mediated by PPARα response elements (PPRE) located in the promoters of the genes, and generally within 1000 nucleotides of the transcriptional start site (55, 56). In other instances transcriptional control by PPARα is indirect, as in the case of the genes encoding the lipogenic and cholesterogenic enzymes, acetyl-CoA carboxylase, fatty acid synthase, and 3-hydroxymethylglutaryl-CoA reductase. These genes exhibit diurnal rhythms of transcription with maximal mRNA levels in the dark period when feeding occurs, contributing to the circadian periodicity of fatty acid synthesis (57, 58) and cholesterogenesis (59). While these genes lack PPRE in their promoters, their normal diurnal rhythms of transcription are markedly suppressed in mice deficient in the PPARα receptor (19, 60). The circadian rhythm of AADA expression is similarly attenuated in the livers of the PPARα knockout mice and may contribute to the disturbance of hepatic TAG metabolism (61, 62).

To determine whether the PPARα dependent control of the AADA gene is direct or indirect, the PPARα ligand, Wy-14,643, was used. A two-fold rise in intestinal AADA mRNA was observed following oral administration to the wild type mice. No increase in hepatic AADA mRNA was observed, while changes in expression of liver acyl CoA oxidase (this study) and other hepatic genes (Patel DD, Knight BL and Gibbons GF unpublished observations) were clearly demonstrable in the wild type animals. It would be reasonable to suppose that if the AADA gene had an associated PPRE it would be located in the 2.4kb upstream region shown here to have promoter activity. However, only a few weak matches with authentic PPRE were detected within the mouse sequence and no PPRE were identified in the rat sequence. A potential COUP-TF/HNF4 site was predicted in both rodent promoters, which may be capable of binding PPARα weakly under certain circumstances (63). The
increase in intestinal AADA mRNA content apparent in wild type mice may have arisen from activation of the weak PPRE-like sequences in the mouse promoter by prolonged high gut concentrations of fibrate associated with the oral route. The absence of a similar effect in liver may reflect efficient competition for these sites by liver-specific factors such as HNF4. Irrespective of the cause of events in the gut, there is no evidence that hepatic AADA expression is sensitive to PPARα agonists. Thus, direct control through the PPARα pathway cannot account for the diurnal cycling observed in the liver.

Regulation of the diurnal cycling of acetyl CoA carboxylase, fatty acid synthase and 3-hydroxyacyl-CoA dehydrogenase may involve sterol regulatory elements (SRE) in their promoters (15, 19). The Matinspector program detected weak SRE-like sequences in the AADA gene in each species, although no strong conserved sites were found. Of possible interest, a potential glucocorticoid response element (GRE) was detected in both the rat and mouse promoters approximately 1.7 kb upstream of the translational start codon (Fig. 4). Diurnal variations in glucocorticoid concentrations (64) could be suggested to explain the cycling in AADA mRNA levels. However, since glucocorticoids are reported to be higher than PPARα in the hierarchy of diurnal regulation (64), this cannot account for the dependence of the AADA diurnal rhythm on PPARα expression. Moreover, in preliminary experiments, the addition of dexamethasone did not alter luciferase expression from the AADA promoter in transfected HepG2 cells. Therefore at present there is no simple explanation for the diurnal regulation of the AADA gene.

It is not a straightforward matter to predict the changes in lipase levels that would be expected under all conditions of altered VLDL-TAG secretion. In addition to regulation of lipolysis, control may also be exerted over the proportion of mobilized fatty acids that are incorporated into VLDL-TAG as opposed to those recycled to the storage pool (3). This may prove to be the site of regulation in
response to simple dietary manipulations, since the increased secretion of hepatic VLDL associated with fructose feeding (18) and the suppression with fish-oil consumption (16) occurred with no apparent change in hepatic AADA mRNA content. In contrast, both dietary orotic acid and the establishment of insulin-deficient diabetes lead to a severe decrease in the secretion of hepatic VLDL-TAG and were accompanied by 40% decreases in hepatic AADA mRNA content. Run-on assays indicate that the decreased steady state mRNA content could result from a decreased rate of transcription. In the case of diabetes the decreased transcription rate was reversible by administration of insulin, although insulin-response elements were not predicted in the AADA promoters from any species. Orotic acid treatment is known to cause a 50% decrease in the fractional turnover of TAG in cultured hepatocytes (17). Thus, the decrease in AADA mRNA expression could account for the decrease in lipolysis following orotic acid treatment and in insulin-dependent diabetes, assuming that a comparable fall in enzyme levels occurs. The ability to measure changes in AADA mRNA will allow us to assess the relative contributions of the two processes to the control of VLDL secretion.
References


Figure legends

Fig. 1 Alignment of the predicted amino acid sequences of rat (RA), human (HA), and mouse (MA) AADA. The presumed active site and an upstream his-gly-gly-gly box, which is characteristic of lipases, are shown in bold. A partial alignment with two sequences of human HSL (HH residues 350-387 and 681-724) and mouse HSL (MH residues 349-386 and 682-725) is also shown, (i) containing the his-gly-gly-gly box and the active site gly-x-ser-x-gly motif and (ii) containing the aspartic acid and histidine residues of HSL which complete the catalytic triad (29).

Fig. 2A Alignment of the 3’ regions of the rat (RA) human (HA) and mouse (MA) AADA cDNA sequences. Termination codons and polyadenylation sequences are shown in bold and are underlined. A potential, but apparently unused, polyadenylation signal in the rat is also underlined. B) Total RNA from adult rat liver (lane1) and mouse liver (lane2) was resolved on a 1% formaldehyde agarose gel and a blot was probed with a cDNA for mouse AADA. The mouse AADA cDNA recognises bands of similar intensity in mouse and rat liver although the rat transcript migrated more slowly than the mouse. Similar results were obtained when the blot was reprobed with the rat AADA cDNA (not shown). The apparent sizes of the rat and mouse transcripts are 1.8kb and 1.5kb respectively which correspond to the deduced cDNA lengths plus ~200 nucleotides of poly (A) tail (30).

Fig. 3 The genomic arrangement of (A) the rat AADA gene and (B) the mouse AADA gene was deduced from sequencing reactions (arrowheads) of subcloned restriction fragments, (hind=HindIII, bam=BamHI, bgI=BglII, eco=EcoRI, pvu=PvuII, sac=SacI). Subclones were obtained by hybridisation with cDNA probes except mE and mH4 which were obtained with genomic probes derived from
subclones mB1 and mH1 respectively. Clone mB1 derived from a \(Bgl\) II digestion but had an atypical end (*), which did not correspond to a restriction site.

Fig. 4 Nucleotide alignments of 3 selected regions of the 5’ flanking sequences of the AADA gene. The human AADA gene sequence was derived from clone AC068647 (chromosome 3q) (65), from the total human genome database. The initiating ATG codon is shown in bold and is shown as nucleotide (+1). Italicised bases (-2408 to -2369) show the point at which homology disappears at the start of simple repeat sequences in the mouse (MA), rat (RA) or human (HA). The first nucleotides of the 5’UTR sequences of independent cDNA clones are in bold and underlined. Where more than one clone starts on a particular nucleotide, the frequency is shown underneath. In the rat these represent either \(\lambda\) triplex clones or individual RACE clones. In the mouse and human, these are the available EST clones. Possible promoter elements were detected with the Matinspector program. The closest predicted TATA box (present in rat and mouse but not in human) is between nucleotides -515 and -503. A potential Inr sequence which is present in the rat only is shown in bold. A predicted glucocorticoid response element is present at nucleotide -1710 in the rat and mouse with a conserved COUP-TF/HNF 4 site 150 bases downstream. A similar motif (GRE in the opposite orientation followed by a COUP-TF/HNF 4 site) is also present in the human sequence at a downstream site. The human COUP-TF/HNF 4 site overlaps (TGA underlined) with a predicted aryl hydrocarbon response element (AHR), however this element is not present in the rat or mouse.

Fig. 5 AADA promoter activity \textit{in vitro}. Human hepatoma cells, HepG2, were transfected with reporter constructs containing different-sized fragments of the 5’- flanking region of the mouse (m) AADA gene cloned upstream of the luciferase gene in the pGL3 basic vector. The sizes of the
fragments are given in bp. In absence of a defined transcriptional start site, constructs extended to a common site within the AADA 5’UTR, 9 nucleotides upstream of the initiating ATG codon. Luciferase activity was corrected for transfection efficiency using co-transfected β-galactosidase vector. Within each experiment luciferase values were expressed as a percentage of that observed with the 941bp fragment. Results represent the means ± SEM of 4 separate transfection experiments for each of the 4 constructs and for the promoterless vector. The m941 construct conferred significantly more activity than the pGL3 basic vector or m66 (p<0.0001 in each case). The m1679 construct conferred significantly more activity than m2180 or m941 constructs (p<0.02 in each case).

Fig. 6A A human RNA master blot (BD Clontech) representing 50 tissues (rows A to G) and 8 controls (row H) was probed with a human cDNA representing the complete coding sequence of human AADA. At the high stringency employed no cross reaction was observed with yeast total RNA (H1), yeast tRNA (H2), E coli rRNA (H3), poly (rA) (H5), or cot1 DNA. Weak reaction was observed with E coli DNA (100ng) (H4), and human DNA at 100 ng (H7) and 500ng (H8). Evidence for strong AADA expression was found in adult liver (E2), adult adrenal gland (D5) and in foetal liver (G4). 14 separate neural tissues (rows A and B) were negative for AADA expression. Expression was not detected in heart, aorta, skeletal muscle, colon, bladder, uterus or prostate (row C 1-7): in testis, ovary, pituitary, thyroid gland or salivary gland (row D 1, 2, 4, 6, 7): in spleen, thymus, leukocytes, lymph node, or bone marrow (row E 4-8): in appendix, trachea, placenta (row F 1, 3 ,4): or in foetal brain heart kidney spleen or thymus (row G 1, 2, 3, 5, 6). A weak signal, (comparable to controls in row H but above the background from negative tissues), was apparent in some tissues including stomach (C8), pancreas (D3), mammary gland (D8), kidney (E1), small intestine (E3), lung (F2), and foetal lung (G7). (B) Multiple tissue Northern blots representing human adult tissues were probed with human AADA
cDNA. A band of the expected size was present in liver (track 1), adrenal cortex (6), adrenal medulla (8), and pancreas (9), but not in stomach (2), thymus (4), testis (5), or thyroid (7). A very weak signal at the limits of detection was present in small intestine (3). HSL mRNA was detected in testis, adrenal medulla and cortex and in pancreas. β Actin RNA was present in all samples examined.

Fig. 7A Mouse liver (lanes 1 and 3) and HepG2 cells (2, 4) total RNA was resolved on 1% formaldehyde agarose gels and blots were probed with the cDNA for lysosomal lipase (1, 2) and for human AADA (3, 4). Both human cDNAs hybridised with their cognate mouse mRNA. AADA mRNA was absent from this and 3 other independent preparations of HepG2 RNA. (B) Total RNA from 6 pooled foetal livers (lanes 1 and 5), adult rat liver (4, 8), and 2 separate neonate rat livers (2, 3, 6, 7) was resolved on 1% formaldehyde agarose gels and blots were probed with cDNA for mouse TGH (1-4) and for rat AADA (5-8). TGH was only strongly expressed in adults while AADA mRNA was apparent in at all stages. β Actin RNA was present in all samples examined.

Fig. 8 The diurnal variation in AADA mRNA measured by real time RT-PCR in control mice (open circles) and in PPARα deficient mice (filled circles) (n= 5, in each case). Each mRNA assay point has been adjusted for the concentration of a coamplified internal standard and is expressed relative to a standard mouse liver preparation. The diurnal maximum in AADA mRNA concentration occurs 10h into the light phase (L10). There is a highly significant elevation in AADA mRNA at L10 in the control animals relative to both the control animals at D10 (P=0.029) and the knockout animals at L10 (P=0.002).
Fig. 9 Run-on assays of nascent nuclear AADA RNA transcripts. Rats were treated as indicated and nuclei prepared from the livers. Nuclei from 2 livers were pooled, lysed and subjected to run-on assays as outlined in Methods. Labelled transcripts were hybridised to membranes containing 3 spots of a full-length AADA cDNA probe (obtained during the present study) and 3 spots of a full length GAPDH cDNA probe (kindly supplied by Dr David Carling, MRC Clinical Sciences Centre, UK). After extensive washing the spots were cut out and the radioactivity assayed. A blank (32.5cpm), given by an unincubated sample, was subtracted from all values. The value for each spot with the AADA probe was divided by the mean of the 3 GAPDH spots for the same sample. Values with the GAPDH probe were approximately 120cpm and those with the AADA probe were up to 200cpm above background. Points represent the mean for the 3 AADA spots, with the SEM to give an indication of the reproducibility of the assay. Results are given for 2 separate samples (each from 2 livers) for each condition, with the bars showing the averages.
Table I

*Oligonucleotides used for RNA quantitation by RT-PCR*

<table>
<thead>
<tr>
<th></th>
<th>Mouse AADA</th>
<th>Rat AADA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>forward</td>
<td>forward</td>
</tr>
<tr>
<td></td>
<td>5’-GATTTGGCTTCATTTGGTGAACACT-3’</td>
<td>5’-GAAATACACTTCTAAACTTTGGAGGAGAT-3’</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>reverse</td>
</tr>
<tr>
<td></td>
<td>5’-TGGGTGGGACTTTGAAAACACT-3’</td>
<td>5’-ACTGTACCGTGTCATGAAATGAT-3’</td>
</tr>
<tr>
<td></td>
<td>probe</td>
<td>probe</td>
</tr>
<tr>
<td></td>
<td>5’-FAM-CTGTATCGTTCCATGAAATGACTGATTCCCA-TAMRA-3’</td>
<td>5’-FAM-GAGTCCCAAAGGTTACACCAAGAGCCA-TAMRA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse/rat β actin</td>
</tr>
<tr>
<td></td>
<td>forward</td>
<td>forward</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-GAGCTATGAGCTGCTGACG-3’</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>reverse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-AGTTTCATGGATGCCCCAGGA-3’</td>
</tr>
<tr>
<td></td>
<td>probe</td>
<td>probe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-VIC-CATCACTATTTGCAACGAGCGGTCC-TAMRA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse acyl CoA oxidase</td>
</tr>
<tr>
<td></td>
<td>forward</td>
<td>forward</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>reverse</td>
<td>reverse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-TGCACCATTGCCATTCGATA-3’</td>
</tr>
<tr>
<td></td>
<td>probe</td>
<td>probe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-FAM-TTTTACAGACTGGGCACCTCAGACT-TAMRA-3’</td>
</tr>
</tbody>
</table>
Table II

*Organisation of the AADA gene*

The sequences at the intron (lower case) / exon (upper case) boundaries of the rat and mouse AADA genes are shown. The 5’ splice sites resemble the consensus g\(_{(100)}\)t\(_{(100)}\)a\(_{(60)}\)a\(_{(74)}\)g\(_{(84)}\)t\(_{(50)}\), and the 3’ splice sites the consensus (11 x pyr)Nc\(_{(78)}\)a\(_{(100)}\)g\(_{(100)}\). Intron lengths include (a) 6,375 and (b) 1,123 bp of retroposon sequence. The human intron sequences by comparison are 3,065, 2,794, 4,211, and 2,740 bp.

<table>
<thead>
<tr>
<th>species</th>
<th>Exon</th>
<th>Length</th>
<th>3’splice site</th>
<th>5’splice site</th>
<th>Intron length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>1</td>
<td>160</td>
<td>NA</td>
<td>CTTGGAGGAGATTGTgaagttttgatttt</td>
<td>3,855</td>
</tr>
<tr>
<td>Mouse</td>
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<td>154</td>
<td>NA</td>
<td>ATAGGAACAGATTGTgaagttttgatttt</td>
<td>3,834</td>
</tr>
<tr>
<td>Rat</td>
<td>2</td>
<td>223</td>
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<td>GTCTGGGGAAGTGCTGgtatgtgccattgg</td>
<td>1,210</td>
</tr>
<tr>
<td>Mouse</td>
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<td>223</td>
<td>attcgtctacctcagGCTTCTTTTTGGTGAAG</td>
<td>GTTGGGGGAGTGCTGgtatgtccacagttg</td>
<td>7,638 (a)</td>
</tr>
<tr>
<td>Rat</td>
<td>3</td>
<td>70</td>
<td>tttctctggttcagCTTACTTCTTATCGTATG</td>
<td>TGGTTGTCACACTGAgtaagaccttattgg</td>
<td>1,014</td>
</tr>
<tr>
<td>Mouse</td>
<td>3</td>
<td>70</td>
<td>tttctctggttcagCTTACTTCTTATCGTATG</td>
<td>TGGTTGTCACACTGAgtaagaccttattgg</td>
<td>1,014</td>
</tr>
<tr>
<td>Rat</td>
<td>4</td>
<td>172</td>
<td>ttatccttcacacagCATAGGCTTTAGCAG</td>
<td>GCTGTGACACACACAGgttagcttcacagtc</td>
<td>3,494 (b)</td>
</tr>
<tr>
<td>Mouse</td>
<td>4</td>
<td>172</td>
<td>ttatccttcacacagCATAGGCTTTAGCAG</td>
<td>GCTGTGACACACACAGgttagcttcacagtc</td>
<td>3,494 (b)</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>1020</td>
<td>ttaaatccttttcagTTCTACAGGATCACC</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Mouse</td>
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<td>675</td>
<td>ttaaatccttttcagTTCTACAGGATCACC</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

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Table III

*Quantitation of AADA mRNA in rat tissues*

AADA mRNA levels were determined in rat tissues by real time PCR from 50ng of total RNA using a single preparation of adult rat liver total RNA (*) as an external reference standard for all amplification reactions. AADA mRNA content is expressed relative to the concentrations in the reference livers. Replicate measurements on single samples did not disagree by more than 5% of the stated values. Levels of AADA mRNA in each sample were corrected for the content of 18S rRNA determined in parallel PCR reactions rather than internally coamplified β actin mRNA, since this transcript is very high in the intestine.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AADA mRNA content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult rat liver</td>
<td>1.00*</td>
</tr>
<tr>
<td>Foetal rat liver (3 groups of 6 pooled)</td>
<td>0.96 ± 0.63 (n=3)</td>
</tr>
<tr>
<td>Neonate rat liver (3 groups of 2 pooled)</td>
<td>1.55 ± 0.74 (n=3)</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>0.074 (n=2)</td>
</tr>
<tr>
<td>Rat epidydimal adipocytes</td>
<td>0.011 (n=2)</td>
</tr>
<tr>
<td>Rat small intestine</td>
<td>0.95</td>
</tr>
<tr>
<td>Rat intestinal mucosal cells</td>
<td>1.20</td>
</tr>
</tbody>
</table>
Table IV
Effects of fibrates on hepatic and intestinal levels of AADA and acyl CoA oxidase mRNA levels.
AADA and acyl CoA oxidase mRNA levels were determined by real time PCR in the liver and small intestine of untreated (-) and Wy-14,643-treated (+), wild type (wt) or PPARα knockout (ko) mice, (n=4 in each case). Mice were sacrificed at L6. Levels (mean and SEM) are normalised to internally coamplified β-actin mRNA content, (to maximise accuracy), and are shown relative to a single reference wild type mouse liver or intestine as appropriate. Statistically significant changes in mRNA are shown.
An essentially similar elevation in hepatic and intestinal acyl CoA oxidase mRNA and intestinal AADA mRNA was observed in wild type animals sacrificed at D6 (data not shown, but P<0.001 in each case and with other differences not significant).
The concentration of AADA mRNA in the intestine relative to liver is much lower than in shown in Table III. This reflects normalisation to β-actin. Mouse intestinal AADA mRNA is expressed at comparable levels to mouse liver mRNA when normalised to 18S rRNA, (not shown).

<table>
<thead>
<tr>
<th>tissue</th>
<th>Wy</th>
<th>Acyl CoA oxidase</th>
<th>AADA</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt liver</td>
<td>-</td>
<td>0.673 ± 0.053</td>
<td>0.811 ± 0.113</td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>5.879 ± 0.073 **</td>
<td>0.569 ± 0.073</td>
</tr>
<tr>
<td>ko</td>
<td>-</td>
<td>0.598 ± 0.131</td>
<td>0.528 ± 0.077</td>
</tr>
<tr>
<td>ko</td>
<td>+</td>
<td>0.896 ± 0.133</td>
<td>0.752 ± 0.094</td>
</tr>
<tr>
<td>wt intestine</td>
<td>-</td>
<td>0.850 ± 0.025</td>
<td>0.192 ± 0.028</td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>4.562 ± 0.901 *</td>
<td>0.381 ± 0.025 *</td>
</tr>
<tr>
<td>ko</td>
<td>-</td>
<td>0.799 ± 0.035</td>
<td>0.161 ± 0.003</td>
</tr>
<tr>
<td>ko</td>
<td>+</td>
<td>0.861 ± 0.148</td>
<td>0.146 ± 0.017</td>
</tr>
</tbody>
</table>

Significant effects of fibrate are denoted ** (p<0.001, student’s t-test) and * (p<0.01, student’s t-test). Other differences are not significant.
Table V

Steady state AADA mRNA levels in rats under regimes which alter VLDL-TAG secretion

Table V shows the mean and SEM (n=4) for AADA mRNA levels (corrected for 18S rRNA content and relative to a standard rat liver preparation); in two separate groups of control animals and in animals treated as described in materials and methods, (OA=orotic acid).

<table>
<thead>
<tr>
<th>treatment</th>
<th>AADA mRNA content</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>diabetic</td>
<td>0.56 ± 0.07 *</td>
</tr>
<tr>
<td>control</td>
<td>0.99 ± 0.11</td>
</tr>
<tr>
<td>fish-oil fed</td>
<td>1.23 ± 0.24</td>
</tr>
<tr>
<td>OA-treated</td>
<td>0.62 ± 0.07 **</td>
</tr>
<tr>
<td>fructose-fed</td>
<td>0.83 ± 0.24</td>
</tr>
</tbody>
</table>

* denotes a significant effect of diabetes (p=0.030, student’s t-test) and ** denotes a significant effect of orotic acid (p=0.024, student’s t-test). Other differences are not significant.
| RA   | CCATGGAGACCTACCTAACCTTCGTTTGAAAATTACATACAGGAGAGAAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCAAATGCA
Fig. 2B
Fig. 3
Luciferase activity (% of 941bp construct)

- pGL3 basic
- m 2180
- m 1679
- m 941
- m 66
Fig. 9

mRNA synthesised (cpm AADA / cpm GAPDH)

Control  Orotic acid  Diabetic  Diabetic+insulin
MA: AGATTGGCAC TGTGTATGAT TCATTCCAAT GCTGCTTTAA ACCGC..TGA
RA: AGATTGGCAC TGAAGCAGAT TCATCTCGGT GCTGCTTAAA ACCAC..TGA
HA: TCCTTCTCGC TACCTCCACT GCAGCCAAAC TTTGAGAACA CAAGTCTTGG

-1758
MA: AAGGAAGCCA GAGGAAGGAA GAAAAC.TTC CTGATCAATT TGGGATTG
RA: AAGGTAGCCA GT...AGGAG GAAAAT.CTC CTGATCAATT TGATATTA
HA: TAACCAACCA GCTGTTCCCT GCAAATACTC CAGCACTCTT CTCAGACCAA

-1608
MA: TTCTTTATGT TCTA TACCTCTCT CTC....... .......CTA GAGAGCTCAG CATGACAGAA
RA: TACTTTATGT TCTA TAATGTTTCT CCT....... .......CCA GCTGACTCAG CATGACAAAA
HA: TACCATCTTT CCAAGTTAAA AAGAGCAACT CGACGTCGACC CATCACAAC

-1508
MA: TTC.C TGAAC ATTGCCAAG A TTTTAACTGA AAGAACAAAG TCAAGTTGAA
RA: TTC.C TGAAC ATTGCCAAC A TGTTTACTGA AAGAAGAAAG CCATCTTGAA
HA: TACCTACTC TTTGCCATAG GAATATATAC TGTCTTGCCT CCTGGGTGGA

-1408
MA: A..TAAAGAT AACTAGTTTC CCCAAATCAC CAAATCAATT CTAACAACCC
RA: AAATAACAAC TACTAGTTTT TCCAAATCAA CAAATCATTT CTAACAATCC
HA: AA.TGTCCTG TCTTTGCCCA TCCTGGCATT TCACACTCTT CCTTCACTGC

-1308
MA: ACTCATACAA ACAATTGGAT GAAGTTCCTG ATGCAGAATT CTTTTTAATT
RA: TCTCATACAA ATAATTATAT GAAATTCCTG ATGCATAATT CTTTTAAATT
HA: ATCACAGAAA ACCTTTCATG GTCCTACCCA GAACATCCCA CCCTCACAGC

-1208
MA: GAGATGGGAA TGTGTATGAC ATACCTTCCT CTTTGCTTTA ACCGC..TGA
RA: GAGATGGGAA TGTACTCTCG ATCATTCAAT GCTGCTTAAA ACCAC..TGA
HA: TCCTTCTCGC TACCTCCACT GCAGCCAAAC TTTGAGAACA CAAGTCTTGG
MA     GACAGATAAG TGACTTTTGT CGAGATTTTC TGGTGGCAAT TATAAACCTTA
RA     TATAGCAAGAG TTTATCTCT CGAGATTTCC TGGTGGCATG TATAAAGCATG
HA     AAGAATAACA GAAAAAACCC CGAGACAAAC TCTCAAATCT TCAACCC...

-708                                               -659
MA     AGACCCCAAGG AAAATTTTTA AGTTAGAATA TGGCTAAA AGGTGAGAAT
RA     AGACCCCAAGG AAAATTTTTA AATTAGAACA TCAGACTAAA AGGTGATAAT
HA     CCCCCTCCCC ACTTCCCC AA TGCTAT...GGA AATCCCACTG CCTACAAC

-658                                               -609
MA     TATCTAATTA TCAATGAGTC AAAAAATAAA ATGAAGAGGA ATGGGTTTTT
RA     TGTCATATTA TTAGTGACGT CAAATATAAA ATGAAGAGAA AAAGTTTTTT
HA     TGACACAGGC CGGACATAGC CAAGATATAGC CGGGTGGAACA CTTGCCTAAA

-608                                               -559
MA     CAAGGTGAGA ATGTACAGAA TGGATTCAGC TGAGCTGTGA ACAGAAAGGA
RA     CAAGTTGAGA TTGTACCGAC TGGAATCAGC TGACCCATGA CAAGAAAGGA
HA     CCCCGTCCCC ACTTCCC..AA TGCAT..GGA AATCCCACTG CCTACAAC

TATA  -509
MA     A.CATGATAT AATTATTCTG TGTTCTCAAA A.....GAAA TCA
RA     AACATGATAT AATTATACTA TATTCTCAAA ATAATGGAAA TAA
HA     ACCCTCAAAC AAACTACTTA TTGACACAAA A.....CGCT ATCTATGGAC

-508                                               -459
MA     CGGAGTTTTT CCAACGGGAA GGAAAGTAGG GCCCAGAGGC AATTAAGGAA
RA     CTGGAGTTTT TCAGCAGGGA GGAGAGCAAG GCATAGAGGG AATAAAGGGA
HA     ATACACACAC ACATATATAT GTATATATAT GTGTGTGTG TATATATATA

-458                                               -409
MA     AATTGAATTT TTTTAAAAA..TITCATAAA GC..AGCT..TT AAAGGGTGTG
RA     AAAAGGCATT TTCTCAAAA..TCTCATAAA GC..AGCT..TT AAGAGGCTG
HA     ATTTGTTTTT TCCATGATAA GCTATTTTTA AACAAGATT CTGAATTCTG

-408                                               -359
MA     TTGATCTTTCT TTATGTTTCT ACGTCAAAC ATGCTATACTT TTTCTATGAA
RA     TTGACCTTTCT ATTAGTTTCT ACGTCAAAC ATGCTATACTT TTTCTATGAA
HA     ATAGTTTTCT ATACAGATAT ATGCTTAGG ATTTCTATGAA TATATATATA

-358                                               -309
MA     TTTGAGTTTTG TTTTATGTC ACGTCAAAC ATGCTATACTT TTTCTATGAA
RA     TTTGACCTTTCT ATTAGTTTCT ACGTCAAAC ATGCTATACTT TTTCTATGAA
HA     ATAGTTTTCT ATACAGATAT ATGCTTAGG ATTTCTATGAA TATATATATA

-308                                               -259
MA     TTGGAACCTT TTTTATGTC ACGTCAAAC ATGCTATACTT TTTCTATGAA
RA     TTTGACCTTTCT ATTAGTTTCT ACGTCAAAC ATGCTATACTT TTTCTATGAA
HA     ATAGTTTTCT ATACAGATAT ATGCTTAGG ATTTCTATGAA TATATATATA

-258                                               -209
MA     TTTTGTGAT ATTAGTTTCT TTTTATGTC ACGTCAAAC ATGCTATACTT TTTCTATGAA... C
RA     TGGAAGGTTG TTTTATGTC ACGTCAAAC ATGCTATACTT TTTCTATGAA... T
HA     CATTGACAAT ACTTGTGCTT AGTTTCTATG TACTCAT... TATAAAGC
Fig. 4W Nucleotide alignments of the 5’ flanking sequences of the AADA gene. The human AADA gene sequence was derived from clone AC068647 (chromosome 3q) (65), from the total human genome database. The initiating ATG codon is shown in bold and is shown as nucleotide (+1). Italicised bases (-2408 to -2369) show the point at which homology disappears at the start of simple repeat sequences in the mouse (MA), rat (RA) or human (HA). The first nucleotides of the 5′ UTR sequences of independent cDNA clones are in bold and underlined. Where more than one clone starts on a particular nucleotide, the frequency is shown underneath. In the rat these represent either λ triplex clones or individual RACE clones. In the mouse and human, these are the available EST clones. Possible promoter elements were detected with the Matinspector program. The closest predicted TATA box (present in rat and mouse but not in human) is between nucleotides -515 and -503. A potential Inr sequence which is present in the rat only is shown in bold. A predicted glucocorticoid response element is present at nucleotide -1710 in the rat and mouse with a conserved COUP-TF/HNF 4 site 150 bases downstream. A similar motif (GRE in the opposite orientation followed by a COUP-TF/HNF 4 site) is also present in the human sequence at a downstream site. The human COUP-TF/HNF 4 site overlaps (TGA underlined) with a predicted aryl hydrocarbon response element (AHR), however this element is not present in the rat or mouse.
Characterisation of the rodent genes for arylacetamide deacetylase, a putative microsomal lipase, and evidence for transcriptional regulation
Jeffrey I. Trickett, Dilip D. Patel, Brian L. Knight, E. David Saggerson, Geoffrey F. Gibbons and Richard J. Pease

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