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Cloning, sequencing and heterologous expression of a cDNA encoding pigeon liver carnitine acetyltransferase

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Two overlapping cDNA clones encoding pigeon liver carnitine acetyltransferase (EC 2.3.1.7) (CAT) were isolated from a pigeon liver λgt11 cDNA library by gene amplification using oligonucleotide primers based on the N-terminal amino acid sequence of the enzyme. The two clones, which represent the 5′ and 3′ ends of the gene, were spliced together to form a single cDNA construct containing the entire coding sequence for CAT, with an in-frame TGA stop codon 42 bases before the first ATG start site and a 3′-untranslated segment of 1057 bases. The largest open reading frame of 1942 nucleotides predicted a polypeptide of 627 amino acids and a molecular mass of 71.1 kDa. The N-terminus and four internal peptides from the amino acid sequence of pigeon breast muscle CAT were identified in the predicted sequence of the liver cDNA clone. The identity of the CAT cDNA was confirmed by heterologous expression of active recombinant CAT (rCAT) in insect cells using the baculovirus expression system. Western blots of rCAT from infected insect cell lysates and immunodetection with a rabbit anti-CAT polyclonal serum showed an immunoreactive protein band similar in size to native CAT from pigeon breast muscle. Like the native enzyme, rCAT was capable of acylating carnitine with a preference for small-chain acyl-CoAs of carbon chain lengths C_2–C_4.

INTRODUCTION

Carnitine acetyltransferase (CAT) is a member of a small family of enzymes involved in the transfer of acyl groups of different lengths from acyl-CoAs to L-carnitine (Bieber and Farrell 1983; Bieber, 1988). Reactions catalysed by carnitine acyltransferases are reversible because, regardless of chain length, the free-energy potential of the acyl-carnitine product is similar to that of the acyl-CoA substrate (Colucci and Gandour, 1988). The individual enzymes are defined by their particular substrate specificities, with CAT being characterized by its preference for short-chain acyl-CoAs (C_2–C_4) (Chase, 1967; Farrell et al., 1984; Bloisi et al., 1990). Genes encoding some members of the carnitine acyltransferase family from different species and tissues have been cloned and sequenced (Chatterjee et al., 1988; Brice et al., 1989; Woeltje et al., 1990; Finocchiaro et al., 1991; Esser et al., 1993; Kispal et al., 1993). Analysis of these genes and their encoded proteins has demonstrated that these biochemically distinct acyltransferases are remarkably similar (Esser et al., 1993; Kispal et al., 1993).

The biological significance of CAT remains speculative. It has been proposed that CAT activity is required to buffer the free CoASAc/CoASH ratio within cells (Pearson and Tubbs, 1967; Lysiak et al., 1988; White and Scates, 1990). Furthermore, acetylcarnitine may serve as an activated reservoir of acetyl groups, which can be efficiently used as a source of acetate to condense with oxaloacetate and form citrate (Bieber, 1988). In higher eukaryotes, CAT may be involved with the transport of short- or branch-chained acyl moieties out of the mitochondria for fatty acid biosynthesis or urinary excretion (Chalmers et al., 1983; Agius et al., 1987). In contrast with CAT, the biological roles of long- and medium-chain acyltransferases are better understood. The carnitine palmitoyltransferase (CPT) enzymes appear to be involved in shuttling long-chain fatty acids into the mitochondrial matrix to the site of β-oxidation. This transport process involves the transfer of palmitate from palmitoyl-CoA to L-carnitine by CPT-I, translocation across the inner mitochondrial membrane and conversion back into palmitoyl-CoA by CPT-II (Hoppel, 1976; McGarry and Foster, 1980; Bieber and Farrell, 1983; Bieber, 1988; McGarry et al., 1991).

Inherited deficiencies of CPT activity in humans result in metabolic aberrations characterized by hypoketonaemia and hypoglycaemia (Bougneres et al., 1981; Bonnefont et al., 1989; Demaugre et al., 1991; Taroni et al., 1992). Furthermore, studies involving CPT-I inhibitors, e.g. Etomoxir {ethyl 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate}, have shown that these compounds lower blood glucose levels in diabetic animals and humans (Ratheiser et al., 1991; Wolf, 1992). These findings provide evidence that supports the use of CPT inhibitors for the treatment of non-insulin-dependent diabetes. However, the irreversibility of CPT-I inhibition of compounds such as Etomoxir may contribute to undesired side effects observed in animals, e.g. cardiac, renal and hepatic hypertrophy (Wolf, 1992; Foley, 1992), which have hindered further development of such agents for the treatment of type-II diabetes in humans.

A better understanding of the structure/function relationships within the carnitine acyltransferase family should assist the rational development of reversible CPT inhibitors that may be useful for the treatment of non-insulin-dependent diabetes. To this end, studies to define the three-dimensional structure of the closely related CAT enzyme were initiated. Pigeon muscle CAT was selected as a model because it is commercially available, is well characterized biochemically and may be amenable to crystallization (Chase et al., 1965). As the determination of protein structure by X-ray crystallography requires peptide-sequence information for interpretation of the diffraction data,

Abbreviations used: CAT, carnitine acetyltransferase; rCAT, recombinant carnitine acetyltransferase; CPT, carnitine palmitoyltransferase; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; SF-9, *Spodoptera frugiperda* cells.

The nucleotide sequence data reported in this paper appears in GenBank under accession number UO 8229.

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this report describes the isolation and characterization of two overlapping CAT cDNAs from a pigeon liver λgt11 cDNA library which together encode the entire CAT protein.

MATERIALS AND METHODS

Analysis of peptide sequence of CAT

Reduction and alkylation of pigeon breast muscle CAT (Sigma), enzymic digestion with endoproteinase Lys-C (Boehringer-Mannheim), separation of peptides by reversed-phase chromatography and determination of amino acid sequence on an Applied Biosystems 470A protein sequencer were performed as previously described (Lokker et al., 1991) with a minor modification: iodoacetic acid was used in place of 4-vinylpyridine for alkylation.

Microbial cultures and transformation

All transformations of competent XL-1 Blue *Escherichia coli* (Stratagene) were performed using $CaCl_2$ (Sambrook et al., 1989). *E. coli* cultures (XL1-Blue or Y1090) were grown in Luria–Bertani broth (components from Gibco/BRL) or on Luria–Bertani plates containing ampicillin (50 μg/ml) at 37 °C. Plasmid isolation and purification were carried out using Magic Minipreps kits (Promega).

Cloning methodology

Genomic phage DNA from a pigeon liver λ cDNA library (Clonetech) was amplified in plate lysates of Y1090 *E. coli* (Sambrook et al., 1989) and purified using the Magic Lambda Preps kit (Promega). A CAT-specific DNA probe was generated by PCR using degenerate oligonucleotide primers designed to amplify 120 bases encoding the N-terminal peptide. The forward primer was 5′-CA(TC) CA(AG) GA(AG) GC(N) (TC)T(N) CC(N) CA(TC) (TC)T(N) CC-3′, and the reverse primer was 5′-TC (TC)TG (N)GT (AG)TG (AG)TT (N)T(AG) (TC)TC (TC)-TC(TC)TC-3′. The degenerate oligonucleotides were synthesized on an Applied Biosystems, Inc. (ABI) 392 RNA/DNA synthesizer using phosphoramidite chemistry with chemicals supplied by ABI. PCR was performed as described by Perkin–Elmer–Cetus using λ cDNA (0.5 μg) with annealing for 1 min at 50 °C and extension for 1 min at 72 °C. The amplified DNA was recovered by ethanol precipitation (Sambrook et al., 1989) and separated on a 4% 3:1 NuSieve agarose gel prepared with 0.5×TBE (45 mM Tris base, 45 mM boric acid and 1 mM EDTA disodium salt) (Sambrook et al., 1989). The 120 bp product was excised from the gel and the DNA was eluted by the freeze/thaw method (Li et al., 1988). The eluted 120 bp DNA (5 μl) was reamplified, precipitated with ethanol and directly cloned into pCR™II (Invitrogen). Three clones containing 120 bp inserts were isolated and sequenced with universal M13 primers by the dideoxynucleotide method (Sanger et al., 1977) using the Sequenase II kit (U.S. Biochemical) and [α-^{33}P]dATP (New England Nuclear). The dried 8% acrylamide sequencing gels (Gibco/BRL) were exposed to Kodak XAR-5 film for 16 h.

On the basis of the sequence information from the 120 bp clones, a large CAT-specific oligonucleotide of 63 bases was synthesized on the Milligen Cyclone Plus DNA synthesizer, 3′-end-labelled with fluorescein-11 dUTP (Amersham) and used to screen 50000 λ plaques. Hybridization was performed at 42 °C with two 60 °C washes (Amersham, Enhanced Chemiluminescence). Positive plaques from a second screen were amplified and the phage DNA was isolated as described above. A 1400 bp insert was excised with *Eco*RI, separated on a 1% agarose gel (Gibco/BRL), isolated with GeneClean II (Bio101), subcloned into Bluescript II KS (−) (Stratagene) and sequenced using nested sets of oligonucleotide primers (Figure 1).

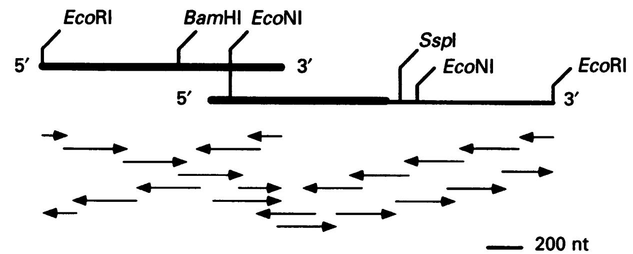

Figure 1 Map of pigeon CAT 1.4 and 2.0 kb cDNA clones

A partial restriction endonuclease map of the two isolated CAT cDNA clones is shown. The thick lines represent the coding region and the scale bar at the lower right indicates the approximate size. The arrows represent regions of sequence data. The *Eco*NI site in the overlapping region of the two clones was used to splice the cDNAs together and the *Eco*RI–*Ssp*I fragment was then transferred to pVL1392 in preparation for expression.

The purified 1.4 kb CAT *Eco*RI fragment (100 ng) was biotin-labelled (Random Prime Images kit; USB) and used to screen 5000 λ plaques as described above (Images kit; USB). Positive plaques were processed as described above. A 2000 bp insert was excised with *Eco*RI, subcloned and sequenced as described above (Figure 1).

The 1.4 and 2.0 kb CAT clones were ligated together to form a single cDNA containing the entire CAT coding region. The 1.4 kb clone was linearized with a single *Eco*NI digestion and then ligated to the *Eco*NI fragment from the 2.0 kb clone (Figure 1) using high-concentration T4 DNA ligase (New England Biolabs) (Sambrook et al., 1989). The entire CAT coding region was excised with *Eco*RI–*Ssp*I and ligated to *Eco*RI–*Sma*I-digested pVL1392 (Invitrogen), pVL1393CAT#4.

Transfection, screening and plaque purification of a recombinant baculovirus

The wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA, *Spodoptera frugiperda* (SF-9) cells and transfer vector pVL1392 were purchased from Invitrogen. The SF-9 cells were maintained as described by Johnson and Li (1991) with Grace's TNM-FH-supplemented medium (Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (Biocell Inc.). Liposome-mediated co-transfection of SF-9 cells with linearized AcMNPV DNA and the cloned pVL1392 CAT construct was carried out using the Transfection Module kit (Invitrogen). The manufacturer's protocol was followed except that HyQ serum-free medium (Hyclone) was used and the transfection was carried out overnight.

The recombinant AcMNPVs CAT-2C.2 and CAT-2C.4 were identified and plaque-purified twice (Johnson and Li, 1991). The plaque assay mixtures were incubated at 27 °C in a humidified container for 6 days and then overlaid with 1 ml of Neutral Red (0.1 mg/ml) in 1% Seaplaque GTG agarose (NuSieve). After 24 h, the recombinant AcMNPV plaques were visualized by light microscopy, excised with a sterile 50 μl glass capillary pipette and stored at 4 °C in 0.4 ml of Grace's complete medium.

Screening for expression of recombinant CAT (rCAT) was carried out as previously described (Johnson and Li, 1991) using 10 μl of cell extract for SDS/PAGE, Western blotting and immunodetection. The first antibody, i.e. rabbit anti-CAT polyclonal serum (see below) diluted 1:3000, was detected with

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   1 GAATTCCGGGCATGACAGACGAGGGAGAGGTTTCCATTCTTTTCTGTCCTCCCAGCCATGGATAGGAAACAGAAGCAAGCAGAGAAGGCCAGGCCCTATG  100
   1                                                         M  D  R  K  Q  K  Q  A  E  K  A  R  P  Y  G   15
 101 GCCTGCTGAAGCCGGCAGCGTTAGGCAAGATCCCGGGCAGATTCCAACTTCATCAGGAAGCGCTGCCCCATCTCCCCGTGCCGCCGCTCCAGCAAACGCT  200
  16  L  L  K  P  A  A  L  G  K  I  P  G  R  F  Q  L  H  Q  E  A  L  P  H  L  P  V  P  P  L  Q  Q  T  L   48
 201 GGACCGTTACCTGCTGGCTCTGCAGCCCATCATCAGCGAGGAGGAGCTGAACCACACGCAGGAGCTGGTGGCCGAGTTCCGCAAGCCGGGCGGTGTCGGG  300
  49  D  R  Y  L  L  A  L  Q  P  I  I  S  E  E  E  L  N  H  T  Q  E  L  V  A  E  F  R  K  P  G  G  V  G   81
 301 GAAAGGCTGCAGAAGGGCCTGGAGAGGAGAGCCAAGAAAACAGACAACTGGCTCTCGGACTGGTGGCTGAAGACCGCGTACCTGGAGTATCGCCTGCCCG  400
  82 E  R  L  Q  K  G  L  E  R  R  A  K  K  T  D  N  W  L  S  D  W  W  L  K  T  A  Y  L  E  Y  R  L  P  V  115
 401 TCGTGGTCCACTCCAGCCCAGGTGTGGTTTACCTAAGCAGGATTTTCAGGATCGACAAGGTCAGCTCAGGTTTGCTGCCAAGCTGATCGAGGGCATCCT  500
 116  V  V  H  S  S  P  G  V  V  L  P  K  Q  D  F  Q  D  R  Q  G  Q  L  R  F  A  A  K  L  I  E  G  I  L  149
 501 GGATTTCAAGACCATGATTGACAATGAGACCCTCCCGGTGGAGTACATGGGTGGGAAGCCCCTCTGCATGAACCAGTACTACCAGATCCTCTCATCCTGC  600
 149  D  F  K  T  M  I  D  N  E  T  L  P  V  E  Y  M  G  G  K  P  L  C  M  N  Q  Y  Y  Q  I  L  S  S  C   181
 601 CGCATCCCTGGGCCCAAGCGGGATTCCATCGTCAACTACGCCAAAGGCAAAAAGCAGTCCAGACACATAACGGTGGTTCACAACTTCCAGTTCTTTGAGC  700
 182 R  I  P  G  P  K  R  D  S  I  V  N  Y  A  K  G  K  K  Q  S  R  H  I  T  V  V  H  N  F  Q  F  F  E  L  215
 701 TGGATGTTTACAACAGCGATGGAAGTCCCCTCACCACTGACCAGCTCTTCATTCAGCTGGAGAAGATATGGAACACCTCCCTCCAAACAAACAAAGAACC  800
 216  D  V  Y  N  S  D  G  S  P  L  T  T  D  Q  L  F  I  Q  L  E  K  I  W  N  T  S  L  Q  T  N  K  E  P  248
 801 TGTTGGGATCCTCACAACAAACCACCGGAACAGCTGGGCAAAAGCCTACAACAACCTGCTGAAAGATAAGACCAACAAGGAATCTGTGCGTACGATTGAG  900
 249  V  G  I  L  T  T  N  H  R  N  S  W  A  K  A  Y  N  N  L  L  K  D  K  T  N  K  E  S  V  R  T  I  E   281
 901 AAGAGCATTTGCACCATCTGCCTCGACGCGCCCATGCCGCGGGTGTCCGACGACATCTACAAGAGCCCGGTGGCCGCCCAGATGCTGCACGGCGGAGGCA  1000
 282 K  S  I  C  T  I  C  L  D  A  P  M  P  R  V  S  D  D  I  Y  K  S  P  V  A  A  Q  M  L  H  G  G  G  S  315
1001 GTCGCTGGAACAGCGGGAACCGATGGTTCGACAAAACCCTTCAGTTCATCATCGCTGAAGATGGCTCCTGTGGTCTCGTTTACGAACACGCTCCTGCAGA  1100
 316  R  W  N  S  G  N  R  W  F  D  K  T  L  Q  F  I  I  A  E  D  G  S  C  G  L  V  Y  E  H  A  P  A  E  348
1101 AGGCCCACCCATTGTTGCTCTTTTGGATCACATTGTGGAGTACACGAAGAAACCTGAGTTGGTAAGATCGCCTATGATTCCTTTGCCAATGCCCAAAAAG  1200
 349 G  P  P  I  V  A  L  L  D  H  I  V  E  Y  T  K  K  P  E  L  V  R  S  P  M  I  P  L  P  M  P  K  K   381
1201 CTGAGGTTTAACATCACCCCAGAAATCAAGAGTGACATAGAGAAAGCGAAGCAGAACCTCAACATAATGGTCGAAGACCTGGATGTCATCGTCTTGGTCT  1300
 382 L  R  F  N  I  T  P  E  I  K  S  D  I  E  K  A  K  Q  N  L  N  I  M  V  E  D  L  D  V  I  V  L  V  F  415
1301 TTCATCAATTTGGGAAAAACTACCCCAAATCTGAGAAGATAAGTCCTGATGCCTTTATCCAGCTGGCCTTGCAGCTGGCGTATTACAGAATGTACGGCCA  1400
 416  H  Q  F  G  K  N  Y  P  K  S  E  K  I  S  P  D  A  F  I  Q  L  A  L  Q  L  A  Y  Y  R  M  Y  G  H  448
1401 CTCCTGTGCCACGTACGAGAGCGCGTCGCTAAGGATGTTCCGCCTGGGCCGCACGGACACCATCCGCTCCACTTCTATAGAATCCCATAAGTTTGTGCAA  1500
 449  S  C  A  T  Y  E  S  A  S  L  R  M  F  R  L  G  R  T  D  T  I  R  S  T  S  I  E  S  H  K  F  V  Q   481
1501 TCAATGGACAGCCCGGACAAATCGGACCAGGAGAAAGCAGACTTGCTGCGGAGAGCTACCCAGGCGCACAAGGAATACACCAATATGGCAATACAGGGCA  1600
 482 S  M  D  S  P  D  K  S  D  Q  E  K  A  D  L  L  R  R  A  T  Q  A  H  K  E  Y  T  N  M  A  I  Q  G  N  515
1601 ACGCAATAGATCGCCACCTCTTAGGCTTGAAGCTTCAAGCCATCGAGGACTTAGTGAGCATACCTGAGCTGTTCATGGACACAGCCTACGCCGTTGCAAT  1700
 516  A  I  D  R  H  L  L  G  L  K  L  Q  A  I  E  D  L  V  S  I  P  E  L  F  M  D  T  A  Y  A  V  A  M  548
1701 GCACTTCAACCTGTCAACCAGCCAGGTCCCAGCAAAGACAGACTGCGTGATGTGTTTTGGTCCCGTGGTTCCAGATGGCTACGGAATCTGTTACAACCCC  1800
 549  H  F  N  L  S  T  S  Q  V  P  A  K  T  D  C  V  M  C  F  G  P  V  V  P  D  G  Y  G  I  C  Y  N  P   581
1801 ATGGGTGAGCACATCAACTTTGCCATTTCAGCCTTCAACAGCTGTGCTGACACCAACGCAGCTCGCATGGCACATTATCTTGAGAAGGCACTGCTAGACA  1900
 582 M  G  E  H  I  N  F  A  I  S  A  F  N  S  C  A  D  T  N  A  A  R  M  A  H  Y  L  E  K  A  L  L  D  M  615
1901 TGAGGAGCTTGCTCCAGTCCGCTCCCAAATCCAAACTGTAAGAGGCAAACGTGATGCAAAACCCCCAGCTAAAGGGGCTGTTCTTCATGCACTGAAGTTC  2000
 616  R  S  L  L  Q  S  A  P  K  S  K  L  *                                                                627
2001 CCAGTTCCTCAGAAAACTATTGAGAGCACAGCTGGCCCTGGAACCTTGGAGGGAATCTATCATTTGTTTTCAGCAAGAGGCTACAGAATAGTCATCTGCA  2100
2101 AATATTACTGGTAAGAGGAAACGCTGTCTTTAGCCGAAGTAGAACCAACTGGTCCTCTCCCATCAATACAAAGCGCACCCTCCTGTCCTCAAAGAGGACT  2200
2201 GAAGTATTATTTTTATGGAACTTACTATAAAGGAATACAGAAAGCACAGTCCTTAGCAGTATGTAAATCCATTTGGTGAGGCAGGTAGTTCTTCTCCAGG  2300
2301 AAAAGCAGGGGACATTCATAATGTGATGGGACTACTGTAAGACCAGTCTTTCTGCTGGTTTCTGAAGAGTGCAGCATCTGTCAAACTTGACTGAGGATCC  2400
2401 TTCTGCTGCTCCAGCAAAACGTCACCATTGAAGTTTGGCAAGGGGAGAAAGAAGTTCTTAAGTGGGAGCAGATCCAGGCTCGTTAGAGTGCCCCGCACTA  2500
2501 CTTATCCCAGGCACTCCAACCATCGCATGCTTTACAGGCCTGGGAACGTTCCCTTTGCTCGTTTTAGGATGCCCTTTCTGTTGCATTGACCCTATCAACA  2600
2601 ACAGAACATCATTTGCTACTAACTAAGGTGTTGTTGTTGGTTTTGTTCTGAGTTTTTTGTTAGGCTTGCTGTTGTGCTCCTAGCCACGGATCTTCAGGCT  2700
2701 CAGCATAAAGATTTTATTCCCCACAAAACTCCTTTCACACAGGTTTTCCCAGCTTTGACTTTATGTACCTTAATGTTTGGAGAGATGAGAAGGAATTGCC  2800
2801 TGTGCAGTTAGCAGGACACTTGCTTCGTTGTACCTTCCTTTGGAACTGAGGAAAAGAACAGATGCTGCCCTTCTGTTTACCACCCAGGGGGCCACATGCG  2900
2901 AACACCATTACATACTTCACTTTCAGAAACAACATATTTGTACATAATGCGTTATGTCTAAGTTGTGGACAAATAAGAATGTTTCTAATGAGGAATTC    2998
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Figure 2 Nucleotide and predicted amino acid sequence of pigeon CAT

The nucleotides and amino acids are numbered at the sides. In-frame stop codons were identified upstream (**bold**/underlined) and 1884 nucleotides downstream (asterisk) of the initiation codon. Regions of predicted amino acid sequence found to be identical with the actual peptide sequence of the native pigeon breast muscle CAT are underlined. The histidine residue at position 344 (**bold**) is conserved among all members of the acyltransferase family.

alkaline phosphatase-conjugated anti-rabbit IgG (1:3000) (Bio-Rad). Alkaline phosphatase blots were developed by Sambrook et al. (1989). Total cellular DNA from infected SF-9 cells was isolated (Johnson and Li, 1991), digested with *Bam*HI–*Eco*RI and separated on a 1% agarose gel. The DNA was Southern transferred (Southern, 1975) to a nylon membrane and probed with biotinylated 1.3 kb *Bam*HI and 0.8 kb *Bam*HI–*Eco*RI fragments from pVL1392CAT#4 (Images kit; USB) (Figure 1). A size marker (1 kb ladder) was purchased from Gibco/BRL.

Development of rabbit anti-CAT polyclonal serum

Rabbit anti-CAT polyclonal serum was developed by Hazleton Research Products. Two female New Zealand White rabbits were injected intradermally with 200 μg of pigeon breast muscle CAT (Sigma) in 1 ml of complete Freund's adjuvant and 1 ml of PBS (Gibco/BRL). After 1 month, booster immunizations were administered every 3 weeks using incomplete Freund's adjuvant, with bleedings every 2 weeks after the first booster. The positive production of antibodies was determined by Western blot and immunodetection (see above) using pigeon breast muscle CAT and serial dilutions of the rabbit serum. Preimmune serum at the lowest dilution (1:1000) was used as a negative control.

CAT assays

SF-9 cells were either mock infected or infected with wild-type or recombinant AcMNPVs at a multiplicity of infection of 10. The cells were scraped from the flasks 72 h after infection and pelleted by centrifugation at 1000 ***g***. The medium was decanted and the cell pellets were immediately frozen on solid CO_2. The cell pellets were lysed by thawing in 400–600 μl of lysis buffer (5 mM potassium phosphate/1% octyl glucoside/50 mM KCl/1 μM pepstatin/10 μM leupeptin/1 mM phenylmethanesulphonyl fluoride, pH 7.5). To evaluate the effect of detergents, 1% Triton X-100 was substituted for octyl glucoside. CAT activity assays, using 2–5 μl of cell extract, were performed as previously described (Bieber and Fiol, 1986) except that final concentrations

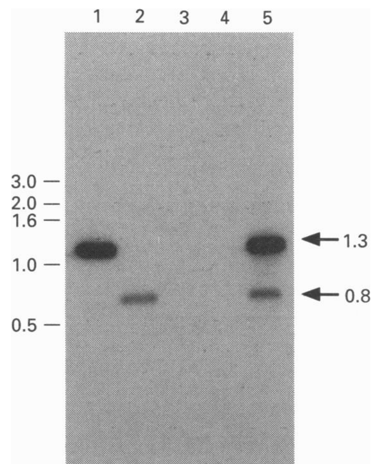

Figure 3 Southern-blot analysis of total DNA isolated from infected SF-9 cells

Total DNA from mock- (lane 3), wild-type- (lane 4) and CAT2C.4- (lane 5) infected SF-9 cells was isolated and digested as described in the Materials and methods section. Lanes 3–5 contained equivalent amounts of total intracellular DNA as determined by ethidium bromide staining. Size markers (1 kb ladder) are indicated on the left. Positive controls (0.1 μg/lane probe DNAs) were loaded into lanes 1 and 2.

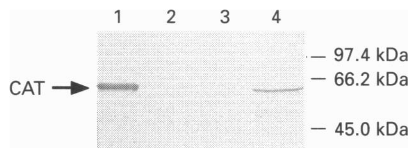

Figure 4 Western blot and immunodetection of CAT in SF-9 cell extracts

Cell extracts were prepared from mock- (lane 2), wild-type- (lane 3) and CAT2C.4- (lane 4) infected SF-9 cells. Lanes 2–4 contained approximately the same amount of total protein, as determined by SDS/PAGE of the same samples (10 μl/0.5 ml lysate) and Coomassie Blue staining. Lane 1 contained 0.5 μg of pigeon breast muscle CAT. The approximate molecular sizes are indicated on the right.

of Hepes and EDTA were 100 mM and 1 mM respectively. All acyl-CoA substrates were added to a final concentration of 0.1 mM. Reactions were monitored for 2 min at 405 nm using a 96-well spectrophotometer (Molecular Devices). Protein concentrations were determined using the DC protein assay kit (Bio-Rad) with BSA as the standard protein.

RESULTS

In the initial cloning effort, a 120-nucleotide fragment was amplified by PCR from a pigeon liver λ cDNA library using degenerate oligonucleotide primers designed to amplify the region encoding the N-terminal peptide of pigeon breast muscle CAT (Figure 2). Sequence analysis of three subclones containing 120 bp inserts revealed that all three contained identical inter-primer sequences but that each was primed by a different oligonucleotide at the 5′ and 3′ ends. The consensus sequence among the three clones comprised sequence encoding the N-terminal peptide of CAT (results not shown). Using this information, a large CAT-specific 65-base oligonucleotide was

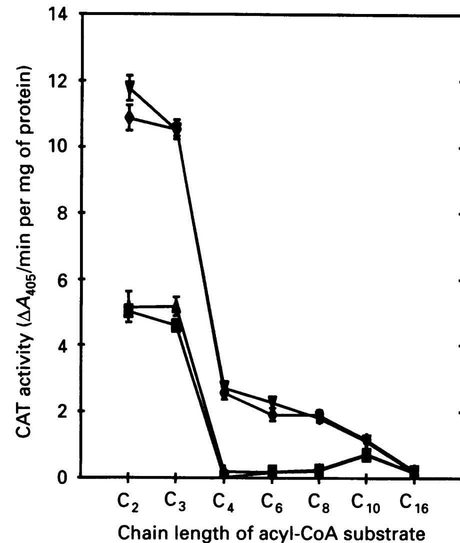

Figure 5 rCAT activity in SF-9 cell extracts

A substrate profile of rCAT activity from CAT2C.2- (▼) and CAT2C.4- (◆) infected SF-9 cell extracts is shown. All specific activities are presented as means ± S.D. of triplicate assays. Acyl-CoA concentrations and assay conditions were as described in the Materials and methods section. Control assays were performed with equivalent samples of mock- (▲) and wild-type- (■) infected SF-9 cell extracts.

synthesized, end-labelled and used to screen 50000 plaques from the same λ cDNA library. The sequence from a 1.4 kb insert indicated that a clone representing the 5′ portion of CAT had been isolated. In turn, the 1.4 kb clone was used to rescreen the library for other CAT clones. Sequence from a second 2.0 kb insert showed that it represented the 3′ end of the CAT gene with sequence overlapping the 1.4 kb clone (Figure 1).

The sequence information from the two clones revealed that the entire construct consisted of 2998 nucleotides with a single long open reading frame of 1941 bp encoding a 627 amino acid polypeptide and a long untranslated 3′ end (Figure 2). The N-terminus and four internal peptides from the amino acid sequence of pigeon breast muscle CAT were identified in the predicted peptide sequence of the liver cDNA clone (Figure 2). The predicted protein was calculated to have a molecular mass of 71.1 kDa. This calculated mass is similar to that predicted for yeast CAT (Kispal et al., 1993) and those estimated by biophysical studies of rat liver, bovine spermatozoa and pigeon muscle CAT enzymes, e.g. mobility of native CAT on SDS/PAGE gave size estimates of 62–63 kDa (Huckle and Tamblyn, 1983; Miyazawa et al., 1983; Colucci and Gandour, 1988; Chung et al., 1991) (see Figure 4, lane 1). However, the actual N-terminal amino acid sequence of the mature pigeon muscle enzyme was identified 31 residues downstream of the first methionine in the predicted amino acid sequence. If the initial 30 amino acids represent a leader sequence and are cleaved during processing of the nascent protein, then a molecular size of 67.7 kDa would be expected for the mature enzyme. The start site is considered to be authentic because of the presence of an in-frame stop codon lying 42 bases upstream from the ATG (Figure 2). However, this CAT cDNA sequence may not be complete because a poly(A) tail was not identified at the 3′ end. The predicted pigeon liver CAT enzyme was similar to the other carnitine and choline acyltransferases in the regions previously described (Kispal et al., 1993) but was no

more homologous to yeast CAT than to the other mammalian long-chain carnitine acyltransferases.

An *Eco*NI site within the overlapping region of the two clones was used to splice the two clones together to form a CAT cDNA containing the entire coding region of the enzyme (Figure 1). To verify the identity of the CAT cDNA, the construct was subcloned into a baculovirus expression vector and heterologously expressed in insect cells. Two identical recombinant baculovirus CAT clones were identified and purified (CAT2C.2 and CAT2C.4). A Southern blot of the total genomic DNA from mock-, wild-type- or recombinant AcMNPV-infected SF-9 cells showed that the recombinant AcMNPV CAT2C.4 contained the entire CAT cDNA (Figure 3). Moreover, the CAT2C.4 infection produced an immunoreactive protein similar in apparent molecular mass to the native pigeon enzyme (Figure 4). The polyclonal anti-CAT serum used in these experiments was raised against the pigeon muscle CAT and did not cross-react with proteins of similar size in the mock- and mild-type-infected insect cell extracts. Similar results were generated when the second recombinant AcMNPV CAT2C.2 was used (results not shown).

Assays for carnitinetransferase activity showed that the insect cells infected with the recombinant baculovirus clones (CAT2C.2 or .4) had $44 \pm 7\%$ ($n = 3$) higher specific activity for CoASAc than the controls and that the activity was clearly more specific for short-chain (C_2–C_4) than long-chain acyl-CoA substrates (Figure 5). The CAT activity in cell extracts treated with 1% Triton X-100 or 1% octyl glucoside was approximately the same.

DISCUSSION

The gene encoding pigeon liver CAT was cloned to provide primary amino acid sequence information to assist in defining its tertiary structure and mechanism of catalysis. The identity of the CAT cDNA was verified by heterologous expression of the cloned gene in insect cells. The expressed rCAT was capable of catalysing the transfer of acetate from CoASAc to carnitine and this reaction appeared to favour short-chain acyl-CoAs over long-chain ones. Since the N-terminus and four internal peptides from the pigeon muscle CAT were identified in the predicted sequence of the liver enzyme, these isoenzymes are presumed to be very similar. This hypothesis is supported by other biochemical studies indicating that CATs from different tissues and species are similar (Edwards et al., 1974). Differences among the muscle and liver enzymes in regions that were not sequenced cannot, however, be ruled out.

The apparent molecular mass of rCAT measured by SDS/PAGE was similar to that of the native pigeon muscle enzyme. However, the apparent size (62–63 kDa) was significantly smaller than that predicted for pigeon liver CAT from the cDNA sequence (71.1 kDa). Part of the discrepancy between the apparent and calculated mass of pigeon liver CAT may be due to proteolytic processing of a leader sequence. Two lines of evidence support the presence of a leader sequence. First, as is characteristic of other leaders sequences, the predicted CAT N-terminus is composed primarily of hydrophobic and positively charged residues. Second, the N-terminus of the mature native muscle protein was identified 31 residues downstream from the initial methionine in the predicted pigeon liver CAT sequence. If the first 30 amino acids are omitted from the molecular mass calculation, a protein of 67.7 kDa would be expected. Even then, the predicted size of mature CAT remains somewhat larger than that observed on SDS/PAGE. This difference may be related to SDS/PAGE rather than to the enzyme itself. Also, the observed molecular size of pigeon CAT was greater than that observed for the mature form of the human liver enzyme (60.5 kDa) (Bloisi et al., 1990). Nevertheless, it is likely that human liver CAT will have significant homology with its pigeon liver counterpart because the amino acid compositions of the two enzymes are very similar.

Alignment of pigeon CAT with other members of the carnitine acyltransferase family showed that these proteins are related, especially in regions defined in other reports (Esser et al., 1993; Kispal et al., 1993). Of particular interest are the highly conserved histidine residues corresponding to His-203, His-311 and His-344 of the pigeon sequence. Biochemical studies with CAT have determined that a histidine and a cysteine residue are involved in transferase activity (Chase and Tubbs, 1970; Nishimura et al., 1982; Venkatraghavan and Smith, 1983). The histidine at position 344 and the negatively charged amino acids that flank it are conserved among all members of the carnitine acyltransferase family and the related enzyme choline acetyltransferase. If a histidine is, in fact, involved in CAT activity, then this particular residue would be the most likely candidate. Although consensus could not be found among cysteine residues within the carnitine acyltransferase family, Cys-170, Cys-285 and Cys-288 were somewhat conserved. Involvement of these residues in CAT activity will ultimately be determined by site-directed mutagenesis.

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