Molecular cloning of rat mitochondrial 3-hydroxy-3-methylglutaryl-CoA lyase and detection of the corresponding mRNA and of those encoding the remaining enzymes comprising the ketogenic 3-hydroxy-3-methylglutaryl-CoA cycle in central nervous system of suckling rat

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We have investigated, by RNase protection assays in rat brain regions and primary cortical astrocyte cultures, the presence of the mRNA species encoding the three mitochondrially located enzymes acetoacetyl-CoA thiolase, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mt. HMG-CoA synthase) and HMG-CoA lyase (HMG-CoA lyase) that together constitute the ketogenic HMG-CoA cycle. As a prerequisite we obtained a full-length cDNA encoding rat HMG-CoA lyase by degenerate oligonucleotide-primed PCR coupled to a modification of PCR–rapid amplification of cDNA ends (PCR–RACE). We report here: (1) the nucleotide sequence of rat mt. HMG-CoA lyase, (2) detection of the mRNA species encoding all three HMG-CoA cycle enzymes in all regions of rat brain during suckling, (3) approximately twice the abundance of mt. HMG-CoA synthase mRNA in cerebellum than in cortex in 11-day-old suckling rat pups, (4) significantly lower abundances of mt. HMG-CoA synthase mRNA in brain regions derived from rats weaned to a high-carbohydrate/low-fat diet compared with the corresponding regions derived from the suckling rat, and (5) the presence of mt. HMG-CoA synthase mRNA in primary cultures of neonatal cortical astrocytes at an abundance similar to that found in liver of weaned animals. These results provide preliminary evidence that certain neural cell types possess ketogenic potential and might thus have a direct role in the provision of fatty acid-derived ketone bodies during the suckling period.

INTRODUCTION

The four-carbon ketone bodies acetoacetate and 3-hydroxybutyrate arise from the catabolism of fatty acids and the ketogenic amino acid leucine. Serum ketone body levels rise significantly as a result of prolonged starvation and high fat diets, and during the suckling period (reviewed in [1]). During suckling, ketone bodies cross the blood–brain barrier and can act as an alternative fuel to glucose, providing up to 75% of the energy requirements of the brain (reviewed in [1,2]). In addition, during the period of intense myelination found in the developing postnatal brain, ketone bodies can act as major substrates for the production of myelin [2]. Diet-derived fatty acids constitute the major source of ketone body precursors, e.g. one molecule of the fatty acid palmitate (C_{16}) can be catabolized, via acetyl-CoA, to four molecules of ketone body (C_4). The major enzymic pathway for the generation of such fatty acid-derived ketone bodies is the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) cycle (reviewed in [3]), which is driven by the three mitochondrially located enzymes acetoacetyl-CoA thiolase (EC 2.3.1.9), mitochondrial HMG-CoA synthase (mt. HMG-CoA synthase) (EC 4.1.3.5) and HMG-CoA lyase (HMG-CoA lyase) (EC 4.1.3.4). This cycle effects the conversion of acetyl-CoA to acetoacetate, part of which can then be converted to the alternative ketone body 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase.

Organs of the body are traditionally regarded as ketogenic (e.g. liver and white adipose tissue), non-ketogenic (e.g. brain, heart and muscle) or both (e.g. kidney and intestine) [3]. The differential ketogenic capacities of body organs are associated with the levels of mt. HMG-CoA synthase mRNA [4] and mt. HMG-CoA synthase protein [4], which is proposed to be a major regulatory step in fatty acid-driven ketogenesis [5]. Changes in circulating fatty acid concentrations seem to regulate the expression of the mt. HMG-CoA synthase gene because rats weaned to a high-fat diet display elevated hepatic abundances of mt. HMG-CoA synthase mRNA [4,6], protein [4], and enzyme activity [5]. The brain, which requires a large input of fuel energy, is regarded as non-ketogenic [3]; this is supported by the inability to detect, so far, mt. HMG-CoA synthase mRNA or protein in the brains of suckling rats [4] or adult mice [7]. In addition, studies attempting to demonstrate the presence of the three enzyme activities necessary to execute the HMG-CoA cycle in mitochondria of whole brain [8–11] or cultured astrocytes [12] are generally equivocal. In contrast, the liver is an overall provider of ketone bodies as it converts most diet-derived fatty acids to ketone bodies that are then released into the blood for

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; mt., mitochondrial; PPARα, peroxisome proliferator-activated receptor α; PUFA, polyunsaturated fatty acid; RACE, rapid amplification of cDNA ends; RPA, RNase protection assay.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y10054.

consumption by other organs such as heart and brain [13]. In keeping with this role, liver expresses high levels of mt. HMG-CoA synthase mRNA and protein, especially during the suckling period when serum free fatty acid levels are high [4,6,14].

However, although the brain as a whole does not show great ketogenic potential, this does not exclude the existence of brain compartments in which significant ketogenesis takes place. Significantly, potentially ketogenic polyunsaturated fatty acids (PUFAs) such as linoleic, arachidonic and docosahexaenoic acids [15] exhibit facile transfer across the blood–brain barrier during the suckling period [16,17]. Furthermore recent hybridization studies *in situ* [18,19] have demonstrated the localization of the peroxisome proliferator-activated receptor α (PPARα), a nuclear transcription factor that is activated by a variety of PUFA ligands [20,21], to certain brain regions. Evidence *in vitro* [22] has demonstrated that the PUFA linoleic acid and PPARα act synergistically to effect a more than 30-fold increase in chloramphenicol acetyltransferase activity in HeLa cells transfected with plasmid containing a chloramphenicol acetyltransferase reporter gene driven by mt. HMG-CoA synthase promoter sequences. Such results, combined with the demonstration that PPARα binds a *cis*-acting DNA sequence, termed the peroxisome proliferator response element, in the promoter region of the mt. HMG-CoA synthase gene [22], indicate that the regulation of mt. HMG-CoA synthase gene expression in response to PUFAs *in vivo* is mediated by PPARα. Therefore such evidence indicates the availability of a potential PUFA fuel source for the developing brain along with the necessary PUFA-responsive transcription factors to enable certain cell types of the brain to exploit the ketogenic potential of such PUFAs.

Thus, as a first step towards investigating the possible existence of ketogenic capacity within local brain regions, we set out to establish whether any or all of the mRNA species encoding the three enzymes of the HMG-CoA cycle are expressed in selected subregions of rat brain, and/or primary cultures of rat astrocytes, by using the highly sensitive and specific RNase protection assay (RPA). As a prerequisite to this work, we obtained partial-length cDNA species encoding acetoacetyl-CoA thiolase and mt. HMG-CoA synthase, and a full-length cDNA encoding HMG-CoA lyase, and report the sequence of the latter cDNA here. The results presented here demonstrate for the first time the presence of mRNA species encoding all the components of the ketogenic HMG-CoA cycle in brain subregions of suckling rats, and further demonstrate significant abundances of mt. HMG-CoA synthase mRNA in primary cultures of neonatal rat cortical astrocytes.

MATERIALS AND METHODS

Animals

Pregnant female Wistar rats were housed in individual plastic cages and fed with a breeding diet (SDS 3; Special Diets Services). Newborn rats were allowed to suckle and were then weaned at 21 days to a high-carbohydrate/low-fat diet (RM1 maintenance diet; Special Diets Services) containing 61.5% (w/w) carbohydrate and 2.6% (w/w) fat.

Primary cell cultures of rat neonatal and adult cortical astrocytes

The methods of Noble and Murray [23] and Peuchen et al. [24] were followed for the isolation of neonatal and adult astrocytes from cortices of 1–2-day-old or 6–8-week-old rats. In both instances cells were harvested after 12–14 days *in vitro*. Both procedures routinely produced astrocyte cultures of more than 95% purity as assessed by staining with a polyclonal antiserum against glial fibrillary acid protein [23,24].

RNA isolation

Three 11-day-old and three 28-day-old rat pups were killed by decapitation; tissues were dissected and immediately frozen in liquid nitrogen. Cells were resuspended into supernatant medium by scraping and recovered by centrifugation at 400 ***g*** for 5 min. After complete removal of the supernatant medium, cells were frozen in liquid nitrogen and stored at -70 °C. Total RNA was isolated by the method of Chomczynski and Sacchi [25] and the concentration of each sample was determined from the absorbance at 260 nm. Aliquots (3 μg) of each RNA sample were subjected to electrophoresis through a denaturing 1% (w/v) agarose gel to ensure RNA integrity.

Isolation of partial-length cDNA species encoding rat mt. HMG-CoA synthase and acetoacetyl-CoA thiolase

Reverse transcription of adult rat (Wistar) liver total RNA (10 μg), primed with 100 ng of random nonamer oligonucleotides (Amersham), was catalysed with 200 units of Superscript II (Gibco-BRL) reverse transcriptase in a total volume of 20 μl, in accordance with the supplier's recommendations. The first-strand cDNA product was then treated with RNase A and RNase H and the reaction volume was increased to 100 μl as previously described [26]. Subsequently, two pairs of oligonucleotide primers, 5′-TGGTGATATCGCAGTCTACC-3′ and 5′-CTGTGAATTCCTCAGGGGAC-3′, and 5′-CACCAAGCTTGGTACTAT-3′ and 5′-GCCTCTGCAGTCATGAGA-3′, were designed from published sequences [27,28] to prime the amplification of approx. 0.8 and 0.7 kb of the coding region sequences of the rat mt. HMG-CoA synthase [27] and rat acetoacetyl-CoA thiolase cDNA [28] respectively. Both pairs of primers contained unique restriction endonuclease sites to facilitate the subsequent ligation of PCR products. PCRs were cycled 35 times at 94 °C for 40 s, 58 °C for 1 min and 72 °C for 70 s, in a Gene E thermocycler (Techne), with 2.5 units of *Taq* DNA polymerase (Promega) and 1 μl of the reverse-transcription reaction product as template.

Isolation of a full-length cDNA encoding HMG-CoA lyase

Reverse transcription of adult rat liver total RNA (10 μg) was performed as described above. PCR amplification was performed with two degenerate oligonucleotide primers [gsp1, 5′-GATCAAGCTTGGGT(AGCT)CC(AGCT)CA(AG)ATG-3′, and gsp2, 5′-AGCTGAATTCGT(AGCT)A(AG)CAT(AG)TC(CT)TTC-3′] designed respectively from two amino acid sequences, Trp-Val-Pro-Gln-Met (residues 81–85) and Met-Lys-Asp-Met-Leu-Thr (residues 214–219), present in the primary sequence of mouse HMG-CoA lyase [29]. PCR conditions were as above except that reactions were cycled at 94 °C for 40 s, 50 °C for 1 min and 72 °C for 70 s for three cycles followed by 32 cycles at 94 °C for 40 s, 58 °C for 1 min and 72 °C for 70 s. PCR products of the predicted size (approx. 400 bp) were isolated [30], eluted, precipitated with ethanol, digested with *Eco*RI and *Hin*dIII restriction endonucleases and ligated into pBSII to give two subclones pcr1.2 and pcr1.5 (Figure 1), each generated from an independent PCR. The subclones were found to be identical by DNA sequencing and to encode 138 amino acid residues with 96% identity with the mouse HMG-CoA lyase.

Subsequently, the extreme 5′ and 3′ stretches of the HMG-CoA lyase cDNA were generated by semi-nested PCRs with

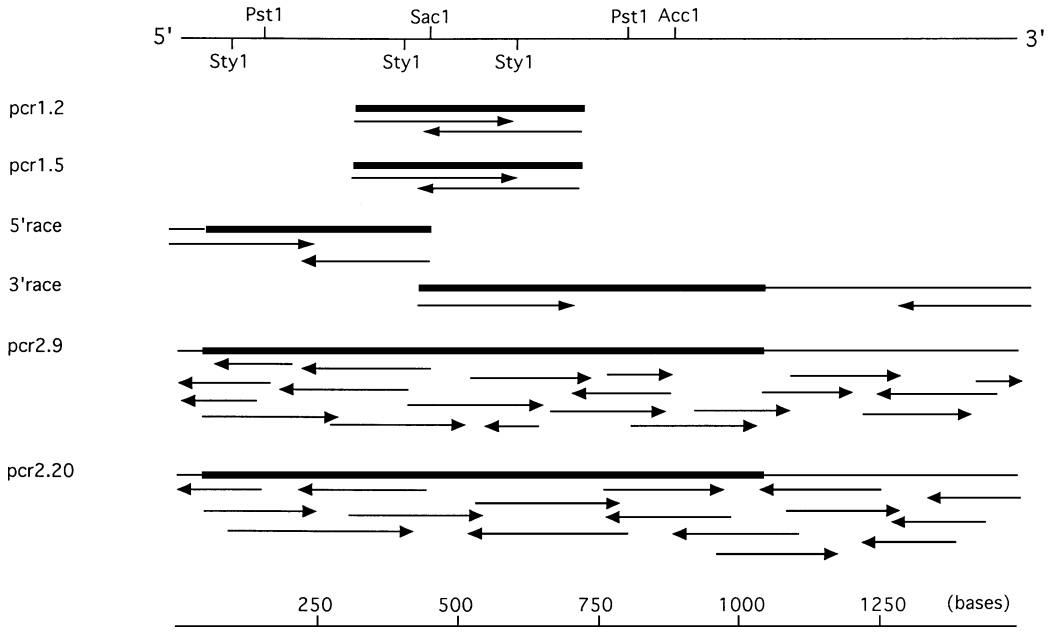

Figure 1 Cloning and sequencing of the cDNA encoding rat HMG-CoA lyase

The partial-length cDNA clones, pcr1.2, pcr1.5, 5′race and 3′race, and the full-length cDNA clones, pcr2.9 and pcr2.20, are represented by horizontal lines (non-coding regions) and solid horizontal bars (protein-coding regions). Arrows below each cDNA indicate the direction and extent of sequence determinations. The positions of restriction enzyme sites and the length of the cDNA species are indicated.

modifications of the 5′ and 3′ PCR–RACE protocols [31] as follows. After synthesis of first-strand cDNA from adult rat liver total RNA as described above, semi-nested 5′ PCR–RACE was performed as described previously [26], except that the reverse degenerate primer gsp2 was used as the gene-specific primer in the first set of PCRs. A 2 μl aliquot of a 1:100 dilution of the product of this first PCR was then used as the template for a semi-nested PCR with a nested gene-specific primer, gsp3 (5′-GGTGAAGAGCTCGGACGCAG-3′), designed from the sequences of pcr1.2 and pcr1.5, and the adaptor primer 365 (5′-CTGATCTAGAATTCGCGAAGC-3′) [26]. For 3′ PCR–RACE, first-strand cDNA was synthesized from adult rat liver total RNA (10 μg) with a mixture of three anchored oligo(dT) oligonucleotide primers as described previously [26]. 3′ PCR–RACE was then performed as described previously [31], except that, as in the 5′ PCR–RACE, the forward degenerate primer gsp1 was used as the gene-specific primer in the initial PCRs. A 2 μl aliquot of a 1:100 dilution of this first PCR was then used as the template for a semi-nested PCR with the nested gene-specific primer gsp 4 (5′-GGCTGCGTCCGAGCTCTTCA-3′) designed from the sequences of pcr1.2 and pcr1.5, and primer 365. Conditions for the first sets of PCRs for both 5′ and 3′ PCR–RACEs were three cycles at 94 °C for 40 s, 50 °C for 1 min and 72 °C for 75 s, followed by 32 cycles at 94 °C for 40 s, 55 °C for 1 min and 72 °C for 75 s. Semi-nested PCR conditions for both 5′ and 3′ PCR–RACEs were 35 cycles at 94 °C for 40 s, 57 °C for 1 min and 72 °C for 75 s. The ends of the 5′ and 3′ PCR–RACE products were rendered blunt with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase (both from New England Biolabs). The products were then purified (Spinbind; FMC Bioproducts) and ligated into pBSII previously digested with *Eco*RV. Two subclones, designated 5′race and 3′race (Figure 1), were sequenced to enable the design of two further oligonucleotide primers, gsp5 (5′-ATAAGAATGCGGCCGCACTCCGGGCGAAGATGGC-3′) and gsp6 (5′-GATCACGTGCGGCCGCTCAAGAATCACGTACAGA-3′), which primed the amplification of the full-length HMG-CoA lyase cDNA. These PCRs were performed as described above except that 10 μl of the reverse transcription product, synthesized previously for the amplification of the initial partial-length cDNA, was used as the template and the reactions were cycled 30 times at 94 °C for 40 s, 56 °C for 1 min and 72 °C for 75 s. Two independent PCR products were separately ligated into pBSII as described for the 5′ and 3′ PCR–RACE products, and two subclones from each ligation, designated pcr2.9 and pcr2.20 (Figure 1), were sequenced (see below).

DNA sequencing

Plasmid DNA was isolated by the method of Lee and Rasheed [32] and sequenced by the dideoxy chain-termination method [33] with a commercial sequencing kit (Sequenase II; United States Biochemicals) and [α-^{35}S]dATP (more than 1000 Ci/mmol; Amersham International). All sequencing reactions were primed with pBSII-specific oligonucleotides. The strategies employed to sequence the partial- and full-length cDNA species encoding rat HMG-CoA lyase are indicated in Figure 1. The inserts of the subclones pcr2.9 and pcr2.20 were sequenced either after shotgun cloning [34] or after further subcloning of restriction fragments (Figure 1). Sequence data were analysed with EDIT SEQ sequence analysis software (DNA Star).

Generation of RPA plasmids and synthesis of anti-sense RNA probes

To enable the synthesis of specific anti-sense transcripts *in vitro*, RPA plasmids were generated by removal of a 319 bp *Hin*cII/*Eco*RI, a 118 bp *Hin*cII/*Pst*I and a 205 bp *Sty*I/*Sac*I restriction

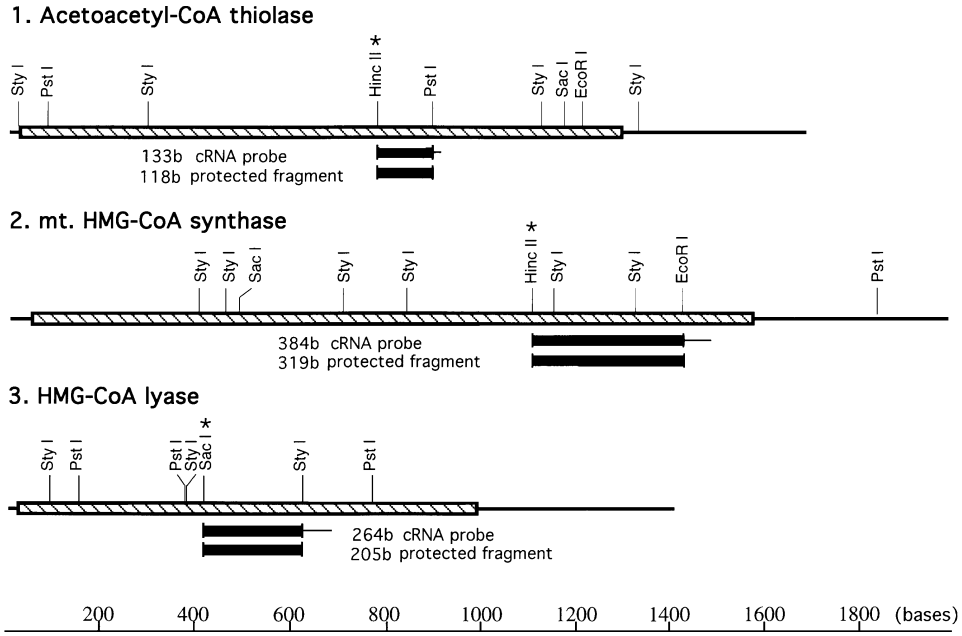

Figure 2 Construction of RPA plasmids and synthesis of cRNA probes

Full-length cDNA species encoding the three enzymes of the HMG-CoA cycle are represented by hatched horizontal bars (protein-coding regions) and horizontal lines (non-coding regions). Restriction enzyme sites used to subclone fragments are shown for each clone. Anti-sense probes and the resulting protected anti-sense RNA species are represented by solid horizontal bars (sequence transcribed from the cDNA insert) and horizontal lines (sequence transcribed from the vector) below the cDNA. Restriction enzyme sites used to linearize the vector are indicated with asterisks.

fragment from within each of the reverse transcription PCR products encoding acetoacetyl-CoA thiolase, mt. HMG-CoA synthase and HMG-CoA lyase respectively, followed by subcloning of each fragment into pBSII (Figure 2). To construct the acetoacetyl-CoA thiolase RPA plasmid, a further *Pst*I–*Sac*I fragment was removed from within the multiple cloning site of the construct. Inserts of all RPA plasmids were fully sequenced to confirm both cDNA identities and insert orientations. RPA plasmids, linearized by digestion at a unique site at the 5′ end of the inserted cDNA fragment (Figure 2), were used to direct synthesis of radiolabelled anti-sense RNA probes by using either T3 or T7 RNA polymerase as described previously [35]. Transcription reaction products were subjected to electrophoresis through a 0.4 mm thick 8 M urea/6% (w/v) polyacrylamide gel ('Mighty small'; Hoeffer) and the location of each full-length anti-sense probe was identified by autoradiography and excised; the RNA was eluted from the gel fragments as described previously [35]. After precipitation with ethanol, probes were resuspended in PES hybridization buffer [25 mM Pipes (pH 6.8)/1 M NaCl/1 mM EDTA] to a final concentration of 5×10^4 cpm/μl and stored in aliquots at -20 °C.

RPA

RPAs were performed by a modification of the aqueous hybridization method [36]: 25 μg of each sample RNA was combined with 5×10^4 c.p.m. of each probe in PES hybridization buffer in a total volume of 30 μl, and the hybridization mix was overlaid with a drop of mineral oil. After a denaturation step at 90 °C for 1 min, the hybridization mix was incubated at 80 °C for 2 h. After hybridization, 150 μl of RNase cocktail [5 μg/ml RNase A and 100 units/μl RNase T1 in 100 mM LiCl/200 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EDTA (pH 7.4)] was added and the mixture was incubated for 30 min at 30 °C. Subsequently samples were treated with proteinase K, extracted with phenol/chloroform, precipitated with ethanol and resuspended in loading buffer, all as previously described [35]. Samples were heated at 80 °C for 3 min and subjected to electrophoresis through a 0.4 mm thick 8 M urea/6% (w/v) polyacrylamide sequencing gel (Gibco/BRL). Radioactive signals were detected by autoradiography and quantified by densitometry (NIH Image). Comparison of the signals generated by the protected species with a standard curve of the appropriate undigested probe permitted the absolute quantification of the specific mRNA species in terms of molecules of mRNA/ng of total RNA [37].

Statistics

Statistical analysis was performed by single-factor analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Isolation and characterization of cDNA species encoding rat HMG-CoA lyase

Because of the selectivity of RPA, we were obliged to obtain partial cDNA fragments derived from the rat cDNA species encoding HMG-CoA lyase, mt. HMG-CoA synthase [27] and acetoacetyl-CoA thiolase [28] by reverse-transcription PCR. As the sequence encoding rat HMG-CoA lyase was not available, we generated the full-length cDNA by a combination of reverse transcription and PCR primed with degenerate oligonucleotide primers, followed by both 5′ and 3′ PCR–RACE. The cloning strategy used was a modification of the RACE method [30] in that one of the degenerate primers used to generate the partial cDNAs was also used as the gene specific primer in the first PCRs of the 5′ RACE and 3′ PCR–RACEs. Subsequently the sequences

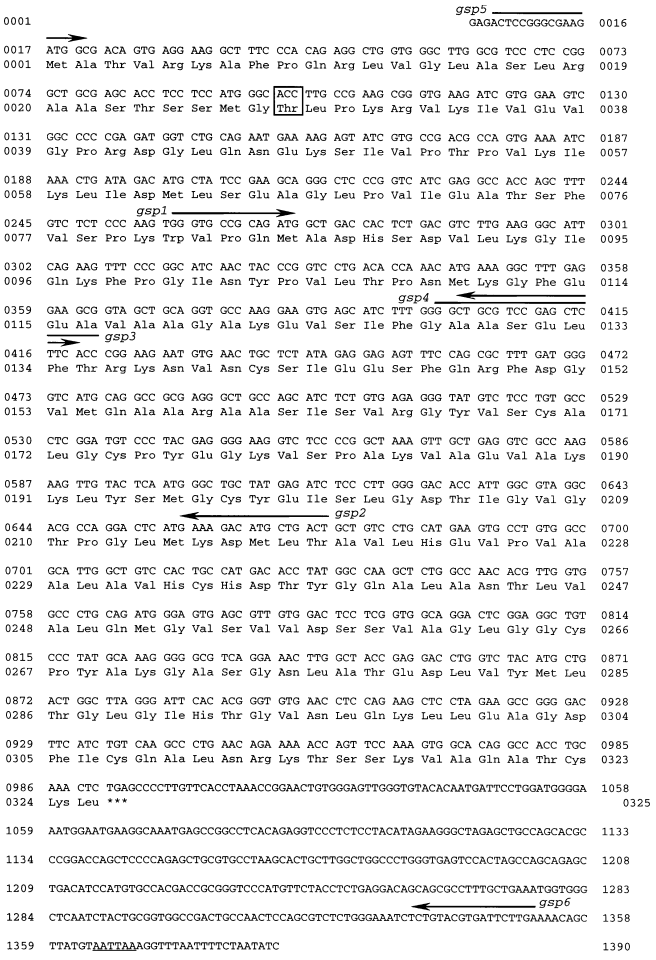

Figure 3 Full-length cDNA and deduced amino acid sequence of the rat HMG-CoA lyase

Length in base pairs or amino acid residues is indicated on either side. The position of a putative polyadenylation signal (AATTAA) is underlined. Thr-28, considered to be the first residue of the mature HMG-CoA lyase peptide, is shown boxed. Arrows above the sequence indicate the oligonucleotides used in the cloning strategy.

of the products of the 5′ and 3′ PCR–RACEs were used to design primers to amplify the full-length cDNA. The full-length cDNA inserts of two subclones pcr 2.9 and pcr2.20, each derived from independent PCRs, were sequenced as illustrated in Figure 1 and the sequences were found to be identical. The nucleotide sequence of the full-length cDNA (Figure 3) contains a 5′ non-coding region of 16 bp followed by an open reading frame of 975 bp, a TGA translation termination codon and a 3′ non-coding region of 399 bp. The open reading frame encodes a polypeptide of 325 residues of calculated molecular mass 34195.40 Da and a predicted pI of 8.413. Analysis of the sequence of the region flanking the initiation codon, GCGAAGATGG, reveals good conformity to a consensus sequence, GCC(AG)CCATGG (initiation codon underlined), identified as being involved in efficient ribosome binding and translation initiation of vertebrate mRNA species [38]. The 3′ non-coding region contains the sequence AATTAA 26 bp upstream of the annealing position of the 3′ ends of the anchored oligo(dT) primers used in the 3′ PCR–RACE, which corresponds to a known variant of the polyadenylation signal sequence [39]. Examination of an alignment of the primary sequences of five HMG-CoA lyase enzymes (Figure 4) reveals that the deduced amino acid sequence of the rat protein exhibits 94.0% and 88.2% identity respectively with the HMG-CoA lyase proteins of mouse [29] and man [40], and 81.4% and 79.3% identity respectively with the available primary sequences of bovine (accession no. U41409) and chicken [40]. The 5′ end of the rat HMG-CoA lyase open reading frame contains a putative mitochondrial importation leader sequence of 27 residues [40], 22 residues of which are identical with the corresponding sequences found in the primary sequences of the HMG-CoA lyases of mouse [29] and man [40]. None of the 27 residues is negatively charged but five are positively charged, which conforms to the consensus charge distribution for mitochondrial importation leader sequences [41].

Construction of RPA plasmids

RPA plasmids were generated from the PCR products for acetoacetyl-CoA thiolase, mt. HMG-CoA synthase and HMG-CoA lyase (see the Materials and methods section). The RPA plasmids for acetoacetyl-CoA thiolase and mt. HMG-CoA synthase were fully sequenced and found to be identical with the previously published [27,28] corresponding stretches of cDNA sequence. As abundances of all three mRNA species were to be determined in the same RNA sample, careful construction of the three RPA plasmids was required, to ensure that any residual undigested probe did not co-migrate with any of the three protected species during electrophoresis. Although RPA is generally highly specific, the specificity can be further enhanced by using a combination of RNase A and RNase T1 [34], which results in the increased cleavage of RNA duplexes formed by non-specific hybridization [42]. In this respect, the use of both RNase enzymes ensured complete discrimination between the mRNA species encoding the cytosolic and mitochondrial isoforms of HMG-CoA synthase, which exhibit 66% [43] and 100% [27] sequence identities respectively with the mt. HMG-CoA synthase anti-sense probe sequence.

Distribution of mRNA species encoding enzymes of the HMG-CoA cycle in brain and extra-neural tissues during the suckling period

A representative autoradiogram of an RPA for the mRNA species encoding the three HMG-CoA cycle enzymes, using total RNA derived from 11-day-old rat organs, is shown in Figure 5. Abundances of each mRNA in different rat organs are presented in Table 1. Liver and intestine isolated from suckling rat exhibit the highest abundances of the mRNA species encoding all three enzymes of the HMG-CoA cycle. This has been observed previously [4,14] and seems to be closely correlated with the high ketogenic capacities of these organs in the suckling rat, as it has been shown [4,44] that mt. HMG-CoA synthase mRNA and protein levels and, in liver, the corresponding enzyme activity and ketone body output [45] are closely coupled. The high abundances of acetoacetyl-CoA thiolase and HMG-CoA lyase mRNA species detected in liver and intestine (Table 1) suggest that high cellular levels of these mRNA species might also be required during suckling, to meet the demands of these highly ketogenic organs. Kidney cortex has been shown to perform active ketogenesis [4]; it has been suggested that the ketone bodies thus generated might provide a local fuel source for tubular and glomerular cells of kidney cortex [4]. The detection of lower abundances of the mRNA species encoding the HMG-CoA cycle enzymes in whole kidney of suckling rats, when compared with the levels present in liver or intestine, is compatible with a more heterogeneous distribution of HMG-CoA cycle-mediated ketogenesis in this organ.

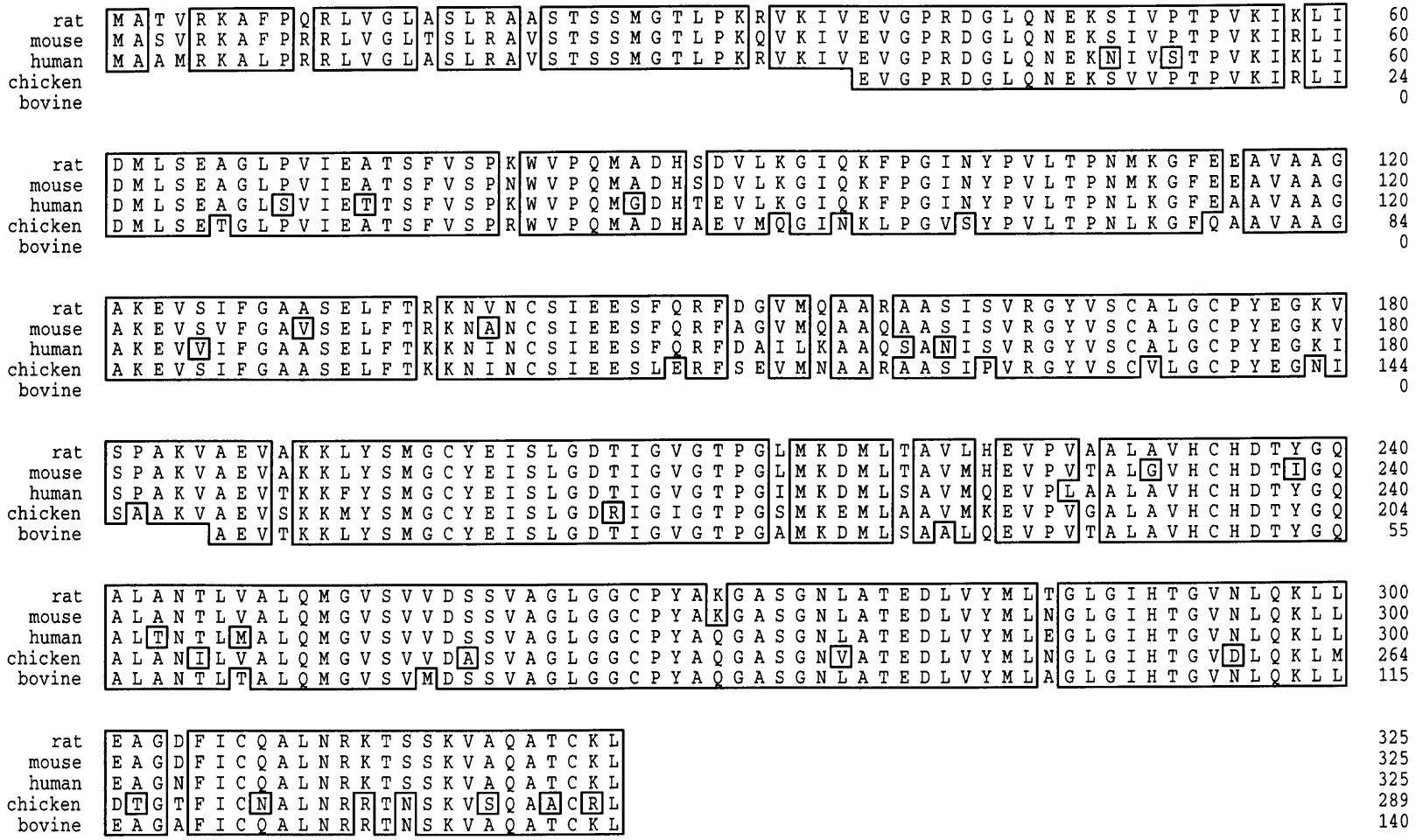

Figure 4 Amino acid sequence alignment of HMG-CoA lyase of rat, mouse, man, chicken and cow

The deduced amino acid sequence of the rat HMG-CoA lyase protein reported here was compared with those of the orthologous mouse, human, chicken and bovine proteins [29,40] and accession no. U41409. Identical or similar residues that are conserved between species are enclosed by a box.

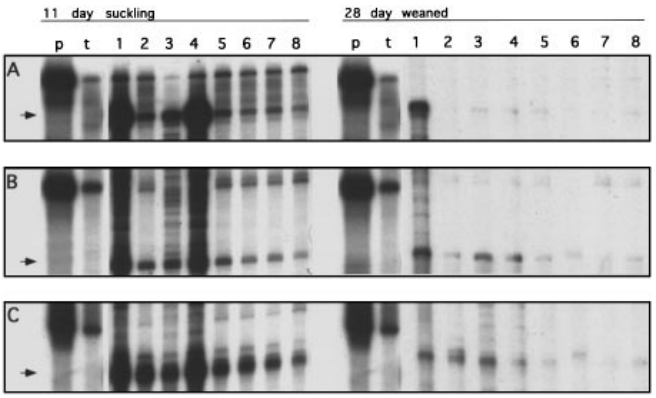

Figure 5 RNase protection analysis of mRNA species encoding the three enzymes of the HMG-CoA cycle during suckling and weaning

RNA samples were co-assayed with anti-sense RNA probes derived from partial cDNA species encoding mt. HMG-CoA synthase (**A**), HMG-CoA lyase (**B**) and acetoacetyl-CoA thiolase (**C**). The origin of the DNA fragments and the derivation of the probes are described in the Materials and methods section and illustrated in Figure 2. Protected species are indicated by arrows at the left. The larger size of the full-length probes is due to the presence of sequences derived from the vector. Residual undigested full-length probe was noted in some lanes. Lanes are annotated as follows: undigested anti-sense probe (p), RNase protection analysis of 25 μg samples of tRNA (t) and of total RNA isolated from 11-day-old suckling or 28-day-old weaned rat liver (lane 1), heart (lane 2), kidney (lane 3), intestine (lane 4), cerebellum (lane 5), cortex (lane 6), medulla oblongata (lane 7) and midbrain (lane 8). Each assay contained 5×10^4 c.p.m. of each anti-sense probe. All lanes for 11-day-old suckling rat organs are derived from an autoradiograph exposed to film for 72 h; all lanes for 28-day-old weaned rat organs are derived from an autoradiograph exposed to film for 36 h.

All three HMG-CoA cycle mRNA species were readily detectable in heart, cerebellum, cortex, medulla oblongata and midbrain of suckling rats at respective mean abundances of 650, 290, 240, 230 and 230 molecules of acetoacetyl-CoA thiolase mRNA/ng of total RNA; 40, 80, 40, 40 and 50 and molecules of mt. HMG-CoA synthase mRNA/ng of total RNA; and 120, 140, 80, 80 and 80 molecules of HMG-CoA lyase mRNA/ng of total RNA. The previous failure to detect mt. HMG-CoA synthase mRNA [4] in whole brain of suckling rats is most probably due to the fact that Northern blotting is approx. 1/20 as sensitive as RPA [34]. Of the remaining HMG-CoA cycle mRNA species, only one previous study has detected acetoacetyl-CoA thiolase mRNA in embryonic mouse brain [46]; to our knowledge, no studies have been performed identifying HMG-CoA lyase mRNA in brain. Notably, whereas the abundances we detect for acetoacetyl-CoA thiolase and HMG-CoA lyase mRNA species in heart and all brain regions investigated are similar to those of kidney, the abundance of mt. HMG-CoA synthase mRNA is approximately an order of magnitude lower. Both heart and brain are regarded as non-ketogenic organs [3], and the low abundances of mt. HMG-CoA synthase mRNA that we detected in these organs compared with those of acetoacetyl-CoA thiolase and HMG-CoA lyase mRNA species serve to emphasize the general association between the cellular abundance of mt. HMG-CoA synthase mRNA and the ketogenic capacity of a given organ. Although our results suggest a low overall ketogenic capacity for the brain, we do observe significant regional differences in abundances of mt. HMG-CoA cycle mRNA species. For example, in cerebellum there is a significantly higher abundance than in cortex of mt. HMG-CoA synthase and HMG-CoA lyase mRNA species [approximate ratios of 1.8-fold ($P < 0.0001$) and 1.8-fold ($P < 0.001$) respectively]. In comparison, the mRNA encoding acetoacetyl-CoA thiolase shows an approximate ratio of 1.3-fold ($P > 0.05$).

Distribution of mRNA species encoding enzymes of the HMG-CoA cycle in brain and extra-neural tissues during weaning

A representative autoradiogram of an RPA for the mRNA species encoding the three HMG-CoA cycle enzymes, with total RNA derived from 28-day-old rat organs, is shown in Figure 5. Abundances of each mRNA in different rat organs are presented in Table 1. With the exception of acetoacetyl-CoA thiolase mRNA in kidney, the abundances of the mRNA species encoding

Table 1 Abundances of mRNA species encoding the three enzymes of the HMG-CoA cycle in organs derived from suckling and weaned rats

RPAs for acetoacetyl-CoA thiolase, mt. HMG-CoA synthase and HMG-CoA lyase were performed on organs derived from 11-day-old suckling rats and 28-day-old weaned rats. Results are means ± S.E.M. for tissues from three separate animals. Abbreviation: n.d., not detected.

		mRNA abundance (molecules of mRNA/ng of total RNA)		
Organ	Age (days)	Acetoacetyl CoA thiolase	mt. HMG-CoA synthase	HMG-CoA lyase
Liver	11	1300 ± 240	4300 ± 950	610 ± 110
Heart	11	650 ± 71	40 ± 6	120 ± 9
Kidney	11	330 ± 72	520 ± 120	160 ± 17
Intestine	11	1200 ± 100	5500 ± 460	400 ± 16
Cerebellum	11	290 ± 5	80 ± 2	140 ± 5
Cortex	11	240 ± 13	40 ± 1	80 ± 3
Medulla	11	230 ± 16	50 ± 1	80 ± 2
Midbrain	11	200 ± 32	50 ± 8	80 ± 8
Liver	28	480 ± 12	500 ± 140	460 ± 78
Heart	28	290 ± 37	20 ± 12	80 ± 38
Kidney	28	450 ± 19	50 ± 10	180 ± 40
Intestine	28	50 ± 20	30 ± 14	120 ± 52
Cerebellum	28	40 ± 18	10 ± 5	80 ± 24
Cortex	28	60 ± 29	n.d.	60 ± 27
Medulla	28	80 ± 36	10 ± 7	70 ± 17
Midbrain	28	50 ± 21	n.d.	60 ± 33

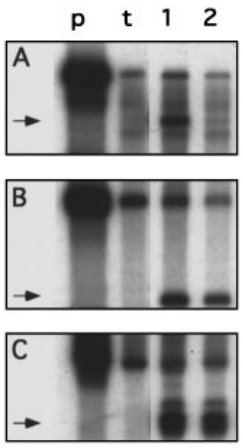

Figure 6 RNase protection analysis of mRNA species encoding the three enzymes of the HMG-CoA cycle in primary cultures of cortical astrocytes

RNA probes, panels and arrows are as described in the legend to Figure 5. Lanes are annotated as follows: undigested anti-sense probe (p), RNase protection analysis of 25 μg samples of tRNA (t) and of total RNA isolated from neonatal cortical astrocytes (lane 1) and adult cortical astrocytes (lane 2). Each assay contained 5×10^4 c.p.m. of each anti-sense probe. All lanes are derived from an autoradiograph exposed to film for 72 h.

all the HMG-CoA cycle enzymes were found to be lower in organs and brain regions derived from weaned rats than in the respective organs derived from suckling animals (Table 1). In any given organ or brain region, the abundance of mt. HMG-CoA synthase mRNA exhibited the greatest decrease from suckling to fully weaned states. Similar decreases in mt. HMG-CoA synthase mRNA have been observed previously [4,14] for liver, intestine and kidney and have been attributed to the change from the high fat levels in milk, ingested by the suckling neonate, to the lower fat levels ingested on weaning to a high-carbohydrate/low-fat diet [4]. With the exception of kidney, we detected decreases in acetoacetyl-CoA thiolase mRNA of between 2.2-fold (heart) and 22-fold (intestine) in all organs and brain regions; this might indicate that dietary fat content also regulates acetoacetyl-CoA thiolase gene transcription. In contrast with either acetoacetyl-CoA thiolase or mt. HMG-CoA synthase mRNA, HMG-CoA lyase mRNA exhibited less pronounced decreases, of between 1.0-fold and 3.4-fold, in all brain regions and organs, apart from kidney, between suckling and weaning. This might reflect the requirement for HMG-CoA lyase to perform 'housekeeping' functions, such as the catabolism of the amino acid leucine, in addition to its role in HMG-CoA cycle-mediated ketogenesis.

Detection of HMG-CoA cycle mRNA species in primary cultures of cortical astrocytes

On the basis of the well-documented complexity of brain cell type and structure [47], and the observations of localized HMG-CoA cycle in kidney [4], we reasoned that specific brain cell types might exhibit a different abundance of mt. HMG-CoA synthase mRNA when compared with tissue in the corresponding brain subregion. Astrocytes are a major neural cell type involved in energy provision in brain [12]; cortical astrocytes can be prepared to a high degree of purity (more than 95 %) in primary culture. Thus RPAs were used to investigate the abundances of HMG-CoA cycle mRNA species in neonatal and adult cortical astrocytes (Figure 6). The abundance of each HMG-CoA cycle mRNA in two independent preparations of neonatal and adult cortical astrocytes is shown in Table 2. Whereas mt. HMG-CoA synthase mRNA was detected near the limit of sensitivity of the RPA (less than 30 molecules of mRNA/ng of total RNA) in adult cortical astrocytes, it was detected at approximately 360 molecules of mRNA/ng of total RNA in neonatal cortical astrocytes.

Conclusions

We have reported here the unequivocal presence of mRNA species encoding the three enzymes of the ketogenic HMG-CoA cycle in brain regions of suckling rats and in primary cultures of neonatal cortical astrocytes. Similarly to the proposed situation in kidney [4], our results imply that HMG-CoA cycle mRNA species are heterogeneously distributed in brain. First, we find significantly higher abundances of mt. HMG-CoA synthase and HMG-CoA lyase mRNA species in cerebellum of suckling rats, suggesting that the cerebellum might have an increased requirement for the latter two enzymes of the HMG-CoA cycle that are essential for ketone body production. Secondly, we find that primary cultures of neonatal cortical astrocytes display approx. 9-fold and 4.5-fold greater abundances of mt. HMG-CoA synthase and HMG-CoA lyase mRNA species respectively than the corresponding abundances in cortex of 11-day-old suckling rats, suggesting a significant astrocytic zonation of mt. HMG-CoA synthase and HMG-CoA lyase mRNA species in cortex *in vivo*. Furthermore the abundance of mt. HMG-CoA synthase mRNA in neonatal cortical astrocyte cultures is comparable to that found in 28-day-old rat liver, a tissue that continues to execute low-level ketogenesis even after the animal has been weaned to a high-carbohydrate/low-fat diet [4]. Thus cortical astrocytes *in vivo* might possess mt. HMG-CoA synthase mRNA at abundances considered significant in terms of cellular ketogenic capacity.

Our results on brain regions derived from rats weaned to a high-carbohydrate/low-fat diet suggest that mt. HMG-CoA

Table 2 Abundances of mRNA species encoding the three enzymes of the HMG-CoA cycle in primary cultures of neonatal and adult cortical astrocytes

RPAs for acetoacetyl-CoA thiolase, mt. HMG-CoA synthase and HMG-CoA lyase were performed on two separate preparations of neonatal and adult cortical astrocytes.

		mRNA abundance (molecules of mRNA/ng of total RNA)		
Culture	Preparation	Acetoacetyl-CoA thiolase	mt. HMG-CoA synthase	HMG-CoA lyase
Neonatal cortical astrocyte	1	280	350	390
Neonatal cortical astrocyte	2	240	370	360
Adult cortical astrocyte	1	230	30	260
Adult cortical astrocyte	2	240	25	270

synthase mRNA in brain might be regulated by dietary fat components. In this regard we have identified PPARα mRNA in cultured neonatal cortical astrocytes [48], indicating the possibility that astrocytic mt. HMG-CoA synthase gene expression might be inducible by PUFAs. However, there might also be developmental programming of mt. HMG-CoA synthase gene expression in brain because, under comparable conditions and time in culture, adult cortical astrocytes exhibited abundances of mt. HMG-CoA synthase mRNA less than one-tenth those of neonatal cortical astrocytes.

The potential of the brain to generate ketone bodies from PUFAs is of significance because the required levels and types of PUFA in infant milk remain controversial [49]. If increasing the PUFA load ingested by the infant results in the stimulation of local ketogenic systems within the infant brain, this might affect overall fuel availability for developing neural systems. In addition, the PUFA composition of the infant brain has been shown to be influenced by the types of PUFA present in milk [50] and, because different PUFAs might be differentially ketogenic [14], this could have implications in the choice of maternal diet ingested during lactation and/or the composition of infant formulas given to the developing infant.

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