Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1863–1866, April 1987
Biochemistry

Optional exon in the 5′-untranslated region of 3-hydroxy-3-methylglutaryl coenzyme A synthase gene: Conserved sequence and splicing pattern in humans and hamsters

(cholesterol synthesis/isoprenoids/3-hydroxy-3-methylglutaryl-CoA reductase/translational regulation)

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ABSTRACT **3-Hydroxy-3-methylglutaryl coenzyme A synthase (hydroxymethylglutaryl-CoA synthase, EC 4.1.3.5) is a negatively regulated enzyme in the synthetic pathway for cholesterol, isopentenyl tRNA, and other isoprenoids. The 5′-untranslated region of the mRNA for Chinese hamster hydroxymethylglutaryl-CoA synthase contains an optional exon of 59 nucleotides located 10 nucleotides upstream of the translation start site. About 50% of the mRNAs contain this exon, and the other 50% lack it owing to differential intron splicing. We show that the two transcripts are found in similar ratios in multiple tissues of the Syrian hamster, including the brain. The relative amounts of the two transcripts in brain and liver are constant from day 0 to day 75 of life. A similar alternative splicing pattern for hydroxymethylglutaryl-CoA synthase was observed in three human tissues: cultured fibroblasts, fetal adrenal gland, and fetal liver. A cDNA for human synthase had 90% homology to the hamster sequence in the region corresponding to the optional exon. This sequence contains a 20 out of 26 nucleotide match with the sequence immediately upstream of the initiator AUG codon in the mRNA for hamster hydroxymethylglutaryl-CoA reductase, the enzyme that follows the synthase in the isoprenoid biosynthetic pathway. These findings raise the possibility that the optional exon plays an important, conserved functional role in humans and hamsters.**

The 5′-untranslated regions of mRNAs from higher eukaryotes are typically short, simple structures that are encoded within single exons. These sequences are generally devoid of AUG codons prior to the one that encodes the start site for translation (1). No regulatory role for these 5′-flanking regions has yet been identified. However, in yeast a function for the 5′-flanking region in one class of genes has been defined. In these genes, which encode enzymes of amino acid biosynthesis, the complex 5′-flanking region contains multiple AUG codons and serves as the target site for feedback suppression of translation (2–4).

In hamsters two negatively regulated genes that catalyze sequential steps in the biosynthesis of cholesterol, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase (hydroxymethylglutaryl-CoA synthase, EC 4.1.3.5) and HMG CoA reductase (hydroxymethylglutaryl-CoA reductase, EC 1.1.1.88), have extraordinarily complex 5′-untranslated regions (5, 6). In the hamster reductase gene, multiple transcription start sites and multiple splice donor sites for an intron in the 5′-untranslated region produce a family of mRNAs with untranslated regions ranging from 68 to 670 nucleotides in length. These mRNAs contain from zero to eight AUG codons upstream of the initiation site for translation (5, 6).

The mRNA for hamster HMG CoA synthase contains an optional exon in the 5′-untranslated region (7). Approximately 50% of the mRNA transcripts in the UT-1 line of cultured Chinese hamster ovary cells contain a 5′-untranslated region of 68 nucleotides that is interrupted by a single intron located 10 nucleotides upstream of the initiator AUG (nucleotide position −10). The other 50% of the mRNAs have a longer 5′-untranslated region of 127 nucleotides, resulting from the insertion of an extra exon of 59 nucleotides at position −10. We have called this the "optional" second exon. Within the sequence of the optional second exon, there is a 20 out of 26 nucleotide identity with the sequence immediately upstream of the initiator AUG in the most abundant HMG CoA reductase mRNA (7).

The complex splicing patterns and nucleotide homologies at the 5′ ends of the HMG CoA synthase and reductase genes are especially intriguing in light of the fact that these two enzymes participate in the synthesis of mevalonate, which is the precursor not only of cholesterol, ubiquinone, and dolichol but also of the isopentenyl group that is attached to certain specific tRNA species (8). Both of these enzymes are subject to feedback repression by sterols and other mevalonate-derived products. This repression is associated with a decrease in the amount of mRNA, resulting from a decrease in transcription (7, 9, 10). The possibility exists, however, that translation of these mRNAs is also regulated. One function of the complex 5′-untranslated region might be to subject translation of some of these multiple mRNAs to feedback suppression by an end-product of the pathway, such as isopentenyl tRNA. Another possible reason for the dual splicing pattern in the HMG CoA synthase gene relates to the two locations of HMG CoA synthase in cells. Some of the enzyme is mitochondrial and the remainder is cytosolic (11). The hamster cDNA that we have isolated (12) encodes cytosolic HMG CoA synthase. However, the predicted protein sequence is strongly homologous to that of the active site of avian mitochondrial HMG CoA synthase (12, 13), raising the possibility that the cytosolic and mitochondrial enzymes are products of the same gene. If this proves true and if there is no distinct gene for mitochondrial HMG CoA synthase gene in hamsters, then it is possible that alternate splicing of the 5′-untranslated region might play a role in targeting some of the HMG CoA synthase to mitochondria.

There is one other gene in which an optional exon in the 5′-untranslated region has been observed, namely, the gene for argininosuccinate synthetase, a cytosolic enzyme of the urea cycle (14). In this case one of the two mRNA species vastly predominates over the other, and the nature of the predominant species differs between animal species. Thus, in

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Abbreviation: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

baboon liver 99% of the argininosuccinate synthetase transcripts contain the optional exon, whereas in human liver 99% of the transcripts lack this exon. This lack of conservation between species raised doubts about the functional significance of the alternative splicing pattern (14).

As an initial step in determining whether the alternative splicing of the HMG CoA synthase mRNA may be of functional importance, we have sought to determine whether the alternate splicing event occurs in multiple tissues, whether it varies with development, and whether it is conserved among species. These results are described.

METHODS

Materials. [α-^{32}P]ATP was purchased from ICN. Reverse transcriptase was purchased from Life Sciences (St. Petersburg, FL). Mevinolin was kindly provided by Alfred Alberts of Merck Sharp & Dohme (Rahway, NJ). Compactin was kindly provided by Akira Endo of Tokyo Noko University (Tokyo, Japan). Other materials were obtained from reported sources (7, 12).

Animals. Male and pregnant female Golden Syrian hamsters (≈100 g) were obtained from Sasco (Omaha, NE). All animals were exposed to a 12-hr light/12-hr dark cycle for at least 7 days prior to use, except for the newborn (day 0) and 2-day-old hamsters. Hamsters were fed a chow diet (Wayne Research Animal Diets) or the same diet supplemented with 0.2% mevinolin for 5 days. Hamsters were sacrificed at the end of the dark cycle.

Primer Extension Analysis. Total RNA was isolated from animal tissues or from cultured cells by homogenization in guanidinium thiocyanate (15) and centrifugation in cesium chloride (16). A synthetic oligonucleotide complementary to nucleotide positions +41 to +100 of the hamster HMG CoA synthase gene (12) was end-labeled with ^{32}P using [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) and polynucleotide kinase (17) to a specific radioactivity of $\approx 10^7$ cpm/pmol. Various amounts of RNA (5–60 μg) and 75 fmol of the ^{32}P-labeled primer were suspended in 24 μl of a buffer containing 50 mM Tris chloride (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, and 20 mM dithiothreitol. The mixture was denatured at 95°C for 4 min and allowed to hybridize for 45 min at 53°C. To initiate primer extension, 6 μl of 1 mM dNTPs and 3 μl (43 units) of reverse transcriptase were added, and the mixture was incubated for 15 min at 40°C. The extended products were precipitated with ethanol, resuspended in formamide, boiled, and resolved on denaturing polyacrylamide gels. The gels were fixed with trichloroacetic acid, dried, and exposed to Kodak XAR-5 film at −70°C for various lengths of time as indicated in the figure legends.

Isolation and Sequencing of Human HMG CoA Synthase cDNA. A cDNA for human HMG CoA synthase was isolated from a human fetal adrenal cDNA library prepared by Yamamoto *et al.* (18). The library was size-fractionated by

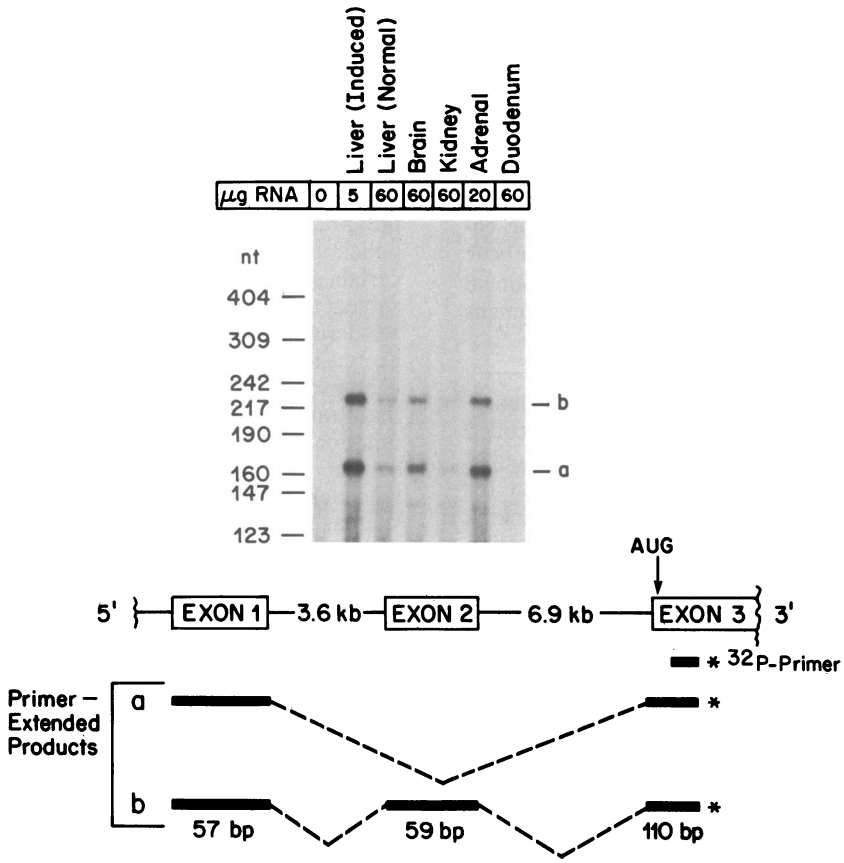

FIG. 1. Alternative splicing of the second exon in the cytoplasmic HMG CoA synthase gene in tissues of the Syrian hamster. The indicated amounts of RNA were hybridized with a ^{32}P-labeled oligonucleotide primer complementary to nucleotide positions +41 to +100 of the hamster HMG CoA synthase gene. The primer was extended with reverse transcriptase in the presence of all four dNTPs, and the products were visualized by autoradiography after size-fractionation on denaturing polyacrylamide gels. The full-length extended products are indicated (a and b) along the right side of the gel. A schematic diagram of the 5′ end of the synthase gene is shown below the gel. The site of hybridization of the ^{32}P-labeled primer is shown: the asterisk marks the labeled site. The two primer-extended products (a and b) are shown at the bottom. Six male Syrian hamsters (≈100 g) were fed a chow diet (normal), and total RNA was isolated from the indicated tissues. One male hamster was fed a chow diet supplemented with 0.2% mevinolin for 5 days, and RNA was isolated only from the liver (induced). The gel was exposed to Kodak XAR-5 film at −70°C for 168 hr. Size standards were generated by electrophoresis of a labeled *Msp* I digest of pBR322 DNA. nt, Nucleotides.

agarose gel electrophoresis and screened under reduced stringency (17) with a ^{32}P-labeled single-stranded hamster HMG CoA synthase cDNA probe containing nucleotides +190 to +401 (antisense strand). The probe was prepared in an M13 vector as described (12). Upon screening of ≈60,000 colonies, three positive clones were isolated, and one of them, pHSyn-22, was further analyzed. An *Acc* I fragment (≈800 base pairs) containing the 5′ end of the human synthase cDNA and part of the vector was rendered blunt-ended with Klenow fragment of DNA polymerase I and dNTPs (17) and then subcloned into the *Sma* I site of the bacteriophage M13mp10 (19). The subclone was used as a DNA template to perform DNA sequencing reactions with either universal or specific oligonucleotide primers (19, 20). Routine sequencing gels contained 10% (wt/vol) polyacrylamide, 100 mM Tris borate (pH 8.3), 7 M urea, and 25% (vol/vol) formamide.

RESULTS AND DISCUSSION

Fig. 1 shows the alternative splicing pattern of Syrian hamster HMG CoA synthase mRNA and illustrates the primer extension assay that was used to monitor this splicing. Total RNA was isolated from various hamster tissues and incubated with a ^{32}P-labeled oligonucleotide primer that hybridizes to exon 3 just downstream of the initiator AUG codon. When hybridized to the mRNA that lacks the optional exon 2, the primer was extended a total of 167 nucleotides. When hybridized to mRNAs that contain the optional exon 2, the primer extension product was 226 nucleotides long. Studies by primer extension sequencing and cDNA sequencing have confirmed (7) that these two primer extension products result from the presence or absence of exon 2. RNA from hamster liver, brain, kidney, adrenal gland, and duodenum all showed evidence for both mRNA transcripts (Fig. 1). The molar ratio of the two transcripts was ≈1:1 in all of these tissues. When hamsters were fed a diet containing mevinolin, an inhibitor of cholesterol synthesis, the amount of HMG CoA synthase mRNA was increased in liver (note that only 5 μg of total RNA from the induced liver was used in Fig. 1). Nevertheless, the 1:1 molar ratio of the two transcripts persisted.

The amount of HMG CoA synthase mRNA was unexpectedly high in the brains of these hamsters as estimated from the intensity of the autoradiograms of the primer-extended products (Fig. 1). The amount of synthase mRNA in the brain was actually higher than that detected in the liver. To confirm this observation and to study the alternative splicing pattern during development, we obtained RNA from the brains and livers of hamsters ranging from 0 to 75 days of age. The amount of HMG CoA synthase mRNA in brain was relatively high at birth and persisted into adulthood (Fig. 2). At all stages of development, the dual splicing pattern was apparent. The amount of HMG CoA synthase mRNA in the livers

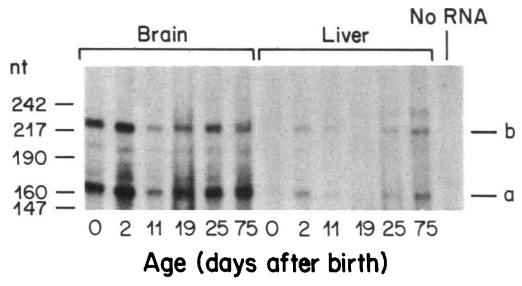

FIG. 2. Alternative splicing of HMG CoA synthase gene in Syrian hamster brain and liver at different stages of development. Aliquots of RNA (60 μg) from eight animals of the indicated age were analyzed by primer extension. Symbols and standards are as in Fig. 1. The gel was exposed to XAR-5 film at −70°C for 92 hr.

of these hamsters was relatively low, but the dual splicing pattern was observed in all samples.

To determine whether alternative splicing of the HMG CoA synthase mRNA is a phenomenon peculiar to hamsters, we examined RNA from human tissues. The HMG CoA synthase mRNA from simian virus 40-transformed human fibroblasts showed evidence for alternative splicing in an ≈1:1 molar ratio (Fig. 3, lane 2). The amount of both transcripts was increased when the cells were grown with compactin, an inhibitor of HMG CoA reductase (lane 1), and both transcripts were suppressed when the cells were grown with sterols (lane 3). The dual splicing pattern was also observed in samples of human fetal adrenal mRNA (lane 4) and human fetal liver mRNA (lane 5).

To determine whether the sequence of the optional exon was conserved in humans as compared with hamsters, we isolated a cDNA for HMG CoA synthase from a human fetal adrenal cDNA library. A restriction fragment containing the 5′-untranslated region was subcloned and subjected to DNA sequencing (Fig. 4). The portion of the human cDNA that corresponded to exon 2 of the hamster gene was strikingly conserved (54 out of 59 nucleotides identical with no gaps). The sequence of the 5′-untranslated portion of exon 3, as well as the protein coding region, was also highly conserved. On

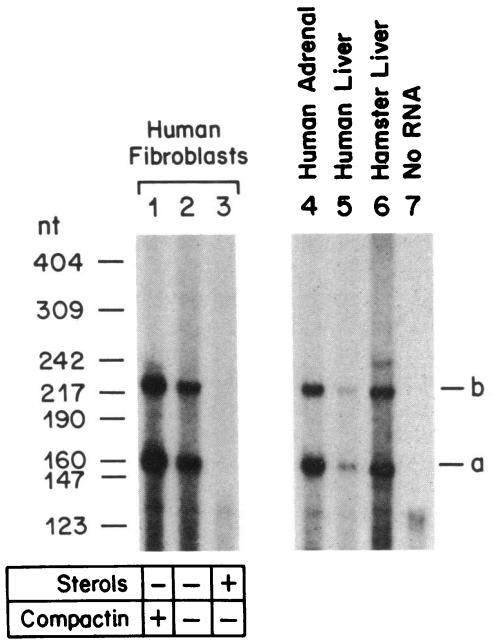

FIG. 3. Alternative splicing of HMG CoA synthase gene in human cells and tissues. RNA was isolated from simian virus 40-transformed human fibroblasts (lanes 1 to 3) that had been grown in the absence of sterols and in the presence of compactin (lane 1), in the absence of both sterols and compactin (lane 2), or in the presence of sterols and in the absence of compactin (lane 3); or from human fetal adrenal gland (lane 4), human fetal liver (lane 5), or liver from hamsters fed mevinolin (lane 6). Simian virus 40-transformed human fibroblasts were set up in roller bottles (3 × 10^6 cells per bottle) and grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum for 48 hr. On day 2 of cell growth, one-third of the cells were maintained in the same growth medium. One-third of the roller bottles were switched to medium containing 10% (vol/vol) calf lipoprotein-deficient serum (21) and 10 μM compactin in the absence of sterols. The other third of the roller bottles were switched to medium containing 10% (vol/vol) newborn calf serum in the presence of 25-hydroxycholesterol (3 μg/ml) plus cholesterol (12 μg/ml). After 24 hr all three groups of cells were harvested for the preparation of RNA. The primer extension reactions were performed using 60 μg of RNA in lanes 1–5 and 20 μg of RNA in lane 6. Symbols and standards are as in Fig. 1. The gel was exposed to XAR-5 film at −70°C for 6 hr.

FIG. 4. Conservation of DNA sequences at the 5′ end of the hamster and human HMG CoA synthase mRNAs. The cDNA sequences for hamster and human HMG CoA synthase were aligned for maximum homology with the use of a Beckman Microgenie Align program. Vertical bars denote identical nucleotides. Position +1 is assigned to the A (indicated by an arrow) of the ATG codon specifying the initiator methionine for the hamster synthase (7); negative numbers refer to the 5′-untranslated sequences. The boundaries of exon 1, 2, and 3 were taken from the hamster gene (7).

the other hand, the sequence corresponding to exon 1 showed much less conservation (33 out of 53 nucleotides identical with three gaps).

The conservation of nucleotide sequence and alternative splicing pattern of exon 2 in the HMG CoA synthase genes among various tissues throughout development and between humans and hamsters, strongly suggests a functional role for this exon in metabolism. Prime candidates for functional roles include the provision of susceptibility to feedback regulation of translation and/or the targeting of the mRNA to a specific cellular site, most likely the mitochondria. The latter mechanism would be in contrast to the general mechanism for mitochondrial targeting of proteins, which depends on the synthesis of a positively charged leader peptide (22, 23). Further studies must now be undertaken to determine which, if either, of these two functions is subserved by the optional exon.

We thank Anna Delaloye for excellent technical assistance and Lavon Sanders for invaluable help in growing cultured cells. This research was supported by a research grant from the National Institutes of Health (HL 20948).

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