The Induction and Characterization of Rat Liver Stearyl-CoA Desaturase mRNA*

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(Fig. 1, 4, and Tables 1 and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 35M-1128, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

* Portions of this paper (including “Experimental Procedures,” “Results,” Figs. 1-6, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 35M-1128, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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Poly(A*) RNA isolated from livers of rats induced for stearyl-CoA desaturase contains elevated levels of mRNA for this enzyme which is translated in a rabbit reticulocyte system. The protein is immunologically and by peptide fingerprinting following Staphylococcus aureus V8 protease digestion identical to the isolated enzyme and, therefore, not synthesized in a detectable larger precursor form. The desaturase mRNA is selectively translated on free cytoplasmic polysomes from rat liver and represents at least a 40-fold increase in translatable mRNA in livers of induced animals. Northern blot analysis, using a cDNA probe complementary to rat liver desaturase mRNA, demonstrated that the desaturase is encoded by a 4960-base mRNA which is elevated approximately 50-fold in induced liver.

Stearyl-CoA desaturase, the terminal component of the liver microsomal stearyl-CoA desaturase system, catalyzes the insertion of a double bond between carbons 9 and 10 in a spectrum of fatty acyl-CoA substrates (1-3). Oshino and Sato (4) originally described the sequence of nutritional manipulation of two cycles of starvation followed by refeeding with a fat-free diet to induce high levels of enzyme activity. In addition to this dietary procedure, insulin and certain carbohydrate metabolites, such as fructose (5), also have a positive effect on the desaturase activity of liver endoplasmic reticulum. Only the terminal desaturase and not the other two components of this oxidative fatty acyl desaturase system appear to be under such hormonal and metabolic regulation (5). Since the half-life of the desaturase in situ has been shown to be only approximately 4 h (6), a combination of induction of translationally active desaturase mRNA and a high turnover rate would provide elevated but transitory desaturase levels in response to physiological needs.

To better understand the molecular basis of the induction phenomenon, we have initiated experiments designed to examine the induced rate of desaturase synthesis by comparison of translationally active hepatic desaturase mRNA levels in normal and induced rats. We report here in vitro translation studies using liver poly(A*) RNA that contains elevated levels of desaturase mRNA following the dietary schedule used to induce desaturase activity in liver tissue. We also have used in vitro translation studies of subcellular fractionated poly(A*) RNA to investigate the cellular location of desaturase mRNA translation. Following similar strategies for the isolation of cDNA complementary to other inducible mRNAs (7-10), we used the substantial difference in desaturase mRNA levels in control and induced animals to screen a liver cDNA library constructed from unenriched poly(A*) RNA from induced rats. The positive identification of a cDNA complementary to desaturase mRNA has been utilized to estimate the desaturase mRNA size and to identify elevated cytoplasmic levels of mRNA as the basic mode of desaturase regulation in rat liver.

EXPERIMENTAL PROCEDURES AND RESULTS

The dietary sequence of starvation and refeeding of a fat-free diet clearly results in a marked increase in translationally active stearyl-CoA desaturase mRNA in liver. Under similar assay conditions we were able to detect only trace levels of translated mRNA in lysates incubated with liver poly(A*) RNA from control rats, which have previously been shown to contain only low levels of enzyme activity (1, 3, 4). Although the experimental protocol probably results in a minimal estimation, the determination that the induction of translationally active desaturase mRNA to at least 0.2% of the total translationally active RNA of liver poly(A*) RNA is consistent with the previous observations of high levels of enzyme activities that are induced by the same dietary manipulation.

Our data clearly implicates soluble cytoplasmic polysomes as the site of desaturase mRNA translation in rat liver and do not support earlier work (4) which suggested that desaturase is synthesized on membrane ribosomes, based upon the movement of desaturase activity from rough to smooth endoplasmic reticulum following cycloheximide treatment. Moreover, immunoprecipitation experiments do not reveal that desaturase mRNA codes for a delectable higher molecular weight precursor peptide species. These findings offer insight into the possible mechanism involved in targeting this very hydrophobic protein into endoplasmic reticulum. Blobel (32) described various mechanisms by which proteins are inserted into and traverse membranes by harboring specific recognition sequences. These recognition sequences can either be proteolytically removed once the protein traverses the membrane or it can be maintained in the mature protein. A second mechanism for protein insertion into membranes (33) proposes the partitioning of hydrophobic helices of de novo
synthesized protein into membranes via a helical hairpin structure. This directly implicates hydrophobic domains in the formation of structural elements to anchor proteins in membranes. Ornithine transcarbamylase, a major mitochondrial matrix protein, is an example of a protein synthesized as a larger precursor on cytoplasmic ribosomes which post-translationally traverses two mitochondrial membranes before reaching the mitochondrial matrix; the additional 4000-dalton amino-terminal peptide involved in targeting the enzyme is removed (34, 35). Unlike ornithine transcarbamylase, cytochrome b$_6$ and phenobarbital-induced cytochrome P-450, two integral membrane proteins, are not synthesized in proenzy form and are inserted into membranes by two different mechanisms. Cytochrome b$_6$ mRNA is translated on free cytoplasmic polysomes (30) and is inserted post-translationally (36) via an insertion sequence (37). In contrast, phenobarbital-induced cytochrome P-450 is synthesized on membrane-bound ribosomes and is cotranslationally inserted into endoplasmic reticulum without the removal of a putative signal sequence (31). The cytoplasmic site of desaturation mRNA translation and the absence of a clear proenzyme form may, as in the case of cytochrome b$_6$, indicate an internal recognition sequence that is responsible for the proper targeting of this very hydrophobic protein into membranes to orient the functional iron center at the cytoplasmic interface. A combination of sequence data and the recognition of nonpolar domains will be required to identify such intramembranous interaction of this enzyme with the endoplasmic reticulum.

The detection of significant levels of translatable mRNA for desaturation in total liver poly(A)$^+$ RNA from induced rats has permitted the construction of a cDNA library and isolation of a cDNA complementary to desaturase mRNA. This cloned desaturase cDNA was used to probe total liver poly(A)$^+$ RNA and to demonstrate, in turn, that dietary induction of this very hydrophobic protein into membranes via a helical hairpin structure of the desaturase mRNA, and the primary structure of the desaturase, should become clear. Moreover, the cDNA segments of the desaturase represent the basic elements required for the isolation of desaturase genomic DNA.

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REFERENCES

The infection and characterization of rat liver Stearyl CoA Desaturase mRNA

By Roh A. Thirode and Phillip E. McCormick

This supplement provides EXPERIMENTAL PROCEDURES and RESULTS.

EXPERIMENTAL PROCEDURES

Preparation and characterization of purified rat liver CoA desaturase - Stearyl CoA desaturase was purified from 0.01 M Tris-HCl, pH 7.5, 0.2 M NaCl, and 0.05 M sodium pyrophosphate by gel filtration on a 2.0 x 40 cm column of Sephadex G-75 equilibrated in 0.1 M Tris-HCl, pH 7.5, and stored in PBS at -70°C.

Antibody specificity was determined by Western Blotting of rat liver cytoplasmic fractions (10) and (11).

Isolation of tissue (a) RNA from rat liver - Two to four male Long-Evans rats (150-190 g) were anesthetized by chloral hydrate and sacrificed by cervical dislocation. The livers were removed and minced in a chilled glass homogenizer. The resulting homogenate was centrifuged at 100,000 x g for 2 h at 4°C. The supernatant was then dialyzed against 0.2 M NaCl, 10 mM Tris-HCl, pH 7.5, and 10% glycerol for 4 h at 4°C. The desaturase activity in this fraction was determined by incubating 0.2 ml of the dialysate with 0.2 ml of 5.0 M urea buffer pH 7.5, 2.0 mM ethylenediaminetetraacetic acid (EDTA), 2.0 mM Tris-HCl, pH 7.5, and stored in PBS at -70°C.

To estimate the percentage of translatable desaturase mRNA in the total liver RNA, gel slices were subjected to electrophoresis. Following electrophoresis, the gel slices were stained with ethidium bromide and excised from the gel to perform Northern blot analysis (11). The excised gel slices were then treated with RNAase-free RNAse A and RNAase D prior to electrophoresis. The RNase treated samples were subjected to Northern blot analysis (11) and the resulting autoradiogram was used to determine the percentage of total liver RNA that was translatable.
To isolate a cDNA clone for desaturase using unenriched liver poly(A+) RNA with a low level of desaturase message as a template, we essentially followed the procedure of Winberry et al. (10). They used total goose uropygial gland poly(A+) RNA in which malic enzyme mRNA constituted 0.2% of the total RNA. To maximize the number of desaturase cDNA recombinants in the library, we used an induced poly(A+) RNA preparation in which desaturase mRNA accounted for 0.21% of the translatable RNA. We synthesized 3 \mu g of double-stranded cDNA from 10 \mu g of poly(A+) RNA, as described in Methods. Following phenol-chloroform and homopolymeric tailing, we recovered approximately 1 \mu g of detailed double-stranded cDNA with a model size of 600 base pairs. Transformation efficiency was approximately 50,000 transformants per \mu g of cDNA.

Replica filters were constructed from transformants generated from a series of transformants. One set of filters were screened with a positive probe of single-stranded [32P] labeled cDNA complementary to poly(A) RNA from induced rats. A second set of filters were screened with a negative probe of single-stranded [32P] labeled cDNA copied from poly(A) RNA from control rats. Colonies containing desaturase sequences would give a signal when hybridized with the positive probe but not with the negative probe. Of 8,000 transformants that were screened in this manner, one repeatedly gave the expected plus-minus signal in several individual hybridization experiments. This clone, Ds36, is depicted in Fig. 4. Plasmid DNA was purified and used for message selection criteria needed to positively identify this cDNA.

Analysis of digests (Fig. 3) of in vitro translation profiles shows a substantial incorporation of [35S] into a 68-kDa peptide in the spectrum of proteins synthesized by membrane associated poly(A+) RNA. Modified et al. (21) and Rat Man et al. (22) have identified a 68-kDa protein synthesized by membrane associated poly(A) RNA. This 68-kDa protein is the translation product of a 21S and 28S polymer. These peptide features confirm the initiation of the two cytidine polyamine fractions.

Immunoprecipitates from livers incubated with either total liver, cytoplasmic, or membrane associated poly(A) RNA were subjected to SDS-gel electrophoresis, and dried gels were stained with Coomassie blue. The gel bands were cut out and subjected to amino acid analysis as described in Methods. Desaturase activity was incorporated into the proteins.}

Fig. 1. In vitro translation and immunoprecipitation of stearyl-CoA desaturase expressed by total liver poly(A) RNA. Translation products were isolated by electrophoresis and analyzed by autoradiography and scintillation counting. A) All lanes were loaded with 10 \mu g of poly(A) RNA. B) The RNA was translated in the absence of added amino acids. C) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. D) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. E) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. F) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. G) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. H) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. I) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. J) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. K) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. L) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. M) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. N) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. O) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. P) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. Q) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. R) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. S) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. T) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. U) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. V) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. W) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. X) The RNA was translated in the absence of added amino acids and 35S labeled amino acid. Y) The RNA was translated in the presence of added amino acids and 35S labeled amino acid. Z) The RNA was translated in the absence of added amino acids and 35S labeled amino acid.
Identification of a desaturase mRNA by message selection - Eukaryotic pDs36 plasmid DNA was bound to nitrocellulose and used to hybridize liver mRNA from induced rats. This mRNA was selected on RNA which directed the synthesis of a peptide that co-comigrated with the desaturase enzyme (Fig. 5). The in vitro translation product was immunoprecipitated by our anti-desaturase rabbit antisera. There was a significant contamination of the selected RNA with other mRNA. To determine unequivocally that pDs36 DNA was selecting desaturase mRNA, we quantitated the fold enrichment of in vitro translated desaturase by selected RNA over that of total poly(A) mRNA. We found that desaturase mRNA was enriched 35 fold when total poly(A) RNA was hybridized to immobilized pDs36 DNA, and that, pDs36 contains desaturase mRNA sequences. Plasmid RNA from two strains containing sequences common to both control and induced poly(A) RNA did not select desaturase mRNA as determined by in vitro translation (data not shown).

pDs36 DNA was digested with PstI and subjected to electrophoresis on a 1.5% non-denaturing agarose gel. The DNA insert contains approximately 910 base pairs with an internal PstI site which gives fragments of about 300 and 540 base pairs (data not shown).

**Fig. 5.** In vitro translation of hybrid selected RNA by pDs36. Total liver poly(A) mRNA was hybridized to denatured sonicated plasmid DNA bound to nitrocellulose (28). Following hybridization and washing of the filters, hybridized mRNA was recovered and translated in a cell free system. The fluorogram above shows in vitro translation profiles of total poly(A) mRNA before (a) and after (b) hybridization and (c) that mRNA hybridized to the bound DNA (d,e). Control hybrid selection experiments were completed with pDs32 and the background translation is seen in lane f. Selected RNA was translated and the resulting [35S] labeled product (lane e) immunoprecipitated using anti-desaturase antisera. Immunoprecipitations from translation of selected mRNA is shown in lane f.

The site of desaturase mRNA as determined by Northern blot analysis - Total liver poly(A) mRNA from either induced or control rats was denatured in the presence of 2.2 M formaldeyde and 5% formaldehyde (28). Electrophoresis was done using a 0.95 agarose gel containing 1.2% ascorbate, 3.0 M urea and 0.5 M base composition. Stearyl-CoA desaturase mRNA is 14463 nucleotides in length. The enzyme contains about 430 amino acids, requiring only 3350 bases for its complete coding sequence. This data suggests that there is a non-translated region(s) in excess of 3150 bases.

**Fig. 6.** Northern blot analysis of desaturase mRNA size. 2 ug of total liver poly(A) RNA from control (lane a) or induced (lane b) was denatured and electrophoresed on a 0.95 agarose gel (28). The separated RNA was electrophoresed to Gene Screen Plus and probed with [32P] labeled nick translated pDs36 DNA. The filters were washed and the blot exposed to tritium for 1 3 h. Lane c contains denatured immobilized plasmid DNA with the indicated number of bases. Desaturase mRNA is enriched in induced poly(A) RNA preparations. In support of in vitro translation studies described earlier in this manuscript, dot blot analysis of [32P] labeled pDs36 DNA was performed using nick translated pDs36 RNA as a probe. 3 ug samples of poly(A) RNA from control and induced rats were spotted on Gen Screen Plus and probed by hybridization with [32P] labeled pDs36 DNA. Following hybridization, areas on the washed blot where RNA was spotted were excised. Quantitation of [32P] labeled plasmid hybridized to pDs36 DNA from induced animals show a 35-60 fold increase in desaturase mRNA levels over those in poly(A) RNA preparations from control rats (Table 1). These data corroborate our findings from in vitro translation studies where it was shown that levels of translatable desaturase mRNA were elevated under the same dietary constraints (Table 2).

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[32P]cpm bound to filter</th>
<th>Fold induction</th>
<th>[32P]cpm bound to filter minus background counts of areas spotted with 5 µl of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>Induced</td>
<td>2770</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Induced</td>
<td>1300</td>
<td>35</td>
<td>35</td>
</tr>
</tbody>
</table>

Values are the average of two trials.