Molecular Cloning of cDNA for Acetyl-coenzyme A Carboxylase*

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Poly(A)* RNA from lactating rat mammary glands was size-fractionated to enrich the relative amount of acetyl-CoA carboxylase mRNA. The enriched mRNA was used to generate a λgt11 cDNA library. Initial screening with polyclonal antiserum to acetyl-CoA carboxylase produced three positive clones. Western blot analysis revealed that two clones, λDH3 and λKH18, synthesized 165,000-dalton proteins that were recognized by antibodies to acetyl-CoA carboxylase and β-galactosidase, indicating that acetyl-CoA carboxylase/β-galactosidase fusion proteins were produced. Competition experiments with purified acetyl-CoA carboxylase further demonstrated that the fusion proteins contained acetyl-CoA carboxylase protein segments. Antibodies which are specific to the fusion proteins were isolated. These antibodies cross-reacted only with acetyl-CoA carboxylase in a preparation of partially purified acetyl-CoA carboxylase. In addition, the antibodies immunoprecipitated enzyme activity from a crude liver homogenate. Northern blot analysis of total RNA revealed two RNA species: one 10 kilobases and the other 3.0 kilobases. The levels of these RNA species increased when starved animals were fed a fat-free diet, indicating that they are coordinately regulated.

Acetyl-CoA carboxylase catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA in the rate-limiting step of long chain fatty acid biosynthesis (1, 2). Short-term regulation is mediated by covalent modification (3) and allosteric mechanisms (1, 4), whereas long-term regulation involving changes in the amount of the carboxylase is controlled by the rates of protein synthesis and/or degradation (5, 6). Long-term regulation of acetyl-CoA carboxylase has been studied in whole animals and in isolated cell culture systems under a variety of hormonal and nutritional states (5--9). Collectively, these studies show that regulation of the amount of acetyl-CoA carboxylase during certain nutritional and hormonal conditions can be accounted for in part by regulation of the rate of enzyme synthesis. Use of inhibitors of RNA synthesis (7, 8, 10) as well as the titration of polysomes involved in the synthesis of acetyl-CoA carboxylase (11) suggests that RNA synthesis is associated with such changes in the rate of enzyme synthesis. However, the relationship between changes in RNA metabolism and control of enzyme synthesis remains unresolved. Identification and isolation of the mRNA for protein as large as acetyl-CoA carboxylase (subunit M, 260,000) has been difficult, hampering progress in the analysis of the metabolism of carboxylase mRNA.

We report here the production of a λgt11 cDNA library using poly(A)* RNA enriched in mRNA for acetyl-CoA carboxylase and identification of a cDNA clone harboring the mRNA sequences for acetyl-CoA carboxylase. Furthermore, using the labeled insert of the clone, we have shown that the level of acetyl-CoA carboxylase mRNA increases when starved animals are fed a fat-free diet.

EXPERIMENTAL PROCEDURES

Materials—Commercial products were obtained as follows: avian myeloblastosis virus reverse transcriptase (Life Science Associates); Escherichia coli DNA polymerase I and 5-bromo-4-chloro-3-indolyl β-d-galactoside (Boehringer Mannheim); T4 DNA ligase, EcoRI methylase, and T4 polynucleotide kinase (New England Biolabs); S1 nuclease, EcoRI linkers, and EcoRI (Bethesda Research Laboratories); and isopropyl-β-d-thiogalactopyranoside (Bio-Rad and Boehringer Mannheim). λgt11 arms and in vitro packaging extracts were purchased from Promega Biotec as the Protoclon UT-Packagen system. The Bio-Rad Express Blot Kit was used to screen the λgt11 library for immunologically reactive peptides. Acetyl-CoA carboxylase was purified (12), and antiserum was prepared as previously reported (13).

Isolation of Total RNA and Poly(A)*RNA—Total RNA was extracted from mammary glands of lactating Wistar rats (14). Poly(A)* RNA was prepared by the method of Aviv and Leder (15). Poly(A)* RNA was size-fractionated on a sucrose gradient (16). Those fractions containing acetyl-CoA carboxylase mRNA were subjected to the sucrose gradient centriugation once more (16).

In Vitro Translation of mRNA—In vitro translation of mRNA using the reticulocyte lysate was carried out as described (17). Immunoprecipitates of the translation products were analyzed on 7% SDS-polyacrylamide gel electrophoresis (18), and the positions of the proteins were identified through autoradiography (17).

Construction of λgt11 Library—The poly(A)* RNA preparation which is enriched for acetyl-CoA carboxylase mRNA was used to construct a cDNA library using the λgt11 vector (19). Production of double-stranded cDNA and digestion with S1 nuclease were performed essentially as described (20) except that hexamer random primers (Pharmacia P-L Biochemicals) were used to prime synthesis of the first strand. The second strand of DNA was synthesized using DNA polymerase I. The cDNA was size-fractionated and separated from free linkers by electrophoresis on a 1% agarose gel. cDNAs larger than 600 base pairs were eluted from the gel using the IBI Unidirectional Electroleuter (International Biotechnologies, Inc.) and ligated into λgt11 which had been digested with EcoRI and dephosphorylated. The DNA was packaged in vitro and used to infect E. coli Y1090r— cells as instructed by Promega Biotec. 3.9 × 10^10 recombinant phage were obtained at an efficiency of 1.6 × 10^8 recombinants/μg of cDNA. Only 5.4% of the total phage generated were non-recombinants, as indicated by their blue color on plates containing 5-bromo-4-chloro-3-indolyl β-d-galactoside.

Screening the cDNA Library—A total of 42,000 phage were screened on six 100-cm LB plates (19). After incubation for 4 h at 42°C, the plates were overlaid with nitrocellulose filters impregnated with 2 mM isopropyl-β-d-thiogalactopyranoside. After 4 h at 31°C, the filters were overlaid with nitrocellulose filters impregnated with 2 mM isopropyl-β-d-thiogalactopyranoside. After 4 h at 31°C, the filters were washed and stained with X-gal. lane 1: λgt11; lane 2: λgt11 containing the insert described in this report; lane 3: λgt11 containing the cDNA inserted after EcoRI digestion (21). The insert is shown to contain a cDNA fragment hybridizing to the probe described in this report. The insert is shown to be different from a cDNA fragment hybridizing to the probe described in this report.
filters and plates were marked, and the filters were removed. The filters were washed for 5 min in TBS/Tween solution (10 mM Tris-
HCl, pH 7.5, 250 mM NaCl, 0.15% Tween 20) and then incubated in
TBS/Tween containing antiserum to acetyl-CoA carboxylase. Incuba-
tion was for 1.5 h at room temperature with gentle shaking. To 
reduce background, the antiserum had been pre-incubated with lysate of E. coli BNN97 which had been bound to nitrocellulose paper. After 
washing five times for 5 min each in TBS/Tween, the filters were 
incubated for 1.5 h with gentle shaking in TBS/Tween containing a 
3000-fold dilution of peroxidase conjugated to goat anti-rabbit IgG.

Filters were then successively washed seven times for 5 min in TBS/
Tween and three times for 5 min in TBS prior to development of the 
peroxidase color reaction in a solution of TBS containing 0.5 mg/ml 
4-chloro-1-naphthol, 15% methanol, and 0.015% H2O2. The reaction 
was stopped by washing the filters in deionized water. Plaque purity 
was obtained on the fourth round of screening.

**Competition Experiments and Production of Fusion Proteins**—The 
ext number of plaques on each filter was recorded. Incubation,
washing, and color development conditions were the same as
described above except that, in the competition experiment, filters were 
incubated with antiserum to acetyl-CoA carboxylase that had been 
preincubated with purified acetyl-CoA carboxylase in TBS/Tween at 
4°C for 10 h. As a control, the antiserum was similarly preincubated 
in TBS/Tween.

Fusion proteins of ADH3 were produced using a plate lysate system, 
by overlaying plaques with 1 ml of 2 mM isopropyl-β-D-galactopyr-
anoside for 3.5 h at 37°C. After induction, the plates were washed 
with 1 ml of Laemmlli (18) buffer, and the proteins were resolved by 
electrophoresis on a 6% SDS-polyacrylamide gel. Western blot 
analysis of these proteins was performed under the same incubation,
washing, and color development conditions described for plaque 
screening. Anti-β-galactosidase IgG (Cappel Worthington) was 
used at a concentration of 1.9 µg/ml.

**Isolation of Antibodies Specific for Fusion Proteins**—Nitrocellulose 
filters containing proteins from λgt11, clone ADH3, or clone λKH18 
were used to isolate the antibodies (21, 22). Acetyl-CoA carboxylase 
activity was assayed in a crude homogenate (23).

**Nutritional Studies and Northern and RNA Dot Blot Hybridiza-
tions**—Total RNA extracted from liver (14) was separated by size on 
a denaturing gel containing glyoxal (24) and transferred to a Zeta-
hybrid (Bio-Rad) membrane by electrophoretting. Hybridiza-
tion and washing conditions were as follows. For prehybridizat-
ion, the membrane was boiled for 5 min in 20 mM Tris- HCl, pH 8.0, 
and incubated at 65°C for 1 h in 0.1 × SSC (1 × SSC is 0.15 M NaCl 
and 0.015 M sodium citrate) and 0.5% SDS. For prehybridization, 
the filter was incubated in prehybridization solution (50% formamide, 5 
× SSC, 25 mM sodium phosphate, pH 6.5, 10 × Denhardt’s solution) 
for 2 h at 65°C. The final concentration of nick-translated probe 
was 1.3 × 106 cpm/ml. For washing, the filter was washed at room 
temperature for 1–2 h in 2 × SSC and 0.1% SDS, at room temperature 
for 30 min in 0.1 × SSC and 0.1% SDS, and at 55°C for 30 min in 
0.1 × SSC and 0.1% SDS. RNA blotted onto nitrocellulose (25) was 
hybridized as described above except that the incubation at 65°C in 
SSC and SDS in the pretreatment step was omitted. Filters were 
exposed to Kodak XAR-5 film at −70°C with intensifying screens. The 
cDNA insert was labeled with 32P using the Amersham Nick-
Translation Kit.

**RESULTS AND DISCUSSION**

**Enrichment of Acetyl-CoA Carboxylase mRNA**—The abundance 
of acetyl-CoA carboxylase mRNA in our preparations of the total RNA or poly(A)+ RNA was such that in vitro translation of these RNAs revealed no detectable amount of 
acetyl-CoA carboxylase. To identify carboxylase mRNA through in vitro translation required further enrichment of the mRNA. Thus, the total poly(A)+ RNA was size-fractionated through isokinetic sucrose density gradient centrifugation, and the various fractions were analyzed by in vitro translation. A typical fraction containing the mRNA for acetyl-CoA carboxylase is shown in Fig. 1. Many different mRNAs for smaller proteins were present (Fig. 1, lane 1), but a prominent single band of acetyl-CoA carboxylase with a molecular weight of 260,000 was obtained following immunoprecipitation (Fig. 1, lane 2), indicating that an apparently full-length acetyl-CoA carboxylase translation product was produced. These mRNAs sedimentsed with a sedimentation constant of about 35 S. In 
a separate in vitro translation experiment using these mRNA 
fractons, we have determined that the size of the carboxylase 
mRNAs was approximtely 10 kb. Such mRNA preparations 
enriched in acetyl-CoA carboxylase mRNA were used for the 
construction of a cDNA library in the λgt11 vector to increase 
the chance of obtaining the phage that contained the DNA sequence complementary to the rare carboxylase mRNA.

**Identification of Acetyl-CoA Carboxylase cDNA Clone**—Ini-
tial screening of 42,000 plaques produced three positive clones. All three clones were plaque-purified. Competition of anti-
genic reactivity with pure enzyme was used to verify that 
positive clones contained a fragment of rat acetyl-CoA carboxylase. Purified acetyl-CoA carboxylase competed with the protein product of ADH3 in the peroxidase-linked immuno-
chemical reaction (Fig. 2). This ADH3 protein product was fused to the β-galactosidase protein coded by the λgt11 vector 
because, in a Western blot assay using anti-β-galactosidase IgG or antiserum to acetyl-CoA carboxylase, a 165,000-dalton protein was recognized by both antibody preparations (Fig. 3A, lanes 2 and 4). E. coli strain Y1909R−infected with λgt11 did not produce protein bands detected with antiserum to

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Fig. 2. Rapid plaque competition experiment. Nitrocellulose filters containing proteins from wild-type Δgt11 (A), ΔDH2 (B), and ΔDH3 (C) plaques were made and screened as described under "Experimental Procedures." Upper panel, filters screened with antibodies to acetyl-CoA carboxylase which had been preincubated for 10 h at 4°C with TBS/Tween; lower panel, filters screened with antibodies to acetyl-CoA carboxylase that had been preincubated for 10 h at 4°C with 2 mg of purified enzyme.

Fig. 3. Western blot analysis of ΔDH3-synthesized proteins. Proteins produced by Δgt11 and ΔDH3 were obtained using a plate lysate system as described under "Experimental Procedures." They were separated on 6% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins reacting with antibodies were visualized as described under "Experimental Procedures." A, lanes 1 and 2, wild-type Δgt11 and ΔDH3, respectively, incubated with antisera to acetyl-CoA carboxylase; lanes 3 and 4, wild-type Δgt11 and ΔDH3, respectively, incubated with 1.9 mg/ml anti-β-galactosidase. B, proteins synthesized by ΔDH3 were separated and transferred as described above: lane 1, normal rabbit serum; lane 2, antiserum to acetyl-CoA carboxylase that was preincubated with purified enzyme in TBS/Tween; lane 3, antibodies to acetyl-CoA carboxylase that were preincubated in TBS/Tween. Authentic β-galactosidase is 116,000 daltons.

acetyl-CoA carboxylase (Fig. 3A, lane 1) but did produce a protein whose molecular weight was 116,000 and cross-reacted with anti-β-galactosidase (Fig. 3A, lane 3). Immunoprecipitation of the 165,000-dalton fusion protein was inhibited when antisera was preincubated with purified acetyl-CoA carboxylase (Fig. 3B, lanes 2 and 3). Normal rabbit antisera did not recognize any of these proteins (Fig. 3B, lane 1). The size difference between authentic β-galactosidase and the fusion protein predicts an acetyl-CoA carboxylase segment of approximate 60 kDa.

Fig. 4. Binding of clone-specific antibodies to partially purified acetyl-CoA carboxylase. Partially purified acetyl-CoA carboxylase was separated on 6% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and then incubated with the isolated clone-specific antibodies for 1.5 h at room temperature with gentle shaking. Incubation with the second antibody and washing were the same as described under "Experimental Procedures." Lane 1, ΔDH3-specific antibodies; lane 2, ΔKH18-specific antibodies; lane 3, Δgt11; lane 4, avidin-conjugated peroxidase; lane 5, Coomassie Blue stain of the partially purified acetyl-CoA carboxylase after electrophoresis in a 7.5% SDS-polyacrylamide gel.

Fig. 5. Immunoprecipitation of acetyl-CoA carboxylase by clone-specific antibodies. Crude rat liver homogenate was incubated with various dilutions of clone-specific antibodies from ΔDH3 (□) and ΔKH18 (●). After primary antibody incubation for 1 h at room temperature, goat anti-rabbit IgG serum was added and the incubation was continued for 10 h at 4°C. Samples were centrifuged at 15,000 × g for 30 min and the supernatants were assayed for acetyl-CoA carboxylase activity. Polyclonal antiserum proteins bound by proteins produced by Δgt11 served as the control (○).
We next used the cDNA as a probe to investigate the effect of diet on the amount of acetyl-CoA carboxylase mRNA in rat liver (Fig. 6). Animals which were fasted for 48 h showed very little or no carboxylase mRNA by Northern analysis (Fig. 6, upper panel). However, in the dot blot analysis, 10 μg of RNA hybridizes an appreciable amount of cDNA probe (Fig. 6, lower panel, lane 5, row a). It is difficult to compare these results directly because the conditions for the hybridization were different and a substantial amount of RNA species migrates in a diffused pattern in Northern analysis. It is interesting to note that CDNA hybridizes to two main RNA species: one 10 kb and the other a somewhat diffused band of 3.0 kb. As mentioned previously, acetyl-CoA carboxylase mRNA is about 10 kb in size. At present, we can offer no information as to the nature of the 3.0-kb RNA species.

An increase in the carboxylase mRNA level was observed in animals fasted for 48 h and then refed a fat-free diet for 24 h (Fig. 6, lane 4) or 48 h (Fig. 6, lane 3). It also should be noted that both species of RNA are affected by the dietary condition. Feeding a normal diet for 72 h to fasted animals had little effect on the carboxylase mRNA level (Fig. 6, lane 2).

In summary, the present clones harboring the nucleotide sequences for acetyl-CoA carboxylase have been obtained using poly(A)+ RNA enriched for acetyl-CoA carboxylase mRNA. That the clones identified actually contain DNA complementary to acetyl-CoA carboxylase mRNA is supported by several lines of evidence: (i) they were identified by screening a agt11 library with polyclonal antiserum to acetyl-CoA carboxylase; (ii) the fusion proteins produced compete with authentic acetyl-CoA carboxylase in immunochemical experiments; (iii) antibodies which bind to the fusion proteins selectively bind to acetyl-CoA carboxylase in a partially purified preparation of the enzyme; and (iv) the antibodies which bind to the fusion proteins immunoprecipitate enzyme activity in a crude liver homogenate. Using the 32P-labeled insert from one of these clones, it has been possible for the first time to show that the increased amount of acetyl-CoA carboxylase in rat liver following fat-free diet feeding is mainly due to an increased amount of acetyl-CoA carboxylase mRNA. Appearance and disappearance of both the 3.0- and 10-kb RNA species are roughly coordinated, and thus the dot blot hybridization studies reflect changes in both forms of RNA.

One of the most interesting results of our studies is the recognition of at least one additional RNA species by the carboxylase clone. However, without further characterization of the 3.0-kb RNA, it is premature to discuss the relationship between the two RNA species which are recognized by the clones.

Availability of the clones for acetyl-CoA carboxylase will provide a means not only to examine transcriptional control, but also to determine the relationship between structure and function of the enzyme.

REFERENCES