Cloning of cDNA to rat mammary-gland fatty acid synthase mRNA

Evidence for the expression of two mRNA species during lactation

Martin BRADDOCK* and D. Grahame HARDIE†

Department of Biochemistry, Dundee University, Medical Sciences Institute, Dundee DD1 4HN, U.K.

A cDNA library was constructed in the expression vector λ gt11, by synthesizing cDNA from size-selected poly(A) RNA from lactating rat mammary gland, using random hexanucleotide primers. Using this library we identified two recombinants which, on addition of a *lac z* inducer, produced proteins recognized by affinity-purified anti-fatty-acid synthase antibody, and which, therefore, contained fatty acid synthase coding sequences. The inserts were subcloned, were shown to be between 500 and 600 base pairs in size, and to cross-hybridize. The cloned DNA was then used in Northern hybridizations with mRNA isolated at various stages throughout lactation. Two mRNA species were identified of approx. 9.7 and 10.4 kilobases, which increased and decreased in parallel during lactation, reaching a peak at 12–13 days. Both mRNA species disappeared rapidly if the pups were removed prematurely. This study provides evidence that, during hormonal induction in lactation, regulation of the level of fatty acid synthase protein can be accounted for by variation in the level of mRNA.

INTRODUCTION

Fatty acid synthase (FAS) of mammalian tissues is a multifunctional enzyme consisting of two identical subunits of 250 kDa [1–3]. Each subunit contains distinct domains for at least six different catalytic activities, plus the acyl carrier function, and together these catalyse the synthesis of palmitic acid using malonyl-CoA, acetyl-CoA and NADPH (for reviews see [4–6]).

Animal FAS is expressed at high levels in the liver of birds and mammals and at particularly high levels in the uropygial gland of waterfowl [2] and the mammary gland [3] of lactating mammals. The activity of this lipogenic enzyme is regulated by dietary and hormonal factors [7,8] which alter the quantity of the enzyme in liver, where it has also been shown that an increase in the expression of FAS mRNA occurs during dietary induction, correlating with the enhanced enzyme level [9–11].

FAS mRNA has been partially purified and characterized from lactating rat mammary gland, using translation *in vitro* as a measure of mRNA activity [12]. Incorporation of radioactive amino acids into polypeptides precipitating with anti-FAS antibody appeared to correlate with a prominent RNA species of $M_r \sim 3.5 \times 10^6$ (~ 10 kb). Recently two FAS mRNA species of 10.8 and 12.2 kb have been identified in avian liver by Northern blotting using FAS cDNA probes [11], with similar results being obtained in rat liver [13]. Southern blots of genomic DNA have suggested, however, the presence of a single gene copy [11,13].

In order to obtain FAS cDNA probes corresponding to the coding region of FAS mRNA, we have isolated cDNAs, by immunological screening, from a size-selected rat mammary gland cDNA library constructed using random primers in λ gt11. We have used these cDNAs to

examine the nature of FAS mRNA in rat mammary gland and changes in its concentration which occur during lactation and weaning.

EXPERIMENTAL

Animals

Lactating Wistar rats with at least eight pups were used for mRNA preparations. Animals were allowed unrestricted access to standard pelleted chow and water.

Materials

RNA and DNA markers and CsCl were from Bethesda Research Laboratories; Ficoll 400, antifoam A and salmon sperm DNA from Sigma Chemical Co.; *EcoRI* linkers, horseradish peroxidase-linked anti-sheep serum and colour development kit were from Amersham International; *EcoRI* methylase, *EcoRI* and 5-bromo-4-chloro-3-indolyl 2-D-galactoside (X-gal) were from Boehringer; bovine serum albumin and polyethylene glycol 6000 were from BDH Chemicals; isopropyl 2-D-thiogalactopyranoside was from Anglian Biotechnology; guanidinium isothiocyanate was from Fluka; nitro-cellulose filters were from Schleicher and Schuell; and Sepharose CL-4B and CNBr-activated Sepharose 4B were from Pharmacia.

Isolation of mRNA

Lactating rats were killed by stunning and cervical dislocation at specified times throughout lactation. mRNA was isolated by the guanidine isothiocyanate method followed by centrifugation through a CsCl cushion [14]. Tissue samples (1–10 g) of rat mammary

Abbreviations used: FAS, fatty acid synthase; SSC, 150 mM-NaCl/15 mM-trisodium citrate (pH 7).

* Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

† To whom correspondence should be addressed.

gland were homogenized in a Polytron (Brinkman Instruments) for 60 s at full speed in a buffer containing 4 M-guanidinium isothiocyanate/0.5% sodium sarcosyl/25 mM-sodium citrate (pH 7.0/0.1 M-2-mercaptoethanol/0.1% antifoam A. After centrifuging down cell debris, this homogenate was pelleted through a cushion of 5.7 M-CsCl in a Beckman SW28 rotor at 23000 rev./min for 20 h at 4 °C. The yield of RNA was about 250 µg/g of tissue.

Purification and labelling of antibodies

Sheep anti-(rabbit FAS) serum was a gift from Dr. A. R. Slabas. Rabbit FAS was purified as described [3] and 30 mg of purified enzyme was coupled to 3.5 ml of CNBr-activated Sepharose according to manufacturers' instructions. Bound polyclonal antibody specific for FAS was eluted from the affinity column and concentrated by ammonium sulphate precipitation according to Paoletti & Aldinucci [15]. Antibody was further concentrated to approximately 100 µg/ml in a Centricon 30 microconcentrator (Amicon) for Western blotting experiments. For screening the cDNA library, antibody was ¹²⁵I-labelled to a specific radioactivity of approximately 2.5×10^6 c.p.m./µg [16].

Electrophoresis and Western blotting

SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli [17]. Samples for Western blot analysis were run in 5–15% polyacrylamide gels and transferred electrophoretically onto nitrocellulose by the procedure of Burnette [18] using a Trans Blot cell (BioRad). The buffer contained 20% methanol/25 mM-Tris/192 mM-glycine, and transfer was for 12–16 h at 30 V. The filters were soaked in 3% (w/v) gelatin in 20 mM-Tris/0.5 M-NaCl (pH 7.5) and were stained using anti-FAS antibody detected by horseradish peroxidase-linked anti-sheep serum.

Construction of the cDNA library in λgt11

RNA was prepared from lactating rats at 13 days *post partum*. After purification by three passages through oligo (dT)-cellulose, poly(A) mRNA was size-fractionated on 5–20% sucrose density gradients and the fraction of mRNA eluting with a molecular mass greater than 28 S ribosomal RNA was retained and used for construction of the cDNA library. Production of 'filled-in' double-stranded cDNA was performed using the protocol of Okayama & Berg [19] as modified by Gubler & Hoffman [20]. A random primed cDNA library was constructed using a Transcriptaid cDNA synthesis kit (P and S Biochemicals). After protection of any internal *Eco*RI sites by *Eco*RI methylase and addition of synthetic *Eco*RI linkers, the cDNA was *Eco*RI digested and size-fractionated on a Sepharose CL4B column. cDNAs larger than 500 bp were ligated into *Eco*RI-digested, dephosphorylated λgt11 arms and packaged *in vitro* using the Proclon GT/Packagene system (Promega Biotechnology).

Screening of the cDNA library

Recombinant phage (6.2×10^6 cells) were obtained at an efficiency of about 7×10^6 recombinants per µg of cDNA. Less than 3% of the total phage generated were non-recombinants, as indicated by their blue colour on plates containing the chromogenic indicator X-gal.

Approx. 7×10^5 independent phage were screened on

LB plates containing 50 µg of ampicillin/ml. Radioiodinated affinity-purified sheep anti-rabbit FAS antibodies at 5 µg/ml in phosphate-buffered saline (170 mM-NaCl/3.4 mM-KCl/4 mM-Na₂HPO₄/2.4 mM-NaH₂PO₄) were used to screen the library plated on *E. coli* Y1090 [21]. Fusion proteins were induced using isopropyl 2-D-thiogalactopyranoside. Two putative positive clones were plaque-purified by four rounds of screening.

Subcloning of cDNA

cDNA inserts from the two positive recombinants, termed λFAS1 and λFAS2, were excised by digestion of the purified phage DNA [22] with *Eco*RI, and sized by gel electrophoresis on 0.8% agarose gels containing 1 µg of ethidium bromide/ml. Inserts were visualized under u.v. light and sized by comparison with markers from a 123-base pair ladder. The cDNA was electroeluted from the gel and subcloned into the unique *Eco*RI site of 'Bluescript' M13 (Stratagene) for large scale preparation of plasmid DNA.

Preparation of radioactively labelled cDNA

*Eco*RI restriction fragments of cloned DNAs were purified by agarose gel electrophoresis followed by electroelution [22]. cDNA was then labelled with [³²P]-dCTP by nick translation [23].

Northern blot hybridization analysis

Total RNA from lactating rat mammary glands was prepared at various stages of lactation as described above, and electrophoresed through 0.8% agarose gels containing 2.2 M-formaldehyde. The gels were then electroblotted onto nitrocellulose in 10 mM-Tris/acetate/5 mM-sodium acetate/0.5 mM-EDTA (pH 7.8) at 100 V for 3 h. After transfer, the filter was baked at 80 °C for 2 h, and the gel stained with Acridine Orange (20 µg/ml) for 30 min to ensure that transfer of RNA was complete. The filter was washed in $0.1 \times$ SSC ($1 \times$ SSC = 150 mM-NaCl/15 mM-trisodium citrate, pH 7)/0.5% (w/v) SDS at 65 °C for 1 h. Prehybridization was in 10 ml final volume containing $6 \times$ SSC/50 mM-NaH₂PO₄ (pH 6.5)/5 × Denhardt's solution (0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/0.1% Ficoll 400)/50% (w/v) formamide/7% (w/v) polyethylene glycol 6000/100 µg of denatured salmon sperm DNA/ml for 12 h at 42 °C. Following prehybridization the ³²P-labelled denatured cDNA probe (specific radioactivity 10^8 c.p.m./µg) was added and the hybridization proceeded for 24–48 h at 42 °C. After hybridization the filter was washed twice in 500 ml of $2 \times$ SSC/0.1% (w/v) SDS for 20 min, and then once more for 1 h at room temperature. Finally a high-stringency wash was achieved in $0.1 \times$ SSC/0.1% SDS (w/v) at 47 °C for 1 h. The filter was air dried and autoradiographed for 36/48 h at 70 °C using Kodak X-omatic intensifying screens.

Slot-blot hybridizations were performed as described previously [24].

Quantification of FAS mRNA

Levels of FAS mRNA from total RNA isolated at various stages throughout lactation were determined by densitometric scanning of autoradiographs of dot blots using an LKB 2202 Ultrosan laser densitometer with associated Apple microcomputer software.

RESULTS AND DISCUSSION

Characterization of FAS antibody

It has been shown previously that the increase in activity of FAS during lactation is brought about by an increase in the amount of enzyme present, with a peak at between 10 and 15 days *post partum* [12]. Protein samples from 100000 *g* supernatants of 12–13 day lactating rat mammary glands were analysed by Western blotting. Fig. 1(a) shows a Coomassie-stained gel of the crude supernatant and of purified rat mammary FAS. A duplicate gel was blotted onto nitrocellulose and probed with affinity-purified anti-FAS antibody and the results are shown in Fig. 1(b). It is worth noting that the very

Fig. 1. Characterization of FAS antibody

(a) Purified rat mammary FAS [27] (5 μ g; lane 1) or total protein from high-speed supernatants (100000 *g*) of rat mammary gland extract (20 μ g; lane 2) were run on a 5–15% SDS/polyacrylamide gel. The gel was stained with Coomassie Blue and destained in 10% methanol/10% acetic acid. Protein markers were: 212 kDa (myosin heavy chain); 116 kDa (β -galactosidase); 97.4 kDa (rabbit muscle phosphorylase *b*); 66 kDa (bovine serum albumin); 45 kDa (hen ovalbumin); 29 kDa (bovine carbonic anhydrase). (b) Western blot of a duplicate gel as shown in (a). Proteins were electroblotted onto nitrocellulose in a buffer containing 20% methanol/25 mM-Tris/192 mM-glycine at 30 V for 12–16 h. After blotting the filter was soaked in 3% (w/v) gelatin in 20 mM-Tris/0.5 M-NaCl, pH 7.5. Visualization of FAS was effected using affinity-purified sheep anti-(rabbit FAS) primary antibody from this laboratory, and biotinylated anti-sheep IgG antibody, streptavidin-peroxidase conjugate, and colour development kit (Amersham International).

large molecular mass of FAS did not appreciably impair transfer of the protein onto nitrocellulose as judged by the lack of Coomassie Blue staining remaining in the gel after transfer (not shown). It is apparent from the Western blot that the antibody prepared by affinity purification of crude serum did not cross-react with any soluble proteins in mammary gland other than the 250 kDa polypeptide of FAS.

In order to quantify the level of protein detectable with this antibody, radio-iodinated anti-FAS antibody was used to probe a filter onto which serial dilutions of a homogeneous preparation of FAS were dotted. Levels of FAS enzyme protein as low as 200 pg were detectable. Such a level of sensitivity should be readily detectable as a fusion protein from any recombinant plaque in λ gt11 [25].

Size selection of mRNA and construction of cDNA library

The previously described FAS cDNA clones from goose uropygial gland [10] and rat liver [26] were constructed using oligo(dT) to prime the reverse transcriptase, and the final screening was carried out in both cases by hybrid-selected translation. This latter method does not depend on the presence of any protein-coding sequences in the cDNAs, and use of oligo-dT primers enriches for cDNAs derived from the 3' end [adjacent to the poly(A) tail] of the mRNA. The largest cloned inserts obtained by both these groups was about ~1.5 kb, out of a total mRNA of ~10 kb, of which ~6.6 kb would be required to code for the FAS subunit. It therefore seemed likely that the existing clones were derived from the 3'-untranslated regions of the mRNA, a suggestion strengthened by DNA sequence work on the goose cDNAs [11]. We were keen to obtain cDNA derived from the coding region of FAS, and therefore adopted a different approach of priming the reverse transcriptase with random hexanucleotides, and cloning in the expression vector λ gt11, using immunological screening with anti-FAS antibody for detection of FAS cDNA.

Purification of intact high molecular mass mRNA was a critical step in this project. Poly(A) RNA was fractionated on a 5–20% linear sucrose gradient, fractions of mRNA larger than 28S mRNA were pooled, and 3 μ g was run on a 1% formaldehyde denaturing agarose gel alongside RNA markers. Fig. 2 shows that a prominent RNA species with a size of around 10 kb was evident in this preparation. This RNA preparation was converted into double-stranded cDNA using random primers as described in the Experimental section. Analysis of the cDNA by agarose gel electrophoresis and autoradiography showed that cDNA species produced ranged from ~100 to ~2000 bases, with the most abundant being 200–500 bases (not shown). Despite the apparent integrity of the RNA preparation (Figs. 2 and 3), and despite considerable experimentation with the source of reverse transcriptase and the incubation conditions, we were unable to produce cDNA larger than this from random primers. Using oligo(dT) as primer, the peak of the cDNA size distribution increased slightly to 1–2 kb, but there was still very little material produced of >3 kb (not shown).

The random primed cDNA was used to construct a cDNA library in λ gt11. Immunological screening of ~ 7×10^5 plaques yielded two positive recombinants (λ FAS1 and λ FAS2) containing inserts of 500–600 base

Fig. 2. Analysis of size-fractionated poly(A) RNA from lactating mammary gland

RNA (3 μ g) from pooled sucrose gradient fractions sedimenting faster than 28S mRNA was loaded on an 0.8% gel and run for 4 h at 70 V. RNA was visualized by staining the gel in 1 μ g of ethidium bromide/ml. Arrows show the position of RNA markers (as in Fig. 3), with sizes given in kilobases.

pairs, detected using the affinity purified anti-FAS antibody. The inserts were subcloned into M13 vectors to produce probes for Northern blotting experiments (pFAS1 and pFAS2). These probes were shown to cross-hybridize, and pFAS1 was selected for further experiments.

Analysis of FAS mRNA levels in mammary gland during lactation

mRNA from 12–13 day lactating mammary gland was run out on a 1% agarose/formaldehyde denaturing gel and probed with 32 P-labelled pFAS1. Two horizontal gel electrophoresis systems were used, a 11 cm $\times$ 14 cm gel bed (Fig. 3a) and a 20 cm $\times$ 25 cm gel tray (Fig. 3b). As has been previously reported for mRNA from goose uropygial gland and rat liver [11], the greater resolution obtained by electrophoresis on a longer gel bed clearly distinguishes two mRNA species. By comparison with RNA standards available (BRL 10 kb ladder) the mRNAs can be estimated to be 9.7 and 10.4 kb respectively (Fig. 3c).

We also used an independently cloned rat-liver FAS cDNA in Northern blot analysis of total RNA from lactating mammary gland. This cDNA was originally

Fig. 3. Northern blot analysis of mammary gland mRNA

(a) Total RNA (10 μ g) blotted from an 11 $\times$ 14 cm gel bed. (b) Total RNA (10 μ g) blotted from a 20 $\times$ 25 cm gel tray. (c) Semi-logarithmic plot of migration of hybridized mRNA (●) compared with RNA markers (BRL 10 kb ladder, ○).

isolated by the group of Porter [26], who employed a hybrid-selection protocol to verify that they had obtained FAS cDNA. They reported that their cDNA hybridized with two RNA species of 8.4 and 9.2 kb, along with a considerable amount of smaller (probably degraded) species, when total poly(A) RNA from rat liver was analysed by Northern blotting [13]. We found that the rat liver cDNA from Porter's group also identifies two distinct mRNA species when total RNA from lactating mammary gland is blotted from a 20 cm $\times$ 25 cm gel, migrating in exactly the same position as found with our cDNA (results not shown). This supports our claim that we have also isolated cDNA for FAS, as the two cDNAs were isolated by independent procedures.

In order to investigate the fluctuation in the level of FAS mRNA during lactation, RNA was prepared at

Fig. 4. FAS mRNA levels during lactation and weaning in rat mammary gland

mRNA levels were quantified by laser densitometry of autoradiograms of slot-blots ($2 \mu\text{g}$ of RNA each). The continuous line (○) shows the variation in FAS mRNA levels when pups were kept with the lactating females throughout lactation; the dotted line (●) shows the effect of removing the pups at 10 days. Due to uncertainties about the exact times of parturition, times quoted are only correct to ± 12 h.

various stages throughout the cycle and analysed by Northern blotting (not shown) and dot blotting (Fig. 4). FAS mRNA is present at very low levels at 3 days *post partum*, and then rises rapidly between 3 and 8 days, reaching a peak at 12–13 days, followed by a decline to 21 days, when the pups are ceasing to suckle. The rapid increase in hybridization intensity up to the peak level demonstrates the rapid induction of these mRNAs, which correlates with the increased level of FAS in the mammary gland [12]. We also observed, on weaning a 10–11 day lactating rat for 2 days, that there was a rapid and dramatic decrease in the level of hybridizing mRNAs. It has been reported previously that FAS activity in mammary gland drops rapidly on weaning [12].

It remains unclear as to why two FAS mRNA species are found in lactating rat mammary gland, although studies on FAS cDNAs from goose uropygial gland have suggested that the two mRNA species result from alternative polyadenylation sites in the transcript from a single gene [11].

This work was supported by a project grant from the Medical Research Council. We are grateful to Drs. M. Jackson and B. Burchell and Mrs. L. McCarthy for helpful discussions, and to Dr. Alan Goodridge for providing the rat liver cDNA from the laboratory of the late John Porter.

REFERENCES

1. Stoops, J. K., Arslanian, M. J., Oh, Y. H., Aune, K. C., Wakil, S. J. & Oliver, R. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1940–1944
2. Buckner, J. S. & Kolattukudy, P. E. (1976) *Biochemistry* **15**, 1948–1957
3. Hardie, D. G. & Cohen, P. (1978) *Eur. J. Biochem.* **92**, 25–34
4. Hardie, D. G. & McCarthy, A. D. (1985) *Trends Biochem. Sci.* **9**, 60–63
5. Hardie, D. G. & McCarthy, A. D. (1986) in *Multi-functional Proteins – Structure and Evolution* (Hardie, D. G. & Coggins, J. R., eds.), pp. 229–253, Elsevier, Amsterdam
6. Wakil, S. J., Stoops, J. K. & Joshi, V. C. (1983) *Annu. Rev. Biochem.* **52**, 537–579
7. Lakshmanan, M. R., Nepokroeff, C. M. & Porter, J. W. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3516–3519
8. Volpe, J. J. & Vagelos, P. R. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 889–893
9. Nepokroeff, C. M. & Porter, J. W. (1978) *J. Biol. Chem.* **253**, 2279–2283
10. Morris, S. M., Nilson, J. H., Jenik, R. A., Winberry, L. K., McDevitt, M. A. & Goodridge, A. G. (1982) *J. Biol. Chem.* **257**, 3225–3229
11. Back, D. W., Goldman, M. J., Fisch, J. E., Ochs, R. S. & Goodridge, A. G. (1986) *J. Biol. Chem.* **261**, 4190–4197
12. Mattick, J. S., Zehner, Z. E., Calabro, M. A. & Wakil, S. J. (1981) *Eur. J. Biochem.* **114**, 643–651
13. Yan, C., Wood, E. A. & Porter, J. W. (1985) *Biochem. Biophys. Res. Commun.* **126**, 1235–1241
14. Chirgwin, J., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5194–5199
15. Paoletti, F. & Aldinucci, D. (1986) *Arch. Biochem. Biophys.* **245**, 212–219
16. Broome, S. & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2746–2749
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
18. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
19. Okayama, H. & Berg, P. (1982) *Mol. Cell. Biol.* **2**, 161–171
20. Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–269
21. Hunyh, T., Young, R. & Davis, R. (1984) in *DNA Cloning: A Practical Approach* (Glover, D., ed.), IRL Press, Oxford
22. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
23. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251
24. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) *Basic Methods in Molecular Biology*, Elsevier, New York
25. de Wet, J. R., Fukushima, H., Dewji, N. N., Wilcox, E., O'Brien, J. S. & Helinski, D. R. (1984) *DNA* **3**, 437–447
26. Nepokroeff, C. M., Adachi, K., Yan, C. & Porter, J. W. (1984) *Eur. J. Biochem.* **140**, 441–445
27. Hardie, D. G., Guy, P. S. & Cohen, P. (1981) *Methods Enzymol.* **71**, 26–33

Received 28 August 1987/23 October 1987; accepted 3 November 1987