Identification of fatty acid synthetase messenger RNA on free polyribosomes isolated from lactating rabbit mammary gland

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The synthesis of fatty acid synthetase on free polyribosomes from lactating rabbit mammary gland was demonstrated by using polyribosome run-off techniques and immunochemical identification of products with synthetase antiserum. Several reproducible and discrete immunoprecipitable polypeptides were observed which were within the molecular-weight range of the synthetase subunit (235 000–252 000), as well as several of lower molecular weight.

Mammalian fatty acid synthetase is a multifunctional enzyme which contains seven different catalytic activities and an acyl-carrier function (see Bloch & Vance, 1977). The enzyme from lactating rabbit mammary gland consists of two polypeptides both of mol.wt. 250 000, which cannot be resolved by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Grunnet & Knudsen, 1978; Hardie & Cohen, 1978). Whether these subunits are identical remains to be established, though evidence based on cleavage of fatty acid synthetase with elastase is consistent with the view that they are (Guy *et al.*, 1978).

Considerable effort has been concentrated on the isolation of mRNA for fatty acid synthetase (Zehner *et al.*, 1977, 1980; Nepokroeff & Porter, 1978; Lau *et al.*, 1979). In conjunction with DNA recombinant and sequencing technology, such studies should ultimately provide the complete amino acid sequence of synthetase subunits, thereby resolving the uncertainties about their identity. This approach would also provide nucleic acid hybridization probes for studies on the regulation of the fatty acid synthetase gene by hormones.

In rabbit mammary gland, prolactin has a profound influence on fatty acid synthetase gene expression and on the stability of the synthetase (Speake *et al.*, 1975, 1976). Sequence-specific hybridization probes for the enzyme would enable the molecular mechanisms involved in this regulation to be investigated. As part of this longer-term study, we describe the identification of fatty acid

synthetase mRNA on free polyribosomes from lactating rabbit mammary gland by run-off in the wheat-germ cell-free translation system. The results are discussed in terms of the structure of the fatty acid synthetase subunits, and the functional distinction between free and membrane-bound polyribosomes in that fatty acid synthetase is synthesized on the former.

Materials and methods

Lactating New Zealand White rabbits were obtained from accredited dealers.

Chemicals

Wheat germ was a gift from Marriage and Sons Ltd., Chelmer Mills, Chelmsford, Essex, U.K. Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Hopkin and Williams, Chadwell Heath, Essex, U.K., sodium deoxycholate was from E. Merck, Darmstadt, W. Germany, and heparin (sodium salt), 2-mercaptoethanol, Triton X-100, aurotricarboxylic acid, phenylmethanesulphonyl fluoride and Coomassie Brilliant Blue were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Electrophoresis-grade acrylamide and *NN'*-methylenebisacrylamide and diethyl pyrocarbonate were obtained from BDH Chemicals, Poole, Dorset, U.K. L-[³⁵S]Methionine (700–1440 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of AnalaR grade supplied by BDH or by Fisons Scientific Apparatus, Loughborough, Leics., U.K. Fuji Rx X-ray film was obtained from Hanimex, Swindon, Wilts., U.K.

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Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the lactating rabbit mammary-gland fatty acid synthetase used in competition assays showed a major band of mol.wt. approx. 260000. There were a number of faint bands of lower molecular weight, which might be due to proteolysis of the synthetase (Paskin & Mayer, 1976). A sample of antibodies raised in sheep against fatty acid synthetase purified from lactating rabbit mammary gland [anti-(fatty acid synthetase)] was a gift from Dr. R. J. Mayer, Department of Biochemistry, University of Nottingham. Immunodiffusion and immunoelectrophoretic analysis of the antiserum showed one precipitin line when the cytosol from lactating rabbit mammary gland was used as the crude source of antigen (Paskin & Mayer, 1976). Antiserum against this antibody [anti-(sheep γ -globulin)] was raised in rabbits.

Solutions and glassware

The preparations of buffers and scintillation fluid and the sterilization of glassware and polyware were as described by Craig *et al.* (1976, 1979).

Preparation of free and membrane-bound polyribosomes

These were prepared from the mammary gland of rabbits at 11–15 days of lactation essentially as described by Houdebine (1977). Fresh tissue, or tissue which had been frozen in liquid N_2 and stored at -70°C for up to 6 months, was used. The preparation was modified slightly by increasing the concentrations of KCl and of heparin in the initial homogenization buffer to 250 mM and 0.5 mg/ml respectively to ensure maximum yields of intact polyribosomes.

Preparation of total and polyribosomal poly(A)-containing RNA

Total poly(A)-containing RNA was prepared from lactating rabbit mammary gland essentially as described by Hall *et al.* (1979), and polyribosomal poly(A)-containing RNA was prepared by the method of Craig *et al.* (1979).

Cell-free translation of free and membrane-bound polyribosomes

The extract of wheat germ used as the cell-free translation system was prepared by the method of Roberts & Paterson (1973) and stored in liquid N_2 . The cell-free translation assay used was based on that described by Craig *et al.* (1976). Sedimented free or membrane-bound polyribosomes were suspended in buffer consisting of 20 mM-Hepes, pH 7.6 at 4°C , 120 mM-KCl, 5 mM-magnesium acetate and 6 mM-2-mercaptoethanol. Each assay contained, in a final volume of 250–1000 μl , 2.5–5.0 μCi of L-[^{35}S]methionine and 1–5 A_{260} units of polyribo-

somes per 50 μl of assay solution. Run-off of polyribosomes occurred in both the presence and absence of 40 μM -aurintricarboxylic acid (which inhibits the initiation of translation; Lodish *et al.*, 1971). The assay mixture was incubated at 22°C for 3–4 h, and then made 1 mM with phenylmethane-sulphonyl fluoride to minimize proteinase activity.

Analysis of polyribosome translation products

L-[^{35}S]Methionine-labelled polypeptides synthesized by polyribosome run-off were analysed by double-antibody immunoprecipitation as described by Craig *et al.* (1976), by using anti-(fatty acid synthetase). The method was modified slightly in that the 0.5% (v/v) Nonidet P-40 in the antibody wash buffer was replaced by 1% (v/v) Triton X-100, and the immunoprecipitate was sedimented through 20% (w/v) sucrose solution before, rather than after, the washing steps used in this method.

In competition assays, anti-(fatty acid synthetase) was mixed with 20–30 μg of fatty acid synthetase purified from lactating rabbit mammary gland before it was added to the immunoprecipitation mixture. The radioactivity in the immunoprecipitates was measured by liquid-scintillation counting, and the immunoprecipitates were analysed by slab-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Pascall *et al.* (1981). In order to separate proteins of both high (≥ 100000) and lower molecular weights in the same experiment, a gel (4 cm) of 6.25% (w/v) acrylamide was cast over a gel (10 cm) of 12.5% (w/v) acrylamide. The following proteins were used as standards in separations which were run in parallel to those with samples from the cell-free translation assays: bovine serum albumin (mol.wt. 67000), *Xenopus* plasma vitellogenin (mol.wt. 215000), and fatty acid synthetase purified from lactating rabbit mammary gland (mol.wt. approx. 500000). The major band obtained when fatty acid synthetase was used had mol.wt. approx. 260000, which is comparable with the value quoted by Grunnet & Knudsen (1978) and by Hardie & Cohen (1978) for the synthetase subunit. Total caseins, and total whey proteins (as a source of α -lactalbumin), were prepared by acid precipitation of skimmed rabbit milk.

The positions of the marker proteins were determined by staining the gel with 0.1% (w/v) Coomassie Brilliant Blue G in acetic acid/methanol/water (1:4:5, by vol.), followed by destaining in acetic acid/methanol/water (1:1:8, by vol.). Fluorographs of the gels were obtained by the method of Bonner & Laskey (1974), and the gels were exposed to Fuji Rx medical X-ray film at -70°C to detect radioactive proteins. The fluorographs obtained after run-off of free and membrane-bound polyribosomes were scanned with a

Corning 750 System Scanning Densitometer, and the areas under the peaks corresponding to fatty acid synthetase peptides were measured.

Results and discussion

Analysis of the total radiolabelled polypeptides obtained when free or membrane-bound polyribosomes were run-off in the cell-free protein-synthesizing system of wheat germ demonstrates that a large spectrum of peptides was synthesized on both polyribosome populations, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1, lanes 3–6). The pattern of peptides obtained from each polyribosome population proved to be distinct and to some extent reflected the situation *in vivo*, since the run-off products were identical in the presence and absence of aurin-

tricarboxylic acid, which inhibits the initiation of protein synthesis (Lodish *et al.*, 1971; see also Craig *et al.*, 1979). Examination of the high-molecular-weight polypeptides [i.e. those that only entered the 6.25% (w/v)-acrylamide section of the gel (>100 000 mol.wt.)] showed that several distinct bands were apparent when free polyribosomes were run-off, but that these were not present after the run-off of membrane-bound polyribosomes. The addition of antibody raised against rabbit fatty acid synthetase resulted in the precipitation of most of these bands, some of which co-migrated with the fatty acid synthetase subunit isolated from lactating rabbit mammary gland (see Fig. 1, lanes 7 and 9). Several bands of lower molecular weight were also precipitated in the 12.5% (w/v)-acrylamide section of the gel. These seem to result from a non-specific interaction with the antibody, since they were the

Fig. 1. Analysis of the total run-off products of free and membrane-bound polyribosomes from lactating-rabbit mammary gland in the cell-free protein-synthesizing system from wheat germ

Polyacrylamide-gel electrophoresis and fluorography are described in the Materials and methods section. Lanes: 1, no added polyribosomes; 2, peptides synthesized after the addition of poly(A)-containing RNA isolated from lactating mammary gland; 3, peptides synthesized by run-off of free polyribosomes prepared from frozen mammary tissue; 4, as lane 3, but in the presence of 40 μ M-aurintricarboxylic acid; 5, peptides synthesized by run-off of membrane-bound polyribosomes prepared from frozen mammary tissue; 6, as lane 5, but in the presence of 40 μ M-aurintricarboxylic acid; 7, peptides synthesized by run-off of free polyribosomes immunoprecipitated with anti-(fatty acid synthetase); 8, as lane 7, but with 30 μ g of purified fatty acid synthetase present in the immunoassay; 9, as lane 7, but in the presence of 40 μ M-aurintricarboxylic acid; 10, as lane 9, but with 30 μ g of purified fatty acid synthetase present in the immunoassay. The lines indicate the positions of non-radioactive proteins used as markers: FAS, fatty acid synthetase purified from lactating rabbit mammary gland (mol.wt. approx. 250 000); Vit, vitellogenin isolated from *Xenopus* plasma (mol.wt. 215 000); BSA, bovine serum albumin (mol.wt. 67 000). The relative mobilities of the major rabbit caseins and of α -lactalbumin isolated from rabbit milk are indicated. The relative mobilities of the primary translation products of rabbit pre-caseins and pre- α -lactalbumin (assumed) as synthesized in the wheat-germ cell-free system are also indicated. The arrow indicates the junction between the 6.25% (w/v)-acrylamide (upper) section and the 12.5% (w/v)-acrylamide (lower) section of the gel.

only bands precipitated (Fig. 1, lanes 8 and 10) when the antiserum was pre-absorbed with highly purified fatty acid synthetase. Alternatively, these bands in the 12.5%-acrylamide section might be due to contaminants in the preparation of synthetase used to raise the antiserum which were absent from the preparation of synthetase used in the competition assays.

Longer exposures to X-ray film of the 6.25% (w/v) region of a polyacrylamide gel from a different series of experiments (see Fig. 2) confirmed that a distinctive pattern of peptides was specifically immunoprecipitated after the run-off of free polyribosomes, and these included a distinctive doublet which essentially co-migrated with the fatty acid synthetase subunit, which has an estimated mol.wt. of 235 000–252 000 (Grunnet & Knudsen, 1978; Hardie & Cohen, 1978). Several other bands ranging in molecular weight from 100 000 to 2 000 000 were also reproducibly immunoprecipitated in six separate experiments. Pre-absorption with bovine serum

albumin did not prevent precipitation by the antibody of the high-molecular-weight bands (see Fig. 2, lanes 5–7).

We have yet to establish why polyribosomal run-off should result in such heterogeneity. Various possibilities exist, such as (a) proteolysis of the nascent synthetase peptides during preparation of the polyribosomes, (b) the synthetase used to raise antibodies contained immunogenic impurities which were also present amongst the polyribosome run-off products, but which were not detected by immunodiffusion and immunoelectrophoretic analysis of the antiserum, (c) the inability of the wheat-germ cell-free system to perform specific (though as yet uncharacterized) co-translational modifications which occur in lactating rabbit mammary-gland cytosol, or (d) additional unscheduled post-translational modifications by the wheat-germ cell-free system which do not occur in lactating rabbit mammary-gland cytosol, a situation similar to other reports of the unscheduled modification of mRNA-directed guinea-pig caseins (Pascall *et al.*, 1981). Although all of these possibilities would explain changes in the mobility of synthetase on sodium dodecyl sulphate/polyacrylamide gels, the possibility cannot be dismissed that the multiplicity of fatty acid synthetase, particularly the very-high-molecular-weight polypeptides observed as a result of polyribosome run-off, reflects the true situation *in vivo*. Fatty acid synthetase purified from rabbit mammary gland is a multifunctional protein consisting of two apparently identical subunits, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and Coomassie Blue staining. It seems reasonable to suppose that the multiple bands that we observed may prove to be variants of the subunits encoded by different mRNA species. This is a situation that, for instance, has been established for vitellogenin (see Wahli & Dawid, 1980).

Fatty acid synthetase mRNA was associated almost exclusively (>90%) with free polyribosomes, which is the expected site of synthesis of a cytosolic enzyme (see Palade, 1975). This observation is in agreement with others (Zehner *et al.*, 1977; Nepokroeff & Porter, 1978; Lau *et al.*, 1979). By contrast, the milk proteins were synthesized by both polyribosome fractions. This again was an expected result, since the initiation of milk-protein synthesis *in vivo* occurs on free polyribosomes, but is followed by attachment of the secretory protein polypeptides to the endoplasmic reticulum (see Craig *et al.*, 1979).

At present we are unable to identify the individual rabbit casein peptides. We have established, however, that the prominent radiolabelled polypeptides, all of which have slightly increased electrophoretic mobility compared with the caseins isolated from rabbit milk (see Fig. 1, lanes 3–6), are caseins as

Fig. 2. Identification of fatty acid synthetase synthesized by run-off of free polyribosomes in the cell-free protein-synthesizing system from wheat germ

Analytical procedures were as described in Fig. 1, except that only the upper half of the gel is shown. Lanes: 1, no added polyribosomes; 2, peptides synthesized after the addition of poly(A)-containing RNA from lactating rabbit mammary gland; 3, peptides synthesized by run-off of membrane-bound polyribosomes prepared from fresh lactating rabbit mammary tissue; 4, as lane 3, but free run-off products of free polyribosomes; 5, as lane 4, but polypeptides immunoprecipitable with antiserum raised against fatty acid synthetase; 6, as lane 5, but antiserum pre-absorbed with purified fatty acid synthetase; 7, as lane 6, but antiserum pre-absorbed with bovine serum albumin. The arrow indicates the junction between the 6.25% (w/v)-acrylamide (upper) section and the 12.5% (w/v)-acrylamide (lower) section of the gel. For abbreviations see Fig. 1.

judged by antibody precipitation (results not shown). Similarly, the predominant mRNA-directed polypeptides in the same molecular-weight range (Fig. 1, lane 2), which can be resolved into four major bands and a series of minor bands, are also caseins. The disparate mobilities of these peptides (Fig. 1, cf. lanes 2, 3 and 5) reflect a combination of unscheduled post-translational modifications by the wheat-germ cell-free protein-synthesizing system (see Pascall *et al.*, 1981), and varying degrees of co-translational modification of the incomplete polypeptides performed *in vivo* before completion of chain elongation *in vitro*. The remaining predominant band(s) probably represent varying extents of processing of pre- α -lactalbumin.

Overall, our results confirm that there is a functional distinction between the two classes of polyribosomes. In particular, they demonstrate the almost exclusive synthesis of fatty acid synthetase, which is a cytosolic enzyme, on free polyribosomes. The results also provide evidence for heterogeneity within the fatty acid synthetase subunits. The isolation of fatty acid synthetase mRNA as described from goose uropygial gland (Zehner *et al.*, 1980) and the subsequent application of recombinant-DNA technology will ultimately resolve such structural possibilities. Mattick *et al.* (1981) have reported the isolation of fatty acid synthetase mRNA from the total poly(A)-rich mRNA of lactating rat mammary gland. It is noteworthy that when the synthetase mRNA was translated in the reticulocyte-lysate cell-free system, immunochemical analysis of the products also showed a spectrum of polypeptides which ranged in molecular weight from about 120 000 up to that of the complete subunit, as we have found.

The availability of suitable plasmids which contain fatty acid synthetase complementary-DNA sequences will enable nucleic acid hybridization procedures to be used to analyse the molecular mechanisms involved in the hormonal regulation of fatty acid synthetase gene expression. The well-established responses of mammary gland when cultured with prolactin and glucocorticoids (see Speake *et al.*, 1975, 1976; Houdebine, 1980) could be analysed by using this approach.

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