Construction of a cDNA encoding the multifunctional animal fatty acid synthase and expression in *Spodoptera frugiperda* cells using baculoviral vectors

Anil K. JOSHI and Stuart SMITH
Children's Hospital Oakland Research Institute, 747 Fifty-second Street, Oakland, CA 94609, U.S.A.

A cDNA encoding the 2505-residue multifunctional rat fatty acid synthase has been constructed and expressed as a catalytically active protein in *Spodoptera frugiperda* (*Sf*9) cells using *Autographa californica* nuclear polyhedrosis virus (baculovirus). The 7.5 kb cDNA was engineered by the amplification and sequential splicing together of seven fragments contained in overlapping cDNAs that collectively spanned the entire rat fatty acid synthase coding sequence. The full-length cDNA was cloned into a baculoviral transfer vector and used together with linearized baculoviral DNA to co-transfect *Sf*9 cells. Recombinant viral clones were purified and identified by Western blotting. The recombinant fatty acid synthase was expressed maximally 2 days after infection of the *Sf*9 cells, constituting up to 20% of the soluble cytoplasm, and could be conveniently separated from the insect host fatty acid synthase by high-performance anion-exchange chromatography. The catalytic properties of the purified recombinant fatty acid synthase are indistinguishable from those of the best preparations of the natural protein obtained from rat liver. These results indicate that, in the insect cell host, all seven catalytic components of the 2505-residue recombinant fatty acid synthase fold correctly, the acyl-carrier-protein domain is appropriately phosphopantetheinylated post-translationally, and the multifunctional polypeptide forms catalytically competent dimers. Thus the baculoviral system appears to be well suited for the expression of specific fatty acid synthase mutants that can be used to explore the mechanism by which the seven domains of this multifunctional homodimer co-operate in the biosynthesis of fatty acids.

INTRODUCTION

The *de novo* synthesis of fatty acids from malonyl-CoA requires seven enzyme activities which in most bacteria and plants exist as discrete monofunctional polypeptides. However, in animals, the seven catalytic activities are integrated into a single polypeptide chain [1–3]. The active form of the animal fatty acid synthase is a homodimer with subunits oriented 'head to tail', and several of the partial reactions appear to require the participation of both subunits [4–6]. To define more clearly how the two subunits co-operate to catalyse the 37 sequential reactions leading to the formation of palmitic acid, we constructed a full-length cDNA encoding the 2505-residue multifunctional polypeptide and sought a convenient host/vector system that would allow us to engineer, express and characterize specific mutants of the recombinant protein. Critical to the successful expression of a catalytically competent recombinant fatty acid synthase is the choice of a host cell which can efficiently perform the post-translational phosphopantetheinylation of Ser-2151. This modification is essential for the production of a functional acyl-carrier-protein domain, as it is the 4′-phosphopantetheine moiety that translocates the growing acyl chain through the six catalytic centres of the complex. Previous attempts to express the acyl-carrier-protein domain of the animal fatty acid synthase as a separate protein in *Escherichia coli* had produced only the apo form (V. S. Rangan and S. Smith, unpublished work), so we sought an animal cell host for expression of the entire fatty acid synthase. We chose an insect cell host for several reasons. Firstly, insect fatty acid synthases have been purified and characterized from several species, both larval and adult forms; they are typical animal fatty acid synthase homodimers of approx. 500 kDa with the usual amino acid composition, carbon-substrate specificity, nicotinamide nucleotide specificity, kinetics and product specificity [7–9] so we expected that insect *Sf*9 cells would probably perform the appropriate phosphopantethenylation at Ser-1251. Secondly, an increasing variety of transfer plasmids are becoming available for the baculovirus, which can accommodate a large insert such as the 7.5 kb fatty acid synthase cDNA, and the virus can be propagated to high titres. Thirdly, the *Sf*9 cells can be adapted readily to suspension culture facilitating relatively large-scale recombinant protein production. Finally, recombinant proteins tend to be produced exclusively as soluble proteins in *Sf*9 cells, thus obviating the need for refolding, a task that might be particularly daunting in the case of a large multifunctional protein. This choice of the *Sf*9/baculovirus system has proved to be highly satisfactory and we now routinely express milligram quantities of catalytically active recombinant fatty acid synthase, as described in the following text.

MATERIALS AND METHODS

Materials

Fatty acid synthase clones were derived from rat mammary gland and rat liver cDNA libraries constructed in λgt11 [2]. The plasmid pUCBM20 was obtained from Boehringer–Mannheim (Indianapolis, IN, U.S.A.), baculoviral transfer vectors pVL1393 and pBlueBac were obtained from Invitrogen (San Diego, CA, U.S.A.) and linearized baculoviral DNA (Baculogold) from PharMingen (San Diego, CA, U.S.A.). A plasmid DNA-purification kit was obtained from Qiagen (Chatsworth, CA, U.S.A.) and 'Sequenase' DNA sequencing kit and 'compact reaction columns' from USB (Cleveland, OH, U.S.A.). Vent polymerase, T4 DNA ligase and restriction enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.) and oligonucleotide primers from Operon (Alameda, CA, U.S.A.). *E. coli* DH5α competent cells and Grace's insect medium were obtained from GIBCO–BRL (Grand Island, NY, U.S.A.) and *Sf*9 cells from Invitrogen. [α-[^{35}S]thio]dATP was obtained from Amersham (Arlington Heights, IL, U.S.A.), dNTPs from Pharmacia LKB (Uppsala, Sweden) and Taq DNA polymerase from Perkin–

Table 1 PCR primers used for amplification of fatty acid synthase fragments

The template cDNAs are identified by the cDNA clone number and their location in the overall fatty acid synthase cDNA sequence is shown in Figure 1. Primers for the sense and antisense strands are denoted as T and B (top and bottom) respectively. Upper-case letters indicate the primer sequence that matches the cDNA sequence. Bases in lower case were incorporated so as to engineer restriction sites at the ends of amplified fragments that could be used conveniently to clone individual fragments into the pUCMB20 vector; these nucleotides are not present in the final cDNA construct. Restriction sites are underlined and the start and stop codons are shown in bold letters. The base numbers are according to fatty acid synthase cDNA sequence [2].

Template cDNA	Primer	Sequence	Location
	5T	5′-ata tgg tac CTA CAT TGA TTG CAT CAA GCA GGT GCA	6927–6948
pFAS 5		*Kpn*I	
	5B	5′-tat agc ggc cgc tag CTT CAT GGT AGG CAG GT**C TA**GC	7617–7595
		*Not*I *Nhe*I	
	1T	5′-GCA TTA TCT TGG AAG CGA TG	6263–6282
pFAS 1			
	1B	5′-atc atc tag aGG AGC ACA TCT CGA AGG CTA C	7000–7020
		*Xba*I	
	200T	5′-CAT GGC CAT CTT CTT GAA GAA	5406–5426
pFAS 200			
	200B	5′-GTG ACG GTG TCG CGT AGA GA	6311–6331
	67T	5′-GAC TGC ATG CTT GGC ATG GAG	4849–4869
pFAS 67		*Sph*I	
	67B	5′-CAT CCA GCA GGA TCC CAT GGA	5435–5455
		*Bam*HI	
	13T	5′-atc tga att cCC GGT TCC CCG AGG GA	2539–2560
pFAS 13		*Eco*RI	
	13B	5′-ACT CCA TGC CCA GCA TGC AGC T	4849–4870
		*Sph*I	
	27T	5′-cat atc tag agc tag CAG ACA GAG AAG AGC C**AT G**G	66–85
pFAS 27		*Xba*I *Nhe*I	
	27B	5′-ata tga att cGC CTA GCT TCA TGA ACT GCA	674–693
		*Eco*RI *Bsp*HI	
	M-13 Universal	5′-cgc cag ggt ttt ccc agt cac gac	–
	M-13 Reverse	5′-agc gga taa caa ttt cac aca gga	–

Elmer (Norwalk, CT, U.S.A.). DEAE-cellulose (DE-53) was purchased from Whatman (Hillsboro, OR, U.S.A.). Rabbit anti-(rat fatty acid synthase) antibodies were prepared as described previously [10] and purified by affinity chromatography on Sepharose–antigen columns, essentially as recommended by Pharmacia LKB. Alkaline phosphatase-coupled goat anti-rabbit IgG antibodies were purchased from BioRad (Hercules, CA, U.S.A.).

Amplification, cloning and sequencing of cDNA fragments

Each of the cDNA fragments, except FAS 54, was amplified by PCR using appropriate pairs of primers (Table 1). The reaction mixtures, in a final volume of 100 μl, contained 20 mM Tris/HCl (pH 8.8 at 25 °C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 100 μg/ml BSA, 0.1 % Triton X-100, 2.5–3.5 mM $MgSO_4$, 0.5 mM of each dNTP, 0.25 μM of each primer, 25–50 ng of template DNA and 2 units of Vent DNA polymerase. The reaction temperature was cycled 20 times, 1 min at 94 °C, 1 min at 52 °C and 1–2 min at 72 °C. Amplified DNA fragments were analysed by agarose-gel electrophoresis, restricted and cloned into suitably digested pUCBM20 vector, using competent DH5α cells [11]. Typically, five to ten ampicillin-resistant colonies were screened for the presence of correct insert, by PCR amplification using whole cells, Taq DNA polymerase and M13 universal and reverse primers (Table 1), and the insert size was determined by agarose-gel electrophoresis [12]. In most cases 80–90 % of ampicillin-resistant clones had the correct insert. Plasmid DNA from at least two positive clones for each fragment was purified and its authenticity confirmed by dideoxynucleotide sequencing. The plasmid pFAS 54, which proved refractory to amplification by PCR was amplified by conventional cloning in *E. coli*, the region of interest was sequenced and then excised with the restriction enzymes *Bsp*HI and *Eco*RI.

To facilitate subcloning of amplified fragments in the pUC vector, some of the primers were constructed so as to encode new restriction sites that would not be present in the final full-length cDNA: *Kpn*I in primer 5T, *Eco*RI in 27B, *Xba*I in 1B and 54T. Restriction sites for *Xba*I–*Nhe*I were introduced via primer 27T and for *Nhe*I–*Not*I via primer 5B so as to be positioned ultimately in the full-length cDNA just upstream and downstream of the start and stop codons respectively.

Engineering the full-length fatty acid synthase cDNA

Construction of a full-length cDNA involved the stepwise ligation of fragments derived from overlapping cDNAs and cloning of the fused DNA fragments into pUCBM20 (Figure 1), a procedure that allowed us to verify proper splicing at each intermediate step in the construction. This strategy has provided us with a series of partial fatty acid synthase cDNA clones that can be used for the introduction of specific mutations and assembly of full-length mutant fatty acid synthase. Authenticity of the spliced DNA was confirmed both by restriction endonuclease mapping and by PCR amplification of contiguous DNA regions contributed in part by both splicing partners. Beginning with FAS 5, the four fragments FAS 1, 200, 67 and 13 were sequentially ligated to generate a 5 kb cDNA extending from the *Eco*RI site to the 3′-non-coding region. FAS 27 and 54 were spliced together to generate the remaining part of the fatty acid synthase cDNA, extending from the 5′-non-coding sequence to the *Eco*RI site. Finally, the two fragments of the fatty acid synthase cDNA were

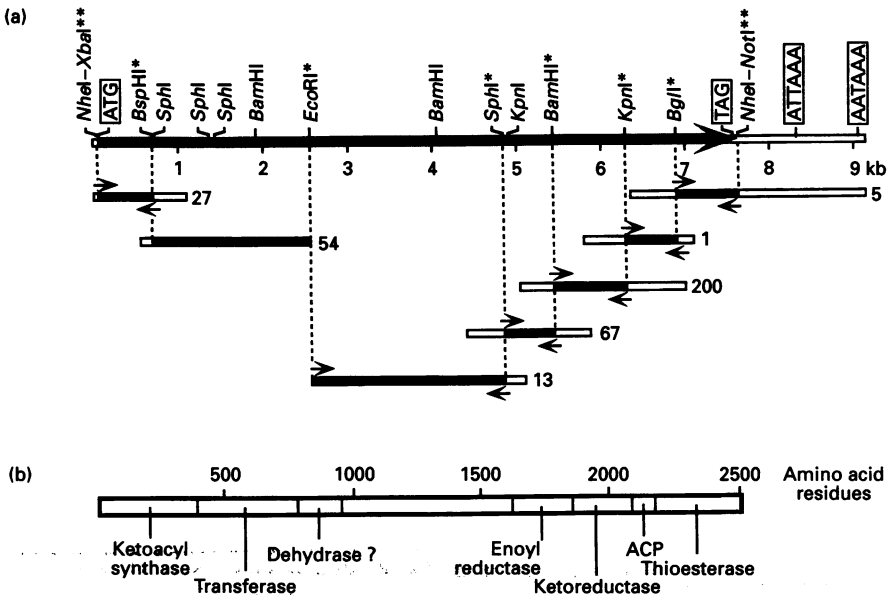

Figure 1 Strategy for engineering of a full-length cDNA clone encoding the seven domains of the multifunctional fatty acid synthase

(**a**) The map shows the location of the seven overlapping cDNA clones used to construct the 7.5 kb full-length cDNA. The regions of each clone that were amplified and ligated together are shaded black and the locations of the PCR priming sites are indicated by arrows (arrow lengths not to scale). The primer sequences are identified in Table 1 according to the fatty acid synthase template cDNA clone number followed by the letter T or B (top or bottom). Restriction sites used in the ligations are marked with a single asterisk. Newly introduced restriction sites that were used subsequently for introduction of the cDNA into the baculoviral transfer vectors are marked with double asterisks. Where restriction sites used in the construction are not unique in the entire sequence, the locations of the other sites are also shown. (**b**) Domain map of the encoded multifunctional polypeptide. The location of the dehydrase domain is considered tentative at this time [13].

excised from their respective plasmids with *Xba*I–*Eco*R1 and *Eco*RI–*Not*I, ligated together and cloned into pUCBM20 to give the full-length rat fatty acid synthase cDNA construct, pFAS 202. Purified pFAS 202 DNA was analysed by restriction mapping.

Expression of recombinant fatty acid synthase

The full-length FAS 202 fragment (approx. 7.5 kb) was excised from pFAS 202 with *Xba*I and *Not*I, or with *Nhe*I alone, purified by agarose-gel electrophoresis and cloned into appropriately digested transfer vectors pVL1393 and pBlueBac, resulting in the recombinant transfer vectors pFAS 203 and pFAS 204 respectively. The procedures adopted for the use of the baculovirus and transfer vectors were essentially as described in detail by O'Reilly et al. [14].

Purified recombinant transfer vector DNA, together with linearized viral DNA was used for co-transfection of *Sf*9 cells, using the calcium-phosphate precipitation method [15]. Recombinant viral clones were purified twice using the end-point dilution procedure. Infected cells were recognized under the microscope by virtue of their unique morphology and then screened for recombinant fatty acid synthase expression by SDS/PAGE, using 7.5% polyacrylamide gels [16] and by Western analysis [17] employing monospecific rabbit anti-(rat fatty acid synthase) antibodies and alkaline phosphatase-coupled goat anti-rabbit IgG antibodies as the primary and secondary antibodies respectively. Immediately before electrophoresis, protein samples were rapidly denatured by dilution into sample buffer that included 7M urea and had been preheated on a boiling-water bath; samples were heated for a further 3 min.

Purification of recombinant fatty acid synthase

Purified recombinant viral stocks, at the third passage, were used at a multiplicity of infection of 3–5, to infect 100–200 ml of *Sf*9 cell cultures (approx. 1×10^6 cells/ml), and the suspension cultures stirred for 48 h at 27 °C. Cells were harvested by centrifugation at 1000 *g* for 5 min, washed once with 10 vol. of PBS, pH 7.5 (137 mM NaCl, 1.47 mM KH_2PO_4, 8.17 mM $Na_2HPO_4,7H_2O$, 2.7 mM KCl) at 0–4 °C, suspended in 5 vol. of lysis buffer (50 mM potassium phosphate buffer, pH 7, 1 mM dithiothreitol, 1 mM EDTA, 0.25 M sucrose), and disrupted by 10–15 strokes in a tight-fitting Potter–Elvehjem homogenizer. The lysate was centrifuged for 35 min at 100000 *g*, and the supernatant (cytosol) was decanted and stored at −80 °C. Purification of the recombinant fatty acid synthase was achieved by direct application of the thawed cytosol to a preparative high-performance anion-exchange column (TSK-DEAE-5PW, 2.15 cm × 15.0 cm, 10 μm, void volume 32 ml) maintained at 20 °C and eluted at a flow rate of 4 ml/min; 2 ml fractions were collected. Unbound and weakly bound proteins were eluted with 100% buffer A (20 mM potassium phosphate buffer, pH 7, 1 mM dithiothreitol, 1 mM EDTA), then the proportion of buffer B (250 mM potassium phosphate buffer, pH 7, 1 mM dithiothreitol, 1 mM EDTA) was increased linearly to 25% over a 15 min period and finally to 100% over a 90 min period. For convenience, fatty acid synthase is routinely located in the column eluate using the keroreductase assay with *trans*-1-decalone as substrate [18] as this compound is considerably less expensive than acetyl-CoA and malonyl-CoA, the substrates for the overall fatty acid synthase reaction, and the sensitivity of the assay is 5-fold higher than that of the overall reaction. Fractions containing recombinant fatty acid synthase are pooled, the potassium phosphate concentration adjusted to approx. 50 mM by dilution, and the proteins rechromatographed on the same TSK-DEAE column. Fractions containing the recombinant fatty acid synthase are again pooled and the enzyme concentrated as follows. Samples are diluted 2-fold with 1 mM dithio-

threitol/1 mM EDTA and applied with a syringe to a small cartridge of DE-53 contained in a 'compact reaction column'. Fatty acid synthase is then eluted from the cartridge by centrifugation in a Microfuge of a small volume of buffer (0.25 M potassium phosphate buffer, pH 7, 1 mM dithiothreitol, 1 mM EDTA) through the cartridge at approx. 400 *g* (2000 rev./min) for 30 s. The volume of the cartridge (typically 1 ml for protein loads in the 1–5 mg range) and the eluting buffer (typically 1 ml) can be adjusted according to the amount of protein in the sample and the final concentration desired. We have used the procedure to concentrate samples of fatty acid synthase over 100-fold with essentially quantitative recovery, the entire procedure taking less than 1 h. For long-term storage of the fatty acid synthase, glycerol is added to 10% (v/v) and the samples frozen at −70 °C.

Enzyme assays

Ketoacyl synthase activity was assessed radiochemically by the condensation–$^{14}CO_2$ exchange reaction [19]. Transferase activity was assayed, using malonyl-CoA as donor and pantetheine as acceptor, by determining spectrophotometrically the free CoA released in a coupled ATP citrate-lyase–malate dehydrogenase reaction [20]. Ketoreductase was assayed spectrophotometrically at 340 nm: assay systems contained 0.1 M potassium phosphate buffer (pH 7), 0.15 mM NADPH, enzyme and either 10 mM *trans*-1-decalone or 0.1 mM acetoacetyl-CoA substrate. Dehydrase activity was assayed spectrophotometrically at 270 nm using *S*-DL-β-hydroxybutyryl *N*-acetylcysteamine as substrate [21]. Enoyl reductase activity was assayed spectrophotometrically at 340 nm essentially as described earlier [22]: assay systems contained 0.1 M potassium phosphate buffer (pH 7), 0.15 mM NADPH, 0.375 mM crotonoyl-CoA, 20 μM CoA and enzyme. Thioesterase activity was assessed radiochemically by extracting and assaying the [^{14}C]palmitic acid formed from [1-^{14}C]palmitoyl-CoA during an incubation of 3 min [23]: assay systems contained in a final volume of 0.1 ml, 25 mM potassium phosphate buffer (pH 8), 20 μg/ml BSA, 10 μM [1-^{14}C]palmitoyl-CoA (20 nCi) and enzyme. Assay of overall fatty acid synthase activity was performed spectrophotometrically as described previously [24]. All enzyme activities were assayed at 37 °C except the transferase, which was assayed at 20 °C. Activity units indicate nmol of substrate used/min. All assays were made at at least two different protein concentrations with the appropriate enzyme and substrate blanks included.

RESULTS AND DISCUSSION

Construction of a full-length fatty acid synthase cDNA

Our strategy for engineering a cDNA construct encoding the 7515 nt open reading frame of the rat fatty acid synthase involved ligating together seven fragments derived from cDNA clones that collectively spanned the entire coding sequence (Figure 1). To facilitate the eventual insertion of the fatty acid synthase cDNA into the baculoviral transfer vectors, during amplification of the constituent fragments we introduced new restriction sites not found elsewhere in the fatty acid synthase DNA, *Xba*I and *Nhe*I at the 5′ end of the fragment encoding the N-terminal region of the fatty acid synthase, *Nhe*I and *Not*I at the 3′ end of the fragment encoding the C-terminal region. By sequentially ligating the seven fragments, we were able to use five convenient splicing sites (*Bgl*I, *Kpn*I, *Bam*HI, *Sph*I and *Bsp*HI) even though they did not represent unique restriction sites in the final construct. The cDNA was assembled in two sections flanking either side of the single *Eco*RI site, one beginning with the C-terminal coding end of the cDNA and consisting of five ligated fragments (FAS 5+FAS 1+FAS 200+FAS 67+FAS 13), the other consisting of two ligated fragments (FAS 27+FAS 54), which constituted the N-terminal coding end of the cDNA. Finally, the two sections of the fatty acid synthase cDNA were spliced together using the unique *Eco*RI restriction site and cloned into pUCBM20 to give pFAS 202. The final product was analysed by restriction mapping with five enzymes; each digestion generated DNA fragments of the predicted size (Figure 2). Sequencing of the seven constituent DNA fragments revealed that only one mutation (C7602G) was inadvertently introduced during the amplification process; fortunately it was inconsequential as it was located in the 3′ non-coding region. These findings indicated that we had engineered a cDNA encoding a 2505-residue polypeptide that matched perfectly the amino acid sequence of the naturally occurring protein.

Expression of recombinant rat fatty acid synthase in *Sf*9 cells

We initially designed the full-length fatty acid synthase cDNA with restriction sites at the 5′ and 3′ ends that would allow insertion into either pVL1393 or pBlueBac and anticipated screening for recombinant virus by identifying infected cells that did not express the polyhedrin protein (occ$^-$), a procedure that, using pBlueBac, is facilitated by identifying infected cells that appear blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). However, the subsequent availability of a linearized form of the baculovirus DNA greatly increased the efficiency of recombination and obviated the necessity for occ$^-$ screening. Thus screening for recombinant virus was ultimately performed by Western immunoblotting, and essentially identical results were obtained regardless of which transfer vector had been used.

The full-length 7.5 kb fatty-acid-synthase-coding fragment constructed in the pUC vector was translocated into the baculovirus transfer vector, either pVL1393 or pBlueBac, and used

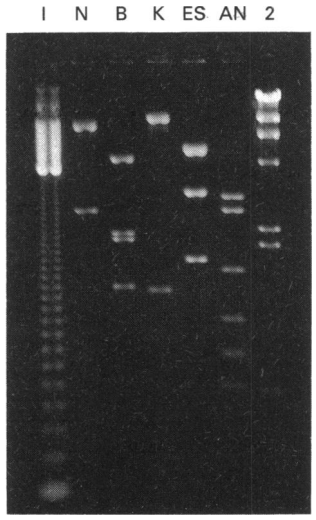

Figure 2 Restriction analysis of pFAS 2505, the plasmid encoding the entire fatty acid synthase

DNA was restricted with *Nhe*I (N), *Bam*HI (B), *Kpn*I (K), *Eco*RI–*Sal*I (ES) or *Apa*I–*Nhe*I (AN) and fractionated by agarose-gel electrophoresis. The DNA size markers are, 123 bp ladder (1) and *Hin*dIII digest of λ phage DNA (2).

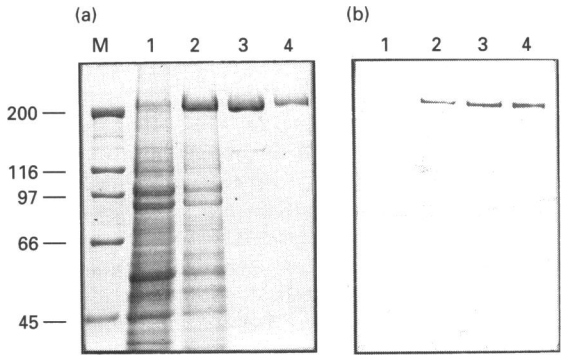

Figure 3 Identification of recombinant fatty acid synthase by SDS/PAGE and Western blotting

(**a**) Coomassie Blue-stained gel. Lane M, molecular-mass standards (values in kDa); lane 1, cytosol from uninfected *Sf*9 cells (8–10 μg of protein); lane 2, cytosol from cells expressing recombinant fatty acid synthase (8–10 μg); lane 3, partially purified recombinant fatty acid synthase (approx. 2 μg); and lane 4, purified rat liver fatty acid synthase (approx. 2 μg). (**b**) Western immunoblot. Lanes 1–4 contained 10-fold less protein than the corresponding samples in (**a**).

together with linearized baculovirus to infect insect *Sf*9 cells. In three different transfection experiments about 20–40% of the clones analysed were found to be positive for fatty acid synthase expression. Recombinant viral clones were purified and used for large-scale expression of the recombinant protein. Cytosols prepared from *Sf*9 cells infected with the recombinant baculovirus contained greatly elevated fatty acid synthase and *trans*-1-decalone reductase activity, typically 20-fold higher than in uninfected cells. Analysis of the cytosols prepared from infected *Sf*9 cells by SDS/PAGE and Western blotting confirmed the presence of a high-molecular-mass protein recognized by the anti-(rat fatty acid synthase) antibodies (Figure 3). Maximal expression of the immunoreactive protein was observed approx. 48 h after infection using recombinant viral constructs derived from either the pVL1393 or pBlueBac vectors. Although fatty acid synthase could be detected by enzyme assay in cytosols from uninfected cells, the insect host enzyme was not recognized by the anti-(rat fatty acid synthase) antibodies.

Purification and properties of recombinant fatty acid synthase

When a cytosol prepared from uninfected *Sf*9 cells was applied to a high-performance ion-exchange column, a single zone of *trans*-1-decalone reductase activity was eluted at approx. 90 mM potassium phosphate (30% buffer B). This zone also contained fatty acid synthase activity and a major protein of approx. 270 kDa which did not react with anti-(rat fatty acid synthase) antibodies (details not shown). Clearly this zone contained the insect host fatty acid synthase. When cytosols prepared from *Sf*9 cells that had been infected with the recombinant baculovirus were applied to the high-performance ion-exchange column (Figure 4a), three zones containing *trans*-1-decalone reductase activity were eluted at approx. 90 mM, 130 mM and 160 mM potassium phosphate (30%, 50% and 62% buffer B respectively). The first zone, eluted at approx. 30% buffer B, typically accounts for less than 5% of the total *trans*-1-decalone reductase activity eluted from the column and contains fatty acid synthase that is not recognized by anti-(rat fatty acid synthase) antibodies; this fatty acid synthase represents the insect host enzyme. The other two zones, which account for the bulk of *trans*-1-decalone reductase activity, both contain immunoreactive fatty acid synthase. When fractions from the two zones of activity were separately concentrated in buffer (0.25 M potassium phosphate

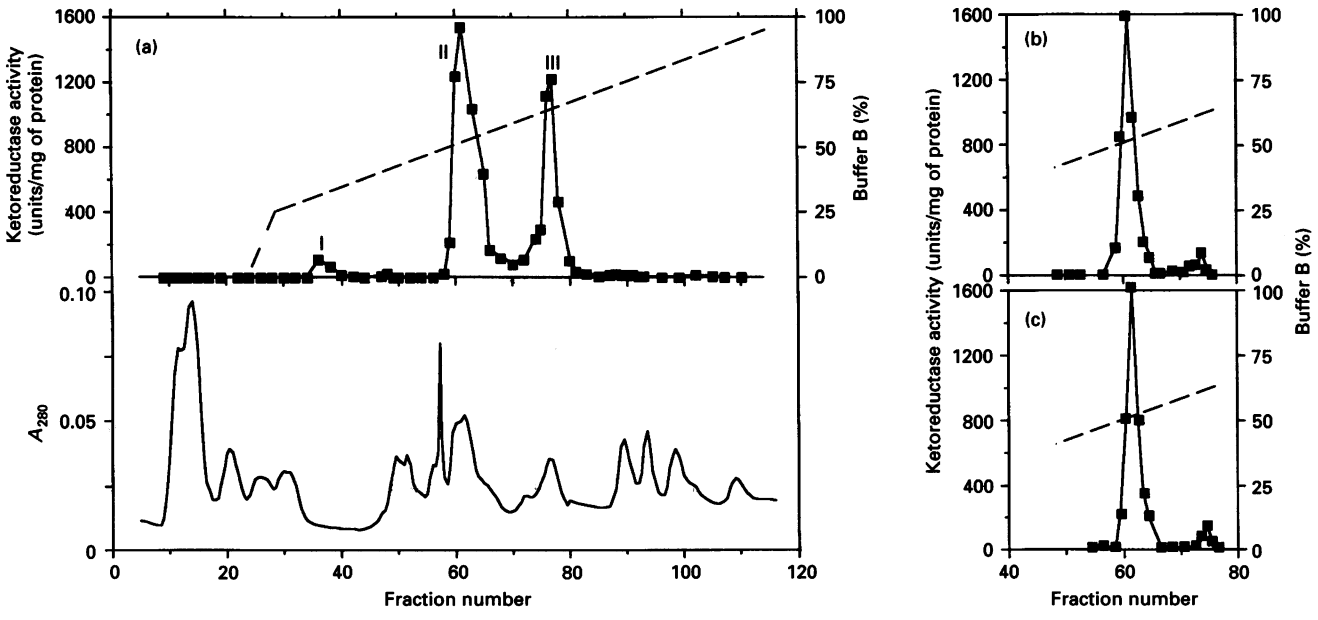

Figure 4 Purification of recombinant fatty acid synthase by high-performance anion-exchange chromatography

(**a**) Chromatogram obtained from application of 10 ml of *Sf9* cytosol to the TSK-DEAE column. Fractions from *trans*-1-decalone reductase zones II and III were separately pooled, concentrated and re-applied to the same column. (**b**) Chromatogram obtained by rechromatography of proteins from zone II. (**c**) Chromatogram obtained by rechromatography of proteins from zone III. The values for concentration of buffer B refer to the buffer entering, rather than leaving, the column at the designated times. Details are provided in the Materials and methods section. ■, Ketoreductase activity; ——, A_{280}; – – – –, [buffer B].

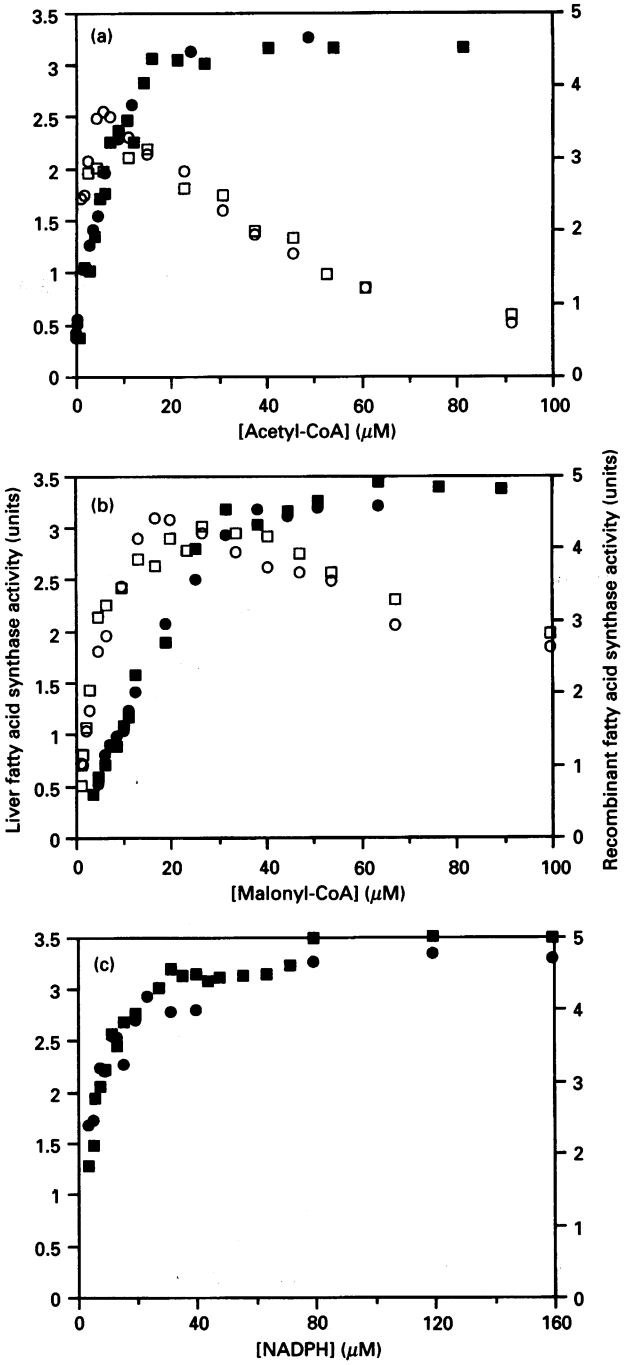

Figure 5 Comparison of the kinetics of the liver and recombinant forms of the rat fatty acid synthase

All reaction mixtures contained 1.5 μg of fatty acid synthase and 0.1 M potassium phosphate buffer (pH 6.6) in a final volume of 250 μl. Reactions were initiated by the addition of malonyl-CoA. (**a**) Plots of initial velocity versus acetyl-CoA concentration for liver (○, ●) and recombinant (□, ■) enzyme at 152 μM NADPH and either 52 μM (●, ■) or 10 μM (○, □) malonyl CoA. (**b**) Plots of initial velocity versus malonyl-CoA concentration for liver (○, ●) and recombinant (□, ■) fatty acid synthase at 152 μM NADPH and either 54 μM (●, ■) or 10 μM (○, □) acetyl CoA. (**c**) Plots of initial velocity versus NADPH concentration for liver (●) and recombinant enzyme (■) at 54 μM acetyl-CoA and 52 μM malonyl-CoA.

(pH 7), 1 mM dithiothreitol, 1 mM EDTA), stored overnight at room temperature, then quickly diluted and re-applied to the column, most of the *trans*-1-decalone reductase activity was eluted at approx. 130 mM potassium phosphate (50% buffer B) in both cases (Figures 4b and 4c). The specific activities of the two enzyme preparations obtained by rechromatography of zones II and III were identical (in the fatty acid synthase and *trans*-1-decalone reductase assays) and both were eluted as the dimer when applied to a high-performance gel-filtration column. As rat fatty acid synthase readily dissociates into its component subunits at low protein concentrations in solutions of low ionic strength and reassociates in solutions of high ionic strength, the activity eluted during the first ion-exchange chromatography step in zones II and III could be attributed to dimer and monomer respectively. The natural rat fatty acid synthase is also eluted as two zones under these conditions [24], so there is nothing unusual in the behaviour of the recombinant enzyme in this regard. Careful identification of the fatty acid synthase present in both zones was undertaken to verify with certainty that both were attributable to the recombinant protein. The two zones of *trans*-1-decalone reductase activity are now routinely pooled after the first ion-exchange chromatography step.

The specific activities of purified recombinant fatty acid synthase preparations are consistently slightly higher than our best preparations obtained from rat liver: otherwise the kinetic properties of the liver and recombinant enzymes, assessed with the three substrates acetyl-CoA, malonyl-CoA and NADPH, are indistinguishable (Figure 5). Both enzymes indicate the typical inhibition by acetyl-CoA and malonyl-CoA due to their competition for a common loading site on the transferase domain [20,25,26]. Similarly when partial activities of one of the recombinant preparations were compared with those of the high-specific-activity rat liver enzyme, again no significant differences were observed (Table 2). The specific activities of animal fatty acid synthases vary considerably, from laboratory to laboratory and from preparation to preparation within the same laboratory. These differences have been attributed, at least in part, to variable proportions of apo- and holo-forms of the enzyme in the preparations. It is very reassuring therefore to find that preparations of recombinant fatty acid synthase can be obtained from the *Sf*9 cells with consistently high specific activity. Apparently in the insect cell host, each of the seven functional domains of the recombinant fatty acid synthase fold correctly, and residue Ser-2151 within the acyl-carrier-protein domain is efficiently phosphopantetheinylated. To our knowledge this is the first

Table 2 Comparison of the activities of natural and recombinant fatty acid synthase

The wide range in the magnitude of the partial activities of the fatty acid synthase reflects the fact that several of the assays can only be conveniently carried out using model substrate of varying effectiveness. Activities were measured on single preparations of liver and recombinant enzymes with a precision of approx. ±6%.

	Activity (units/μg of protein)	
Enzyme	Rat liver	Recombinant
Transferase	17	16
Ketoacyl synthase	3.3×10^{-3}	2.9×10^{-3}
Ketoreductase *trans*-1-decalone	11	10
Acetoacetyl-CoA	1.5	1.6
Dehydrase	4.6×10^{-3}	4.4×10^{-3}
Enoyl reductase	1.7	1.9
Thioesterase	0.74	0.72
Fatty acid synthase	2.0	2.5

demonstration of correct post-translational phosphopantetheinylation of a recombinant protein performed by *Sf*9 cells and the first successful production of a large multifunctional polypeptide as a catalytically active recombinant protein. That the recombinant fatty acid synthase can be expressed to very high levels and can readily be separated from the small amount of host fatty acid synthase found in these cells indicates that the *Sf*9/baculovirus host/vector system is an excellent one for generating mutants that can be used to explore details of the mechanism of action of this multifunctional protein.

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