Expression of a Mammalian Fatty Acid-binding Protein in Escherichia coli*

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Rat liver fatty acid-binding protein (FABP) is a 14,200-Da polypeptide that is abundantly represented in the cytosol of liver and small intestinal epithelial cells. It may play an important role in the intracellular transport and metabolism of fatty acids. We have recently determined its sequence from an analysis of cloned cDNAs (Gordon, J. I., Alpers, D. H., Ockner, R. K., and Strauss, A. W. (1983) J. Biol. Chem. 258, 3356–3363). With the availability of full-length cloned FABP cDNAs, genetic manipulation of the coding region introduced into an appropriate expression vector could provide a powerful tool for analyzing the molecular details of fatty acyl-protein interaction. We, therefore, have inserted the coding sequence of rat FABP cDNA into a prokaryotic expression vector. Synthetic oligodeoxynucleotides were used to “properly” space the initiator methionine residue downstream from a Shine-Dalgarno ribosome-binding site. FABP transcription in the chimeric plasmid was under the direction of the leftward promoter of phage λ. Promoter activity in turn was regulated by a thermolabile repressor specified by a defective λ prophage contained in the host chromosome. When grown at permissive temperatures, rat liver FABP represented approximately 0.8% of radiolabeled soluble bacterial proteins. Edman degradation of FABP purified from Escherichia coli lysates indicated that it was intact. However, its NH₂ terminus was not acetylated as it is in mammalian tissues. A frameshift mutation was introduced in vitro into the coding region of FABP cDNA. The effects of this on fatty acyl-protein interaction were examined using a solid phase (oleic acid-Sepharose) binding assay. Amino acid sequence rearrangements in the COOH-terminal region of rat liver FABP had a significant effect on its ability to bind fatty acids as well as on its stability in bacteria.

Rat liver FABP3 represents approximately 5% of the cytosolic protein mass in hepatocytes (1). It is a small 127-amino acid-long polypeptide that is identical to Z protein (2, 3).

FABP is also found in the small intestinal lining cell (enterocyte). In fact, FABP mRNA is the most abundant translatable RNA sequence in gut epithelium (4). The precise physiologic role played by FABP in gut and liver is unknown. Accumulation of the protein in liver is correlated closely with the rate of hepatic fatty acid utilization and uptake (1, 5–8). The activities of several enzymes that metabolize fatty acids are altered by FABP, at least in vitro (9–11). These observations suggest that liver FABP may be involved in the transport and/or metabolism of fatty acids. Takahashi et al. have proposed that this protein could be an intracellular homologue of serum albumin (12).

This protein has remarkable sequence homologies with another intestinal fatty acyl-binding protein (13) as well as cellular retinoic acid-binding protein (14) and the P2 protein found in peripheral nerve myelin (15). Sixty per cent of long-chain fatty acids found in the cytosolic fraction of hepatocytes are bound to FABP (1). Organic solvent extraction of 1 mol of FABP purified from liver cytosol yields 1 mol of long-chain (C₁₆–C₂₀) saturated and unsaturated fatty acids (12). Its ligand binding site has not been defined. Secondary structure predictions using Chou-Fasman rules indicate that FABP contains a long α-helical domain in the middle portion of the molecule (2). Analysis of fatty acyl binding to albumin has indicated that its primary long-chain fatty acid-binding site is a trough-like structure formed by α helices (16).

Because of its small size, liver FABP may offer a good model system for studying the molecular details of fatty acyl-protein interaction. For this reason, we have initiated a series of investigations designed to study the relationship of FABP structure to function by site-directed mutagenesis of cloned cDNA introduced into a prokaryotic expression vector. This paper describes efficient expression of rat liver FABP in E. coli and outlines an approach that can be used to assay the effects of structural changes on its fatty acid-binding properties.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**

The Escherichia coli expression vectors pLc28 (17) and pLc245 (18) were generously provided by Dr. Erik Remaut (Ghent, Belgium). All pL plasmid constructions were maintained in E. coli strain K12ΔH1Δtrp (19) which is Sm⁸, lacZam, Δbio–wrbB, trpEΔ2 (pNam7Nam5cJ857ΔH1). All experiments involving expression of FABP in bacteria were performed in accordance with the National Institutes of Health guidelines for recombinant DNA research.

**Oligodeoxynucleotide Synthesis**

Oligodeoxynucleotides were manually synthesized on a solid-phase support using the phosphite triester method (20). Diisopropyl derivatives of phosphoramidites (Biosearch) were employed. 5'-OH groups that were not condensed with activated phosphoramidite after each
base addition were capped with acetate using acetic anhydride in a reaction catalyzed by dimethylaminopropidine (21). Following deprotection and cleavage of the 5'-unsaturated linkage to the Vydac support, an aliquot of the completed oligodeoxynucleotide was labeled at its 5' end with T4 polynucleotide kinase and [32P]yrATP and analyzed on polyacrylamide gels containing 7 M urea (22). Because autoradiography of the labeled product showed minimal contamination with failure sequences, the oligodeoxynucleotides were used without further purification.

**DNA Sequencing**

The deoxynucleotide chain termination method of Sanger (23) was used to determine the DNA sequence of restriction fragments subcloned into the M13 phage vector mp18. The reaction products were resolved on thin (0.4 mm) buffer gradient gels (24) which were subsequently dried and subjected to autoradiography.

**Construction of FABP cDNA Containing Plasmid Expression Vectors**

**pJBL 1**

Four pg of plasmid pL-28 were digested with BamHII. The 3'-recognized ends of the linear vector DNA were filled in by incubation with the Klenow fragment of DNA polymerase I (25). Dephosphorylation of the blunt-ended vector with calf intestine alkaline phosphatase was performed exactly as previously described (26). A 676-bp DNA fragment which specifies a portion of the 5'-nontranslated region as well as the entire coding and 3'-nontranslated domain of rat liver FABP mRNA was recovered from plasmid pJG418 by digestion with PvuIII and HaeIII. 300 ng of the purified restriction fragment were ligated to 4 pg of blunt-ended dephosphorylated vector DNA in a 10-1 reaction mixture containing 30 mM Tris, pH 7.5, 8 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 mM spermidine, 10 mM rATP, and 400 units of T4 DNA ligase (New England Biolabs). Following an overnight incubation at 10 °C, the mixture was used to transform E. coli K12HilAtrp. Plasmid DNAs were prepared (26) from several transformants and subsequently digested with Rsi. One plasmid containing a 856-bp Rsa fragment indicative of a properly oriented cDNA insert was designated pJBL 1.

**pJBL 2**

Preparation of pLc245 for Oligonucleotide Linker Addition—2.5 pg of pLc245 DNA were digested with SufI. Mung bean nuclease (P-L Biochemicals) was then used to remove the 5' protruding nucleotides from the SufI restriction terminus. Mung bean nuclease digestion of the gel showed in a 20-μl reaction containing 390 nm NaCl, 50 mm Na acetate, pH 5.0, 1 mm ZnCl2, 2.5 pg of Sall-cut pLc245 DNA, and 200 units/ml of the nuclease. Incubation at 30 °C for 30 min generated a blunt end immediately after the initiator methionine codon of the MS2 replicase gene fragment present in pLc245. One pg of the fragment was ligated to 1 pg of Sall-cut pLc245 DNA ligated to 1 pmol (1 pg) of SalI-cut mung bean nuclease-digested pLc245 DNA, and 200 units/ml of the nuclease. Incubation at 30 °C for 30 min followed by slow cooling to room temperature. The annealing reaction was applied to a Sephadex G-25 column pre-equilibrated with 300 mM ammonium bicarbonate. The excluded fractions containing the synthetic linker were pooled and lyophilized. Approximately 200 pmol of the annealed hemisphosphorylated linker was ligated to 1 pmol (1 μg) of Sall-cut mung bean nuclease-digested pLc245 DNA. Free linkers were separated from linkers that had been ligated to vector DNA by Bio-Gel G1.5 mm column chromatography using 2.5 mM Tris, pH 7.4, 10 mm NaCl, and 1 mM EDTA as buffer.

**Ligation of FABP cDNA to pLc245 DNA Containing Oligodeoxynucleotide Linker**—A 575-bp fragment was isolated from pJBL 1 after digestion with Msl. This restriction fragment contains DNA sequences specifying amino acids 5 through 127 of FABP (see Fig. 1). One μg of the fragment was ligated to 1 μg of Sall-cut pLc245 containing the linker and the reaction mixture used to transform E. coli K12HilAtrp. Transformants harboring plasmids with FABP cDNA inserts were identified by colony hybridization using the 575-bp Rsa fragment, which had been labeled by nick translation, as a probe. Plasmid DNAs isolated from 17 probe-positive transformants were digested with Rsi. Ten plasmids with a 790-bp Rsa fragment characteristic of a properly oriented FABP DNA insert were tested for their ability to direct synthesis of FABP in E. coli (see below). One of the four expression-positive plasmids was designated pJBL 2.

**pJBL 3**

Ten pg of pJBL 2 DNA were digested for 1 h at 37 °C with 20 units of EcoRI in the presence of 75 μg/ml of ethidium bromide (28). Linear plasmid DNA (i.e. cut at only one of the two EcoRI sites) was purified by electrophoresis on agarose gels. "Fill-in" of the 5' protruding EcoRI termini was performed using a Klenow fragment of DNA polymerase.

Two μg of the linear DNA were circularized by incubation with T4 DNA ligase, and the reaction was used to transform E. coli K12HilAtrp. Plasmid DNAs extracted from several transformants were analyzed by digestion with HindIII and EcoRI. One plasmid, designated pJBL 3, contained a 790-bp EcoRI-HindIII fragment, indicating loss of the EcoRI site within the FABP-coding sequence.

**Labeling of Bacterial Proteins**

Cells were grown at 28 °C in LB broth (25) containing ampicillin (100 μg/ml) to an Ab of 0.3. Cells were then washed once in either methionine- or lysine-free media (Difco), resuspended in the same medium (in a volume equal to that of the original culture), and grown for an additional 1 h at 28 °C. The culture was divided in half; one aliquot was grown at 42 °C for 75 min while the other was maintained at 28 °C. Samples of each culture were then labeled for 10 min with 100 μCi/ml of [35S]methionine (1200 Ci/mmol) or [4,5-3H]lysine (100 Ci/mmol). Cells were subsequently collected by low-speed centrifugation and lysed in one of two ways. A detergent lysis of cells was performed by resuspending the cell pellet in a volume of lysis buffer equal to one-fourth that in which they were labeled. The lysis buffer contained 50 mM Tris, pH 7.5, 10 mM EDTA, 10 μg/ml lysozyme, 10 μg/ml DNase, 10 μg/ml RNase, 1 mM phenylmethylsulfonyl fluoride, 50 mM Na acetate, pH 5.0, 1 mM ZnCl2, 2.5 pg of SalI-cut pLc245 DNA, and 200 units/ml of the nuclease. Incubation at 30 °C for 30 min followed by slow cooling to room temperature. The annealing reaction was terminated by heating to 90 °C for 30 s. Incorporation of [%]methionine was terminated by the addition of unlabeled methionine and chloroamphenicol (75 pg/ml). Control experiments were performed by resuspending the cell pellet in a volume of lysis buffer equal to one-fourth that in which they were labeled. The lysis buffer contained 50 mM Tris, pH 8.0, 10% sucrose, and the two serine protease inhibitors. Following 3 cycles of freezing and thawing, NaCl was added to 20 mm, spermidine HCl to 3 mm, diethiothreitol to 10 mm, and lysozyme to 200 pg/ml. Lysates were subjected to 45 min on ice and then for 30 min at 30 °C. 100,000 × g supernatants of each lysate prepared in an Airfuge and stored at −86 °C.

**Protein Stability Measurements**

Cells were grown to midlog phase at 28 °C in LB broth containing ampicillin. Cells were transferred to methionine-free broth as described above and grown at the nonpermissive temperature (28 °C) for an additional 1 h. Aliquots were then incubated for 5 min at the permissive temperature (42 °C). [35S]Methionine (1200 Ci/mmol, 100 μCi/ml of culture) was rapidly added and the cells labeled at 42 °C for 30 s. Incorporation of [35S]methionine was terminated by the addition of unlabeled methionine (300 μg/ml) and chloramphenicol (75 μg/ml) and centrifuged at 100,000 × g. Control experiments demonstrated virtually no incorporation of [35S]methionine into protein after the addition of unlabeled methionine and chloramphenicol (data not shown). Aliquots were withdrawn from 42 °C cultures at various times after the pulse labeling and quickly frozen in liquid N2. Samples were then deproteinized and fractionated by electrophoresis through discontinuous polyacrylamide slab gels containing SDS (29).

**Solid-phase Oleic Acid Binding**

Preparation of Oleylumirwalkylumirw Sepharose—Oleic acid was coupled to α-aminohexylamine Sepharose 4B using a water-soluble carbodiimide. The coupling was performed exactly as described by
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**Fig. 1.** Construction of rat liver FABP expression vectors using pL plasmids. Details are provided under “Materials and Methods.”
Peters et al. (30) except that free amino groups present after oleic acid coupling were acetylated with acetic acid during a second carbodiimide reaction (31). Fifty pCi of [3H]oleic acid (specific activity 5.7 Ci/mmol) were included in the 5.4-ml coupling reaction along with the unlabeled sodium soap of oleic acid (60 mM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (17 mg/ml), and 1.8 ml of \( \text{oleic acid} \) incorporated into the matrix, we estimated that 8 pmol of oleic acid were bound per ml of gel. A control matrix was also prepared in which acetate was coupled to aminoexohexylamine Sepharose 4B. This matrix was used to assay for the presence of nonspecific binding of proteins to the Sepharose support or to the acetylated spaces.

Solid-phase Binding Assay—Aliquots of detergent-free [35S]methionine-labeled lysates were delipidated with diisopropyl ether (32). Typically a 200-ml aliquot (1.5 \( \times \) 10\(^6\) dpm from 3 \( \times \) 10\(^6\) cells) of delipidated lysate was adsorbed to 100 ml (packed volume) of oleylaminohexyl Sepharose or "mock" matrix. The mixture was incubated for 1 h at room temperature in Eppendorf tubes. The matrix was subsequently washed with phosphate-buffered saline, and the bound proteins eluted with a solution containing 75 mM sodium phosphate, pH 6.8, and 50% ethanol.

Purification of FABP—FABP was purified from cell lysates or from oleylaminohexyl Sepharose 4B eluants by immunoprecipitation with monospecific rat liver FABP antibody (32). After addition of SDS to a final concentration of 1%, samples were boiled for 5 min and diluted 15-fold into a solution containing 300 mM NaCl, 50 mM Tris, pH 7.5, 10 mM EDTA, 5 mM methionine or lysine, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, and 10 mg/ml of antiserum. Antigen-antibody complexes were recovered by incubation with formalin-fixed Staphylococcus aureus cells (Bethesda Research Laboratories), extensively washed, dissociated, and fractionated on SDS-polyacrylamide gels (33). FABP was eluted from gel slices as previously described (33).

The amount of antisera used was sufficient to quantitatively bind all the radiolabeled FABP from small-scale lysates and eluants. This was established by performing a second immunoprecipitation on the supernatant obtained after spinning down antigen-antibody-Staph A complexes.

Aliquots of the eluted radiolabeled protein were subjected to automated sequential Edman degradation using a Beckman 890C sequenator and 0.33 M Quadrol program (13). Alternatively, they were counted directly (33) and the results expressed as a fraction of total bacterial protein synthesis (determined by trichloroacetic acid precipitation).

RESULTS

Strategy for Expression of FABP cDNA—Expression of rat liver fatty acid-binding protein in a prokaryotic host offers the opportunity to manipulate easily the ligands presented to this polypeptide, to study the effects of the protein on lipid metabolism in a variety of auxotrophs and to employ host strains that have reduced proteolytic activities when site-directed mutagenesis of the protein affects its stability. We chose the pl vectors constructed by Remaut et al. (17, 18) for FABP expression. These plasmid vectors contain the very strong leftward promoter of phage \( \lambda \) which can be controlled by a thermolabile repressor (c \( \beta \)57) specified by a defective \( \lambda \) prophage present in the chromosome of the host strain. In cells grown at 28 °C, the repressor effectively blocks transcription initiated at the PL promoter (17). Cells can be grown to moderate densities at this nonpermissive temperature and, because the c \( \beta \)57 repressor is unstable at 42 °C, shifted to the permissive temperature for efficient transcription of sequences downstream from the promoter. The system has obvious advantages if the foreign protein has a deleterious effect on bacterial growth.

Construction of an FABP Expression Vector—The schemes used for construction of chimeric FABP cDNA/pl vectors are detailed in Fig. 1. One of the key points of the construction was to "properly" space a Shine-Dalgarno ribosome-binding site upstream from the start site of FABP mRNA translation. The 5' nontranslated region of FABP mRNA represented in our original full-length rat cDNA clone (pJG418) has a purine-rich region (AGGA), similar to a Shine-Dalgarno sequence, 15 nucleotides upstream from the initiation codon. This spacing was maintained during construction of our first chimeric vector, pJBL 1. A 670-bp PvuII-HindIII fragment from pJG418 was cloned, in the proper orientation, into a "filled-in" BamHI site of plasmid pHPlc26 (Fig. 1). This restriction fragment contains DNA sequences representing the complete coding sequence and 26 nucleotides of the 5' nontranslated region of rat liver FABP mRNA. We tested the ability of plasmid pJBL 1 to direct synthesis of FABP in E. coli strain K12DH1txp. No immunoreactive FABP was detectable in bacterial lysates prepared after labeling cells with [35S]methionine during growth at permissive temperatures (data not shown).

One explanation for the lack of detectable FABP was that the distance between the Shine-Dalgarno sequence and initiation codon in the FABP mRNA transcript generated from the plasmid was too great for efficient translation by E. coli (34). Therefore, synthetic oligodeoxynucleotides were used to reduce the spacing between the Shine-Dalgarno sequence and initiation codon to 6 nucleotides (see Fig. 1 and "Materials and Methods"). This construction (pJBL 2) was designed to preserve the nucleotide sequence of the MS2 polymerase gene fragment proximal to the FABP translational start site. The net result would be a ribosome-binding site (derived from the MS2 polymerase gene) 6 nucleotides upstream from the initiator AUG of the FABP transcript. There were two reasons why we anticipated that pJBL 2 would generate an efficiently translated FABP mRNA. First, this 6-nucleotide distance was identical to the spacing found in the MS2 polymerase gene transcript which is efficiently translated in E. coli (35). Second, at least one other eukaryotic sequence has been expressed when similarly placed in this vector (18).

Cells containing pJBL 2 were labeled during growth at 42 °C, lysates prepared, and subjected to electrophoresis through denaturing SDS-polyacrylamide gels. A 14,000-Da polypeptide was detected easily among the [35S]methionine-labeled proteins (Fig. 2A, lane 2). It was not found in E. coli containing vector (pPLc245) alone even when grown under identical conditions (compares lanes 2 and 3 of Fig. 2A). This 14,000-Da protein was immunoprecipitated by monospecific rat liver FABP antiserum (Fig. 2B, lane 2). It co-migrated with the primary translation product of FABP mRNA recovered from wheat germ lysates that had been programmed with intestinal epithelial RNA (Fig. 2B, lane 1). No immunoreactive protein was detected in lysates prepared from a pJBL 2-containing host grown at 28 °C (Fig. 2B, lane 3) or from pPLc245-containing hosts grown at either 42 °C (lane 6) or 28 °C (data not shown).

The 14,000-Da protein appeared to be confined to the bacterial cytosol. No protein was detected in the 12,000 \( \times \) g pellet obtained after lysis of pJBL 2-containing cells, whether that lysate occurred in the presence or absence of detergent. Moreover, no immunoreactive radiolabeled sequences were detected in the media during growth at 42 °C (data not shown).

To prove that this polypeptide was FABP, we purified the [35S]methionine or [3H]lysine-labeled 14,000-Da protein from cell lysates and subjected it to automated sequential Edman degradation (Fig. 3A). Peaks of methionine were obtained at cycles 1, 19, and 22, in agreement with the known protein sequence of rat liver FABP. Similarly, radioactive peaks were obtained at cycles 6, 20, 31, 33, and 36 when the [3H]lysine-labeled polypeptide was sequenced. The distribution of lysine residues exactly matched that in liver FABP (Fig. 3A). The
Expression of rat liver FABP in E. coli. Bacteria were pulse labeled with [35S]methionine for 10 min, lysed with detergent, and either analyzed directly by SDS-polyacrylamide gel electrophoresis or immunoprecipitated with monospecific antiserum raised against rat liver FABP. A, lane 1, lysate prepared from bacteria containing pJBL 2 after growth at 28°C (nonpermissive temperature); lane 2, lysate prepared from bacteria containing pJBL 2 after growth at the permissive temperature (42°C); lane 3, lysate prepared from bacteria containing vector alone (pPLc245), grown at 42°C; lane 4, lysate prepared after the bacteria containing pJBL 3 (mutated FABP cDNA) were grown at 42°C. B, lane 1, primary translation product of rat liver FABP mRNA purified from wheat germ lysates programmed with intestinal epithelial RNA (2); lane 2, immunoprecipitation of [35S]methionine-labeled lysate prepared from bacteria containing pJBL 2 grown at 42°C; lane 3, immunoprecipitation of pJBL 2 lysates obtained from bacteria grown at the nonpermissive temperature; lane 4, immunoprecipitation of pJBL 3 lysates prepared from bacteria grown at the permissive temperature; lane 5, immunoprecipitation of pJBL 3 lysates generated after growth at 28°C; lane 6, immunoprecipitation of pPLc245 containing bacterial lysates prepared after growth at 42°C. Molecular weight markers used include ovalbumin (43,000), α-chymotrypsinogen (25,700), lysozyme (14,300), and cytochrome c (12,400).

very high yield of 35S at cycle 1 (55% of the input disintegrations per min/residue) indicated that the protein recovered from bacterial lysates was not blocked. This contrasts with FABP isolated from rat liver or from eukaryotic cell-free translation systems. FABP isolated from these sources is NH₂-terminally acetylated (2, 3).

Finally, we determined the DNA sequence of pJBL 2 in the vicinity of the FABP translation start (Fig. 3B). This region includes the sequences contributed by the synthetic oligonucleotide. The predicted DNA sequence was verified, and the derived protein sequence matched the known NH₂-terminal sequence of rat liver FABP.

Expression of a Mutant Rat Liver FABP Generated by in Vitro Site-directed Mutagenesis—One of the potential values of this expression system is its ability to provide mutant rat liver FABPs that can offer insights about structure-function relationships in the wild-type protein. A simple structural change in the COOH terminus of FABP can be introduced by a frame-shift mutation. The nucleotides specifying residues 103 and 104 of FABP form an EcoRI site. No other EcoRI sites are present in the full-length FABP double-stranded cDNA. pPLc245 contains a unique RI site (Fig. 1). When we digested pJBL 2, which contains wild-type FABP cDNA, with this restriction endonuclease in the presence of ethidium bromide, we were able to generate a heterogeneous population of DNA molecules (some cut uniquely at the vector RI site)

FIG. 3. Sequencing of pJBL2 and recombinant rat liver FABP. A, NH₂-terminal sequence analysis of radiolabeled rat liver FABP purified from bacterial lysates. Rat liver FABP, labeled with either [35S]methionine or [3H]lysine was purified from lysates of E. coli containing pJBL 2 using techniques described under “Materials and Methods” section and subjected to automated sequential Edman degradation. The NH₂-terminal sequence of the “authentic” cytosolic protein is shown. The curves drawn are normalized to the first radioactive peak and represent the predicted recovery of counts if the repetitive yield were 96%. 13,700 dpm of [35S]methionine-labeled and 10,400 dpm of [3H]lysine-labeled FABP were introduced into the sequenator cup. B, alignment of a Shine-Dalgarno sequence with the start site of FABP translation using synthetic oligodeoxynucleotides. A 150-bp EcoRI-ResI fragment isolated from pJBL 2 was subcloned into mp 8 and sequenced using the dideoxychain termination technique of Sanger et al. (23).
and some cut within the cloned double-stranded cDNA. The EcoRI sites were filled in with a Klenow fragment of E. coli DNA polymerase, the DNA fragments religated and used to transform E. coli K12ΔH11trp. We identified several recombinants that had retained their vector RI site but had lost their insert's restriction site. One of these was called pJBL 3. The effect of the in vitro fill-in reaction was to generate a frameshift in the FABP-coding sequence due to insertion of 4 nucleotides at the former EcoRI site. This insertion was verified in pJBL 3 by determining the DNA sequence across the mutation site (Fig. 4). The wild-type and predicted mutant COOH-terminal sequences are quite different. The frameshift results in deletion of the COOH-terminal 10 amino acids of FABP (residues 118-127) and alters every amino acid from position 104 to 117 (Fig. 5A). These substitutions change the predicted secondary structure of the mutant protein's COOH terminus from β sheet to random coil (Fig. 5B). The frameshift also alters the Kyte-Doolittle hydropathy index (37) of the COOH terminus. Fig. 5C shows the increase in COOH-terminal hydrophilicity of the mutant compared to the wild-type protein.

We predicted from the mutant DNA sequence that a single truncated FABP would accumulate in bacteria grown at permissive temperatures. Bacteria harboring the mutant plasmid DNA were pulse labeled with [35S]methionine, lysates prepared, and incubated with liver FABP antiserum. Two different sized polypeptides were immunoprecipitated (Fig. 2B, lane 4). The largest was approximately 1000 Da smaller than the wild-type protein, exactly as we had predicted. The other was approximately 2000 Da smaller. Edman degradation of each protein labeled with [35S]methionine and [3H]lysine showed that they had identical NH₂-terminal amino acid sequences (data not shown) and that these were the same as those found in wild-type FABP. Our conclusion was that the larger immunoreactive protein represented intact "mutant" FABP. The origins of the smaller polypeptide were less clear. Its COOH terminus was not defined. Considering the location and type of mutation introduced into the FABP mRNA transcript, it probably was derived from proteolytic cleavage of mutant FABP by bacterial proteases, although we could not rule out premature termination of transcription or translation.

Relative Abundance and Stability of the Expressed FABPs—The results shown in Fig. 24 indicated that the concentration of the mutant FABP in E. coli lysates was lower than that of the wild-type protein (compare lanes 2 and 4). We determined the relative abundance of the mutant and wild-type polypeptides by measuring the incorporation of [35S]methionine into immunoreactive FABP during a 10-min pulse and expressing the results as a percentage of total radiolabeled protein accumulation in bacterial lysates. The results are shown in Table I. During the labeling period, the accumulation of the wild-type protein in detergent-free delipidated lysates was 13-fold greater than the accumulation of the intact mutant protein (0.86 versus 0.067%). It was 19-fold more abundant than the truncated 12,000-Da NH₂-terminal peptide. These abundance estimates varied somewhat when different conditions were used for lysate preparation. The calculations would be influenced by factors which affect the solubility of FABP relative to total E. coli proteins. The presence or absence of detergent or bound ligands may produce such changes in solubility.

One of the reasons for the reduced accumulation of the mutant polypeptides could be protein instability. To assess protein stability we performed a pulse-labeling experiment with bacteria containing either wild-type or mutated FABP DNA. The intensity of the wild-type FABP band remained virtually unchanged for 90 min after termination of labeling (Fig. 6, lanes 3–5). On the other hand, the mutant 13,000-Da FABP band became undetectable within 5–7 min (lanes 7–12). We concluded that part of the difference between the accumulation of the recombinant FABPs reflects these differences in their intracellular stabilities. Expression of both wild-type and mutant FABP during growth at permissive temperatures had no effect on E. coli growth kinetics when compared to strains containing vector DNA alone (data not shown).

Fatty Acid-binding Properties of Wild-type and Mutant FABPs—The effect of the frameshift on FABP function was tested using a rapid solid-phase fatty acid-binding assay. We prepared detergent-free delipidated extracts of bacteria that had been labeled for 10 min at 42 °C with [35S]methionine. The bacteria contained either vector alone (pPLc245), wild-type FABP DNA (pJBL 2), or mutated FABP DNA (pJBL 3). The radiolabeled polypeptides were applied to oleylaminohexylamino Sepharose 4B. After extensive washing with phosphate-buffered saline the bound peptides were eluted. The results are shown in Fig. 7. Several bacterial proteins bound to the matrix including a conspicuous 21,000-Da polypeptide. Wild-type FABP was also prominently represented among the bound peptides (compare lanes 1–3 with 7 and 8). We quantitated the efficiency of binding of wild-type FABP to oleylaminohexylamino Sepharose by immunoprecipitating aliquots of the lysate applied to the matrix and aliquots of the bound and eluted proteins with monospecific antibody. Immunoprecipitates were fractionated on SDS-polyacrylamide gels and radiolabeled FABP eluted from gel slices. In this way we determined that a minimum of 51% of

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**Fig. 4. Confirmation of the frameshift mutation in pJBL 3.** A 485-bp EcoRI-AcI fragment containing the mutation was purified from pJBL 3, subcloned into mp 8 and sequenced as described under "Materials and Methods."
FIG. 5. Effects of the frameshift mutation on FABP structure. A, comparison of the COOH-terminal amino acid sequences of wild-type and mutant FABPs. Residue numbers are listed. B, secondary structure analyses using Chou-Fasman rules (36). The prediction for the entire wild-type protein is shown. Only that region of the mutant polypeptide which has a different predicted secondary structure is included. α, α helix; β, β bend; γ, γ sheet; empty spaces or flat lines are shown in areas where secondary structure predictions could not be definitely made. C, Kyte-Doolittle hydration potential plots (37) of wild-type (—) and mutant (---) proteins. The COOH terminus of the mutant 12,000-Da protein is hydrophilic compared to the COOH-terminal domain of wild-type FABP.

TABLE I
Accumulation of rat liver FABP in E. coli during pulse-labeling experiments

| Plasmid               | Method of preparing bacterial lysate | Total radiolabeled lysate proteins (%) |
|-----------------------|-------------------------------------|---------------------------------------|
| pJBL 2 (wild-type FABP) | Detergent                          | 0.42*                                  |
|                       | Detergent free                      | 0.57*                                  |
|                       | Detergent free, delipidated         | 0.86*                                  |
| pJBL 3 (frameshift mutation) | Detergent                          | 0.061*                                |
|                       | Detergent free                      | 0.059*                                 |
|                       | Detergent free, delipidated         | 0.067*                                 |

*a 14 kDa.
*b 13 kDa.
*c 12 kDa.

the wild-type FABP had bound to the matrix (Table II). To establish whether the wild-type FABP had specifically bound to oleic acid or nonspecifically bound to the acetylhexylamino blocking groups present on the support, portions of the same lysate were applied to an acetylaminohexylamino Sepharose matrix. No protein binding to the control matrix was detectable (see lane 9 of Fig. 7). Furthermore, no protein was eluted from the control matrix even after “harsh” stripping with 50 mM NaOH, 50% ethanol (data not shown). We, therefore, concluded that the 14,000-Da wild-type protein had bound to the oleylaminohexylamino Sepharose matrix by interacting with the long alkyl chain of the coupled oleic acid. The percent of binding measured represents a minimal estimate given the number of steps involved in the assay (adsorption and elution from the matrix, immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis and elution). Similar binding of FABP to the matrix was observed over a 5-fold range of fatty acid to protein concentration.

Lysates obtained from strains harboring the mutant plasmid (pJBL 3) also were applied to the oleic acid-containing matrix. Fifteen per cent of the 13,000-Da mutant FABP and 12% of the 12,000-Da peptide bound to and were eluted from the matrix (Table II). No additional protein was recovered from the column after NaOH-ethanol stripping. The change

FIG. 6. Comparison of the stabilities of wild-type and mutant FABPs in E. coli. Bacteria containing either pPLc 245, pJBL 2, or pJBL 3 were labeled during growth at 42 °C for 30 s with [35S]methionine. Following the pulse, labeling was terminated by adding cold amino acid plus chloramphenicol. Aliquots were withdrawn at various times after pulse labeling. Lysates were prepared and analyzed by electrophoresis through a 15% polyacrylamide gel containing 0.1% SDS. An autoradiograph of the gel is shown. Lane 1, lysate prepared from pPLc245-containing bacteria 1 min after termination of labeling; lane 2, purified wild-type FABP; lane 6, purified mutant FABP.
The matrix was washed, and bound peptides were analyzed on a 15% polyacrylamide gel containing SDS. An autoradiogram is shown. Lane 1, immunoprecipitation of lysate prepared from bacteria containing pJBL 2 with liver FABP antiserum; lane 2, total pJBL 2 lysate applied to oleylaminohexyl Sepharose; lane 3, bound and eluted polypeptides from pJBL 2 lysate; lane 4, proteins immunoprecipitated from pJBL 3 lysate with the monospecific antiserum; lane 5, total pJBL 3 lysate; lane 6, oleylaminohexyl Sepharose bound and eluted proteins from pJBL 3 lysate; lane 7, lysate from bacteria containing vector DNA (pPLc245); lane 8, proteins from pPLc245 lysates that were bound and eluted from the oleic acid-containing matrix; lane 9, result obtained after applying pJBL 2 lysate to a control matrix consisting of acetylatedaminohexyl Sepharose; lane 10, results obtained with pJBL 3 lysate and the control matrix.

| Table II | Relative binding efficiencies of wild-type and mutant FABPs to oleylaminohexyl Sepharose |
|----------|------------------------------------------------------------------------------------------|
| FABP     | Oleylaminohexyl Sepharose | Acetylaminohexyl Sepharose |
| Wild type (14 kDa) | 57 | ND* |
| Mutant   | 13 kDa | 15.1 | ND |
|          | 12 kDa | 11.6 | ND |

*Delipidated detergent-free lysates were prepared from E. coli containing pJBL 2 (wild type) or pJBL 3 (mutant) after labeling with [35S]methionine for 10 min at 42 °C. Immunoreactive FABP was purified from aliquots of these lysates and from affinity matrix eluants as described under "Materials and Methods."

**Discussion**

We have described efficient synthesis of a small mammalian fatty acid-binding protein by E. coli. The rationale for undertaking these experiments was based on several factors. First, there is physiologic evidence that this protein plays an important role in the intracellular transport and metabolism of fatty acids (1, 5–12). The molecular details of fatty acyl-protein interactions are to a large extent undefined. Because of its small size, FABP may represent a less complex model for analyzing the structural basis of this interaction than, for instance, albumin. Second, in vitro site-directed mutagenesis of cloned cDNAs introduced into expression vectors represents a powerful method for analyzing protein-ligand interactions. Finally, manipulation of lipid metabolism in prokaryotes is easier to achieve than in eukaryotes. Wild type strains of bacteria contain a population of fatty acids smaller (C12–C18) and more homogeneous than that encountered in higher organisms. E. coli synthesize saturated, cis-unsaturated, and β-hydroxy fatty acids from acetate. Synthesis proceeds along a common pathway to β-hydroxydecanoyl thioester which can be elongated to give saturated fatty acids and β-hydroxymyristic acid. Alternatively, β-hydroxydecanoylthioester can be converted to cis-5-decenoylthioester and the chain extended to cis-monoenoic acids. Temperature-sensitive mutants in unsaturated fatty acid biosynthesis (e.g. fabA and fabB) are available (38). Their growth can be supported by fatty acids possessing unusual structural features. Hence, the potential exists to control the delivery of defined ligands to this protein in vivo.

Insertion of FABP cDNA into the plasmids of Remaut et al. (17, 18) permitted us to regulate FABP gene expression by means of a temperature-sensitive trans-acting repressor. Although we did not systematically study several of the variables known to affect synthesis of mammalian proteins in E. coli, several observations were noteworthy. We found that the efficiency of expression was affected by the distance between the Shine-Dalgarno ribosome-binding site and the translational start in the plasmid's FABP mRNA transcript. A spacing of 15 nucleotides did not result in any detectable FABP accumulation. When we maintained the 6-nucleotide spacing found in an efficiently expressed prokaryotic gene (MS 2 replicase), high levels of FABP synthesis could be induced. The presumed translational start for FABP RNA in our construct (pJBL 2) occurs 246 nucleotides upstream from the codon specifying the initiator methionine (17, 18, 39). However, secondary structure analysis of these 246 nucleotides failed to predict any stable hairpin structures which might interfere with efficient initiation of translation (40). Specifically, neither the Shine-Dalgarno sequence nor the translational start point occurred in the stem of a (predicted) stable hairpin (data not shown). Finally, computer analyses of codon usage in our FABP cDNA showed no clustering of codons that are associated with inefficient translation in prokaryotes (2, 41, 42).

Many eukaryotic proteins expressed in E. coli have short intracellular half-lives (43). Presumably these polypeptides are recognized as abnormal sequences by one or more of the E. coli proteases which have been described (44). It appears that following translation, rat liver FABP is remarkably stable in E. coli (Fig. 6). This stability was documented by pulse-labeling experiments in a strain of bacteria that was not deficient in protease activity (e.g. it was not deg" (45)). Edman degradation of FABP purified from E. coli indicated that it differed in one respect from the polypeptide purified from rat liver cytosol; its NH2 terminus was not blocked. Like most eukaryotic cytoplasmic proteins, FABP possesses an acetylated NH2-terminal amino acid. E. coli are apparently not competent to perform this modification on FABP. Although the precise function of NH2-terminal blockade of cytosolic
proteins is not known in eukaryotes, in this prokaryotic strain, absence of an NH2-terminal blocking group (formyl or acetyl) was not associated with loss of the initiator methionine residue.

To illustrate an approach that can be used to analyze fatty acyl-protein interactions with the expression system, we performed a simple frameshift mutation. This altered the COOH-terminal sequence of FABP, decreasing the hydrophobicity and changing its predicted secondary structure (Fig. 5). The sequence alteration had several effects. First, the mutant protein was relatively unstable, having a disappearance rate that was at least 10-fold greater than wild-type FABP. Second, the mutant protein bound less efficiently to oleic acid-Sepharose. If bound fatty acids “protect” FABP from bacterial proteases, it is conceivable that these two functional changes are interrelated. Takahashi et al. have recently reported that fatty acids confer stability on Z protein when they are bound, resulting in remarkable resistance to trypsin (3).

Although the change in COOH-terminal sequence adversely affected the ability of the protein to bind to an immobilized long-chain fatty acid, we cannot conclude that the COOH terminus itself forms all or part of the binding site. It is possible that a change in conformation results from the mutation which perturbs an NH2-terminal binding site. An unexpected finding was the accumulation of an additional 12,000-Da NH2-terminal FABP peptide in E. coli containing the frameshift mutant (pJB Bl). This peptide may have arisen from proteolytic processing of the “intact” mutant protein. It exhibited less competent binding to the oleylaminohexyl-Sepharose column than wild-type FABP. Although this raises the possibility that the NH2-terminal domain of FABP does not have all the structural features necessary for optimal ligand binding, much more subtle mutations will have to be introduced into FABP before such a conclusion can be made. Rational design and interpretation of future in vitro mutagenesis studies will require information about the tertiary structure of rat liver FABP. These studies are currently in progress using FABP purified from bacterial lysates.

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