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Liver fatty acid binding protein is the mitosis-associated polypeptide target of a carcinogen in rat hepatocytes

(Z protein/chemical carcinogenesis/*N*-2-fluorenylacetamide/"2-acetylaminofluorene")

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ABSTRACT **Hepatocytes in normal rat liver were found previously to contain a cytoplasmic 14,000-dalton polypeptide (p14) that is associated with mitosis and is the principal early covalent target of activated metabolites of the carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene). The level of immunohistochemically detected p14 was low when growth activity of hepatocytes was low, was markedly elevated during mitosis in normal and regenerating livers, but was very high throughout interphase during proliferation of hyperplastic and malignant hepatocytes induced in rat liver by a carcinogen (*N*-2-fluorenylacetamide or 3′-methyl-4-dimethylaminoazobenzene). We report here that p14 is the liver fatty acid binding protein. The nucleotide sequence of p14 cDNA clones, isolated by screening a rat liver cDNA library in bacteriophage λgt11 using p14 antiserum, was completely identical to part of the sequence reported for liver fatty acid binding protein. Furthermore, the two proteins shared the following properties: size of mRNA, amino acid composition, molecular size according to $NaDodSO_4$ gel electrophoresis, and electrophoretic mobilities in a Triton X-100/acetic acid/urea gel. Their pI values overlapped in 2-dimensional isoelectric focusing/$NaDodSO_4$ gel electrophoresis and showed the same response to delipidation. Either polypeptide reacted with and blocked the antiserum raised against the other polypeptide. The two polypeptides bound oleic acid similarly. Finally, identical elevations of cytoplasmic immunostain were detected specifically in mitotic hepatocytes with either antiserum. The collected findings are suggestive that liver fatty acid binding protein may carry ligands that promote hepatocyte division and may transport certain activated chemical carcinogens.**

A 14,000-dalton cytoplasmic polypeptide (p14) that is the principal early covalent target of activated metabolites of the carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene; FAA) (1–3) appears to be closely coupled to normal mitosis and carcinogen-induced proliferation of hepatocytes in rat liver. In hepatocytes of normal and regenerating livers, the cytoplasmic level of immunodetected p14 is markedly elevated only during mitosis as compared to that in adjacent interphase hepatocytes (4–6). In contrast, the concentration of discernible p14 is greatly increased throughout the cell cycle in proliferating hepatocytes of hyperplastic foci and hepatocellular carcinomas in livers of rats fed either of the carcinogens FAA or 3′-methyl-4-dimethylaminoazobenzene (4, 7). Moreover, the level of immunostained p14 is very low in the surrounding liver parenchyma (4, 7), where responsiveness to mitotic stimuli is inhibited by the carcinogens (reviewed in ref. 8). Considered altogether, the level of detectable p14 appears to be positively associated with the growth activity of rat hepatocytes.

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Rat liver fatty acid binding protein (L-FABP), also termed Z protein, is an abundant protein that is thought to be involved in the uptake, intracellular transport, compartmentalization, and metabolism of free fatty acids and their acyl-CoA esters (reviewed in refs. 9–11). L-FABP belongs to a family of eight polypeptides: fatty acid binding proteins (liver, heart, and intestinal), cellular retinol binding protein, cellular retinol binding protein II, cellular retinoic acid binding protein, P2 protein of peripheral nerve myelin, and the adipocyte-specific 422 protein (reviewed in ref. 12). In these homologous proteins, the amino-terminal sequence corresponding to the first exon is conserved (13). Other proteins have been reported to be closely similar to L-FABP or in the same family—namely, sterol carrier protein (14), minor aminoazo dye binding protein A (15), phosphatidylcholine exchange protein (16), band C protein (17), and heme binding protein (18).

The present study identifies p14 as L-FABP and, thus, L-FABP as the principal early covalent target of activated metabolites of FAA in rat liver. The conclusion that derives from this finding is that the level of L-FABP is positively associated with the growth activity of rat hepatocytes.

MATERIALS AND METHODS

Rats, Liver Cytosols, and 2S Proteins. Liver cytosols of normal adult Fischer 344 CDF rats (Charles River Breeding Laboratories) were prepared and subjected to gel filtration in order to isolate 2S proteins (1–3).

p14, L-FABP, and Antisera. Previously isolated p14 from normal rat liver was homogeneous in molecular size and charge (3, 5). Rabbit antiserum raised against p14 was immunologically specific in reaction with normal rat liver cytosol (3, 7). In immunoaffinity purifications of p14 for use in the present study, rabbit anti-p14 immunoglobulins were coupled to Affi-Gel 10 (Bio-Rad). Nonspecifically bound 2S proteins were removed by successively washing the column with (*i*) 25 mM Tris·HCl (pH 7.5) containing 0.25% Triton X-100 and 0.5 M NaCl, (*ii*) 25 mM Tris·HCl, pH 7.5/0.15 M NaCl, (*iii*) 25 mM Tris·HCl, pH 7.5/2 M NaCl/10 mM EDTA, and (*iv*) water. Specifically bound p14 was eluted with 0.05% redistilled formic acid (pH 2.2) and lyophilized to near dryness. Purified L-FABP from normal rat liver and its rabbit antiserum were kindly provided by Nathan M. Bass and Robert K. Ockner, University of California (San Francisco) (19).

Gel Electrophoresis and Isoelectric Focusing. Proteins were resolved by $NaDodSO_4$ electrophoresis in 15% polyacrylamide slab gels, stained with Coomassie brilliant blue R-250, and

Abbreviations: p14, 14,000-dalton polypeptide; L-FABP, liver fatty acid binding protein; FAA, *N*-2-fluorenylacetamide (2-acetylaminofluorene).

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                                          1          10          20
5'                                        gcc acc atg aac ttc tcc ggc aag tac
CTG TTG GTG GCA GCT GGG AAA GGA AAC CTC ATT GCC ACC ATG AAC TTC TCC GGC AAG TAC   60
                                                  ***

  30          40          50          60          70          80
caa gtg cag agc caa gag aac ttt gag ccc ttc atg aag gcg atg ggt ctg cct gag gac
CAA GTG CAG AGC CAA GAG AAC TTT GAG CCC TTC ATG AAG GCG ATG GGT CTG CCT GAG GAC  120

  90          100         110         120         130         140
ctc atc cag aaa ggg aag gac atc aag ggg gtg tca gaa atc gtg cat gaa ggg aag aaa
CTC ATC CAG AAA GGG AAG GAC ATC AAG GGG GTG TCA GAA ATC GTG CAT GAA GGG AAG AAA  180

  150         160         170         180         190         200
gtc aaa ctc acc atc acc tat ggg tcc aag gtg atc cac aat gag ttc acc ttg ggg gag
GTC AAA CTC ACC ATC ACC TAT GGG TCC AAG GTG ATC CAC AAT GAG TTC ACC TTG GGG GAG  240

  210         220         230         240         250         260
gag tgc gaa ctg gag acc atg act ggg gaa aag gtc aag gca gtg gtt aag atg gag ggt
GAG TGC GAA CTG GAG ACC ATG ACT GGG GAA AAG GTC AAG GCA GTG GTT AAG ATG GAG GGT  300

  270         280         290         300         310
gac aat aaa atg gtg aca act ttc aaa ggc ata aag tcc gtg act  EcoR1
GAC AAT AAA ATG GTG ACA ACT TTC AAA GGC ATA AAG TCC GTG ACT GAA TTC AAT GGA GAC  360

ACA ATC ACC AAT ACC ATG ACA CTG GGT GAC ATC GTC TAC AAG AGA GTC AGC AAG AGA ATT  420
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FIG. 1. Comparison of the p14 and L-FABP cDNA sequences of rat liver. The 312-base sequence indicated in lowercase letters is that of the p14 cDNA (this report); the 420-base sequence shown in uppercase letters is from the 491-base sequence reported for L-FABP cDNA (cf. ref. 30). The overlapping sequences show 100% homology. A redundant ambiguity of T and C was encountered at base 12 of the p14 cDNA. ***, Reported translation initiation and termination codons of L-FABP (13).

compared with matched immunoblots (3, 5). Proteins were similarly analyzed after electrophoresis in 12% polyacrylamide slab gels containing 6 mM Triton X-100, 5% acetic acid, and 7.5 M urea (Triton/acetic acid/urea gels) (20).

Proteins were isoelectric focused in tube gels containing 5% polyacrylamide, 0.2% methylene bisacrylamide, and Ampholines (0.8% pH 3.5–10/0.2% pH 5–8/0.2% pH 9–11; LKB), with or without 6.25 M urea (deionized). In experiments involving prior delipidation, proteins were incubated at 37°C for 15 min and immediately denatured in 0.5% $NaDodSO_4$. Then by using two-dimensional gel electrophoresis (21), proteins were isoelectric focused, with or without urea as above (no incubation at 37°C and without $NaDodSO_4$). Following migration in a $NaDodSO_4$-containing slab gel (above), the proteins were silver stained or immunoblotted.

Sequence Determination of Cloned p14 cDNA. A donated cDNA expression library in bacteriophage λgt11 was previously generated from liver poly(A)$^+$ RNA of normal adult Fischer rats (22). Recombinant phage clones were screened with p14 antiserum (3, 5, 23, 24). Clones containing p14 cDNA were subcloned in plasmid vector pUC9 and then in bacteriophage M13mp18 (24).

Blot-Hybridization Assay of Rat Liver Polysomal RNA. Liver polysomes from normal CDF rats yielded RNA, which was electrophoresed in agarose/formaldehyde gels (25), blotted onto nitrocellulose filter paper, and hybridized to a nick-translated p14 cDNA probe.

Immunological Assays of p14 and L-FABP. In most immunoblots, p14 and L-FABP were subjected to $NaDodSO_4$ gel electrophoresis and were electrotransferred onto nitrocellulose filter paper (0.1 μm) (3, 5). In other experiments, proteins in Triton/acetic acid/urea gels were transferred similarly, except for the addition of 0.1% $NaDodSO_4$. Paper blots were incubated with p14 antiserum or L-FABP antiserum and processed with ^{125}I-labeled protein A for autoradiography (3, 5).

To test competitive immunoreactivities, 8 μl of L-FABP antiserum was mixed with 65 μg of p14 or L-FABP, diluted to 1.0 ml with Tris-buffered saline, and shaken for 2.5 hr at room temperature.

Fatty Acid Binding Assay of p14 and L-FABP. p14, L-FABP, sterol carrier protein 2 (26), and ribonuclease A (2 μg of each) were separately incubated with 18 μM [1-^{14}C]oleic acid (56 mCi/mmol; 1 Ci = 37 GBq; New England Nuclear) in 0.1 ml of 10 mM potassium phosphate buffer (pH 7.4) for 10 min at 37°C as reported (19). Each mixture was resolved using a new column (3.5 × 0.5 cm) of Sephadex G-10 equilibrated in 50 mM potassium phosphate buffer (pH 7.4) at 4°C.

Immunohistochemistry of p14 and L-FABP. Histological sections of 2-day regenerating rat livers were stained employing L-FABP antiserum, without or with previous absorption by p14 or L-FABP, developed with peroxidase-antiperoxidase complex (DAKO; Accurate Chemicals, Westbury, NY) and 3,3′-diaminobenzidine, and counterstained with hematoxylin (4–7).

RESULTS

Sequence Homology of Cloned p14 and L-FABP cDNAs. An initial plaque screening of 10^5 independent recombinants with p14 antiserum identified 13 putative p14 clones. To examine their diversity, one of the cloned cDNAs was used as a probe against the *Eco*RI-digested DNAs of the other clones. The probe hybridized to all of the digested clones, indicative of their close relatedness or identity (data not shown). The insert DNA of one of the clones was incorporated into M13mp18 and was sequenced (27). As revealed by comparison with the GenBank Database‡ using the algorithm of Wilbur and Lipman (28) as implemented by Young and Cael (29), the sequence of the 312-base insert was identical to that of 312 of the 491 bases of the cDNA reported for L-FABP (cf. ref. 30). Alignment of the two sequences begins 6 bases 5′ from the translation initiation codon of L-FABP mRNA and ends at the beginning of an *Eco*RI site 75 bases 5′ from the end of the translation termination codon (Fig. 1). The cloned p14 cDNA spans all of exons I and II, 66 bases of the 5′ region of exon III, and none of exon IV of the L-FABP gene (cf. ref. 13). Blot-hybridization analyses of polyadenylylated liver RNA, using the p14 cDNA clone as probe, revealed an mRNA species of approximately 700 nucleotides. This is

‡EMBL/GenBank Genetic Sequence Database (1986) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 42, Locus RATFABPL; accession no. J00732.

similar to the 600–700 nucleotides previously reported for the mRNA of L-FABP (13, 30).

In order to determine whether or not the above immunoselection and identification of the cDNA clones possibly resulted from a cross-immunoreactivity of p14 with another member of the L-FABP family of proteins or from a spurious reactivity of the p14 antiserum, we carried out direct comparisons between the p14 and L-FABP proteins and also their antisera.

Chemical and Physical Homologies of p14 and L-FABP. There was a close similarity between the reported amino acid compositions of p14 (3), L-FABP (19), and that derived from the cDNA sequence of L-FABP (30). The observed minor differences could be the result of adventitious contaminations. In addition, the molecular sizes of p14 and L-FABP were the same according to $NaDodSO_4$ gel electrophoresis (Fig. 2, lanes 2 and 3). Furthermore, p14 and L-FABP demonstrated identical electrophoretic mobilities in Triton X-100/acetic acid/urea gels (data not shown).

p14 and L-FABP migrated almost identically in two-dimensional gel electrophoresis. Isoelectric focusing of undelipidated proteins in 6.25 M urea followed by migration in $NaDodSO_4$-containing buffer resolved each protein into four isoforms, all of which reacted with either antiserum (not shown). Three isoforms shared virtually identical pI values (p14: **8.6, 8.4**, 7.4 and 6.6; L-FABP: **8.2, 7.4**, 6.6, and 5.7). Principal forms are in boldface type. Isoforms of L-FABP result mainly from diversity of bound ligands (reviewed in refs. 9–11). After delipidation, both proteins focused at pH 6.8 in the absence of urea, in agreement with the pH 6.9 value previously reported for delipidated L-FABP (19) (see also refs. 9–11). A minor band was observed at pH 7.5 for p14 and at pH 7.4 for L-FABP. In 6.25 M urea, both p14 and L-FABP focused only at pH 8.0, analogous to the results at pH 8.3 with p14 (3).

Immunological Homology of p14 and L-FABP. The p14 and L-FABP antisera reacted with both proteins in immunoblots following polyacrylamide gel electrophoresis in either $NaDodSO_4$ buffer (Fig. 2) or in the Triton X-100/acetic acid/urea gel (data not shown). Furthermore, both p14 and L-FABP individually blocked the reaction of either antiserum with its respective antigen (Fig. 2).

Identical Binding of Fatty Acid by p14 and L-FABP. Both p14 and L-FABP complexed with [1-^{14}C]oleic acid and eluted in the void volume of a column of Sephadex G-10 (Table 1). Bovine serum albumin complexed to a lesser degree, whereas ribonuclease A and sterol carrier protein 2 did not.

Identical Immunostaining of p14 and L-FABP in Mitotic Hepatocytes. In 2-day regenerating livers, 36 of 40 (90%) mitotic hepatocytes exhibited specific elevations of immunostained L-FABP, as compared to adjacent interphase hepatocytes (Fig. 3). This behavior closely resembled that encountered with p14 antiserum (4, 6). Moreover, staining of both dividing and interphase hepatocytes by L-FABP antiserum was inhibited by preincubation with either p14 or L-FABP but not with ribonuclease A or sterol carrier protein 2 (data not shown).

DISCUSSION

L-FABP is the mitosis-associated polypeptide that is the covalent target of activated metabolites of the carcinogen FAA in rat hepatocytes. The concentration of cytoplasmic immunodetected p14 was shown previously to be low when growth activity is low, markedly elevated only during mitosis in normal and regenerating livers, and very high during interphase in hyperplastic and malignant hepatocytes brought about by the carcinogen FAA or 3′-methyl-4-dimethylaminoazobenzene (4–7). This report presents strong evidence that p14 is L-FABP. The known actions of p14 as the principal early covalent target of metabolites of FAA (1–3) and as the minor covalent target of derivatives of aminoazo dyes (15, 31) during liver carcinogenesis enhance the significance of these relationships and the identification of p14 as L-FABP. The findings point collectively to an involvement of L-FABP in normal mitosis and the carcinogen-induced proliferation of hepatocytes in rats.

The nucleotide sequence of the partial cDNA clone of p14 was identical to approximately two-thirds of the sequence

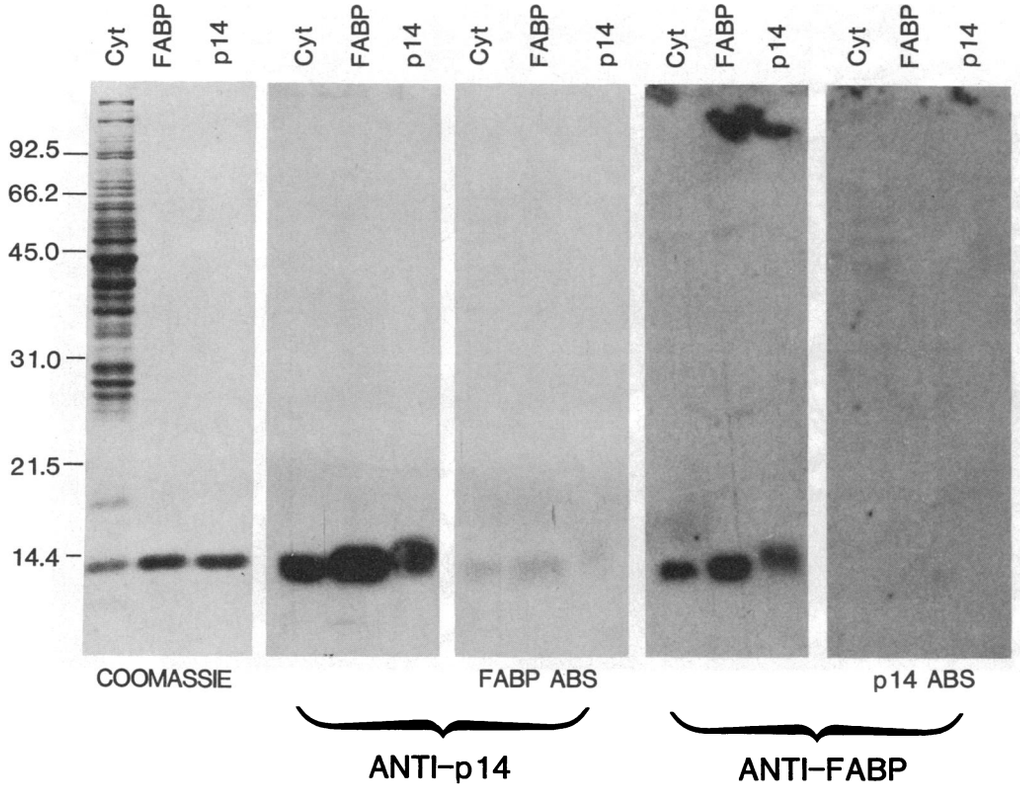

FIG. 2. $NaDodSO_4$ gel electrophoretogram of rat liver cytosol (Cyt), p14, and L-FABP and their reactions with antiserum against either p14 or L-FABP in matching immunoblots. The left triad of lanes demonstrates the molecular size purity of p14 and L-FABP, which are stained with Coomassie blue. The middle group contains the immunoblots of their reactions against p14 antiserum, with or without prior absorption (ABS) by L-FABP. The loss of reaction of p14 antiserum with both p14 and L-FABP due to prior blockage of p14 antiserum by L-FABP is shown. The right group of lanes presents the converse immunoblot with L-FABP antiserum and the loss of its reactions with p14 and L-FABP caused by prior blockage by p14. Molecular size markers (in kDa) are given at the left.

Table 1. [1-^{14}C]Oleic acid binding to p14 and L-FABP

Fraction	Counts per min					
	p14	L-FABP	BSA	SCP_2	RNase A	Buffer
1	70	55	40	51	41	74
2	3264	3345	2261	581	115	165
3	2933	2685	1811	376	147	173
4	1779	1549	623	232	124	121
5	666	436	238	174	90	104
6	598	275	156	169	60	89
7	345	270	155	167	66	78

Protein-bound [1-^{14}C]oleic acid eluted in the void volume (fractions 2 and 3) of a column of Sephadex G-10. BSA, bovine serum albumin; SCP_2, sterol carrier protein 2; RNase A, ribonuclease A; buffer, 50 mM potassium phosphate (pH 7.4).

reported for L-FABP (cf. ref. 30). In addition, both polypeptides have analogous amino acid compositions, the same molecular size, identical electrophoretic mobilities in Triton/acetic acid/urea (reflecting matching hydrophobic properties), overlapping pI values, the same increase in pI values and the same decrease in the number of isoforms brought about by delipidation, cross-reactions and common blocking of immunological reactions, identical binding of [^{14}C]oleate according to gel exclusion chromatography, matching purifications by oleo-affinity chromatography, and finally, the same specific elevated immunostains of mitotic hepatocytes. Collectively, the evidence strongly indicates that not only does p14 belong to the L-FABP family of proteins but that p14 is L-FABP.

The ability of hepatocytes to modulate the concentration of immunodetected L-FABP within the relatively short time interval spanned by mitosis is not compatible with its reported half-life of 3.1 days in normal rat liver. Different endogenous ligands have little effect on the half-life (reviewed in ref. 9). However, Dempsey (14) has inferred a half-life of less than 2 hr for sterol carrier protein, which is reported to be identical or similar to L-FABP. Taken together, it remains to be resolved whether in the rare dividing hepatocytes (1 out of >10,000) the increased immunodetection of L-FABP may reflect an intracellular relocation, reprocessing, or conformational change of that protein or whether its turnover in the mitotic cells may be much faster than in resting hepatocytes.

The actions of L-FABP make it an attractive possible carrier of certain hydrophobic reactants in their passage from cytoplasm to chromatin. The two carcinogens, FAA and aminoazo dyes, form covalent adducts of p14 (1–3, 15, 31). It is noteworthy that FAA, itself, does not bind to p14/L-FABP to any discernible extent (2). Rather, the covalent adducts arise secondarily after activated forms of the carcinogens complex (presumably hydrophobically) with L-FABP, in analogy to its association with long-chain fatty acids. In a similar fashion, L-FABP may bind and transport peroxides and hydroperoxides of fatty acids and related icosanoids. These prooxidants cause cleavage of chromosomes, stimulate DNA synthesis (32), and are tumor promoters (reviewed in ref. 33). Noteworthily, certain hypolipidemic drugs bring about proliferation of peroxisomes, marked increases in L-FABP concentration, hepatocyte multiplication, hepatomegaly, and hepatocellular carcinomas in rats (reviewed in refs. 34 and 35). Interactions between the hydrophobically

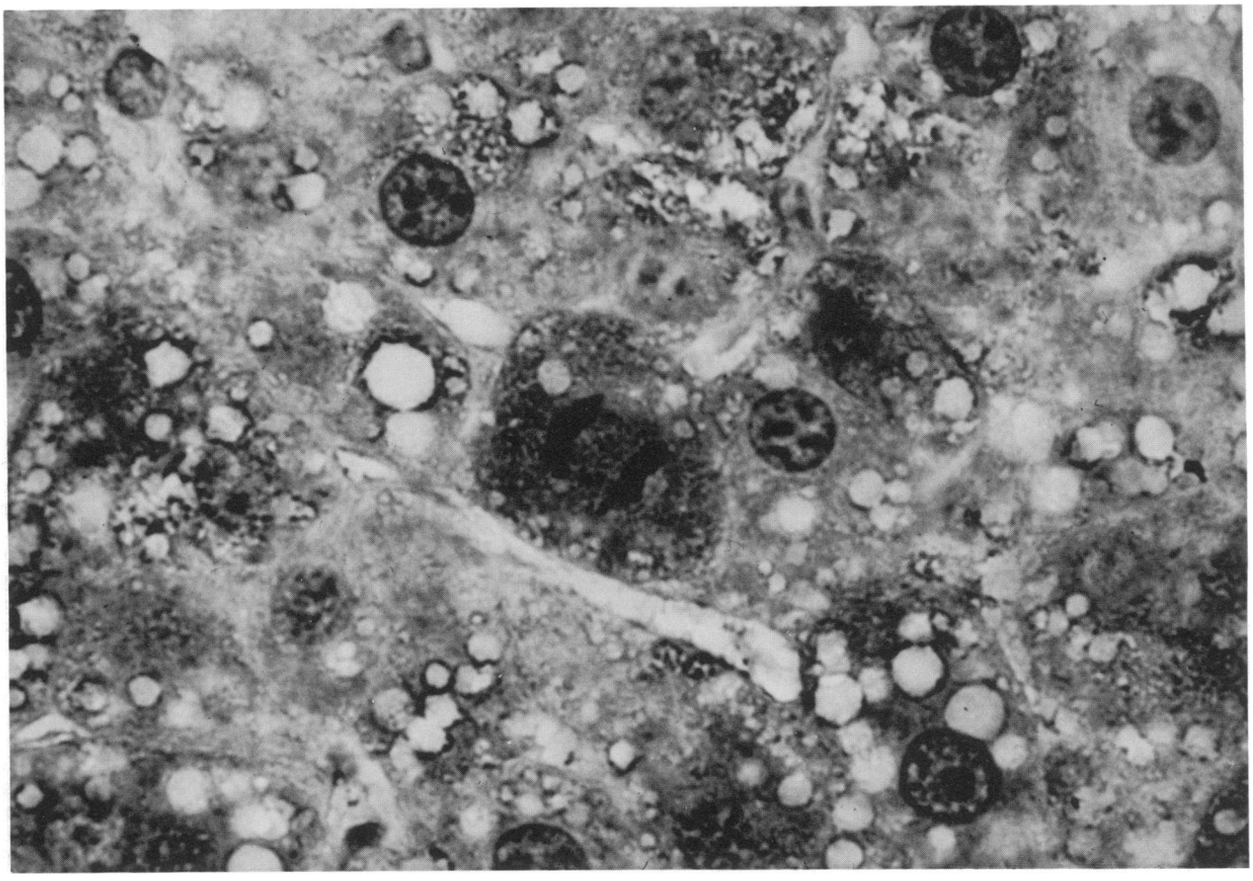

FIG. 3. Specifically increased expression of L-FABP in the cytoplasm of a mitotic hepatocyte in 2-day regenerating rat liver. The mitotic hepatocyte (anaphase) shows greater immunostain of L-FABP than do adjacent interphase hepatocytes. The liver section was stained with L-FABP antiserum, peroxidase-antiperoxidase complex, 3,3'-diaminobenzidine, and then hematoxylin. The enhancement of the L-FABP immunostain specifically during mitosis matched that of p14, which was reported previously with normal and regenerating livers (4, 6).

associated protein-bound reactants and DNA may presumably be facilitated by the contact of L-FABP with chromatin at the time of mitosis, when the level of L-FABP is apparently maximal and the nuclear membrane is disrupted.

The current findings are suggestive that ligands of L-FABP may be involved in normal mitosis and carcinogen-induced cell proliferation of rat hepatocytes. The principal known ligands of L-FABP in normal adult rat liver are palmitic, oleic, linoleic, and arachidonic acids (reviewed in refs. 9–11). These fatty acids appear to play important roles in cell proliferation. Specific interactions of palmitic acid and myristic acid mediate, in part, the attachment of the cytoskeleton and other proteins to the plasma membrane (36–38). Furthermore, oleic, linoleic, and arachidonic acids enhance protein kinase C activity (39, 40), which has been implicated as playing a key role in the transduction of mitotic signals from the cell membrane to the nucleus (reviewed in ref. 41). It is noteworthy, in view of the observed association of p14/L-FABP with mitosis of rat hepatocytes, that mitosis-specific phosphorylations occur in histones, nonhistone chromosomal proteins, high mobility group proteins, nucleolar proteins, nuclear matrix proteins, nuclear laminae, and intermediate filament proteins (reviewed in ref. 42). Moreover, signal transduction releases membrane-bound arachidonic acid, which with linoleic acid is a precursor of prostaglandin I_2 (prostacyclin) and thromboxane A_2, which have been implicated in modulation of growth (43, 44). It is interesting that the L-FABP family of proteins includes three intracellular retinol- and retinoic acid-binding proteins, whose ligands play important roles in differentiation (see above). It seems, therefore, important to determine whether or not changes in the kinds and levels of ligands of L-FABP are related specifically to the growth of hepatocytes.

If it is assumed that indeed L-FABP does function in normal mitosis and carcinogen-induced proliferation of rat hepatocytes, then that role combined with its known actions as targets of the two liver carcinogens would seemingly confer on L-FABP opposite promotional and protective effects in the overall process of liver carcinogenesis. Promotion would pertain to its presumed operation at the level of cell proliferation. On the other hand, protection against the reactive carcinogens would result from their covalent combinations with L-FABP, with the accompanying inactivation of that protein and consequent inhibition of mitosis. In fact, chemical carcinogens do in general initially suppress cell division (reviewed in ref. 8). The effect of the suppression of mitosis would be to reduce further exposure of the DNA to attack by additional activated carcinogens.

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