Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4830–4834, June 1992
Cell Biology

Specific growth stimulation by linoleic acid in hepatoma cell lines transfected with the target protein of a liver carcinogen

(liver fatty acid binding protein/fatty acids/eicosanoids/2-acetylaminofluorene)

TIBOR KELER, CHRISTOPHER S. BARKER, AND SAM SOROF*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

Communicated by Sidney Weinhouse, February 28, 1992

ABSTRACT **The hepatic carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene) was shown previously to interact specifically with its target protein, liver fatty acid binding protein (L-FABP), early during hepatocarcinogenesis in rats. In search of the significance of the interaction, rat L-FABP cDNA in the sense and antisense orientations was transfected into a subline of the rat hepatoma HTC cell line that did not express L-FABP. After the transfections, the basal doubling times of the cells were not significantly different. However, at 10^{-5}–10^{-7} M, linoleic acid, which is an essential fatty acid, a ligand of L-FABP, and the precursor of many eicosanoids and related lipids, stimulated the incorporation of [^{3}H]thymidine in three randomly isolated and stably transfected cell clones that expressed L-FABP, but virtually did not stimulate the incorporation of [^{3}H]thymidine in three L-FABP-nonexpressing clones transfected with the antisense DNA. Linoleic acid at 10^{-6} M increased cell number almost 3-fold (38% vs. 14%; $P < 0.0001$) and thymidine incorporation nearly 5-fold (23.2% vs. 4.9%; $P < 0.001$) in the L-FABP-expressing cells compared to that in the transfected nonexpressing cells. L-FABP acted specifically and cooperatively with linoleic acid, inasmuch as all the proteins other than L-FABP in the transfected L-FABP nonexpressing cells and four other fatty acids (γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, and palmitoleic acid) were unable to effect a significant elevation or difference in the level of DNA synthesis that was attributable to the transfection. Metabolism of the linoleic acid to oxygenated derivatives was apparently necessary, since the cyclooxygenase inhibitor indomethacin partly inhibited and the antioxidant lipoxygenase inhibitors nordihydroguaiaretic acid and α-tocopherol completely abolished the growth stimulation. The evidence supports the idea that L-FABP, the target protein of the liver carcinogen, acts specifically in concert with oxygenated metabolites of linoleic acid to modulate the growth of hepatocytes.**

The target protein of the liver carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene; FAA) has previously been implicated in the modulation of hepatocyte growth. Metabolites of FAA react principally (i.e., specifically) with a cytosolic protein in rat liver early during hepatocarcinogenesis (1–3), less in the later stages (1, 2), and virtually not at all in the subsequent primary and transplanted hepatomas (2, 4). The target protein was isolated (3), and the level of its immunohistochemical stain was found to be markedly increased in the cytoplasm of normal hepatocytes during mitosis and throughout the cell cycle in hyperplastic nodules and hepatocarcinomas produced by the carcinogen (5–7). The protein was identified as liver fatty acid binding protein (L-FABP) (8), formerly termed Z protein, which is conventionally considered to be an intracellular carrier of fatty acids (9–12). By using hybrid reporter DNA constructs in transgenic mice, the activity of the L-FABP promoter was then shown to be elevated during mitosis in hepatocytes of normal livers (13). L-FABP of mouse liver was also identified as a target of selenium compounds (14), which reversibly block cell multiplication in cultures and inhibit carcinogenesis in animals, and whose levels correlate inversely with cancer mortality rates in humans (reviewed in refs. 15 and 16). Further, L-FABP is thought to participate in the biosynthesis of phospholipids through regulation of the transport of lysophosphatidic acid from mitochondria to endoplasmic reticulum for conversion to phosphatidic acid (17, 18, 48). Moreover, when applied extracellularly, the two phospholipids are highly mitogenic (cited in ref. 19). Evidence strongly suggests that phosphatidic acid is a second messenger and that the mitogenic effect of phosphatidic acid is mediated by activated ras protein (reviewed in ref. 47). Likewise, L-FABP appears to be involved in the efflux of heme from mitochondria in the formation of cytochromes P450 and b_5 (20, 21) and also in the intracellular translocation of retinyl palmitate, the most abundant retinoid in liver (22). In addition, L-FABP binds *in vitro* both growth stimulatory and growth inhibitory eicosanoids specifically and with ≈10-fold greater affinities than fatty acids (23, 24). Last, L-FABP belongs to a protein family (25) in which several members have been associated with suppression of cell multiplication (cited in ref. 24).

More direct evidence that L-FABP may play a role in the modulation of hepatocyte growth is presented here. It was reasoned that introduction of encoded rat L-FABP into rat hepatoma cells that do not endogenously express that protein might reveal hepatocyte-dependent roles of L-FABP. We therefore transfected rat L-FABP cDNA into a rat hepatoma cell line that does not express L-FABP. Linoleic acid stimulated the growth and DNA synthesis of the cell clones that expressed L-FABP but had virtually no effect on the nonexpressing cell clones transfected with the antisense-oriented DNA. The stimulations were specific for linoleic acid and appeared to require metabolic oxygenation of the fatty acid. Linoleic acid is an essential fatty acid, a ligand of L-FABP (9–12), and the precursor of many biologically active eicosanoids and related lipids (see *Discussion*). The findings imply that L-FABP, the target of the carcinogen, acting specifically in concert with linoleic acid, may modulate the growth of hepatocytes.

MATERIALS AND METHODS

Reagents. Fatty acids from Biomol Research Laboratories (Plymouth Meeting, PA) and Nu-Check-Prep (Elysian, MN) were stored in ethanol under argon at −75°C for up to 3 weeks.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: L-FABP, liver fatty acid binding protein (Z protein); FAA, *N*-2-fluorenylacetamide (2-acetylaminofluorene); NDGA, nordihydroguaiaretic acid.

*To whom reprint requests should be addressed at: Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.

Indomethacin, nordihydroguaiaretic acid (NDGA), geneticin (antibiotic G-418), controlled process serum replacement type 1, and α-tocopherol were from Sigma; [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was from NEN; and RPMI 1640 and calf serum were from GIBCO.

Cell Cultures. The parental cell subline HTC-R_3T_2, donated by Gary M. Williams, arose from the HTC cell line (26), which was derived from the Morris rat differentiated hepatoma 7288c. HTC-R_3T_2 cells and their transfected clones were routinely maintained in RPMI 1640, 10% calf serum, sodium bicarbonate (2 g/liter), penicillin (50 units/ml), streptomycin sulfate (50 μg/ml), and kanamycin sulfate (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2. All cells were mycoplasma free.

L-FABP Expression Vector. Rat L-FABP cDNA was generated by PCR using rat liver RNA and synthetic terminal oligodeoxynucleotide primers derived from the sequence of L-FABP mRNA (27). The prepared 403-base-pair cDNA was inserted at the *Bam*HI site of plasmid Bluescript SK (Stratagene), sequenced, and subcloned as a 450-base-pair fragment in both orientations at the *Xba* I and *Hin*dIII sites of the expression vector pRc/RSVneo (Invitrogen, San Diego). The resultant plasmid vectors, pRSV-Z-S and pRSV-Z-A, contained the L-FABP coding sequence in the sense and antisense orientations, respectively, under the promoter control of the Rous sarcoma virus long terminal repeat.

Transfection of Cells. Subconfluent cultures of HTC-R_3T_2 hepatoma cells in 60-mm dishes containing Dulbecco's modified Eagle's medium and 10% calf serum were treated overnight with the calcium phosphate precipitates of 10 μg of plasmid vector DNA containing the L-FABP inserts, and after 48 hr the cultures were exposed to the antibiotic geneticin (active concentration of 400 μg/ml) for 14 days. Cell clones were isolated from the resultant geneticin-resistant mass cultures at limiting dilution. Three clones (HTC-Z-S7, -S9, and -S12) were randomly chosen that expressed L-FABP following transfection with the coding sequence in the sense orientation. Likewise, three clones (HTC-Z-A1, -A2, and -A4) contained the insert in the antisense orientation. The clones are hereafter referred to as S7, S9, S12, A1, A2, and A4. Back-up stores of the parent HTC-R_3T_2 cell line, the transfected cell clones, and *Escherichia coli* carrying the plasmid vectors have been deposited in the Cell Culture Facility of this Institute.

Southern and Northern Analyses. For Southern assays, genomic DNAs were completely digested with the restriction endonuclease *Hin*dIII, resolved in a 0.9% agarose gel, transferred to nitrocellulose paper (0.1 μm; Schleicher & Schuell), probed with a ^{32}P-labeled 403-base-pair cDNA of L-FABP, and processed under high stringency (28). DNA sizes were based on *Hin*dIII fragments of λ bacteriophage DNA. For Northern analyses (28), total RNAs were extracted from the cultured cells by the acid/phenol method (29) and electrophoresed in 1.5% agarose/0.66 M formaldehyde gels. Blots were processed as for Southern analyses, except that the hybridizations were at 45°C.

Cell Cytosols, Western Blots, and Quantification of L-FABP. L-FABP from cultured cells and liver cytosols was detected in Western blots (3, 8). Cultured cells (5×10^7) were homogenized in a glass/Teflon Potter–Elvehjem apparatus (size 0; Thomas) in 2 ml of 0.25 M sucrose solution containing 1 mM phenylmethylsulfonyl fluoride and 200 kallikrein inactivator units of aprotinin (Mobay Chemical). The resultant suspensions were centrifuged at 100,000 × *g* for 2 hr. The concentrations of the supernatant proteins were assayed (Bio-Rad); the proteins were electrophoresed through an SDS/15% polyacrylamide gel and electrotransferred to nitrocellulose paper; and the blot was probed with rabbit antiserum against purified rat L-FABP (3, 6, 23). Immunoreactive L-FABP was detected autoradiographically after incubation with ^{125}I-labeled protein A, scanned with a radioanalytic imaging system (Ambis Systems, San Diego), and quantified by comparisons with purified L-FABP in adjacent electrophoretic lanes.

Measurements of DNA Synthesis and Cell Growth (Standard Conditions). Cells (1×10^4) were seeded in 1 ml of the supplemented RPMI 1640 medium (as described above) and 10% calf serum in 24-well plates (Corning). After incubation overnight, all cells were provided fresh medium and 1% calf serum in the presence or absence of test additives [e.g., fatty acids in <0.1% ethanol (final)]. After 24 hr the medium and test additives were renewed by replacement. For measurements of thymidine incorporation in quadruplicate cultures (below half-confluency), 1 μCi of [^{3}H]thymidine in 20 μl of phosphate-buffered saline was added at 45 hr, and the amount of trichloroacetic acid-insoluble [^{3}H]thymidine was assayed at 48 hr. For measurements of cell growth, triplicate cultures were treated as above, except that the medium and additives were renewed by replacement at 24 hr and 48 hr. Cells were harvested by trypsinization at 72 hr and counted in a Coulter Counter.

Basal doubling times were determined from five daily Coulter cell countings of triplicate cultures growing exponentially in Dulbecco's modified Eagle's medium and 2% bovine reduced-lipid serum (controlled process serum replacement type 1) without added fatty acids.

Statistical significance was determined by the two-tailed Student *t* test.

RESULTS

Cell Characteristics. The L-FABP gene was present in the rat hepatoma cell line HTC-R_3T_2 but was not expressed. L-FABP mRNA was not detected in Northern blot assays or after PCR amplification of L-FABP mRNA followed by Southern blotting (data not shown); L-FABP itself was not evident (Fig. 1). Because of the absence of L-FABP expression, the hepatoma cell line was chosen for homologous transfections with the coding sequence of rat L-FABP cDNA in the sense or antisense orientation. Stable cell clones resistant to geneticin were isolated and expanded. Southern blot analyses detected the transfected integrated DNA in all cell clones and confirmed that the individual cell clones were not identical (data not shown).

Three transfected sense clones, chosen at random from among those that expressed L-FABP (below), contained L-FABP mRNA (≈0.6 kilobases). In contrast, three transfected and randomly selected L-FABP nonexpressing cell clones had no detectable RNA that hybridized with the L-FABP DNA probe (data not shown). Presumably, the RNA in the latter cells was too unstable to allow detection.

Only the three sense clones had detectable L-FABP. In Western blot analyses, the cells contained one-fifth to one-tenth of the L-FABP present in liver cytosol from normal

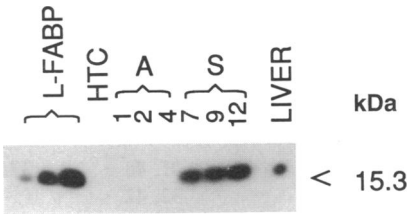

FIG. 1. Autoradiogram of a Western blot of L-FABP expression in transfected hepatoma cell lines. Rat liver cytosol (2 μg), cytosol of the parental HTC-R_3T_2 hepatoma cell line and its transfected clones (40 μg each), and purified L-FABP (5, 20, and 60 ng) were electrophoresed in an SDS/polyacrylamide gel, and the blot was probed with rabbit L-FABP antiserum and then ^{131}I-labeled protein A (see *Materials and Methods*). The L-FABP from the sense clones S7, S9, and S12 comprised 0.10%, 0.15%, and 0.22% (wt/wt) of the cytosolic proteins, respectively. L-FABP was not detected in the antisense transfected cells (clones A1, A2, and A4).

male rats, as referenced to the purified protein (3, 6, 23) and normalized to total cytosolic proteins on a weight basis (Fig. 1). These levels remained virtually unchanged during 25 passages in culture.

Expression of L-FABP did not alter the basal doubling times of the transfected cells. During exponential growth, the expressing and nonexpressing cells doubled in number after 21–26 hr in medium without supplemented fatty acid (see *Materials and Methods*).

Stimulation of Cell Growth by Linoleic Acid. Two distinctions and types of comparisons are generally made in this report: (*i*) the *relative effects* of the presence vs. absence of fatty acid or other additive and, more importantly, (*ii*) these relative effects in L-FABP expressing vs. nonexpressing transfected cells. The first describes the actions of the additives; the second describes the roles of L-FABP.

Linoleic acid stimulated the growth of the L-FABP-expressing cells significantly more than that of the nonexpressing cells. Importantly, the three L-FABP-expressing clones increased in cell number almost 3-fold more than did the nonexpressing clones over 72 hr in 10^{-6} M linoleic acid (37.8% ± 3.3% vs. 14.0% ± 2.4%; see Table 1). This difference brought about by L-FABP expression was highly significant at $P < 0.0001$.

Stimulation of DNA Synthesis by Linoleic Acid. L-FABP expression also resulted in elevated DNA synthesis in medium supplemented with linoleic acid in the standard assay (Fig. 2). In L-FABP-expressing cells, [^{3}H]thymidine incorporation was significantly stimulated in medium containing 10^{-7}–10^{-5} M linoleic acid, relative to the incorporation in the same medium without supplemented linoleic acid (Table 2). Importantly, the maximal stimulation at 10^{-6} M linoleic acid was nearly 5-fold greater than that in the nonexpressing cells (23.2% ± 1.4% vs. 4.9% ± 1.2%). This difference brought about by L-FABP expression was also highly significant ($P < 0.001$).

Specificity of Linoleic Acid-Induced Stimulation. Linoleic acid was unique among the five fatty acids in its ability to elevate DNA synthesis in L-FABP-expressing cells, both in the magnitude of the enhancement relative to that in control medium without added fatty acid and in the differential responses between the expressing vs. nonexpressing cells (Fig. 3). Compared to the stimulatory response of the expressing clones to linoleic acid (23.2% ± 1.4%), the fatty acids γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, and palmitoleic acid at 10^{-6} M all brought about in both the expressing and nonexpressing cells smaller increases of thymidine incorporation relative to that in control medium (Table 2). Some of the increases were statistically significant. However, only with linoleic acid was the enhancement of relative thymidine incorporation significantly greater or different in the L-FABP-expressing cells compared to that in the nonexpressing cells ($P < 0.001$).

Table 1. Growth stimulation by linoleic acid in L-FABP-transfected cells

Cell line	% relative increase in cell number: Individual values	Mean ± SE for group
L-FABP expressing		
S7	31, 48	37.8 ± 3.3*
S9	32, 42	
S12	35, 39	
L-FABP nonexpressing		
A1	18, 16	14.0 ± 2.4*
A2	11, 22	
A4	5, 12	

Transfected cells were cultured without or with 10^{-6} M linoleic acid for 72 hr under standard conditions (see *Materials and Methods*). SE, standard error.

*Significance of the near 3-fold growth increase of L-FABP expressing vs. nonexpressing cells has a P value of <0.0001.

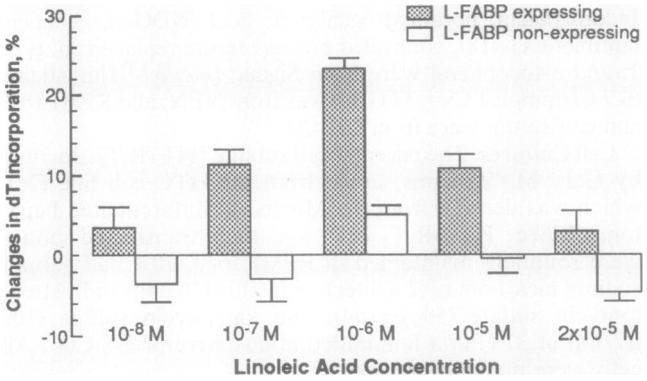

FIG. 2. Dependence of the stimulation of DNA synthesis on the linoleic acid concentration in L-FABP-expressing cells. Transfected cell clones that expressed L-FABP (S7, S9, and S12) and those that did not (A1, A2, and A4) were incubated without or with linoleic acid at the indicated concentrations for 48 hr under the standard conditions. Shown are the changes and standard errors of the thymidine incorporations at different concentrations of linoleic acid, relative to incorporations in same medium without supplemented linoleic acid, in the two cell types (see Table 2).

Inhibition of the Linoleic Acid-Induced Stimulation of DNA Synthesis. Inhibitors were used as probes of the metabolic pathways involved in the stimulation of growth by linoleic acid in the L-FABP-expressing cells. Indomethacin, an inhibitor of cyclooxygenase (30), and NDGA and α-tocopherol, both antioxidant inhibitors of lipoxygenases (30, 31), at concentrations that were by themselves not inhibitory, all depressed the elevation of relative thymidine incorporation induced by linoleic acid at 10^{-6} M (Table 2). With the three L-FABP-expressing cell lines, indomethacin at 10^{-5} M inhibited the linoleic acid-induced increase by 64%, NDGA at 10^{-7} M suppressed it by 80%, and α-tocopherol at 10^{-5} M blocked it by 94%. Considered another way, linoleic acid in the presence of indomethacin still brought about a small but significant stimulation (12%; $P < 0.05$) above that of indomethacin alone, whereas correspondingly NDGA (0.8%) and α-tocopherol (3%) each did not (Table 2). The inhibitions indicate that the stimulation of DNA synthesis by linoleic acid in the L-FABP-expressing cells results from the production of oxygenated metabolites of that fatty acid by lipoxygenases and to lesser extent by cyclooxygenase.

DISCUSSION

Linoleic acid specifically stimulated the growth and DNA synthesis of the hepatoma cell clones that were stably transfected with the coding sequence of the target liver protein L-FABP of the hepatic carcinogen FAA. Cell growth was increased ≈3-fold ($P < 0.0001$), and relative thymidine incorporation was increased ≈5-fold ($P < 0.001$) in the L-FABP-expressing cells compared to that in the nonexpressing cells. These sizeable differences in responses, especially in cell number, are remarkable, considering that they were brought about by the expression of one protein, that the enhancement was above the already high level of growth of the hepatoma cells, that the process of cell growth is complex, that the level of L-FABP expression is relatively low, and that complementation of L-FABP expression is probably less than perfect in the hepatoma cells. The present findings constitute the most direct and strongest evidence thus far implicating L-FABP, the target liver protein of the carcinogen, as a possible modulator of hepatocyte growth.

The enhancement of DNA synthesis was specific in regard to both L-FABP and linoleic acid. L-FABP needed linoleic

Table 2. Stimulation of DNA synthesis by linoleic acid in L-FABP-transfected HTC cells

Additive(s)	L-FABP-expressing cells: % increase in thymidine incorporation mean ± SE	*n*	L-FABP-nonexpressing cells: % increase in thymidine incorporation mean ± SE	*n*	Expressing vs. nonexpressing *P* value
Linoleic acid					
10^{-8} M	3.3 ± 2.5	7	−3.6 ± 2.4	6	
10^{-7} M	11.2 ± 2.1*	7	−3.0 ± 3.0	6	<0.01
10^{-6} M (reference conc.)	23.2 ± 1.4**	23	4.9 ± 1.2*	17	<0.001
10^{-5} M	11.0 ± 1.9*	10	−0.4 ± 1.9	9	<0.001
2×10^{-5} M	2.8 ± 2.7	3	−4.6 ± 1.2	3	
γ-Linolenic acid (10^{-6} M)	10.3 ± 1.1**	18	16.3 ± 3.0**	16	
Dihomo-γ-linolenic acid (10^{-6} M)	9.2 ± 2.3*	21	9.0 ± 2.5*	22	
Arachidonic acid (10^{-6} M)	7.5 ± 1.0**	18	5.4 ± 3.1	17	
Palmitoleic acid (10^{-6} M)	7.1 ± 2.8	6	8.6 ± 1.8*	6	
Indomethacin (10^{-5} M)	−3.9 ± 1.1*	7			
Indomethacin (10^{-5} M) + linoleic acid (10^{-6} M)	8.4 ± 1.9	7*			
NDGA (10^{-7} M)	3.8 ± 1.7	4			
NDGA (10^{-7} M) + linoleic acid (10^{-6} M)	4.6 ± 2.7	4			
α-Tocopherol (10^{-5} M)	−1.5 ± 2.9	4			
α-Tocopherol (10^{-5} M) + linoleic acid (10^{-6} M)	1.5 ± 1.2	4			

The three L-FABP-expressing cell lines (S7, S9, and S12) and the three L-FABP-nonexpressing cell lines (A1, A2, and A4) were incubated without or with the above additives in RPMI 1640 and 1% calf serum for 48 hr under the standard conditions (*Materials and Methods*). The percent increases of thymidine incorporation, their standard errors (SE), and *P* values (indicated by * and **) of the cultures containing the additives are relative to those of the same cells in the control medium without additive. *n*, number of replicate assays. Two comparisons are shown: with vs. without additive and with vs. without L-FABP expression. *, $P < 0.05$; **, $P < 0.01$; the absence of these notations (in columns 2 and 4) or *P* values (in column 6) indicates no statistical significance.

acid, and linoleic acid needed L-FABP. L-FABP was unique in that all the proteins other than L-FABP in the transfected nonexpressing cells were unable to effect the growth enhancement that was achieved by L-FABP. In addition, among the five fatty acids, linoleic acid brought about the greatest and the only significant elevation of DNA synthesis that required L-FABP. DNA synthesis was maximally elevated by linoleic acid at 10^{-6} M, a concentration that approximates the dissociation constant (K_d) of the binding of fatty acids to L-FABP *in vitro* (9–12). It appears that L-FABP acting specifically in concert with linoleic acid and/or its metabolites may play a unique role in the growth modulation of hepatocytes.

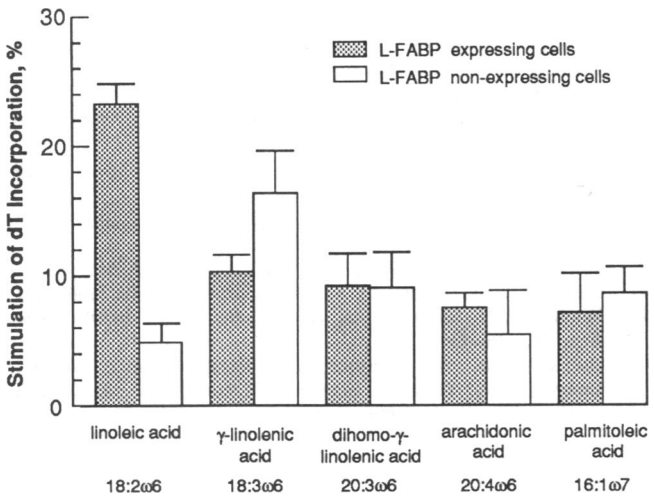

FIG. 3. Linoleic acid specificity of the stimulation of DNA synthesis. Shown are the stimulations of thymidine incorporation brought about by five fatty acids at 10^{-6} M in the standard assays of L-FABP-expressing and -nonexpressing cells (see Table 2).

The presence and absence of L-FABP expression likely resulted in secondary phenotypic effects in the transfected cells. Altered compositions of membrane lipids and changed levels of lipid-metabolizing enzymes have recently been associated with transfection of L-FABP in fibroblasts (32, 33). Such consequences, as well as changes in the amounts, protection, or intracellular targeting of the growth-enhancing metabolites of linoleic acid, may all have contributed to the differential responses of the transfected cells to linoleic acid in the present study. The present zero-background homologous model of transfection appears to constitute a suitable and controlled system for study of the biological and biochemical actions of L-FABP and their consequences in cells, in which the effects may be dependent on the natures and functions of the different ligands bound to L-FABP.

Metabolism of linoleic acid was apparently essential for the growth stimulation. This need is in accord with an observed lack of significant difference in uptake of linoleic acid at 4°C and 28°C in the expressing and nonexpressing cells (34). Linoleic acid is the most abundant and important essential fatty acid. As the fountainhead of the C18 ω6 and C20 ω6 eicosanoids and related lipids, linoleic acid is successively converted to γ-linolenic acid, dihomo-γ-linolenic acid, and arachidonic acid (30, 35). Each then undergoes enzymatic lipoxygenations, epoxidations, and, in the cases of the latter two eicosanoids, also cyclooxygenations. All can be further metabolized to multiple ω6 derivatives, of which many are biologically active (30, 35). In the present study, maximal growth stimulation specifically required both L-FABP and linoleic acid. This specific interrelationship was supported in part by the nonparticipation of L-FABP in the smaller elevations produced by γ-linolenic acid and dihomo-γ-linolenic acid, which are present in animals at much lower concentrations than is linoleic acid (35), and by the lack of any significant effect by arachidonic acid (Table 2 and Fig. 3). These data also argue against a direct conversion of these fatty acids to mediators of the stimulation by L-FABP. On the

other hand, the stimulation by linoleic acid was partly inhibited by indomethacin and completely abolished by the antioxidants NDGA and α-tocopherol, at concentrations that individually by themselves minimally affected incorporation of thymidine. The findings indicate that oxygenated metabolites of linoleic acid, formed by lipoxygenases and to lesser extent by cyclooxygenase, act as mediators of the growth enhancement (cf. refs. 36–38).

Oxygenated metabolites of linoleic acid have previously been implicated in the stimulation of cell growth. Linoleic acid can promote the growth of tumors (39, 40) and of various cells in culture (41–43), and these effects have been blocked in some cases by inhibitors of cyclooxygenase (42, 43) and reconstituted by prostaglandin E_2 (42). In addition, oxygenated metabolites of linoleic acid (i.e., its 13-hydroxy, 13-hydroperoxy, and 13-keto derivatives) stimulated cell proliferation of colon mucosa when instilled intrarectally in rats (38, 44). Further, 9- and 13-hydroxylinoleic acids (i.e., 9- and 13-hydroxyoctadecadienoic acids) are produced by BALB/c 3T3 fibroblasts, and the 13-hydroxy and its 13-hydroperoxy precursor markedly potentiate the thymidine incorporation effected by epidermal growth factor in those cells (37). It is noteworthy that *in vitro* L-FABP avidly binds lipoxygenase metabolites of arachidonic acid—namely, hydroperoxyeicosatetraenoic acids and hydroxyeicosatetraenoic acids (23)—and hence may do likewise with these linoleic acid-derived C_{18} homologs. The need exists to examine whether the transfected hepatoma cells produce particular oxygenated metabolites of linoleic acid and whether they mediate the growth stimulation in those cells.

Early events during chemical carcinogenesis usually involve a growth inhibition, which in effect confines the proliferation of the few escaping chemically transformed cells to regions of focal hyperplasia, surrounded by growth-suppressed nontransformed cells. FAA is especially effective in bringing about such a growth inhibition and has been used widely for that purpose in the rapid induction of focal hyperplasia in rat liver early in the chemical carcinogenic process (reviewed in refs. 45 and 46). The present finding that L-FABP expression is associated with a specific growth stimulatory activity may seemingly provide a basis by which the carcinogen may usurp and/or inhibit the normal growth modulatory function of its target protein.

We are grateful to Dr. Philip N. Tsichlis for his discussions and Dr. Gary M. Williams for a gift of the HTC-R_3T_2 hepatoma cell subline. This study was supported in part by National Institutes of Health Grant CA-05945, Institutional Grants CA-06927 and RR-05539 from the National Institutes of Health, and an appropriation from the Commonwealth of Pennsylvania.

1. Blackburn, G. R., Andrews, J. P., Rao, K. V. K. & Sorof, S. (1980) *Cancer Res.* **40,** 4688–4693.
2. Blackburn, G. R., Andrews, J. P., Custer, R. P. & Sorof, S. (1981) *Cancer Res.* **41,** 4039–4049.
3. Blackburn, G. R., Schnabel, S. J., Danley, J. M., Hogue-Angeletti, R. A. & Sorof, S. (1982) *Cancer Res.* **42,** 4664–4672.
4. Sorof, S., Young, E. M., Coffey, C. B. & Morris, H. P. (1966) *Cancer Res.* **26,** 81–88.
5. Custer, R. P. & Sorof, S. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6738–6742.
6. Vinores, S. A., Churey, J. J., Haller, J. M., Schnabel, S. J., Custer, R. P. & Sorof, S. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2092–2096.
7. Sorof, S. & Custer, R. P. (1987) *Cancer Res.* **47,** 210–220.
8. Bassuk, J. A., Tsichlis, P. N. & Sorof, S. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7547–7551.
9. Sweetser, D. A., Heuckeroth, R. O. & Gordon, J. I. (1987) *Annu. Rev. Nutr.* **7,** 337–359.
10. Bass, N. M. (1988) *Int. Rev. Cytol.* **111,** 143–184.
11. Kaikaus, R. M., Bass, N. M. & Ockner, R. K. (1990) *Experientia* **46,** 617–630.
12. Veerkamp, J. H., Peters, R. A. & Maatman, R. G. H. J. (1991) *Biochim. Biophys. Acta* **1081,** 1–24.
13. Sweetser, D. A., Birkenmeier, E. H., Hoppe, P. C., McKeel, D. W. & Gordon, J. I. (1988) *Genes Dev.* **2,** 1318–1332.
14. Bonsal, M. P., Cook, R. G., Danielson, K. G. & Medina, D. (1989) *J. Biol. Chem.* **264,** 13780–13784.
15. Ip, C. (1986) *J. Am. Coll. Toxicol.* **5,** 7–20.
16. Willett, W. C. & Stampfer, M. J. (1986) *J. Am. Coll. Toxicol.* **5,** 29–36.
17. Vancura, A., Carroll, M. A. & Haldar, D. (1991) *Biochem. Biophys. Res. Commun.* **175,** 339–343.
18. Haldar, D. & Vancura, A. (1992) *FASEB J.* **6,** A494 (abstr.).
19. Van Corven, E. J., Van Rijswijk, A., Jalink, K., Van Der Bend, R. L., Van Blitterswijk, W. K. & Moolenaar, W. H. (1992) *Biochem. J.* **281,** 163–169.
20. Vincent, S. H., Bass, N. M., Snider, J. M. & Muller-Eberhard, U. (1987) *Biochem. Arch.* **3,** 443–451.
21. Liem, H. H., Grasso, J. A., Vincent, S. H. & Muller-Eberhard, U. (1990) *Biochem. Biophys. Res. Commun.* **167,** 528–534.
22. Fukai, F., Kase, T., Shidotani, T., Nagai, T. & Katayama, T. (1987) *Biochem. Biophys. Res. Commun.* **147,** 899–903.
23. Raza, H., Pongubala, J. R. & Sorof, S. (1989) *Biochem. Biophys. Res. Commun.* **161,** 448–455.
24. Khan, S. H. & Sorof, S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9401–9405.
25. Sweetser, D. A., Lowe, J. B. & Gordon, J. I. (1986) *J. Biol. Chem.* **261,** 5553–5561.
26. San, R. H. C., Shimada, T., Maslansky, C. J., Kreisler, D. M., Laspia, M. F., Rice, J. M. & Williams, G. M. (1979) *Cancer Res.* **39,** 4441–4448.
27. Gordon, J. I., Alpers, D. H., Ockner, R. K. & Strauss, A. W. (1983) *J. Biol. Chem.* **258,** 3356–3363.
28. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
29. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
30. Willis, A. L., ed. (1987) *Handbook of Eicosanoids: Prostaglandins and Related Lipids* (CRC, Boca Raton, FL), Vol. 1, Parts A and B.
31. Reddanna, P., Rao, M. K. & Reddy, C. C. (1985) *FEBS Lett.* **193,** 39–43.
32. Jefferson, J. R., Powell, D. M., Rymaszewski, Z., Kukowska-Latallo, J., Lowe, J. B. & Schroeder, F. (1990) *J. Biol. Chem.* **265,** 11062–11068.
33. Jefferson, J. R., Slotte, J. P., Nemecz, G., Pastuszyn, A., Scallen, T. J. & Schroeder, F. (1991) *J. Biol. Chem.* **266,** 5486–5496.
34. Keler, T., Barker, C. & Sorof, S. (1992) in *Eicosanoids and Other Bioactive Lipids*, Second International Conference, Berlin, September 1991, eds. Nigam, S., Honn, K. V., Marnett, L. J. & Walden, T. (Kluwer, Boston), pp. 435–438.
35. Mead, J. F. & Willis, A. L. (1987) in *Handbook of Eicosanoids: Prostaglandins and Related Lipids*, ed. Willis, A. L. (CRC, Boca Raton, FL), Vol. 1, Part A, pp. 85–98.
36. Funk, C. D. & Powell, W. S. (1985) *J. Biol. Chem.* **260,** 7481–7488.
37. Glasgow, W. C. & Eling, T. E. (1990) *Mol. Pharmacol.* **38,** 503–510.
38. Earles, S. M., Bronstein, J. C., Winner, D. L. & Bull, A. W. (1991) *Biochim. Biophys. Acta* **1081,** 174–180.
39. Welsch, C. W. (1987) *Am. J. Clin. Nutr.* **45,** 192–202.
40. Sauer, L. A. & Dauchy, R. T. (1988) *Cancer Res.* **48,** 3106–3111.
41. Wicha, M. S., Liotta, L. A. & Kidwell, W. R. (1979) *Cancer Res.* **39,** 426–435.
42. Balakrishnan, A., Cramer, S., Bandyopadhyay, G. K., Imagawa, W., Yang, J., Elias, J., Beattie, C. W., DasGupta, T. K. & Nandi, S. (1989) *Cancer Res.* **49,** 857–862.
43. Rose, D. P. & Connolly, J. M. (1990) *Cancer Res.* **50,** 7139–7144.
44. Bull, A. W., Nigro, N. D. & Marnett, L. J. (1988) *Cancer Res.* **48,** 1771–1776.
45. Farber, E. & Cameron, R. (1980) *Adv. Cancer Res.* **31,** 125–226.
46. Erikson, L. C. & Anderson, G. N. (1992) *Crit. Rev. Biochem. Mol. Biol.* **27,** 1–55.
47. Kiss, Z. (1990) *Prog. Lipid Res.* **29,** 141–166.
48. Vancura, A. & Haldar, D. (1992) *J. Biol. Chem.*, in press.