ALPHA$_1$-FETOPROTEIN BIOSYNTHESIS DURING THE GROWTH CYCLE OF DIFFERENTIATED FETAL RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE

H. L. LEFFERT and S. SELL. From the Cell Biology Laboratory, Armand Hammer Center for Cancer Biology, The Salk Institute, San Diego, California 92112 and the Department of Pathology, University of California at San Diego, School of Medicine, La Jolla, California 92112

INTRODUCTION

Serum proteins associated with malignancies ("oncoproteins") have attracted considerable attention, especially because of their possible use in the early clinical detection of cancer (1, 2). Perhaps the most intensively studied of these oncoproteins are the embryonal carcinogenic antigen (3), associated mainly with gastrointestinal malignancies (4–6), and alpha$_1$-fetoprotein (α_1F) (1, 2), associated with primary hepatocellular carcinoma (1, 7–10), and teratoblastomas of gonadal origin (11, 12).

However, oncoproteins may also be found in nonmalignant states (5, 13–18). This is especially the case of the α_1F, which appears in the serum of pregnant and growing (neonatal) rats (18, 19) as well as in the serum of adult rats induced by partial hepatectomy to regenerate their liver (20, 21). These observations imply that biosynthesis and release of α_1F are related to the cell cycle. In the studies described here, in which a well-characterized monolayer culture system of fetal rat hepatocytes is used (22–24), direct evidence is presented which supports this hypothesis.

MATERIALS AND METHODS

Reagents

Tissue culture media and supplies required to grow fetal rat hepatocytes have been described in detail

elsewhere (22–24). 9.0-cm tissue culture dishes were obtained from NUNC (Roskilde, Denmark). Radioisotopes (CH_3-[^{3}H]thymidine[dT], sp act 20 Ci/mM; and L-[4,5-^{3}H]leucine, sp act 30–50 Ci/mM) were obtained from New England Nuclear, Boston, Mass. Hepatoma 7777 sera and goat anti-α_1F antisera were obtained or prepared as previously described (25, 26).

Fetal Rat Hepatocyte Tissue Culture

In the experiments described in this report, arginine-free plating medium containing 10% (vol/vol) dialyzed fetal bovine serum (22) was supplemented with L-ornithine (0.2 mM, final concentration) previously shown to enhance fetal hepatocyte proliferation in vitro (24). This medium will be referred to as standard medium.

The procedures used to establish *growth cycling primary monolayer cultures* have been described elsewhere in detail (24). After the repeated collagenase digestions, freshly isolated packed cell pellets were washed twice with serum-free medium (10 ml) before resuspension in plating medium. Because of the proliferative requirement for conditioned medium (22, 24), the medium was not changed during 8 days of culture (unless specified) and at the end of this time greater than 90% of the attached cells were viable as determined by exclusion of an aqueous solution of 0.4% trypan blue. The observed recovery efficiency (22) of 10% at 24 h postplating under these plating conditions (2–3 $\times$ 10^6 cells/2-ml plating medium/30-mm diameter tissue culture dish) probably resulted from both the stringent conditions of the arginine-free, ornithine-supplemented selection medium (22) and the presence in dialyzed fetal bovine serum of a macromolecular fraction (40–80,000 daltons) which antagonizes growth.[1]

Step-down conditions were used to generate *quiescent*[2] *primary monolayer cultures* in diluted conditioned medium (CM) prepared without exogenously added serum.[3] CM was prepared by plating freshly isolated hepatocyte cell suspensions (4×10^6 cells/10-ml serum-free standard medium) into 9.0-cm plastic tissue culture dishes. CM was collected 44 h postplating, centrifuged (International Clinical Desk-Top, International Scientific Instruments Inc., Palo Alto, Calif., 2,000 rpm, 10 min), and the supernate sterilized by filtration (0.45-μm Nalgene filter, Nalge Co., Nalgene Labware Div., Rochester, N. Y.) to remove debris. CM was stored at 4°C for periods of up to 6–8 wk without detectable losses of activity. Fetal rat hepatocytes were plated (2.0–2.5 $\times$ 10^5 cells/dish) together with standard medium (2 ml/30-mm diameter dish). 34-h postplating, the medium was aspirated and replaced with fresh standard culture medium (2 ml). 56-h postplating, the medium was aspirated and fresh CM diluted 1:1 with serum-free standard medium (2 ml). The average concentration of arginine in the culture medium under these conditions did not exceed 10 μM as determined by amino acid analysis (22). During the next 7 days, cell multiplication occurred as indicated by an 80–100% increase in the number of attached cells (day 2, 0.12–0.25 $\times$ 10^5 cells/30-mm dish; day 9, 0.24–0.50 $\times$ 10^5 cells). By the 9th day postplating—although the cells were not confluent at this time—cell multiplication ceased, the rate of DNA synthesis had declined to a steady low value (20 cpm/dish), and the percentage of DNA synthesizing cells was less than 2% as determined by autoradiography.

Radioimmunoassay for Rat α_1F

The procedure for detecting rat α_1F in small quantities (ca. 5 ng/ml) by radioimmunoassay has been described in detail elsewhere (26, 28).

Specific Immunoprecipitation of Labeled Amino Acids Incorporated into Rat α_1F

For quantitation of specific incorporation of labeled amino acids into α_1F, a specific immunoprecipitation of label by goat anti-α_1F and carrier hepatoma 7777 sera was used after elimination of nonspecific coprecipitating material by reaction of carrier human albumin (Calbiochem, San Diego, Calif.) with goat antialbumin at equivalence. The following reagents were mixed together: 0.25 ml of a human albumin solution containing 0.4 mg/ml (albumin carrier), 0.5 ml of tissue culture fluid, and 0.25 ml of a goat antiserum specific to human albumin. This mixture was allowed to stand for 2 h at room temperature and then overnight at 4°C. The precipitate was removed by centrifugation at 3,000 *g* for 40 min, washed (three times) in cold saline, dissolved in standard scintillation fluids containing Triton-X and toluene, and counted. For specific precipitation of α_1F, the supernate of the albumin-antialbumin precipitation was treated with 0.2 ml of a 1:64 dilution of hepatoma 7777 serum (α_1F carrier) and 0.25 ml of goat anti-α_1F (previously demonstrated to give precipitation equivalence with the α_1F in 0.2 ml of 1:64 hepatoma 7777 serum). The precipitate was obtained, washed, and

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[1] Leffert, H. L. 1974. *J. Cell Biol.* Manuscript submitted for publication.

[2] 1–2% of the cells are in S phase as judged by autoradiography (22) after a 12–24-h pulse with CH_3-[^{3}H]dT (1.25 μCi/ml culture medium). It has been shown, however, that quiescent states developed by different culture conditions are not necessarily biochemically identical with respect to RNA metabolism (27); thus, the extent to which these cultured quiescent hepatocytes differ biochemically from nongrowing hepatocytes in vivo remains to be determined.

[3] The conditioning phenomenon in this culture system has already partly been described elsewhere (22, 24) and will be described in detail (Leffert, H. L., and K. Koch. 1974. *J. Cell Biol.* Manuscript submitted for publication).

counted. Preprecipitation with the albumin-antialbumin system once or repeatedly usually brought down only 3% of the total counts. If more than 10% of the counts were preprecipitated by antialbumin, a second antialbumin preprecipitation step was carried out before specific anti-α_1F precipitation. Under these conditions, post-precipitation with antialbumin consistently failed to produce any precipitable counts over background. This system was tested using ^{125}I-labeled α_1F added to normal rat serum. Specific precipitation with anti-α_1F brought down 95–98% of the counts precipitable with anti-α_1F under standard conditions using 18% wt/vol Na_2SO_4 (26).

DNA Synthesis Assays

The *rate* of incorporation of CH_3-[^{3}H]dT was determined by 2-h pulse-labeling of triplicate cultures with 0.05 ml of a cocktail such that the final thymidine concentration in the medium was 3×10^{-6} M, 1.25 μCI/ml (23). The rate of incorporation under these conditions was linear for at least 2 h. The medium was aspirated and the dishes were washed twice with Tris-saline, pH 7.4 (23), 2 ml/wash. Ca^{++}- and Mg^{++}-free trypsin solution (23) was then added (2 ml/dish) and the dishes were incubated at 37°C for 30 min. Vigorous hand-pipettation was required to ensure removal of greater than 95% of the attached cells; this material was then directly filtered (Whatman GF/C 2.4-cm glass fiber filters presoaked in 5% trichloroacetic acid [TCA]). The filters were washed with ice-cold 5% TCA and ice-cold ethanol and air-dried in plastic scintillation vials. Liquifluor scintillant (5 ml, New England Nuclear) was used for counting with a Beckman counter model LS-233 (Beckman Instruments, Inc., Fullerton, Calif.). Errors of measurement were ±5–10%.

Preparation of Culture Media for α_1F Determinations

Unlabeled or labeled (5 μCi/ml of L-[4,5-^{3}H]leucine) culture media were aspirated from the monolayer cultures, sterilized by filtration (Swinnex, Millipore Corp., Bedford, Mass.), and frozen (−20°C) in polyethylene tubes until the time of assay (1–2 wk). Under these conditions, immunoreactive α_1F is stable at least up to 6 mo. Volumes recovered per culture were usually of the order of 1.7 ml (plating volume = 2 ml); evaporation which occurs over long time periods (greater than 8 days) without medium changes was negligible (−10%) in the studies reported here (culture media less than 8-days old). Triplicate samples of culture media were assayed; errors of measurement were ±10.0%.

Cell Counts

The number of attached cells per dish, which has been shown to be a valid measurement of cell multiplication, was measured with a Coulter counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) as previously described (24).

RESULTS

A typical growth curve[1] (cycle) of fetal rat hepatocytes plated under conditions which maximize growth (2×10^5 cells/30-mm diameter dish; 2 ml arginine-free, L-ornithine-supplemented [0.2 mM] medium containing dialyzed fetal bovine serum [10% vol/vol]; no medium change) is characterized by: a lag phase of about 48 h (during which the numbers of attached cells drop to about $1.0–1.5 \times 10^4$ cells/dish); logarithmic growth through the 8th-day postplating with a population doubling time of about 20–24 h; and a subsequent stationary phase during which the fraction of DNA-synthesizing cells declines, cell multiplication declines, and the number of attached cells remains relatively constant at about 10^5 cells/dish. Culture media were sampled during this growth cycle and the quantity of accumulated α_1F was determined by radioimmunoassay. These results are shown in Table I.

Between the 1st- and the 6th-day postplating, about a 10-fold increase of accumulated radioimmunoassayable α_1F was detectable in the culture medium under the above conditions; similar increases were not detected in the culture media derived from fetal rat fibroblasts (dispersed cell suspensions obtained from the body wall) although some α_1F was detectable (ca. 70–80 ng/ml) in these culture media (control ≤5 ng/ml). This probably was due to adsorbed material carried over from fetal fluids in the original cell platings because incubation of these cultures with excess tritiated leucine (5 μCi/ml) failed to yield synthesized material as indicated by the failure to incorporate the labeled amino acid into α_1F-specific immunoprecipitable material (≤10 cpm/ml). This is also shown in Table I. In contrast, after similar 24-h incubations with tritiated leucine, hepatocyte cultures in lag phase released a small quantity of synthesized material (45 cpm/ml); logarithmically growing cultures released about six times as much (265 cpm/ml); and, as net population growth subsided (day 8), the amount of synthesized material released declined to about twice the lag phase level (90 cpm/ml).

The incorporation of labeled leucine into specific immunoprecipitable α_1F was increased, as expected, when fresh leucine-free standard medium was added to cultures 168–192 h postplating,

TABLE I

Synthesis of α_1F by Primary Monolayer Cultures of Fetal Rat Hepatocytes

Medium assayed	Labeling interval (hours post-plating)	α_1F	
		Specific immuno-precip-itable radio-activity	Radio-immuno-assayable
		cpm/ml	*ng/ml*
Complete medium (no cells)			
+ or − dFBS (10% vol/vol)	96–120	15	≤5
+ or − dARS (10% vol/vol)	96–120	8	≤5
Fetal rat liver culture medium (no medium changes)			
+ dFBS (10% vol/vol)			
Day 1	0–24	45	121
Day 6	120–144	265	≥1,000
Day 8	168–192	90	≥1,000
Fetal rat liver culture medium minus L-leucine (fresh medium change, 168–192 h post-plating)			
+dFBS (2.5% vol/vol)			
Day 8	168–192	900	180
− serum			
Day 8	168–192	945	142
Fetal rat fibroblast culture medium (no medium change)			
Day 1	0–24	10	81
Day 8	72–96	3	74

Fetal rat hepatocytes were plated (2.0×10^6 cells/30-mm diameter dish) into arginine-free, L-ornithine-supplemented (0.2 mM) medium ("standard medium") containing dialyzed fetal bovine serum (dFBS, 10% vol/vol). Fetal rat fibroblasts (body wall) were plated similarly except that the plating medium was supplemented with L-arginine (0.4 mM) to ensure fibroblast viability. Some dishes (controls) without cells contained medium supplemented with either dFBS (10% vol/vol) or dialyzed adult rat serum (dARS, 10% vol/vol). Parallel dishes set up for labeling studies received L-[4,5-^{3}H]leucine (5 μCi/ml, sp act 30–50 Ci/mM) during the indicated labeling intervals. Usually, the medium was not changed; however, in some parallel cultures, before labeling, the spent medium was aspirated, the dishes were washed with arginine-free and serum-free medium, and then these cultures received 2-ml fresh leucine-free medium with or without dFBS (2.5% vol/vol) but supplemented with L-arginine (0.006 mM). All labeling periods were for 24 h. The accumulated radioimmunoassayable α_1F (unlabeled parallel cultures) and the α_1F specific immunoprecipitable radioactivity were determined as described in Materials and Methods. All values represent the average of triplicate determinations whose errors of measurement were ±10%.

but this stimulatory effect was independent of the presence or absence of dialyzed serum under the conditions of this particular determination (Table I). Radioimmunoassayable α_1F also was released (140–180 ng/ml) into leucine-free medium 168–192 h postplating but, as expected, this quantity of released α_1F was considerably less than the amount present in day 8 culture medium (accumulated from zero time) because of the shorter incubation time (24 h) and the likelihood that leucine deprivation arrests growth (20).

The appearance of increasing amounts of radioimmunoassayable α_1F in the culture media most likely indicated net synthesis because, as shown in Fig. 1, a proportionate increase of α_1F-specific immunoprecipitable radioactivity was also observed. In these experiments, logarithmically growing cultures (day 7) of fetal rat hepatocytes were exposed to fresh medium changes (after washes [two times] with 2-ml fresh, serum-free medium) designed to arrest growth differentially. Under these conditions, both the growth rate of the cell population and the induction of new rounds of DNA synthesis were suppressed.[1, 3] Thus, cultures received fresh serum-free, L-ornithine-supplemented (0.2 mM) medium with or without L-arginine (0.4 mM). The culture media were sampled immediately (time zero) and every 12 h thereafter for the next 48 h. Parallel cultures were incubated under identical conditions together with an excess of tritiated leucine (5 μCi/ml), and media similarly withdrawn. Radioimmunoassayable α_1F accumulated in the culture media derived from both sets of cultures, although the rate and the quantity of material appearing in the media of L-arginine-supplemented cultures was elevated three- to fourfold compared to cultures incubated without L-arginine. Under both conditions, however, the increase of radioimmunoassayable material was paralleled by a strict proportionate increase of α_1F-specific immunoprecipitable counts. In separate control experiments (not shown), these increases could be blocked 85–95% by cycloheximide (5 μg/ml).

To determine the relationship between the appearance of synthesized α_1F in the culture medium and the position of fetal hepatocytes in the cell cycle, suggested from the results of Table I and Fig. 1, quiescent fetal rat hepatocytes were induced to grow by a change to fresh medium supplemented with L-arginine (0.4 mM) and dialyzed fetal bovine serum (10% vol/vol). Simultaneously, quantitative determinations of DNA synthesis rates and accumulated radioimmunoassayable α_1F were made. These results are shown in Fig. 2.

α_1F was detected in the culture medium before 20 h had elapsed after the medium change, but at

least 8–10 h after the first wave of DNA synthesis had been initiated. The quantity of accumulated α_1F increased after 20 h, and was highest at about 30 h (40–45 ng/ml), at least 5–6 h after the first mitotic figures appeared (determined by phase microscope examination of the cultures, ×100). α_1F levels remained elevated (about fourfold) although somewhat reduced (20 ng/ml) as additional rounds of DNA synthesis proceeded (Fig. 2), presumably indicative of subsequent rounds of cell

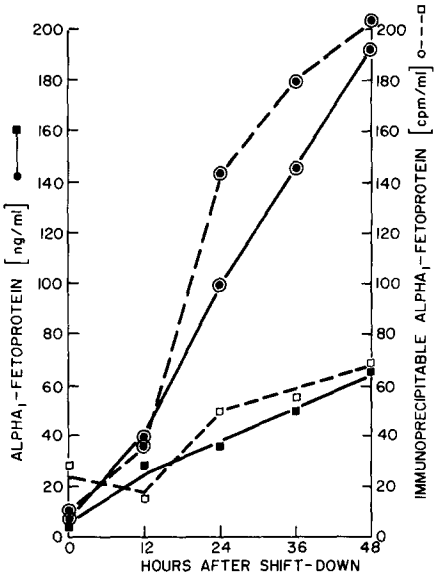

FIGURE 1 Synthesis and release of α_1F by growth-arrested cultured fetal rat hepatocytes. Fetal rat hepatocytes were plated (3×10^5 cells/30-mm diameter dish) into 2-ml arginine-free medium containing L-ornithine (0.2 mM) and dialyzed fetal bovine serum (10% vol/vol). On the 7th-day postplating, the culture media were aspirated and the dishes washed (two times) with arginine-free medium. Dishes were then divided into two groups: one group received fresh arginine-free, ornithine-supplemented medium (squares); the other group received similar medium supplemented with L-arginine ([circles], 0.4 mM). All culture media contained L-leucine (0.8 mM) but, within each group, half of the dishes received 5 μCi/ml L-[4,5-^{3}H]leucine (sp act 30–50 Ci/mM). Unlabeled (solid lines) and labeled (dashed lines) culture media were sampled immediately (time zero) and then 12, 24, 36, and 48 h thereafter. Media were filtered (Swinnex, Millipore Corp.) and assayed for α_1F by radioimmunoassay (solid lines) or specific radioimmunoprecipitation (dashed lines) as described in Materials and Methods. Abscissa: time (hours) after medium changes. Left ordinate: α_1F (nanograms per milliliter culture medium) by radioimmunoassay. Right ordinate: α_1F-specific immunoprecipitable counts per minute per milliliter culture medium.

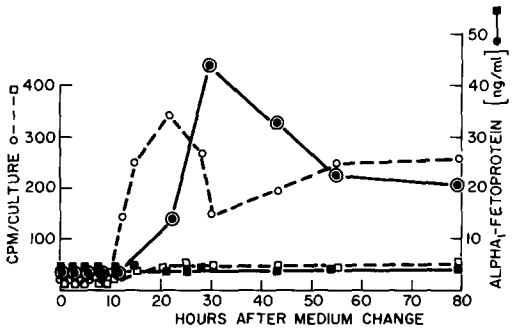

FIGURE 2 Appearance of α_1F in the culture medium of quiescent fetal rat hepatocytes after the induction of DNA synthesis. Quiescent fetal rat hepatocyte cultures were prepared by step-down conditions as described in Methods. At time zero (9-days postplating), the spent culture medium was aspirated and the cultures were washed (two times) with 2-ml arginine-free medium. These cultures (0.29×10^5 cells/dish) then received either 2-ml fresh medium containing dialyzed fetal bovine serum (10% vol/vol) and L-arginine (0.4 mM) (circles); or similar fresh medium without serum (squares). At varying times thereafter, DNA synthesis rates were determined in one set of cultures labeled with CH_3-[^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) for 2 h and by measuring the radioactivity incorporated into TCA- (5% vol/vol) insoluble material (dashed lines). In the parallel set of cultures, media were sampled from unlabeled cultures at identical times and worked up as described in Fig. 1 for the measurement of radioimmunoassayable α_1F (solid lines). During the 80-h interval of this experiment, there was no detectable increase in radioimmunoassayable α_1F in culture media derived from control dishes (i.e., quiescent cultures remaining in day 9 spent medium). Abscissa: time (hours) after the medium change. Left ordinate: counts per minute CH_3-[^{3}H]dT incorporated per culture per 2-h pulse. Right ordinate: α_1F (nanograms per milliliter culture medium) by radioimmunoassay.

multiplication. Quiescent cultures that received fresh serum-free media and, consequently, were not induced to synthesize DNA or to divide to any significant amount failed to release detectable α_1F into their culture medium.

DISCUSSION

This fetal hepatocyte tissue culture system is being used as a model system to study hepatocellular growth control (22–24). Because pancreatic, pituitary, adrenocortical, and thyroid hormones are implicated,[4] it seemed pertinent to explore the capac-

[4] A preliminary discussion of the "[insulin]/[glucagon] ratio" hypothesis of liver regeneration, based upon

ity of these cultured cells to synthesize α_1F (1, 10), an oncoprotein (1, 2) that has been reported to have a high binding affinity for estrogens (30, 31).

The initial observations described in this report suggest that the appearance of synthesized α_1F is related to the position of cultured fetal hepatocytes in both the growth and the cell cycle. Thus, the amount of radioactivity incorporated into released α_1F was highest in culture media derived from logarithmically growing cells (Table I). Synthesized material continued to appear in the culture media in proportion to radioimmunoassayable α_1F as these cells entered stationary phase (Table I) or were arrested by step-down conditions (Fig. 1) in their rate of growth and in their ability to proceed through additional rounds of growth. Therefore, the studies described here provide direct evidence for *net* biosynthesis in a growing in vitro hepatocyte system, although other groups have previously shown qualitative biosynthesis of α_1F in vitro using (dying) liver tissue slices and the combined techniques of immunoelectrophoresis and autoradiography (19, 32).

In vitro studies with this system (Fig. 2) indicate that quiescent hepatocytes release detectable α_1F only after being induced to grow (G_0 exit) by serum factors in the presence of a nutritionally adequate medium (24). Serum per se does not affect release of synthesized α_1F as indicated by the observation (Table I) that under conditions of growth arrest (leucine-free medium [30]) the presence or absence of serum in the culture medium failed to raise or to lower, respectively, the quantity of α_1F-specific immunoprecipitable counts. It was further indicated (Fig. 2) that released α_1F becomes detectable after DNA synthesis has been initiated and concomitant with the onset of cell division. These observations suggest that α_1F is synthesized in G_2; but the possibility cannot yet be excluded that α_1F is synthesized during G_1 and then released by the cells during the $G_2 \rightarrow M$ boundary and/or postmitotically. Problems of interpretation would appear to be compounded by the possibility, as suggested by in vitro results (Fig. 2), that parenchymal liver cells might be catabolic sites for α_1F in that α_1F levels were observed to decline (44–55%) as cell multiplication proceeded.

Although evidence has been presented that these cultured hepatocytes synthesize and secrete albumin (22), it has not yet been determined whether in vitro biosynthesis of albumin or other proteins normally synthesized and secreted rapidly by the liver also would occur in the same relationship to the growth (or cell) cycle as α_1F. Evidence from in vivo studies with rats suggests that this will not be the case because (*a*) during ontogenesis and postnatal life serum albumin levels continuously rise as α_1F levels fall precipitously (33), and (*b*) during liver regeneration, serum α_1F levels rise (20, 21) and there is evidence that the liver remnant might actually synthesize albumin at elevated rates (34). Further in vitro studies with this system are in progress to determine the relationship between secretion and synthesis of albumin and α_1F.

α_1F may be an intracellular mediator of hepatocellular proliferation. It is unlikely that α_1F is itself a stimulatory serum growth factor (at least for cultured fetal rat hepatocytes) because adult rat sera—which contain about 30 ng/ml of α_1F (28)—were found to stimulate in vitro hepatocellular growth as effectively as pregnant rat sera (Leffert, unpublished observations) which contain over 100-fold greater quantities of α_1F (35). Alternatively, the function of α_1F as it relates to hepatocellular proliferation may be extracellular, perhaps by controlling hepatocellular utilization of estrogens or estrogen-like material. Estrogen-binding "transitional" hepatocytes (31) have been tentatively identified as the putative α_1F producing cells in the liver. However, results of experiments with this in vitro system support the hypothesis that hepatocytes with urea cycle functions (parenchymal cells, which comprise about 45% of the cells in the adult rat liver [36]) are identical with α_1F-synthesizing hepatocytes, because the majority of these cultured cells synthesize arginine (22) and multiply in arginine-deficient medium (24). Thus, transitional estrogen-binding hepatocytes which are present in very low frequencies in the adult liver may represent that small fraction of hepatocytes which traverse the cell cycle under normal conditions (37).

Finally, it is notable that hepatocarcinogens are capable of stimulating α_1F production in vivo before (or without inducing) detectable DNA synthesis and/or cell division (38). These observations imply that under special conditions α_1F production can be dissociated from the program of cell cycle events and in this regard be unrelated to growth control. Further studies of hormonal and carcinogen interactions with these cultured fetal rat hepatocytes may help to elucidate this paradox.

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studies with this culture system, has been submitted (Leffert, H. L. 1974. *J. Cell Biol.*).

SUMMARY

Primary monolayer cultures of different fetal rat hepatocytes synthesize and release α_1F into their culture medium, as determined both by radioimmunoassay and by the incorporation of L-[4,5-^{3}H]leucine into specific immunoprecipitable material. Similar synthesized material was undetectable in the culture media derived from primary cultures of fetal rat fibroblasts. The release of synthesized α_1F into the culture medium is related to the growth rate of the cultured fetal hepatocytes as well as to their position in the cell cycle. This culture system should facilitate studies of functional and developmental relationships between biosynthesis and release of this particular oncoprotein and the hepatocellular cell cycle.

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This is paper no. IV in a series of studies relating to the growth control of cultured fetal rat hepatocytes (H. L. Leffert) and paper no. VIII in a series of rats α_1F (S. Sell). Papers numbered V–VII in the fetal rat hepatocyte in vitro series have been submitted to *The Journal of Cell Biology*.

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