Regulation of Growth and Differentiation of a Rat Hepatoma Cell Line by the Synergistic Interactions of Hormones and Collagenous Substrata

ZENAIDA GATMAITAN,* DOUGLAS M. JEFFERSON,‡ NELSON RUIZ-OPAZO,‡ LUIS BIEMPICA,*§ IRWIN M. ARIAS,* GREG DUDAS,* LESLIE A. LEINWAND,‖ and LOLA M. REID‡

*The Liver Center, and the Departments of ‡Molecular Pharmacology, §Pathology, and ‖Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT Serum-free, hormonally defined media have been developed for optimal growth of a rat hepatoma cell line. The cells' hormonal requirements for growth are dramatically altered both qualitatively and quantitatively by whether they were plated onto tissue culture plastic or collagenous substrata. On collagenous substrata, the cells required insulin, glucagon, growth hormone, prolactin, and linoleic acid (bound to BSA), and zinc, copper, and selenium. For growth on tissue culture plastic, the cells required the above factors at higher concentrations plus several additional factors: transferrin, hydrocortisone, and triiodothyronine.

To ascertain the relative influence of hormones versus substratum on the growth and differentiation of rat hepatoma cells, various parameters of growth and of liver-specific and housekeeping functions were compared in cells grown in serum-free, hormonally supplemented, or serum-supplemented medium and on either tissue culture plastic or type I collagen gels. The substratum was found to be the primary determinant of attachment and survival of the cells. Even in serum-free media, the cells showed attachment and survival efficiencies of 40–50% at low seeding densities and even higher efficiencies at high seeding densities when the cells were plated onto collagenous substrata. However, optimal attachment and survival efficiencies of the cells on collagenous substrata still required either serum or hormonal supplements. On tissue culture plastic, there was no survival of the cells at any seeding density without either serum or hormonal supplements added to the medium. A defined medium designed for cells plated on tissue culture plastic, containing increased levels of hormones plus additional factors over those in the defined medium designed for cells on collagenous substrata, was found to permit attachment and survival of the cells plated into serum-free medium and onto tissue culture plastic.

Growth of the cells was influenced by both substrata and hormones. When plated onto collagen gel substrata as compared with tissue culture plastic, the cells required fewer hormones and growth factors in the serum-free, hormone-supplemented media to achieve optimal growth rates. Growth rates of the cells at low and high seeding densities were equivalent in the hormonally and serum-supplemented media as long as comparisons were made on the same substratum and the hormonally supplemented medium used was the one designed for that substratum. For a given medium, either serum or hormonally supplemented, the saturation densities were highest for tissue culture plastic as compared with collagen gels.

Differentiative functions, as measured by various enzymatic assays and by analyses of RNA populations for liver-specific and housekeeping mRNA, were also influenced by synergies between hormones and substratum. Some known liver-specific functions, such as albumin,

© The Rockefeller University Press · 0021-9525/83/10/1179/12 $1.00

were not expressed at all by these rat hepatoma cells under any culture conditions tested. Those that were expressed were either maintained at equivalent levels (uridine diphosphate glucuronyl transferase and CHOB) in the various culture conditions or were significantly augmented by the serum-free, hormonally supplemented media in conjunction with collagenous substrata (β-actin, α-tubulin, glutathione-*S*-transferase, and tyrosine aminotransferase).

We have been exploring the relevance of the extracellular matrix and its components in conjunction with hormones and other soluble factors in the regulation of cellular physiology (1). Towards this end, we developed serum-free, hormonally supplemented media for a rat hepatoma cell line, $H_4A_zC_2$. These media permit the cells to grow and to express differentiated functions similar to those observed in the same cells grown in serum-supplemented media. The serum-free, hormonally defined media (DM)[1] were developed using a clonal growth assay according to the procedures of Ham and McKeehan (2) and Ham (3) and Barnes and Sato (4). The DM were then compared with serum-supplemented medium (SSM) and with unsupplemented, serum-free medium (SF) for their influence on the growth and physiological behavior of the rat hepatoma cells when cultured on substrata of tissue culture plastic versus Type I collagen gels.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (150–180 g) were purchased from Charles River Breeding Laboratories, Inc., (Wilmington, MA) and used immediately for purification of Type I collagen and for the preparation of normal rat hepatocytes.

Cell Line

A partially differentiated rat hepatoma cell line, $H_4A_zC_2$, was obtained from Dr. Nadel-Ginard (Albert Einstein College of Medicine). It has been found to be positive for tyrosine aminotransferase (TAT) inducibility, conjugation of bilirubin, liver-specific alcohol dehydrogenase production, and ornithine decarboxylase production (5, 6). The subline we received does not express albumin, although the parental cell line has been found to express this liver-specific function (5). The stock cultures were kept in tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) and were subcultured and fed once per week. The cells were routinely checked for mycoplasma by the method of Chen (7).

Culture Conditions

Since the assay for the defined media was based on measurement of the number and size of clones, careful attention was given to the following: (*a*) the need for uniform suspension of fully dissociated, viable cells; (*b*) high purity of hormones and growth factors to be tested; and (*c*) the use of triple distilled water for the preparation of the media. Water was a major source of toxicity problems. Considerable effort was made to ensure its purity.

Media

The stock cells were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. In preliminary studies, RPMI 1640, when compared with minimal essential medium, Dulbecco's modified Eagle's medium, Hams-F12 (all from Gibco Laboratories) and a 1:1 ratio of Dulbecco's modified Eagle's medium and Hams-F12, was found to be the optimal basal medium for the hepatomas and hepatocytes (Reid, L., Z. Gatmaitan, and D. Shouval, unpublished results). In brief, these results indicated that no clonal growth or growth at high seeding densities (>10^4 cells per 60-mm dish) occurred in any unsupplemented basal medium when cells were plated on tissue culture plastic. However, if the cells were plated onto collagenous substrata, kept in the basal medium with serum supplementation for 24 h and then switched to one of the basal media without serum supplements, clonal growth did occur in all of the media. However, the highest number of clones occurred in RPMI 1640. Without any further supplements, the RPMI 1640 was referred to as unsupplemented, serum-free medium (SF). When it was further supplemented with 10% fetal bovine serum, the medium was called serum-supplemented medium (SSM). As described in Results and given in summary form in Table II, serum-free media supplemented with a defined mixture of trace elements, hormones, and growth factors were developed optimizing the growth of the rat hepatoma cells on various substrata. These media are referred to as "defined media" (DM). Although some of the factors in the DM contain some impurities (those in Table I and II listed in terms of units of activity), the factors used are the most pure available. In a few experiments, cells were cultured in SSM to which was added the purified factors in DM, i.e., a combination of the two media: serum-supplemented medium and defined medium (SSM/DM).

Serum

Fetal bovine serum was purchased from Gibco Laboratories. Serum lots were screened on several cell lines and primary cultures prior to selection of a serum lot. All serum was heat inactivated (56°C for 30 min) and stored at −20°C until needed.

Hormones and Trace Elements Used in the Defined Medium

The hormones, growth factors, and trace elements and their commercial sources, stock, and working concentrations are listed in Table I. Stock solutions of insulin and of factors prepared in ethyl alcohol were stored at 4°C. Factors prepared in PBS or other solutions were aliquoted and stored at −20°C.

Substrata

Two types of substrata were used: tissue culture plastic (Falcon 60-mm tissue culture dishes) or type I collagen gels. Type I collagen gels were prepared by the procedures of Michalopoulos and Pitot (8). The collagenous substrata were sterilized with 10,000 rads of gamma irradiation (cesium 135) and were then stored at 4°C until used.

Influence of Culture Conditions on the Morphology and Growth of Hepatoma Cells

Attachment Efficiency

The cells were seeded at various seeding densities between 10^4–10^6 cells per plate onto 60-mm tissue culture dishes or onto petri dishes with a coat of Type I collagen in SF, SSM, or DM. 24 h later the plates were rinsed with PBS and the cells were detached with 0.5% trypsin–0.2% EDTA in PBS for cells on tissue culture plastic or with 0.2% collagenase in serum-free RPMI 1640 for cells on collagenous substrata. The number of viable cells was ascertained by the trypan blue exclusion assay using hemocytometer counts.

Clonal Growth Assay

The cells were seeded at 100 cells per 60-mm plate under the appropriate culture conditions and left undisturbed for 3 wk. They were fixed with 100% methanol and left unstained. The clones of cells were readily recognizable as white patches against a clear background (tissue culture plastic) or a translucent yellow background (collagen gels). Clonal growth efficiency was calculated as the number of clones per number of cells seeded × 100.

[1] *Abbreviations used in this paper:* CHOB, Chinese hamster ovarian cells-probe B; DM, serum-free, hormonally defined media; DM (C), DM designed for cells on collagenous substrata; DM (TCP), DM designed for cells on tissue culture plastic substrata; GST, glutathione-*S*-transferase; SF, unsupplemented, serum-free medium; SSM, serum-supplemented medium; TAT, tyrosine aminotransferases; and UDPGT, uridine diphosphate gluronyl transferase.

TABLE I

Factors Found Influential to Clonal Growth of Rat Hepatoma Cells Plated on Collagen

Hormone/factors	Sources*	Solvent	Final concentration	Presence of colonies
Proteins and peptides				
Adrenalin	Parke, Davis	PBS	250 ng/ml	−
Adrenocorticotropin	Armour Pharmaceutical Co.	PBS	5 μU/ml	−
Ceruloplasmin	Sigma Chemical Co. (Sigma)	PBS	50 mU/ml	−
Epidermal growth factor	Collaborative Research	PBS	50 ng/ml	−
Follicle stimulating hormone	Sigma	PBS	20 mU/ml	+
Glucagon	Sigma	0.01 N NaOH	10 μg/ml	+
Growth hormone	Sigma	0.01 N HCL	10 μU/ml	+
Gly-His-Lys	Sigma	PBS	0.5 μg/ml	−
Insulin (24 U/mg)	Sigma	0.01 N HCl	240 mU/ml	+
Luteinizing hormone	National Institutes of Health (NIH)	PBS	3,000 μg/ml	−
Luteinizing hormone releasing factor	Sigma	PBS	10 ng/ml	−
Multiplication stimulating activity	Collaborative Research	PBS	50 ng/ml	−
Noradrenalin	Sigma	PBS	0.1 μg/ml	−
Oxytocin	Sigma	PBS	5 μU/ml	−
Parathyroid hormone	Calbiochem-Behring Corp.	PBS	0.5 ng/ml	−
Prolactin (20 U/mg)	Sigma	0.01 N HCl	2 mU/ml	+
Secretin (20 CRICK U/mg)	Sigma	PBS	200 μU/ml	−
Somatostatin	Sigma	PBS	0.01 ng/ml	−
Somatotropin releasing inhibitory factor	Sigma	PBS	10 ng/ml	−
Thyrocalcitonin	Sigma	PBS	0.1 μg/ml	−
Thyrotropin-releasing factor	Calbiochem-Behring Corp.	PBS	10^{-11} M	+
Thyrotropin stimulating hormone	Sigma	PBS	500 μU/ml	−
Thyroxine	Sigma	0.01 N NaOH	10^{-11} M	+
Triiodothyronine	Sigma	0.01 N NaOH	10^{-10} M	+
Transferrin	Sigma	PBS	10 μg/ml	+
Vasopressin	Sigma	PBS	5 μU/ml	−
Steroids*				
Aldosterone	Sigma	Ethanol	50 ng/ml	−
Deoxycorticosterone	Sigma	Ethanol	10^{-9} M	−
Dexamethasone	Sigma	Ethanol	10^{-7} M	−
Dihydrotestosterone	Sigma	Ethanol	10^{-7} M	−
17-β-estradiol	Sigma	Ethanol	10^{-9} M	−
Hydrocortisone	Sigma	Ethanol	10^{-9} M	+
Trace elements				
Copper ($CuSO_4 \cdot 5H_2O$)	Johnson Matthey	H_2O	10^{-7} M	+
Manganese ($MnSO_4 \cdot 5\ H_2O$)			10^{-1} M	+
Molybdate ($NH_4Mo_6O_{24} \cdot 4H_2O$)			10^{-8} M	+
Nickel ($NiSO_4 \cdot 6\ H_2O$)			5×10^{-15} M	+
Selenium (H_2SeO_3)			3×10^{-10} M	+
Silicon ($Na_2SiO_3 \cdot 9\ H_2O$)			5×10^{-9} M	−
Tin ($SnCl_2 \cdot 2\ H_2O$)			5×10^{-10} M	+
Vanadium (NH_4VO_3)			10^{-13} M	+
Zinc ($ZnSO_4 \cdot 7\ H_2O$)			10^{-11} M	+
Other factors				
Inosine	Sigma	PBS	10 μg/ml	+
Linoleic Acid	Sigma	1 mg/ml BSA†	10 μg/ml	+
Prostaglandin E_1	Upjohn Co.	Alcohol	10 ng/ml	−
Prostaglandin E_2	Upjohn Co.	Alcohol	10 ng/ml	−

* Location of sources: Parke, Davis & Co., Detroit, MI; Armour Pharmaceutical Co., Tarrytown, NY; Sigma Chemical Co., St. Louis, MO; Collaborative Research Inc., Lexington, MA; National Institutes of Health, Bethesda, MD; Calbiochem-Behring Corp., San Diego, CA; Johnson Matthey Chemicals, London; and Upjohn Co., Kalamazoo, MI.

† Fatty acid-free BSA (Pentex Biochemical, Kankakee, IL).

DOUBLING TIME AND SATURATION DENSITY

Growth curves of the cells were initiated by seeding 10^5 cells per 60-mm dish coated with or without the collagenous substrate and in either SSM or DM. Triplicate plates were assessed for cell number at each time point. On day 1 and every other day thereafter, time points were taken by rinsing the plates with PBS, detaching the cells from their substrata with 0.2% crude collagenase (Type I, Sigma Chemical Co., St. Louis, MO), and counting them by trypan blue exclusion assay for viability. The growth curves on plastic and collagen gel substrata were plotted as cell counts per dish against length of time in culture.

TABLE II

Constituents of "Defined Media" (DM) for Optimal Clonal Growth of Rat Hepatoma Cells on Tissue Culture Plastic or Collagen Gels

Factor	Source*	Optimal concentration for clonal growth on: Tissue culture plastic:DM (TCP)	Collagenous substrata:DM (C)
Insulin	Sigma	100 μg/ml	1–5 μg/ml
Glucagon	Sigma	10 μg/ml	5 μg/ml
Hydrocortisone	Sigma	1×10^{-8} M	Not required
Transferrin	Sigma	10 μg/ml	Not required
‡Linoleic acid	Sigma	10 μg/ml	5 μg/ml
Triiodothyronine	Sigma	1×10^{-9} M	Not required
Prolactin	Sigma or NIH	2 mU/ml	2 mU/ml
Growth hormone	Sigma or NIH	10 μU/ml	10 μU/ml
Trace elements	Johnson Matthey Chemicals		
Zinc	Johnson Matthey Chemicals	nt	5×10^{-11} M
Copper	Johnson Matthey Chemicals	nt	1×10^{-7} M
Selenium	Johnson Matthey Chemicals	nt	3×10^{-10} M

Pituitary hormones were purchased from commercial sources when possible. An alternate source was through the NIH hormone distribution service.
The trace elements were also required on tissue culture plastic. However, the concentration required for optimal clonal growth is unknown (*nt*, not tested). The concentrations used were the same as for cell cultures plated on collagenous substrata.
* For location of sources, see footnote (*) to Table I.
‡ Linoleic acid must be present in combination with fatty acid-free BSA (Pentex Biochemical) at a 1:1 molar ratio. If added alone, it is quite toxic to the cells.

MORPHOLOGICAL STUDIES

The hepatoma cells were studied by both light and phase-contrast microscopy and compared with adult rat hepatocytes in vivo and in vitro. Cultures were monitored by phase-contrast microscopy for their cell shape, granularity, vacuolar formation, organelle content, polarity, and other such parameters. The stability of the cultures was documented by phase-contrast microscopy at various durations after plating.

Assays of Differentiated Functions

ASSAYS OF LIVER-SPECIFIC PROTEINS

To ascertain the functional state of the hepatoma cells, several liver-specific markers were assayed. Uridine diphosphate glucuronyl transferase (UDPGT) was assayed by the procedures of Billings et al. (9). Glutathione-*S*-transferase (GST) was assayed by the method of Grover and Sims (10). Medium changes on the cells were done every other day. Where relevant, a medium change was done 24 h prior to the assay to enable determination of the amount of the product per unit time per cells. At various time points after plating, a set of plates was rinsed and taken for assessment of the basal levels of TAT. A duplicate set was maintained an additional 24 h under the "induction conditions": SSM, DM designed for cells on collagenous substrata (DM (C)), or SF with and without 10^{-5} M dexamethasone. TAT levels were assessed by the procedures of Diamondstone (11).

DNA ASSAYS

Data on differentiative functions was normalized to the DNA content of the cultures. On the same plates (where medium was being assayed) or on replicate plates (where the cells were being assayed), the plates were rinsed and the cells plus any substratum (collagen gel) were scraped from the dish. The scraped material was put into test tubes and homogenized with a polytron homogenizer. For all culture conditions, the DNA was isolated from the cells by the procedures of Blin and Stafford (12), and the purified DNA concentration assessed by the fluorometric assay of Burton (13).

ASSAYS OF MRNA LEVELS FOR CLONED GENES (NORTHERN BLOT ASSAYS)

PREPARATION OF CYTOPLASMIC RNA: Cells were grown for 5–6 d to confluency in monolayer cultures on 100-mm dishes under the culture conditions specified above. In each of three experiments, cells were pooled from 5–10 dishes per culture condition. The cells were washed two times with 15 ml of cold PBS. Cells grown on tissue culture plastic were harvested by scraping the plates with a rubber policeman. Those cells grown on Type I collagen coated dishes were released from the gel using a solution of Type I collagenase (0.5 mg/ml, Sigma Chemical Co.). All cells were then washed two times in 10 ml of cold PBS and pelleted, and RNA was isolated using the guanidinium/hot phenol method of Feramisco, et al. (14).

NORTHERN BLOT ANALYSIS: RNA samples were resolved by electrophoresis through 1% agarose, denaturing gels. RNA was transferred to gene screen (New England Nuclear Boston, MA) and then prehybridized and hybridized according to Method II in the gene screen instruction manual (15).

NICK TRANSLATION OF CDNA PROBES: The cDNA clones complementary to specific mRNA were radioactively labeled by nick translation as described by Rigby et al. (16) and as modified by Rajan et al. (17). ^{32}P-labeled cytidine triphosphate ([^{32}P]dCTP, sp act 3,000 Ci/mmol) was included to obtain a specific activity of 1.5–4.5 × 10^8 cpm/μg of DNA. cDNA clones used were complementary to (*a*) mouse liver-specific mRNA, pα-1-antitrypsin (α-1-antitrypsin probe) and pIII1b (18); (*b*) a Chinese hamster ovary "housekeeping" mRNA, pCHOB (19); (*c*) β-actin (20) and α-tubulin (21); and (*d*) rat mRNA for albumin, palb-149 (22), and for GST, pGST94 (23). The clones pα-1-antitrypsin, pIII1b, pCHOB, α-tubulin, and β-actin were generously provided by Dr. James E. Darnell, Jr. (Rockefeller University); pGST94 was provided by John M. Taylor (Gladstone Foundation Laboratories); palb-149 was obtained from Mark A. Zern (Albert Einstein).

RESULTS

Development of Defined Media for Rat Hepatoma Cells

The ability of hepatoma cells to attach and survive (the number of colonies) as well as to grow (the diameter of the colonies) was used to ascertain which factors were active as survival factors or mitogens. The clonal growth assay, developed by Ham and McKeenan (2) and Ham (3) for development of nutritional components of the medium, proved useful as an assay of hormones and growth factors. As indicated in Fig. 1 and Table II, the most critical variable proved to be substratum. The serum supplement was essential for colony formation on tissue culture plastic but not on the collagen gels. Some comparisons could not be made between the substrata. As shown in Fig. 2, the cells on tissue culture plastic formed flat epithelial or epithelioid cells. On the collagenous substrata, the cells were more tightly packed and more three-dimensional and cuboidal. Therefore, comparisons of mitogenic potential were made only on cells on the same substratum.

SCREENS FOR ACTIVE FACTORS

The factors were tested systematically by the following screens:

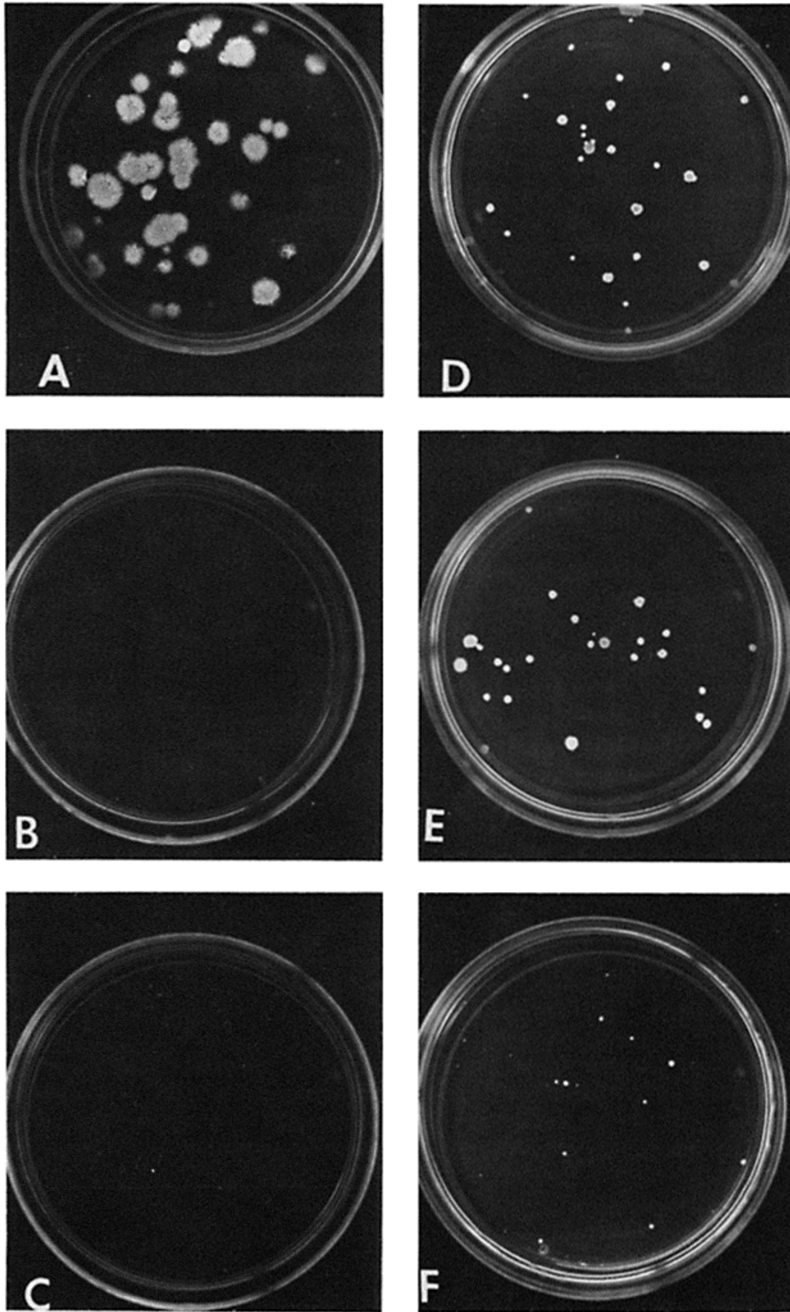

FIGURE 1 Clonal growth of rat hepatoma cells: The influence of culture conditions. The cells were plated at 100 cells per 60-mm dish and cultured for 3 wk. The plates were then rinsed, and the cells were fixed with 100% methanol. The cells were left unstained but were readily recognizable as white patches against a clear (plastic) or a translucent, yellow background (collagen). The cell morphology and the colony number were assessed under each of the conditions given below. Shown are photographs of cultures representative of critical aspects of the results. A summary of the clonal growth behavior is clarified further in the text and in Tables II and III. (*A*) Tissue culture plastic. RPMI 1640 supplemented with SSM. The cells assumed a squamous shape (see also Fig. 2*A*). The plating efficiency was 10–15%. (*B*) Tissue culture plastic plus DM (C). Atlhough this medium permits growth at high seeding densities (above 10^5 cells per 60-mm dish) on tissue culture plastic, it did not permit clonal growth of the cells, i.e., growth at very low seeding densities (100–200 cells per 60-mm dish). Typically, as shown in this figure, no clones survived under this condition. (*C*) Tissue culture plastic plus SF. No clones survived. (*D*) Type I collagen gel plus SSM. The cells assumed a cuboidal shape (see Fig. 2*C*). The plating efficiency was 29–36%. (*E*) Type I collagen gel plus DM (C). The DM is as designated for collagenous substrata. See Table II. The plating efficiency was 37–41%. (*F*) Type I collagen gel plus SF. The plating efficiency was 10–14%.

THE PRIMARY SCREEN: These involved assays of factors suggested by or found active in previous investigations (24–28): insulin, hydrocortisone, transferrin, triiodothyronine, fatty acids (linoleic acid coupled to albumin), glucagon, and inosine. When the factors were added individually on tissue culture plastic, only linoleic acid coupled to albumin gave any viable clones. In contrast, on collagen gel substrata, many factors (see Table I) were able to stimulate colony formation when added individually to SF. Combinations of factors, especially insulin plus transferrin and insulin plus linoleic acid/albumin stimulated colony formation on tissue culture plastic. However, no combination of these factors was able to achieve the clone numbers or sizes seen on collagen gel substrata either in SF or in RPMI 1640 supplemented with insulin and glucagon. From these studies, two new control conditions were chosen: collagen gel substrata plus SF and collagen gel substrata plus medium supplemented with insulin and glucagon. The latter gave colony numbers and sizes ~80% of that seen on collagen gels plus SSM.

GENERAL SCREEN: The factors were tested on cells plated on collagen gels and in SF supplemented with glucagon and insulin or on cells on collagen gels and in SF. There were also five control conditions: (*a*) tissue culture plastic and SSM, (*b*) collagen gels and SSM, (*c*) tissue culture plastic and SF, (*d*) collagen gels and SF, and (*e*) collagen gels and RPMI 1640 plus insulin (10 μg/ml) and glucagon (10 μg/ml).

In Table I are listed factors that were active in the general screen. Activity was assessed as an increase in the number of clones or an increase in the diameter of the clones over that found in the control conditions. Of the 46 factors tested, 20 were positive in stimulating clonal growth, especially follicle stimulating hormone, thyrotropin-releasing factor, prolactin,

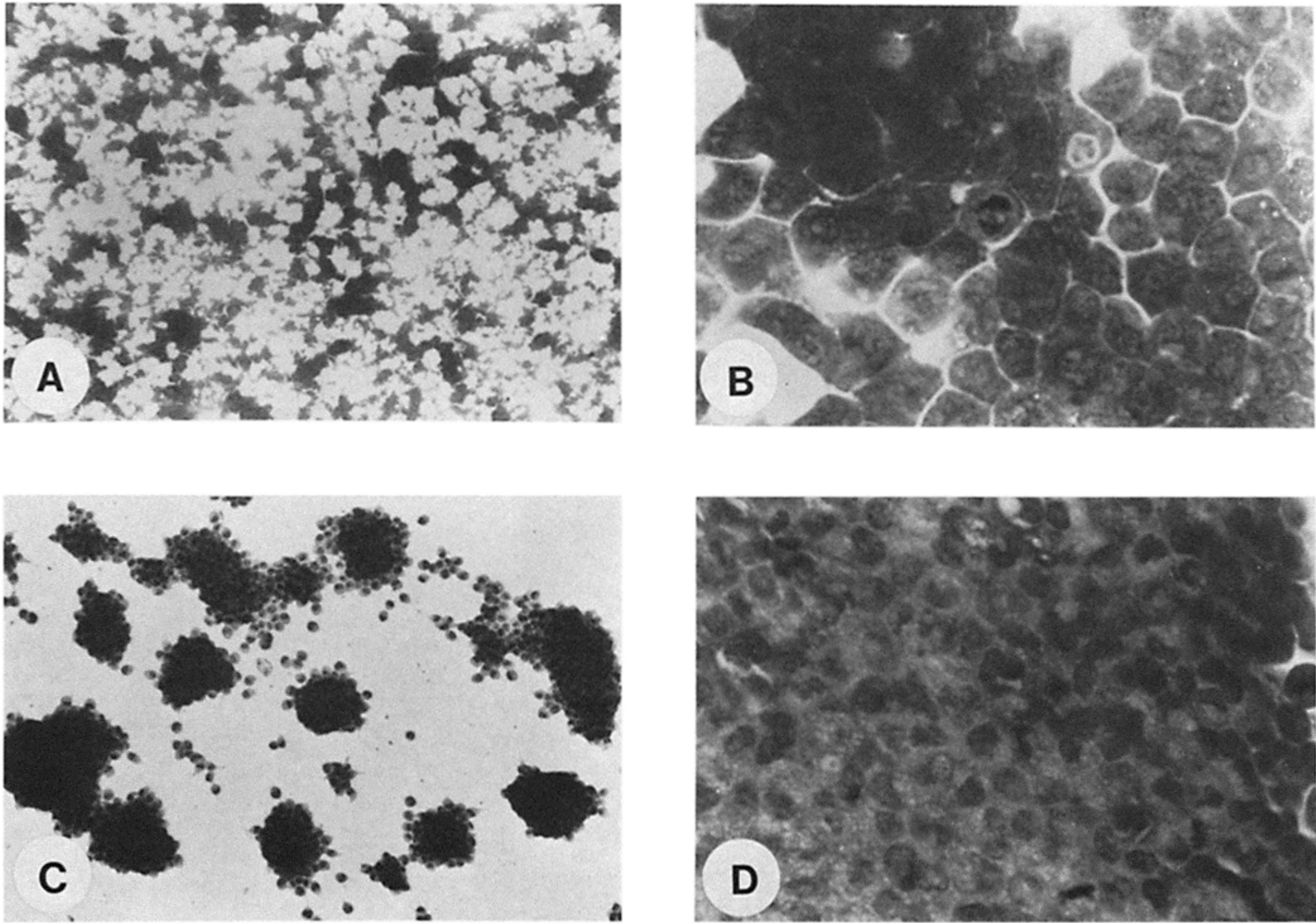

FIGURE 2 Influence of substratum and hormones on cell morphology. The cells were plated under conditions specified below. They were then fixed with 100% methanol and stained with hematoxylin/eosin (those on tissue culture plastic) or with periodic acid schiff (those on collagen gels) and then photographed with a Zeiss microscope and camera. (*A* and *B*) Hepatoma cells on tissue culture plastic and in SSM. The cells assumed a flattened, polygonal shape, the "cobblestone" pattern typical of epithelial cells in culture. (*A*) × 130. (*B*) × 260. (*C* and *D*) Hepatoma cells on type I collagen gels and in DM (C) as listed in Table II for cells on collagenous substrata. Under these conditions the cells assumed a more three-dimensional, cuboidal shape and formed tightly clumped colonies. (*C*) × 130. (*D*) × 260.

and growth hormone, and several trace elements including zinc, copper, manganese, tin, vanadium, selenium, molybdate, and nickel.

OPTIMAL CONCENTRATIONS OF THE ACTIVE FACTORS: The optimal concentrations of each active factor were tested over a four to five logarithmic concentration range. The initial concentrations chosen were those found appropriate for rat liver cells in previous studies (1–3, 24–28). As shown in Table II, the concentration requirements were markedly affected by the substratum. On collagen gels, the concentration of a factor required for optimal growth of the cells dropped significantly, especially that of insulin. The requirements for hydrocortisone, transferrin, and triiodothyronine were essential for cells on plastic but were not essential for cells plated on collagen gels.

SCREENS FOR SYNERGISTIC EFFECTS OF THE HORMONES AND GROWTH FACTORS: Studies on synergies among the growth factors resulted in a reduction of the active factors from 20 to the 11 indicated in Table II. Selection of the factors to be included in the final hormonally supplemented medium was based on the criteria that clonal growth with the factor(s) had to be better than that observed with the control conditions. Although some factors were active when added alone to RPMI 1640, they were not sufficiently active to improve the clonal growth when added to insulin and glucagon in RPMI 1640. Thus, these factors were eliminated as unnecessary in the final version of the hormonally supplemented medium.

The combination of follicle stimulating hormone, growth hormone, thyrotropin-releasing factor, and prolactin produced a greater response than those seen by each of the four factors alone. Various combinations of subsets of these four hormones did not yield as much response as did the four together. However, when tested in RPMI 1640 with insulin and glucagon, only growth hormone and prolactin permitted a higher clonal growth efficiency than observed with insulin and glucagon. Doubling or halving the concentration of prolactin or growth hormone did not produce the same response as seen in assays with the two together.

The addition of trace elements did not alter the number of clones but only the colony size. Of the five cations found active when added individually, the combination of copper and zinc was found as effective as all five cations together. The effect of the combination of these two cations could not be replaced by doubling the concentration of either. In fact, reduced concentrations (less than half) of copper and zinc in

combination produced clone diameters more than double those seen at optimal concentrations of either copper or zinc alone. Combinations of the other cations with the copper and/or zinc resulted in reduced colony sizes.

Of all the trace elements, selenium proved the most potent, when added individually, in stimulating colony formation and increased clone diameters. The influence of selenium, whether added alone or in combination with other trace elements, resulted in colony diameters more than double those observed with other trace elements. In some instances there was actually inhibition of selenium's effect, if it was coupled with other trace elements. For example, vanadium or molybdate alone proved active; but in combination with each other or with selenium, produced clone sizes smaller than those for selenium alone.

Finally, a mixture of the hormones and trace elements (indicated in Table II) was tested. This mixture proved as effective as that seen with SSM. The optimal concentration of each factor in DM was measured while holding the concentration of all others constant. With each adjustment, the screens were run again. From an extensive series of experiments on concentration effects in which concentrations varying over a two logarithmic range were tested, (data not shown), the concentrations of active factors resulting in optimal colony growth were chosen and are listed in Table II.

Synergies Between Substrata and Defined Media Regulating Growth

In Fig. 3 and Table III, data are shown from growth curves of rat hepatoma cells plated onto one of the two substrata and in SF, SSM, DM (C), or DM (C) in which one or the other factors is omitted, in order to learn that factor's relative role in influencing the growth of the cells. The attachment of the cells and the clonal growth efficiency were most affected by the substratum. On tissue culture plastic and collagen gels, the attachment efficiency was zero in SF and in DM (C). If the cells were seeded in SSM for 24 h and then switched to a test medium (SSM, DM [C], or SF) and assessed for colony formation, they produced clones on collagen gels with any of the test media, but they were not able to produce clones on tissue culture plastic except in SSM. Thus, some factor(s) in

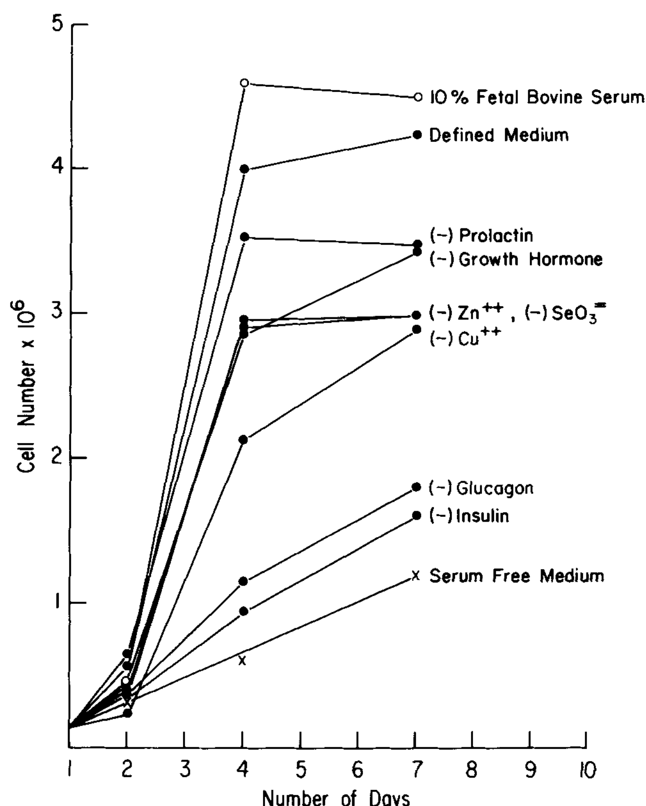

FIGURE 3 Growth curves of rat hepatoma cells on collagen gels and in defined media (C). Rat hepatoma cells were seeded at 5×10^5 cells per 60-mm tissue culture dish coated with 2 ml of type I collagen gel (1 mg/ml). The medium, RPMI 1640, was either SF, SSM, supplemented with hormones according to the listing in Table II for DM (C), or supplemented with the hormones for the DM (C) minus the factor indicated. Thus, (−) prolactin means the medium consisted of RPMI 1640 supplemented with insulin, growth hormone, glucagon, linoleic acid, zinc, selenium, and copper. Triplicate plates were assayed for cell numbers on days 1, 2, 4, and 7. At each time point, the plates were rinsed and the cells were freed from the collagenous substrates by treatment with 0.2% collagenase (Sigma Chemical Co., type I) in SF. The cells were counted by trypan blue exclusion assay for number of viable cells. The growth curves above are representative of results from four separate experiments.

TABLE III

Growth Parameters of Rat Hepatoma Cells Cultured under Various Substrata and Medium Conditions

Substrate / Medium	Growth parameters: Attachment efficiency	Plating efficiency	Doubling time	Saturation density
			h	*cell no.* $\times 10^6$
Tissue culture plastic				
SF	0	$0^{1,2}$	0^1; 57 ± 2.3^2	0^1; 0.8 ± 0.2^2
SSM	91 ± 4	12.0 ± 3^1	25.3 ± 4.6^1	8.5 ± 1.3^1
DM (C)	0	$0^{1,2}$	0^1; 28.6 ± 1.2^2	0^1; 2.7 ± 0.1^2
Type I collagen				
SF	0	0^1	75.0 ± 1.2^1	1.0 ± 0.04^1
	0	12 ± 2^2	49.2 ± 2.7^2	1.8 ± 1.1^2
SSM	88.0 ± 6	32 ± 4^1	21.0 ± 4.7^1	5.3 ± 1.5^1
DM (C)	0	0^1; 39 ± 2^2	25.0 ± 4.6^2	5.6 ± 1.5^2

Rat hepatoma cells were plated under the specified conditions at 100 cells per 60-mm plate for the plating efficiency assays and at 10^5 cells per 60-mm plate for all other assays. The attachment efficiency indicates the percentage of viable cells that attached to the substratum within 24 h after seeding. The plating efficiency indicates the number of clones per number of cells seeded × 100 3 wk after seeding the cells. The doubling time (in hours) and the saturation density (in cell number $\times 10^6$) of the cultures were obtained from growth curves of the hepatoma cells grown under each of the conditions indicated. All cells were plated and grown in the same medium except where indicated: [1]The cells were plated and grown in the test medium. [2]The cells were plated in SSM and 24 h later changed into the test medium.

serum was essential for the initial process of attachment and survival of cells but could be introduced by incubating the cells for a few hours in SSM during the initial seeding. Since the serum factor(s), if introduced during a 24-h period during the initial seeding, was not able to permit colony formation of the rat hepatoma cells on tissue culture plastic, it must require collagenous substrata to stabilize the factor and/or its effect.

The growth rate of the hepatoma cells was affected significantly by the collagenous substrata and especially by insulin and glucagon (Fig. 3 and Table III). In the presence of SSM, the growth rate of the cells was equivalent on both tissue culture plastic and collagen gels. However, in DM (C) the growth parameters were significantly affected by whether the cells were plated onto tissue culture plastic or collagen gels. When components in DM (C) were tested for their relative influence, only the omission of glucagon or insulin (Fig. 3) resulted in a reduced doubling time.

The saturation density was also dramatically affected by substratum. The highest saturation densities observed (8.5×10^6 cells per 60-mm dish) were seen on tissue culture plastic and with SSM. With SF, the cells did not survive on tissue culture plastic. In contrast, the cells did survive in SF if plated onto collagen gels. There was, though, almost no growth on collagen gels and in SF. Use of DM in conjunction with tissue culture plastic resulted in no survival of the cells unless the DM (TCP) designed for cells on this substratum was used. Then the saturation density in DM (using the DM designed for the substratum used, see Table II) was equivalent to that observed in SSM. Although all of the soluble factors in the DM contributed towards saturation density effects, the trace elements, in particular, produced dramatic effects in producing higher saturation densities.

Influence of Media and Substrata on Morphology

As seen in Figs. 1 and 2, the substrata showed dramatic effects on the morphology of the cells. Those plated onto tissue culture plastic showed a flat epithelioid-like shape with pleomorphic nuclei. Those plated on the collagenous substratum exhibited a more cuboidal shape. Over time on the collagenous substrata, the cells formed tightly packed colonies in which the cellular membranes became difficult to visualize at light microscopic levels. The cells also darkened in the cytoplasmic regions. The cells plated into DM (TCP) and onto tissue culture plastic did not change significantly in morphology. However, use of the DM (C) in conjunction with the collagenous substrata accentuated the changes in shape noted above.

Influence of Media and Substrata on the Expression of Liver-specific and Housekeeping Functions

ENZYMATIC ASSAYS: Three liver-specific functions were assayed: UDPGT, GST, and TAT. For two of the enzymes studied, UDPGT and GST, the levels observed in SSM could be maintained at equivalent levels in DM (C) (see Table IV). The collagenous substrata did not significantly augment the levels above those seen in SSM or in DM (C).

TAT was affected both by substrata and by medium conditions. Thus, the TAT levels were significantly augmented by collagenous substrata, by DM (C), and especially by the combination of the two. The realization of the influence of the DM (C) was not made initially, since the standard TAT assay involves incubating the cells under the test conditions and then switching them into serum-free medium with and without dexamethasone for 24 h. As shown in Table V, cells cultured for up to 1 wk in DM (C) and then switched into SF for 24 h for the TAT assay show lowered basal levels of TAT and no induction of TAT by dexamethasone. However, if the cells were cultured for the 24-h assay in DM (C) (or in the controls in SSM) with and without dexamethasone, the basal and induced levels of TAT in DM (C) were found to be the equivalent of or higher than those seen in SSM.

ANALYSES OF SPECIFIC MRNA IN CULTURES: Whereas the enzymatic assays described above required separate cultures for each assay, the assessment of mRNA levels for any number of functions could be made on a single set of cultures per condition. Therefore, a transition was made to evaluate mRNA levels for liver-specific and "housekeeping" functions. We are defining "housekeeping" function as a mRNA that is produced in all tissues; the expression of

TABLE IV

UDPGT and GST Levels in Rat Hepatoma Cells Cultured under Various Conditions

Substratum Medium	UDPGT	GST
	mM PNP/µg DNA per min	*Δ OD/µg DNA per min*
Tissue culture plastic		
SSM	393 ± 50	0.1 ± 0.007
DM (C)	460 ± 57	0.1 ± 0.007
Type I collagen gels		
SSM	490 ± 80	0.1 ± 0.004
DM (C)	463 ± 70	0.1 ± 0.007

The results are the averages from four (UDPGT) or six (GST) experiments in which 10^6 rat hepatoma cells were plated under the conditions specified. The values are listed ± the SEM. In each experiment, there were a minimum of triplicate plates per experimental condition. The cells were maintained under these conditions for 4 d and then assayed for the factor indicated. The levels of the enzymes in normal rat liver are 448 mM PNP/µg DNA per min for UDPGT and 0.321 Δ OD/µg DNA per min for GST. PNP, paranitrophenol.

TABLE V

TAT Levels in Rat Hepatoma Cells. Influence of Hormones Versus Collagenous Substrata

Medium	+ or − dexamethasone	TAT levels (µM HBA/min per µg DNA): Tissue culture plastic	Collagen
SSM	−[1,2]	6.6 ± 2	8.5 ± 1.6
	+[1,2]	19.2 ± 6.2	24.9 ± 6.9
DM (C)	−[1]	3.9 ± 0.8	10.0 ± 1.8
	+[1]	10.1 ± 2	10.0 ± 0.2
	−[2]	4.4 ± 0.9	4.8 ± 0.6
	+[2]	20.3 ± 3.6	25.9 ± 4.8

The data are the averages of six experiments ± SEM. In each experiment, 10^6 rat hepatoma cells were plated onto the appropriate substratum and in one of the two media. The cells were cultured under the conditions for 4 d. The plates were rinsed and then cultured for 24 h either in SF[1] or again in the test medium, SSM or DM (C)[2], supplemented with and without 10^{-6} M dexamethasone. The plates were scraped, and the cells were collected, sonicated in 0.14 M KCl, pH 7.4, and centrifuged at 20,000 g for 30 min. The resulting supernatant was assayed for TAT by the method of Diamondstone (11). An aliquot of each sample was used by assessing the DNA content. TAT levels in normal rat liver are 6.3 µM HBA/min per µg DNA. HBA, *p*-hydroxybenzaldehyde.

"housekeeping functions" is not restricted to a particular cell type, developmental stage, or tissue. Fig. 4 shows the results of analyses of total RNA isolated from cells grown either on tissue culture plastic plus SSM or on collagen gels plus DM (C). RNA was hybridized to four liver-specific functions: albumin (22), GST (23), III1b (18), and α-1-antitrypsin (18), and three housekeeping functions: CHOB (19), α-tubulin (21), and β-actin (20). In *A*, the steady state levels of GST mRNA were greater in cells grown on collagen gels in DM (C) than in those grown on tissue culture plastic in SSM. Though the use of DM (C) plus collagenous substrata resulted in augmented levels in the cells of GST mRNA, the levels were still below those seen for normal rat liver. Albumin, α-1-antitrypsin, and III1b were not expressed under either of the culture conditions tested but are normally expressed in the liver (represented by the blot for α-1-antitrypsin in *B*) (for comparison, see references 18 and 22). CHOB mRNA levels (*C*) were augmented under culture conditions over the levels observed in vivo but were similar under the two culture conditions tested. CHOB was shown previously (19) to represent an mRNA found in Chinese hamster ovary cells and subsequently in all cell types tested. There were greater levels of β-actin and α-tubulin mRNA in cells grown in culture than in rat liver, and the highest levels of these two species of mRNAs were observed in cells in DM (C) on collagen gels as opposed to those in SSM on tissue culture plastic. This finding is represented in *D* by the hybridization of the RNA to β-actin to the DNA clone.

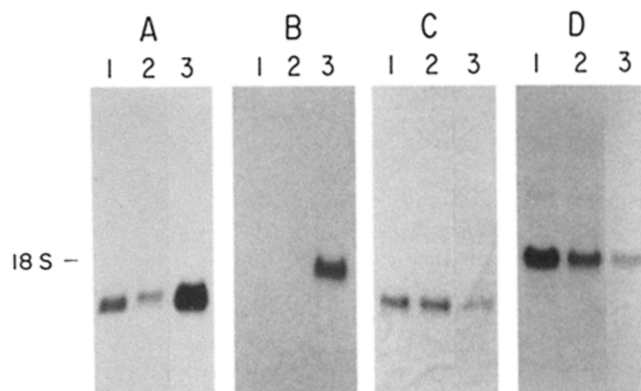

FIGURE 4 Northern blot analyses for housekeeping and liver-specific functions in rat hepatoma cells. Each panel shows hybridization patterns of RNA from normal rat liver or from rat hepatoma cells cultured under different conditions. The hybridization pattern from each function, housekeeping or liver-specific, is shown for the cells under the following conditions: (1) rat hepatoma cells plated on Type I collagen gels and in DM (C), (2) rat hepatoma cells plated on tissue culture plastic and in SSM, and (3) rat liver control (RNA isolated directly from normal liver). For the hepatoma, the cells were cultured for 6 d at which time the total RNA was isolated as described in Material and Methods. The cells were assessed for liver-specific and housekeeping functions with a number of cDNA probes. Some of the liver-specific functions, represented by the findings with GST (*A*), showed much higher steady state levels of mRNA in normal rat liver than in the hepatoma and showed an augmentation in these levels in hepatoma cells plated on collagenous substrata and in defined media. Several of the liver specific functions (albumin, α-1-antitrypsin, and III1b) were never expressed in the hepatoma cells under any condition tested, as represented in *B* in the blots for α-1-antitrypsin. C shows the finding with CHOB, a housekeeping function (19), which is expressed at higher levels in the hepatoma cells than in normal rat liver and which is expressed at equivalent levels under the culture conditions tested. The other housekeeping functions tested (β-actin and α-tubulin), represented by the hybridization pattern in *D* for β-actin, were expressed at higher levels in the hepatoma cells than in normal liver and were significantly augmented by the defined media and collagenous substrata.

DISCUSSION

Cells in culture require serum supplements to the medium to survive and grow. The role of serum in culture media is to supply the cells with hormones, growth factors, and assorted trace elements and nutrients necessary for cell division and/or differentiation (2–4). We used the approaches of Barnes and Sato (4) and of Ham and McKeenan (2) and Ham (3) to systematically test more than 60 factors and hormones in the development of serum-free, hormonally supplemented media for growth in culture of rat hepatoma cells. The development of such media has been done for many cell types (reviewed by Barnes and Sato, reference 4). Therefore, developing serum-free, hormonally supplemented media for hepatoma cells, although extremely useful, is not new theoretically. What proved new and of interest in these experiments was the finding that the composition of the serum-free, hormonally supplemented media is dependent upon the type of substratum used for the cultures and that the growth and/or differentiation of the cells is effected by synergies between hormones and the substrata. For example, none of the hormones or factors tested permitted either clonal growth or growth at high seeding density of the rat hepatoma cells when that factor was added alone to cells plated on tissue culture plastic. This was because cells could not survive on tissue culture plastic and in serum-free medium supplemented with any of the known soluble growth factors or hormones when they were added individually; multiple hormones and growth factor supplements were required. However, if the cells were plated onto collagenous substrata, they could at least survive even in a totally serum-free medium devoid of any of the purified factors. On collagenous substrata, 20 of the factors (Table I), when added alone to serum-free medium, proved active in permitting clonal growth of the cells. Thus, the most critical variable dictating the attachment and survival, and, thereby, the responsiveness of cells to hormones was the substratum. Furthermore, the composition of a serum-free, hormonally supplemented medium necessary to permit optimal growth of the cells was different depending upon whether the cells were on collagenous substrata or tissue culture plastic. In general, when plated onto collagenous substrata, the cells' hormonal requirements for growth were qualitatively and quantitatively reduced (Table II). However, we have not addressed in these experiments the mechanism(s) by which the substratum alters the hormone requirements. Possible interpretations include the following: (*a*) Some factors needed for growth of cells on plastic are perhaps needed for the production of an extracellular matrix by the cells (28–30), and, therefore, when plated on an appropriate matrix, the factor(s) is not needed or needed in reduced amounts. (*b*) The cells may acquire the ability to produce the required factor when plated onto an appropriate substratum (in the rat hepatoma cells, this is a likely explanation for the loss of the transferrin requirement when the cells were plated onto collagenous substrata). (*c*) The substratum may alter the stability of hormone receptors or other aspects of hormonal mechanisms thereby altering the responsiveness of the cells to those

factors (31, 32). Whatever the explanation(s), it is clear that two sets of factors, the soluble hormones and the insoluble components of the extracellular matrix such as collagen, work in synchrony in the regulation of cellular physiology. Although prior studies (31–35) both in vivo and in vitro have hinted at the idea, the principle becomes especially clear from studies with defined culture conditions.

A discussion of the potential mechanism of action of each of the components in the serum-free, hormonally supplemented media on the growth and/or differentiation of the cells seems unwarranted, since there are excellent reviews (2–4) on whatever is known of the relevance of such components in serum-free, hormonally supplemented media for many different cell types including parenchymal liver cells. However, in brief, there are studies both in vivo and in vitro suggesting roles for a number of these factors in the regulation of growth of normal and neoplastic hepatocytes, especially insulin and glucagon (24–28), and the pituitary hormones, growth hormone and prolactin (36, 37). The relevance of trace elements such as copper, zinc, and selenium, which proved so influential in augmenting the saturation density of the hepatoma cultures, is undoubtedly related to their well known ability to act as critical co-factors for various enzymes, functioning as activators or inhibitors and some as integral parts of the enzyme structure (2, 3, 38–46). It has been suggested by Ham and McKeehan (2) and Ham (3) that such factors as trace elements which influence the saturation density are likely to facilitate uptake and utilization of nutrients or to regulate metabolic activity in general. Of the trace elements tested, selenium, SeO_3^-, was the most active in promoting clonal growth. Noguchi et al. (45) and others (44, 46, 47) have postulated that both selenium and vitamin E protect biological membranes from oxidative degradation. Selenium has been shown to act as a component of cytosolic glutathione peroxidase to destroy peroxides before they can attack cellular membranes.

The serum-free, hormone supplemented media, used in conjunction with the appropriate substratum, gave growth curves equal to those with serum-supplemented medium. Because these media were defined empirically, they may not be the only ones enabling the hepatoma cells to grow nor necessarily the unique mixture that stimulates parenchymal cell growth in vivo. Even so, the defined media, with no serum, enables one to deduce potential mitogens for hepatomas under conditions where there is a minimum of undefined variables.

The most important variable in cell attachment, shape, survival, and ability to respond to hormones in growth or differentiation proved to be the substratum, a fact most dramatically indicated in the serum-free, hormonally supplemented media. As shown in Figs. 1 and 2, the collagenous substrata significantly altered cell shape. A popular current hypothesis is that the critical variable regulating growth and/or differentiation and induced by the collagenous substrata is shape change via an influence on specific cytoskeletal patterns (48–51). The cytoskeleton (microtubules, intermediate filaments, and actin) are thought to be coupled through transmembrane proteins to the extracellular matrix which can alter the cell shape and, thereby, the spatial integration of the cytoplasm and the placement of organelles within the cytoplasm (51). The relevance of placement of organelles and cellular components, i.e., the cellular architecture, to growth and/or differentiation is unclear but may contribute in some way to regulation of metabolic activity. It is likely that the matrix influence on the cell is more than mechanical and may include specific and nonspecific chemical interactions between components in the cell membrane (for example, hormone receptors) and in the substratum. These possible interactions are undefined at present but are implied by the change in hormonal responsiveness of the cells depending upon the type of substratum.

The synergies evident between substrata and hormones in regulating growth were also evident in the regulation of cellular functions, both liver-specific and housekeeping functions. That is, liver-specific and housekeeping functions were influenced by hormones, by types of substratum, and often, most effectively of all, by combinations of collagenous substrata and hormones. In prior studies (1, 52), we found that normal cells express tissue-specific and housekeeping functions that are influenced both by type of substratum and by the hormonal constituents in the medium, a finding now corroborated by other similar studies (53). Prior studies (8, 31–33, 47, 54–56) have long implicated the relevance of simple, Type I collagenous substrata to differentiation of mammalian cells. Therefore, in these studies, we contrasted the influence of the collagenous substrata and of the serum-free, hormonally supplemented media on a number of housekeeping and liver-specific functions. In contrast to the past studies on normal cells (1, 52, 53), these hepatoma cells were found to have functions that (*a*) were not expressed at all (and the culture conditions we tested were unable to alter that lack of response (e.g., albumin and α-1-antitrypsin), (*b*) were expressed constitutively at some level that did not change with any culture condition tested (e.g., CHOB), (*c*) were maintained by both collagenous substratum and defined media but only to the same level as observed in SSM (e.g., TAT and UDPGT), or (*d*) could be augmented by collagenous substratum and defined media to levels higher than those observed with classical cell culture conditions (e.g., at least the mRNA for GST, see below). The level of UDPGT, TAT, and GST were found to be significantly reduced or lost altogether in cells cultured in serum-free medium (with no hormonal supplements) whether the cells were plated onto tissue culture plastic or on collagenous substrata. Although the cells were dying when plated onto tissue culture plastic and in serum-free medium, and, therefore, the loss of functions correlated with cell death, a significant percentage of cells survived on collagenous substrata and in serum-free medium. Thus, these functions required both a collagenous substratum and the hormones to be maintained even to the levels observed under classical cell culture conditions.

An important technical note with respect to use of these defined conditions was realized in the assays for TAT. It was discovered that the influence of serum, but not hormone supplements, could be maintained through the 24-h incubation in serum-free medium, the usual conditions for the TAT assay. By assessing the TAT levels with and without dexamethasone in the presence of either serum or the hormones, it was seen that the stability of hormonal factors or their effects were affected by component(s) in serum, and/or the levels of relevant factors in cells were more stable in serum-supplemented medium. Thus, the use of serum-free, hormonally defined media requires modifications in how cell cultures are handled (the frequency of medium changes) and in how they are assayed for particular functions in order to take into account the reduced stability of the factors and/or their effects

under serum-free conditions.

By Nothern blot analysis, all the RNA levels measured were different for the hepatoma cells as compared with normal rat liver. In normal rat liver, the steady state levels of mRNAs for liver-specific functions (GST, albumin, III1b, and α-1-antitrypsin) were high and the housekeeping functions (CHOB, β-actin, and α-tubulin) low. In contrast, in the rat hepatoma cells, the steady state level of mRNA for three liver-specific proteins was either too low to be detected or not transcribed at all (albumin, α-1-antitrypsin, and III1b) or at lower levels (GST) than in normal rat liver, whereas the mRNAs encoding housekeeping functions (CHOB, α-tubulin, and β-actin) were at much higher levels than found in normal rat liver. The various liver-specific and housekeeping functions responded variably to the two culture conditions tested. As noted above, three liver-specific mRNA were not expressed at all. Of those functions, whether liver-specific or housekeeping, that were expressed, one, CHOB, had mRNA levels that were equivalent in the two conditions, whereas the other three (GST, α-tubulin, and β-actin) exhibited increased steady state levels of mRNA when the rat hepatoma cells were grown in DM (C) and on collagen gels as compared with those cultured on tissue culture plastic dishes in serum-supplemented medium. The increased levels of GST mRNA in cells grown on collagen gels and in hormone-supplemented medium was not expressed at the level of the protein (Table IV). This difference may be due to some posttranscriptional control mechanism, or the enzymatic assay may not be as sensitive as analysis of RNA in discerning the differences in GST levels that occur under the culture conditions. Since several liver-specific functions were not expressed at all with any of the culture conditions tested, these conditions can modulate the mRNA steady state levels of transcriptionally active genes, either liver-specific or housekeeping, but do not induce a transcriptionally silent gene.

In summary, this rat hepatoma cell line was found to be only slightly differentiated. It has retained some liver-specific functions (GST) and some housekeeping functions (β-actin and α-tubulin) which were significantly augmented by the hormones in the defined media and/or by collagenous substrata; three functions tested (CHOB, TAT, and UDPGT) were found to be maintained but not augmented by the defined culture conditions; and several liver-specific functions (e.g., albumin and α-1-antitrypsin) were not expressed under any condition tested. Thus, the culture conditions were able to alter levels of expression of those functions already being transcribed but were not able to induce de novo transcription of functions not being expressed. Furthermore, the studies indicated that growth parameters of the cells showed dramatic responses to the serum-free, hormonally defined conditions as compared with the serum-supplemented medium and tissue culture plastic. Further corroborating evidence has been provided by the studies of Goto, et al. (57) who found that these defined conditions dramatically enhanced ferritin synthesis in the presence of iron, a parameter found correlated with growth rates of the hepatoma cultures.

The use of defined culture conditions including serum-free, hormonally supplemented media and collagenous substrata provide culture conditions that can modulate levels of expression of various functions, both liver-specific and housekeeping, in these rat hepatoma cultures. The defined media developed for these slightly differentiated hepatoma cells have led the way to development of culture conditions for growth of normal rat hepatocytes in culture (58). Thus, the studies should enable one to systematically analyze the relevance of substrata and hormones alone or in combination on normal and neoplastic hepatocellular physiology.

We would like to thank Dr. John M. Taylor (Gladstone Foundation Laboratories, San Francisco, CA) for the cDNA probe for GST, Dr. Mark A. Zern (Albert Einstein, New York) for the probe for albumin, and Dr. J. E. Darnell, Jr. (Rockefeller University) for providing the clones, α-1-antitrypsin, III1b, rat α-tubulin, chicken β-actin, and CHOB. Excellent technical assistance of Clifford Liverpool, Dorothy Occhino, Rosina Passela, and Manya Shafran is gratefully acknowledged. Critical readings of the manuscript were provided by Dr. Diana Chiuten and Dr. John Sninsky.

This research was supported by grants from the American Cancer Society (BC-249 and NP-348) and from the National Institutes of Health (AM17702, AM2019, P30CA 1330-66, GM-29090, CA 30117).

Received for publication 24 November 1982, and in revised form 27 June 1983.

REFERENCES

1. Reid, L. 1982. Regulation of growth and differentiation of mammalian cells by hormones and extracellular matrix. *In* From Gene to Protein: Translation into Biotechnology. Fourteenth Miami Winter Symposium. W. J. Whelan, editor. Academic Press, Inc., New York. 53–73.
2. Ham, R., and W. McKeehan. 1979. Media and growth requirements. Cell Culture. *Methods Enzymol.* 58:44–93.
3. Ham, R. 1981. Survival and growth requirements of nontransformed cells. *In* The Handbook of Experimental Pharmacology. Springer-Verlag, Berlin. 1–139.
4. Barnes, D., and G. Sato. 1980. Serum-free medium. A review. *Cell.* 22:649–655.
5. Leinwand, L., R. Strair, and F. Ruddle. 1978. Phenotypic and molecular expression of albumin in rat hepatoma X L cell hybrids. *Exp. Cell Res.* 155:261–268.
6. Strair, R. K., S. H. Yap, B. Nadel-Ginard, and D. A. Shafritz. 1978. Identification of a high molecular weight presumptive precursor to albumin mRNA in the nucleus of rat liver and a rat hepatoma cell line, $H_4A_2C_2$. *J. Biol. Chem.* 253:1328–1331.
7. Chen, T. R. 1977. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* 104:255–262.
8. Michalopoulos, G., and H. C. Pitot. 1975. Primary cultures of parenchymal liver cells on collagen membranes. *Exp. Cell Res.* 94:70–78.
9. Billings, R. E., T. R. Tephly, and R. H. Tukey. 1978. *In* Conjugation Reaction in Drug Biotransformation. A. Aitio, editor. Elsevier/North Holland Biomedial Press, Amsterdam. 365–376.
10. Grover, P. L., and P. Sims. 1964. Conjugation with glutathione. *Biochem. J.* 90:603–606.
11. Diamondstone, T. I. 1966. Assay of tyrosine transaminase activity by conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde. *Anal. Biochem.* 16:395–401.
12. Blin, N., and D. W. Stafford. 1976. Isolation of high molecular weight DNA. *Nucleic Acids Res.* 3:2303.
13. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62:315–323.
14. Feramisco, J. R., J. E. Smart, K. Burridge, D. M. Helfman, and G. P. Thomas. 1982. Co-existence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle. *J. Biol. Chem.* 257:11024–11031.
15. New England Nuclear. 1983. Gene Screen Hybridization Transfer Membrane Instruction Manual. Catalog No. NEF-972. New England Nuclear.
16. Rigby, P. W. J., M. Kieckmann, C. Rhodes, and P. Berg. 1977. Labelling of deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237–251.
17. Rajan, T. V., E. D. Halay, T. A. Potter, G. A. Evans, J. G. Seidman, and D. H. Margulies. 1983. H-2 hemizygous mutants from a heterozygous cell line: role of mitotic recombination. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.
18. Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. E. Darnell, Jr. 1981. Transcriptional control in the production of liver-specific mRNA. *Cell.* 23:731–739.
19. Harpold, M. M., R. M. Evans, M. Salditt-Georgieff, and J. E. Darnell. 1979. Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell.* 17:1025–1035.
20. Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner. 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. *Cell.* 20:95–105.
21. Wilde, C. D., C. E. Crowther, KT. P. Cripe, M. G. Lee, and N. J. Cowan. 1982. Evidence that a human β-tubulin pseudogene is derived from its corresponding mRNA. *Nature (Lond.).* 297:83–84.
22. Zern, M. A., P. R. Chakraborty, N. Ruiz-Opazo, S. H. Yap, and D. A. Shafritz. 1983. Development and use of a rat albumin cDNA clone to evaluate the effect of chronic ethanol administration on hepatic protein synthesis. *Hepatology (Baltimore).* 3:317–322.
23. Kalinyak, J. E., and J. M. Taylor. 1982. Rat glutathione-s-transferase. *J. Biol. Chem.* 257:523–530.
24. Leffert, H., K. Koch, T. Moran, and B. Rubalcava. 1979. Hormonal control of rat liver regeneration. *Gastroenterology* 76:1470–1482.
25. Bucher, N., and T. Swaffield. 1975. Regulation of hepatic regeneration in rats by synergistic action of insulin and glucagon. *Proc. Natl. Acad. Sci. USA.* 72:1157–1160.
26. Monato, U., E. Draghi, and P. Andreis. 1978. Effect of glucagon and insulin on the

growth of neonatal rat hepatocytes in primary tissue culture. *Endocrinology* 102:1155–1166.

27. Motwani, N., N. Unakar, and A. Roy. 1980. Multiple hormone requirements hepatocytes in long-term primary culture. Endocrinology 107:1606–1613.
28. Rieutort, M., M. Lombard, C. Nadal, and M. Strosser. 1980. Rat liver cell cycle and variations of growth hormone (GH) levels in vivo. *Eur. J. Cell Biol.* 22:493 (Abstr. S1467).
29. Weiss, R., A. Reddi, and E. Lenk. 1980. Insulin regulates the fibronectin mediated collagenous matrix-cell interaction. *J. Cell Biol.* 87(2, Pt. 2):124. (Abstr.)
30. David, G., and M. Bernfield. 1981. Type I collagen reduces the degradation of basal lamina proteoglycan by mammary epithelial cells. *J. Cell Biol.* 91:281–286.
31. Salomon, D. S., L. A. Liotta, and W. R. Kidwell. 1981. Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. *Proc. Natl. Acad. Sci. USA.* 78:382–386.
32. Gospodarowicz, D., G. Greenburg, and C. R. Birdwell. 1978. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res.* 38:4155–4171.
33. Gospodarowicz, D., D. Delgado, and I. Vlodavsky. 1980. Permissive effect of the extracellular matrix on cell proliferation *in vitro*. *Proc. Natl. Acad. Sci. USA.* 77:4094–4098.
34. Clark, G., M. Stroker, A. Ludlow, and M. Thernton. 1970. Requirement of serum for DNA synthesis in BHK 21 cells. Effects of density, suspension and virus transformation. *Nature (Lond.).* 227:738–801.
35. McKeehan, W. L., and K. A. McKeehan. 1980. Serum factors modify the cellular requirements for Ca^{2+}, K^{+}, Mg^{2+}, phosphate ions, 2-oxocarboxylic acid for multiplication of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 77:3417–3421.
36. Nicoll, C. S. 1978. Comparative aspects of prolactin physiology: is prolactin the initial growth hormone in mammalian species also? *In* Progress in Prolactin Physiology and Pathology. Elsevier/North Holland Biomedical Press, New York. 175–187.
37. Wong, B. S., M. E. Chenoweth, and A. Dunn. 1980. Possible growth hormone control of liver glutamine synthetase activity in rats. *Endocrinology.* 106:268–274.
38. Swierenga, S., J. Whitfield, A. Boynton, J. MacManus, R. Rixon, R., M. Sikorska, B. Tsang, and P. Walker. 1980. Regulation of proliferation of normal and neoplastic rat liver cells by calcium and cyclic AMP. *Ann. NY Acad. Sci.* 341:294–311.
39. Chen, R., D. Eakin, and P. Wanger. 1974. Biological function of metallothioein. II. Its role in liver metabolism in the rat. *Nutr. Rep. Int.* 10:195–207.
40. Fuijioka, M., and I. Lieber. 1964. A zinc requirement for the synthesis of deoxyribonucleic acid by rat liver. *J. Biol. Chem.* 239:1164–1167.
41. Ohtake, H., K. Hasegawa, and M. Koga. 1978. Zinc-binding protein in the liver of neonates: normal and partially hepatectomized rats. *Biochem. J.* 174:999–1005.
42. Van Campen, D. R., and P. U. Scaife. 1967. Zinc interference with copper absorption in rats. *J. Nutr.* 91:473–476.
43. Evans, G. 1973. Copper homeostasis in the mammalian system. *Physiol. Rev.* 53:535–570.
44. Rotruck, J., A. Pope, H. Gunther, A. Swanson, D. Hofeman, and W. Hockstra. 1973. Selenium: biochemical role as a component of glutathione peroxidase. *Science (Wash. DC).* 179:588–590.
45. Noguchi, T., A. Cantor, and M. Scott. 1973. Mode of action of selenium and vitamin E in prevention of exudative diathesis in chicks. *J. Nutr.* 103:1502–1511.
46. Lewko, W., and K. McConnell. 1982. Biphasic influence of selenium on cell growth and the synthesis of collagen in cultured mammary tumor cells. *Fed. Proc.* 41:623.
47. McKeehan, W. L., W. G. Hamilton, and R. G. Ham. 1976. Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 73:2023–2027.
48. Emmerman, J., and D. Pitelka. 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro.* 13:346–348.
49. Pardoll, D. M., B. Vogelstein, and D. S. Coffey. 1980. A fixed site of DNA replication in eucaroytic cells. *Cell.* 19:527–536.
50. Ben-Ziev, A., S. Farmer, and S. Penman. 1980. Protein synthesis requires cell surface contact while nuclear events respond to cell shape in anchorage-dependent fibroblasts. *Cell.* 21:365–372.
51. Condeelis, J. 1981. Microfilament-membrane interactions in cell shape surface architecture. *In* International Cell Biology. H. Schweiger, editor. Springer-Verlag, New York. 306–320.
52. Rojkind, M., Z. Gatmaitan, S. Mackensen, M. Giambrone, P. Ponce, and L. Reid. 1980. Connective tissue biomatrix: its isolation and utilization for long-term cultures of normal rat hepatocytes. *J. Cell Biol.* 87:255–263.
53. Wicha, M. S., G. Lawrie, E. Kohn, P. Bagavandross, and T. Mahn. 1982. Extracellular matrix promotes mammary epithelial growth and differentiation *in vitro*. *Proc. Natl. Acad. Sci. USA.* 79:3213–3217.
54. Sirica, A., W. Richards, Y. Tsukada, C. Sattler, and H. Pitot. 1979. Fetal phenotypic expression by adult rat hepatocytes on collagen gel-nylon meshes. *Proc. Natl. Acad. Sci. USA.* 76:283–287.
55. Bissell, D., and P. Guzelian. 1981. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann. NY Acad. Sci.* 349:85–98.
56. Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473–485.
57. Goto, Y., M. Paterson, and I. Listowsky. 1983. Iron uptake and regulation of ferritin synthesis by rat hepatoma cells in serum-free, hormone supplemented media. *J. Biol. Chem.* 258:5248–5255.
58. Enat, R., D. M. Jefferson, N. Ruiz-Opazo, Z. Gatmaitan, L. Leinwand, and L. Reid. 1983. Hepatocyte proliferation *in vitro*: its dependence on serum-free hormonally defined medium and on substrata of extracellular matrix. *Proc. Natl. Acad. Sci. USA.* In press.