GROWTH CONTROL OF DIFFERENTIATED FETAL RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE

VII. Hormonal Control of DNA Synthesis and Its Possible Significance to the Problem of Liver Regeneration

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ABSTRACT

The initiation of DNA synthesis has been studied in quiescent primary cultures of fetal rat hepatocytes using defined hormones and chemically defined medium.

Preparations of crystalline insulin (0.01–10 μg/ml) or 20,000-fold purified somatomedin (0.01–1 μg/ml) are stimulatory. Growth hormone (0.025 μg/ml) and hydroxycortisone (0.025 μg/ml), 3′:5′-GMP! (10^{-5} M) fail by themselves to initiate DNA synthesis but added together with insulin, enhance the stimulatory response by 50–100%. Thyroid hormones (L-T_3, L-T_4, 7.5–30 ng/ml) are by themselves without effect, but when they are added to thyroid hormone-depleted serum, the reconstituted mixtures show an enhanced capacity to initiate DNA synthesis. In contrast, glucagon (0.01 μg/ml) inhibits the insulin-stimulated response by about 50% without reducing basal DNA synthesis rates.

The results described here and in the accompanying two reports indicate that environmental control over the initiation of DNA synthesis is complex, and can involve the participation of many factors. The in vitro findings are consistent with the concept that liver regeneration is hormonally controlled and raise the possibility that alterations of the intrahepatic ratio of insulin to glucagon are growth regulatory.

INTRODUCTION

Growth control studies with fetal rat hepatocyte cultures (1–4) indicate that a macromolecular insulin-enriched serum fraction, SFI (5), promotes initiation of DNA synthesis. This may be brought about, as suggested in the preceding report (6), by elevating protein synthesis rates as a consequence of, or coupled with SFI-stimulated utilization and/or synthesis of L-arginine, a growth-regulatory nutrient also shown to be identical with an obligatory growth-limiting conditioning factor produced by these cells (6). Because insulin was found under similar conditions to stimulate the rate of arginine incorporation into protein (6), it seemed likely that hormones involved with regulat-

ing hepatic nitrogen balance such as insulin, growth hormone, and hydroxycortisone (7–10), or that hormones with insulin-like activity, such as somatomedin (11–15), might also promote in vitro initiation of DNA synthesis. Studies described in this report provide direct evidence for this hypothesis, extend the concept that hepatocellular growth is susceptible to control by a multiplicity of serum factors (5), including known hormones (16–20), and raise the possibility that in vivo alterations of the intrahepatic ratio of insulin to glucagon are growth regulatory.

MATERIALS AND METHODS

Reagents

The following hormone preparations were purchased from Sigma Chemical Corp. St. Louis, Mo. Prolactin (sheep, 20–50 IU/mg), growth hormone (porcine, 0.7 IU/mg), thyrotropic stimulating hormone (bovine, 0.5–1.0 IU/mg), insulin (bovine, 24.3 IU/mg), 3,3′,5-L-thyronine (T_3)[1], 3,3′,5,5′-L-thyronine, hydroxycortisone-21-sodium succinate, and crystalline porcine glucagon. Bovine luteinizing hormone was the gift of the Endocrinology Study Section, The National Institutes of Health, Bethesda, Maryland. Purified follicle-stimulating hormone and FGF (21) were the generous gifts of W. Vale and D. Gospodarowicz (Salk Institute), respectively. Somatomedin C-enriched preparations (15) were the kind gifts of J. J. Van Wyk (20,000-fold pure, 0.25 units/μg, $\leq$0.25 microunits immunoreactive insulin/μg). Adrenal corticotrophic hormone (porcine, 84 units/mg, Grade B) was purchased from Calbiochem, San Diego, Calif. The following nucleotides and nucleosides were also obtained from Sigma: guanosine 5′-monophosphoric acid, guanosine 2′:3′-cyclic monophosphoric acid, guanosine 3′:5′-cyclic monophosphoric acid, N^2, O^2-dibutyryl guanosine 3′:5′-cyclic monophosphoric acid, N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (Grade II), and guanosine. Tissue culture supplies were obtained as previously described (1–5). CH_3-[^{3}H]thymidine (sp act 20 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. Insulin radioimmunoassay kits were obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

Assays

DNA synthesis initiation assays were performed with 9–11-day old quiescent cultures. 1–2% of the cells are in S-phase as judged by autoradiography following a 12–24-h pulse with [^{3}H]dT established as previously described (4, 6). Unless noted, all experiments were conducted by addition to the cultures of 2 ml fresh, arginine-supplemented (0.4 mM) medium together with substances to be tested (0.1–0.3 ml), the indicated concentrations of which represent the final concentration initially present in the medium. Substances for testing were prepared fresh before use. All hormone dilutions were made using plastic pipettes and tubes (Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif.).

Preparation of Thyroid Hormone-Free Serum

Dialyzed fetal bovine serum was alkalinized to pH 13.0 with 12 M NaOH to dissociate thyroid hormone-protein complexes, and dextran-coated charcoal was added (6.6 g Norite A per 100 ml serum) to adsorb free hormone (22). The supernate was collected and the pH adjusted to 8.6 with 6 N HCl. This material was then centrifuged three times (Ivan Sorvall, Inc., Newtown, Conn., 4°C, 10 min, 20,000 g) to remove charcoal, dialyzed by standard procedures vs. isotonic NaCl, sterilized by filtration (0.45-μm Nalgene filter; Nalge Co., Nalgene Labware Div., Rochester, N. Y.), and stored at −20°C. This procedure removed >99% of the serum tetraiodothyronine (initial concentration ca. 14.5 μg %) as determined chemically by a T_4-protein binding assay (23), and >99% of the serum L-T_3, as determined by the recovery of radioactivity associated with L-T_3 after incubation of dFBS with 1×10^5 cpm L-T_3 (Abbott Scientific Products Div., Abbott Laboratories, South Pasadena, Calif., 365 μCi/ml, 5 μg/ml) and subsequent extraction as described above.

RESULTS

Hormonal initiation of fetal hepatocellular DNA synthesis in chemically defined medium is shown in Figs. 1 and 2. Under these conditions, as indicated previously (6), greater than 90% of the responding cell population is derived from G_0 or early G_1.

[1] *Abbreviations used in this paper:* ACTH, adrenal corticotrophic hormone; 3′:5′-AMP!, N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid; db3′:5′-GMP!, N^2O^2-dibutyryl guanosine 3′:5′-cyclic monophosphoric acid; dFBS, dialyzed fetal bovine serum (3); FGF, 3T3-fibroblast growth factor (21); FSH, follicle-stimulating hormone; GH, growth hormone; GMP, guanosine 5′-monophosphoric acid; 2′:3′-GMP!, guanosine 2′:3′-cyclic monophosphoric acid; 3′:5′-GMP!, guanosine 3′:5′-cyclic monophosphoric acid; [^{3}H]dT, CH_3-[^{3}H]thymidine; LH, luteinizing hormone; L-T_3, 3,3′,5-L-thyronine (tri-iodothyronine); L-T_4, 3,3′,5,5′-L-thyronine.

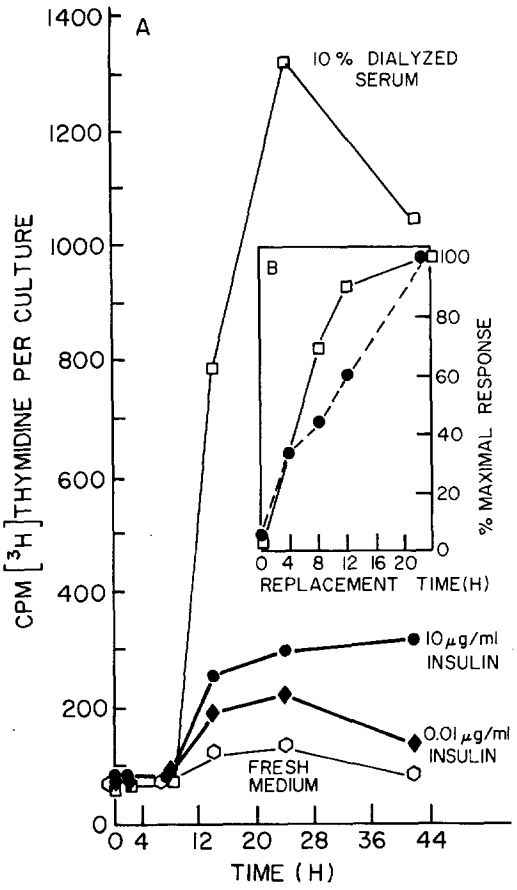

FIGURE 1 Initiation of DNA synthesis by insulin in chemically defined medium. (A) Time course. Fresh medium containing insulin (◆—◆, 0.01 μg/ml; ●—●, 10.0 μg/ml), 10% dFBS (□—□), or 0.2 ml isotonic NaCl (hexagons) was added to quiescent cultures (0.5×10^5 cells/dish). At varying times, [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) was added, and after a 2-h pulse (points shown) the incorporation of TCA-precipitable radioactivity determined. Abscissa: time (hours) after fresh medium change. Ordinate: counts per minute [^{3}H]dT incorporated per culture per 2-h pulse. (B, inset) Insulin or serum-exposure times required to stimulate DNA synthesis. Fresh medium containing insulin (10 μg/ml) or 10% vol/vol dFBS was added to quiescent cultures (time zero [cpm/culture/2-h pulse] = 80). At 4, 8, or 12 h thereafter, the dishes were washed twice with insulin-free and serum-free medium and replaced with similar medium. Control dishes received continuous exposure. All cultures were pulse labeled and assayed as in Fig. 1 A. Absolute incorporation rates (100% maximal response) for dishes receiving continuous exposure were: insulin, 240; 10% serum, 1,640. Abscissa: replacement time (hours), i.e., time at which dishes were washed and replaced with fresh insulin-free and serum-free medium. Ordinate: percent of maximal response.

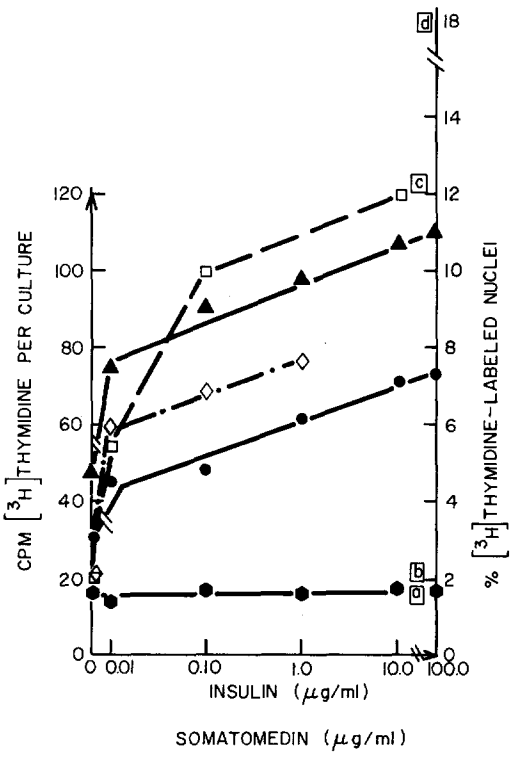

FIGURE 2 Quantitative initiation of DNA synthesis by insulin and somatomedin; enhancement by growth hormone and hydroxycortisone and by L-arginine. Fresh medium containing 0.4 mM L-arginine was added to quiescent cultures (0.29×10^5 cells/dish) together with insulin (□---□) or somatomedin (◇-·-·-◇) at the concentrations (μg/ml) indicated by the abscissa. These cultures were incubated for 6 h with [^{3}H]dT (1.25 μCi/ml) between 18 and 24 h later, and the proportion of DNA-synthesizing cells was determined by autoradiography (1,000 cells/dish scored [6]) and indicated by the right ordinate. Similar experiments were conducted with the following additions: growth hormone (0.025 μg/ml) and hydroxycortisone-succinate (0.025 μg/ml), ([a]); growth hormone (0.025 μg/ml) *or* hydroxycortisone-succinate (0.025 μg/ml), ([b]); growth hormone (0.025 μg/ml), hydroxycortisone-succinate (0.025 μg/ml), and insulin (0.05 μg/ml), ([c]); growth hormone (0.025 μg/ml), hydroxycortisone-succinate (0.025 μg/ml), and insulin (1.0 μg/ml), ([d]); and 10% vol/vol dFBS (not shown, = 60%). In separate experiments (solid lines), fresh medium containing L-arginine (solid hexagons, zero; ●—●, 0.004 mM; and ▲—▲, 0.4 mM) and insulin was added at varying concentrations to quiescent cultures as indicated on the abscissa. These cultures were pulse labeled with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} dT) between 22 and 24 h and the radioactivity incorporated in TCA-precipitable material per culture was determined (left ordinate).

Kinetics of DNA Synthesis After Hormone Addition

Preparations of crystalline bovine insulin (0.01 μg/ml and 10.0 μg/ml), or 20,000-fold purified, insulin-deficient somatomedin (1 μg/ml) were added to quiescent cultures together with fresh medium. The kinetics of [^{3}H]dT incorporation into DNA (2) were measured for 0–43 h and the results, for the insulin addition, are shown in Fig. 1 A. It is seen that a 10-h lag period ensued, after which the initial incorporation rates, at 10–12-h, increased in proportion to the final concentrations of hormone initially added to the medium. DNA synthesis rates remained elevated in cultures which had received high doses, but not low doses of insulin, suggesting that additional cells were being recruited to synthesize DNA (4, 6). Similar kinetics were observed with cultures that received somatomedin (1 μg/ml, not shown). As expected from previous observations (4, 6), DNA synthesis rates were stimulated slightly in the absence of hormones, a response which represented under these conditions $\leq$1–2% increase in the basal nuclear labeling index, as determined by autoradiography.

Commitment to Synthesize DNA

To determine whether continuous exposure to insulin or to 10% vol/vol dFBS-containing medium was required to obtain maximal DNA synthesis rates, quiescent cultures were incubated for varying periods of time (0–12 h), after which the medium was changed to fresh, hormone-free and serum-free medium. Cultures were then pulse labeled with [^{3}H]dT (22–24 h) and the quantity of label incorporated into DNA was determined. The results, shown in Fig. 1 B, indicated that serum was more efficient than insulin in "committing" a cell population to synthesize DNA after either 8 or 12 h of continuous exposure. In both cases, however, about 30% of the maximal DNA synthesis occurred with incubation times of 4–8 h, but little DNA synthesis was detectable after incubation times of 4 h. Similar results using serum additions have been reported with 3T3 cells (24) and secondary chick embryo (25) fibroblasts.

Recruitment of DNA-Synthesizing Cells

Autoradiographic measurements (18–24 h post-addition) indicated that the stimulation of DNA synthesis by preparations of both insulin and somatomedin resulted from the initiation of DNA synthesis in a small but significant fraction of the cells. This is shown in Fig. 2.

Quantitative comparisons between efficiencies of stimulation of each of the hormone preparations cannot be made until homogeneously pure somatomedin C has been obtained. Dose-response curves (Fig. 2) indicated that neither of the hormone preparations was capable of replacing serum, which would imply that additional factors, perhaps specific carriers (5, 26), are required to obtain a maximal response. The presence of 1 mg/ml crystalline albumin did not enhance hormonal stimulation, so that adsorption to the plastic tissue culture dish would be unlikely to have caused the high requirement. Alternatively, heterogeneity of the responding cell population might explain these observations, with respect to either cell type or position in G_1 (25). These possibilities seem unlikely, however, owing to the selection procedures employed (1, 3, 6), and to the observation (Fig. 2) that increasing amounts of hormone increased the nuclear labeling index which would be expected from a noncommitted, G_0 cell population responding to increasing amounts of serum (25). Finally, in considering the possibility that crystalline insulin contains a minor contaminant, preliminary use of guinea pig anti-insulin antiserum, the generous gift of E. A. Arquilla (University of California at Irvine), in attempts to block insulin-stimulated DNA synthesis has been inconclusive owing to the simultaneous presence in unfractionated normal guinea pig serum of possibly specific (5) inhibitory material.

Visual inspection of the cultures by phase microscopy revealed the presence of mitotic figures only after hormone addition and no sooner than 23–24 h. Scarcity of these dividing cells, however, made difficult the direct attempts to determine whether mitotic cells had previously synthesized DNA, although the kinetics of DNA synthesis seen in Fig. 1 A would indirectly lend support to this interpretation.

Enhancement of Insulin-Initiated DNA Synthesis

Stimulation of insulin-initiated DNA synthesis was enhanced synergistically by the additional presence of 0.1–1.0% vol/vol dialyzed fetal bovine serum as shown by the increasing slopes of the dose-response curves in Fig. 3. Because the concentrations of immunoreactive insulin (1–10 pg) present in these concentrations of sera failed to stimu-

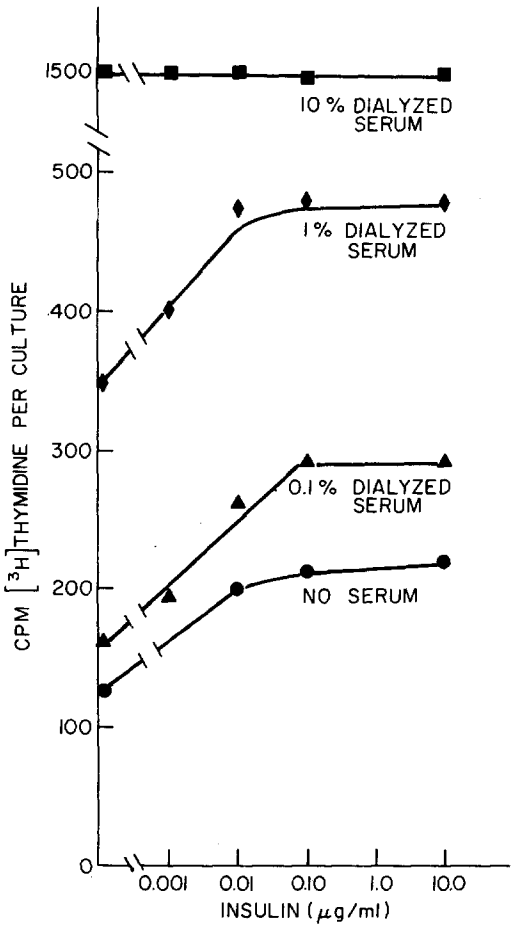

FIGURE 3 Serum enhancement of insulin-initiated DNA synthesis. Fresh medium containing varying concentrations of insulin (0–10 μg/ml) and supplemented with either isotonic saline (●—●, 0.2 ml/dish), or dFBS (▲—▲, 0.1%, vol/vol containing 1–2 pg immunoreactive insulin; ◆—◆, 1.0%, vol/vol containing 10–20 pg immunoreactive insulin; ■—■, 10%, vol/vol containing ca. 300 pg immunoreactive insulin was added to quiescent cultures (0.53×10^5 cells/dish). All cultures were pulse labeled with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) between 22 and 24 h and the radioactivity incorporated into TCA-precipitable material per culture was determined. Abscissa: insulin added to culture medium (μg/ml). Ordinate: cpm [^{3}H]dT culture.

late detectable DNA synthesis (Fig. 2), the results of Fig. 3 further indicated that additional serum factors are involved with initiating DNA synthesis (5, 27) which, if present, might also function to reduce the insulin requirement.

In regard to this possibility, combinations of somatomedin and insulin (μg/ml, respectively, 1:1, 1:10, and 10:1), failed to produce synergistic effects. However, the combined addition of preparations of GH (0.025 μg/ml) and hydroxycortisone (0.025 μg/ml), previously reported to stimulate, in the presence of insulin, protein synthesis in the isolated perfused liver (7, 8), lowered the insulin requirement for initiating DNA synthesis and synergistically enhanced by about 50% the insulin-stimulated response. This is shown in Fig. 2, boxes C and D, respectively. GH and hydroxycortisone were not stimulatory when added either together (0.025 μg/ml, Fig. 2, box A) or separately (0.025 μg/ml, Fig. 2, box B). Higher concentrations of hydroxycortisone inhibited DNA synthesis (≥ 1 μg/ml), which is qualitatively and quantitatively consistent with results observed for in vivo hepatocellular growth studies (28).

In light of recent reports which have suggested that cyclic guanosine nucleotides may be involved with growth stimulation of mammalian cells (29–31), it was of interest to determine whether one or more of these compounds could replace insulin. The results of these experiments are shown in Fig. 4. The stimulatory effects of insulin could not be replaced by any one of the compounds tested (3′:5′-GMP!, N^2,O^2-dibutyryl 3′:5′-GMP!, 2′:3′-GMP!) including GMP and guanosine (not shown) within a concentration range of 10^{-7}–10^{-3} M. In the presence of excess insulin (10 μg/ml), however, the rate of DNA synthesis was enhanced optimally at 10^{-5} M by 3′:5′-GMP! (100%), N^2,O^2-dibutyryl 3′:5′-GMP! (60%), and 2′:3′-GMP! (50%). No prior estimate of the extent of contamination with butyric acid of the dibutyryl compound was made which might account for the reduced enhancing effect observed with this compound. At present, this appears an unlikely possibility because suppression of insulin-stimulated DNA synthesis with derivatives not containing dibutyryl groups also was observed at higher concentrations (10^{-4} M).

The efficiency of insulin-stimulated DNA synthesis also was enhanced by L-arginine, as shown in Fig. 2, much the same as the interaction between serum and L-arginine was shown to be a determinant of DNA synthesis capacity in these cultures (6). Stimulation of DNA synthesis required the simultaneous addition of insulin (or SFI (5)) and L-arginine because in separate experiments it was found that pretreatment of the cultures with arginine-free, insulin-supplemented, or 10% vol/vol SFI-supplemented medium for varying periods of time (0–24 h) before arginine-addition (0.4 mM) failed to shorten the 10-h onset time.

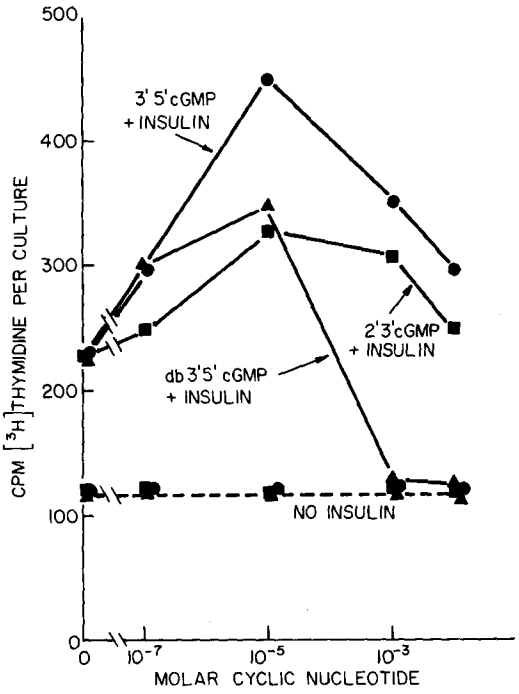

FIGURE 4 Cyclic guanosine nucleotide enhancement of insulin-initiated DNA synthesis. Fresh medium containing varying concentrations of cyclic guanosine nucleotides (■—■, guanosine 2':3'-cyclic monophosphoric acid; ▲—▲, N^2,O^2-dibutyryl guanosine 3':5'-cyclic monophosphoric acid; ●—●, guanosine 3':5'-cyclic monophosphoric acid) with or without insulin (0.1 μg/ml) was added to quiescent cultures (0.39×10^5 cells/dish). All cultures were pulse labeled with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) between 22 and 24 h and the radioactivity incorporated into TCA-precipitable material per culture was determined. Abscissa: cpm [^{3}H]dT per culture. Ordinate: molar concentration of cyclic nucleotide.

A spectrum of additional pituitary-derived polypeptide hormones was also tested (0.01–1.0 μg/ml) for DNA synthesis-stimulating capacity, using chemically defined complete media and quiescent cultures. Hormones tested included LH, TSH, FSH, ACTH, prolactin, and a recently purified fibroblast growth factor, FGF (21). None of these hormones was found to stimulate or to enhance insulin-stimulated DNA synthesis.

DNA Synthesis Requirement for Thyroid Hormones

In view of past (16, 17) and recent studies (20) implicating thyroid-derived hormones in in vivo hepatocellular growth control, as well as recent in vitro studies demonstrating growth-controlling effects of thyroid hormones with cultured pituitary tumor cells (32, 33), it was of interest to determine whether or not cultured fetal hepatocytes would also demonstrate a similar growth requirement.

In preliminary experiments using chemically defined complete media, little stimulation of DNA synthesis was observed (Table I) with either L-T_3 or L-T_4 (≤20% above control and therefore not statistically significant under the conditions of the assays used). Combinations of L-T_3 and glucagon (20) (0.01–1.0 μg/ml) similarly failed to stimulate DNA synthesis. In addition, insulin-stimulated DNA synthesis was not enhanced by L-T_3. However, if either L-T_3 (7.5–15 ng/ml), or L-T_4 (10–30 ng/ml) was added to quiescent cultures together with thyroid hormone-free dFBS (2.5–15% vol/vol), then 60–70% of the DNA synthesis-stimulating capacity was restored, with L-T_3 appearing about twice as efficient as L-T_4. This also is shown in Table I.

Inhibition of Insulin-Stimulated DNA Synthesis by Glucagon

With regard to the catabolic effects of glucagon (34), many of which antagonize insulin-mediated functions (10, 35–37), as well as recent surgical studies implicating glucagon as a possible circulating inhibitor of hepatocellular proliferation (19), it was of interest to determine the direct effects of glucagon upon the initiation of DNA synthesis in cultured fetal rat hepatocytes.

Chemically defined complete medium supplemented with insulin (10.0 μg/ml), and varying amounts of porcine glucagon (0.001 μg/ml–1.0 μg/ml) were added together to quiescent cultures, and the rate of DNA synthesis was measured 22–24-h later. The results are shown in Fig. 5. It may be seen that the basal rate of [^{3}H]dT incorporation into DNA was unaffected by glucagon throughout the concentration range tested. Independent measurements (trypan-blue exclusion and cell attachment [3]) also showed that no detectable cytotoxic effects had occurred as a result of exposure to the hormone for 24 h. However, the stimulatory effect of insulin was suppressed by about 50% as the concentration of glucagon was increased ($\geq 2 \times 10^{-9}$ M). Autoradiography also revealed diminished nuclear labeling: basal level (2%); insulin, 10.0 μg/ml (10.3%); insulin, 10.0 μg/ml plus glucagon, 0.5 μg/ml (5.8%).

N^6,O^2-dibutyryl 3':5'-AMP! ($\geq 10^{-4}$ M) was

TABLE I

Stimulation of DNA synthesis in fetal rat hepatocyte cultures by thyroid hormone-depleted fetal bovine serum reconstituted with L-T_3 or L-T_4

		Percent vol/vol initial serum				
L-T_3	L-T_4	0	2.5	5.0	15.0	
ng/ml	*ng/ml*					
0	0	350	900	1,750	2,200	Untreated dFBS
0	0	340	390	490	940	Depleted dFBS
0	10	350	590	ND	1,100	
0	30	370	780	ND	1,540	
7.5	0	390	ND	1,000	ND	
15.0	0	415	ND	1,270	ND	

Fetal rat hepatocytes were prepared for DNA synthesis initiation assays, and thyroid hormone-depleted dFBS (A_{280} = 22/ml) was prepared as described in Materials and Methods. The additions were made to 11-day old quiescent cultures (0.62×10^5 cells/30-mm diameter dish) as 0.1–0.3-ml aliquots in 2 ml fresh medium supplemented with 0.4 mM L-arginine. The values given represent the rate of DNA synthesis (cpm [^{3}H]dT per culture) as determined by 2-h pulse-labeling 22–24 h later with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT). The amount of A_{280} nm material present in untreated dFBS was 25.1/ml.

ND, not determined.

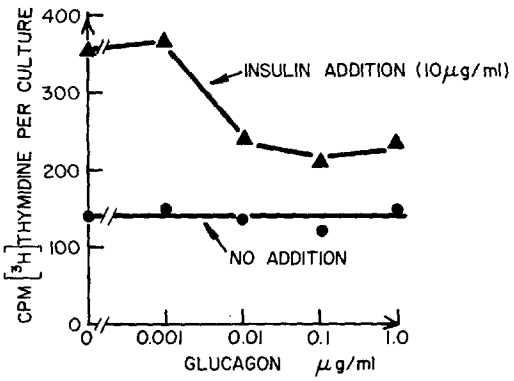

FIGURE 5 Inhibition of insulin-initiated DNA synthesis by glucagon. Fresh medium containing varying concentrations of glucagon (0–1.0 μg/ml) with or without insulin (10 μg/ml) was added to quiescent cultures (0.57×10^5 cells/dish). All cultures were pulse labeled with [^{3}H]dT (1.25 μCi/ml, 3×10^{-6} M dT) between 22 and 24 h and the radioactivity incorporated into TCA-precipitable material per culture was determined. Abscissa: glucagon added to culture medium (μg/ml). Ordinate: cpm [^{3}H]dT incorporated per culture per 2-h pulse.

found to inhibit both insulin-stimulated DNA synthesis and the basal DNA synthesis rate, an expected finding in view of the known cytotoxic effects of this drug (38), as well as its capacity to inhibit thymidine transport (39). At high concentrations, glucagon (10.0 μg/ml) also reduced (50–80%) the basal DNA synthesis rate. Therefore, it may be that in vitro suppression by glucagon (3×10^{-9} M) of insulin-stimulated initiation of DNA synthesis is mediated by intracellular cyclic AMP because: (*a*) these cultured cells do show increased intracellular cyclic AMP levels within 1–2 min in response to glucagon (H. Leffert and W. Seifert, unpublished observations); and (*b*) in growth cycle studies (5), intracellular cyclic AMP levels are inversely correlated with lag, log, and stationary growth states (H. Leffert and W. Seifert, unpublished observations).

Physiological concentrations (10^{-8} M) of L-epinephrine, suggested to act upon the liver via cyclic AMP-mediated reactions (40), although less efficiently than glucagon (41), did not inhibit insulin-stimulated DNA synthesis in this culture system. Much higher concentrations (10^{-4} M) were required to produce effects similar to those seen with toxic levels of cyclic AMP (10^{-4} M).

DISCUSSION

The initiation of DNA synthesis by serum factors in quiescent fetal rat hepatocyte cultures has been shown, in this report, to involve at least six hormones. Included in this group are insulin, growth hormone, hydroxycortisone, the thyroid hormones L-T_3 and L-T_4, enriched preparations of somatomedin-C(15), and glucagon.

Two main conclusions are to be derived from these studies: First, an *interaction* among many

different serum factors can control the initiation of DNA synthesis. This was indicated by the observation that although none of the hormones detectably promoted significant initiation of DNA synthesis, except for microgram levels of insulin and somatomedin, conditions could be found whereby insulin effects were enhanced. For example, in the presence of nanogram levels of growth hormone and hydroxycortisone (Fig. 2, box C), nanogram levels of insulin were found to stimulate DNA synthesis sixfold over background, but in the absence of these "permissive" hormones, 200 times as much insulin was required to achieve a similar effect.

Enhancement of insulin-stimulated DNA synthesis also was brought about by 10^{-5} M 3′:5′-GMP! (Fig. 4). In contrast, glucagon (1–1000 ng/ml) had neither detectable stimulatory nor inhibitory effects, but when combined with insulin, partially inhibited (50%) the stimulation of DNA synthesis (Fig. 5).[2] A further example of multiple serum factor interactions is suggested from the finding that thyroid hormones (L-T_3, L-T_4) were by themselves nonstimulatory at nanogram levels, but were stimulatory in the presence of the appropriate additional serum factors Table I. The "permissive" factor(s) which thyroid hormones apparently require for expression of their DNA synthesis-promoting capacity remain to be determined.

Second, if a multiplicity of factors including those yet to be identified (5, 6) controls initiation processes, then the problem of initiating DNA synthesis becomes a problem in understanding *concerted* hormone action (42–50).

For example, some evidence with this system suggests that both insulin and SFI (5) stimulate increased utilization and/or synthesis of a growth-limiting nutrient (arginine) required to increase protein synthesis rates which precede the induction of DNA synthesis (6). However, as indicated in this report, since pretreatments of the cells with either insulin or SFI fail to reduce the onset time for DNA synthesis, since insulin or SFI must be added simultaneously, together with arginine, and since only insulin or insulin-like material (somatomedin [11–15]) appear to be the most limiting of the hormones studied thus far, one of the earliest prereplicative events would appear to be an interaction between arginine, insulin, and the cell membrane (42, 43, 51, 52). Whether or not this proposed interaction is modulated (53) by additional hormones including those described in this report remains to be determined, although with regard to recent reports (37, 54), the results here suggest a possible link between thyroid hormones, the regulation of cholesterol metabolism (55), membrane permeability changes (56–58), and DNA synthesis.

In light of the above conclusions, it is puzzling that only a fraction of the cells responds to either single or multiple hormone additions. The evidence tends to argue against a trivial explanation such as monohormonally responsive (nonparenchymal) subpopulations (see Results), (3). Alternatively, if heterogeneity exists amongst G_0 cells in regard to their steady state interactions and/or "preactivation" with or by any one of a set of limiting serum factors, then this could explain the "quantized" nature of the response. This interpretation is consistent with similar in vitro findings using a cloned 3T3-fibroblast cell line (59), and would help to clarify the observed differences between serum and insulin in regard to commitment-time kinetics (Fig. 1 B). This interpretation is also consistent with in vivo evidence that L-T_3 can preactivate quiescent liver, assuming that the hormone acts directly, in preparation for subsequent stimulation (20). However, differences in the cellular topology of cultured hepatocytes, the more peripherally situated cells (1) being more available or sensitive to interactions with one or more specific hormones, remains an additional explanation (60). This possibility has been ruled out, however, in the fibroblast system (59).

Finally, it is of interest to consider whether or not the results described for this hepatocyte system (5, 6) have any bearing upon the problem of liver regeneration. Of possible significance is the finding that a partially purified, insulin-enriched serum fraction (SFI) which promotes DNA synthesis in vitro also was found to promote some detectable hepatocellular DNA synthesis in vivo (5). In addition, thyroid hormones which have been shown to promote in vivo hepatocellular growth (16, 17, 20) also appear to play a role in the in vitro control of DNA synthesis in this system. Moreover, although ablation of no single endocrine gland has yet abolished liver regeneration, those ablations examined thus far, including parathyroidectomy (61), hypophysectomy (62), and

[2] These observations have been confirmed in similar studies carried out by: Paul, D., and S. Walter. 1974. *J. Cell. Physiol.* Submitted for publication.

adrenalectomy (63), all significantly alter the response. So, too, does partial chemical ablation by alloxan of pancreatic β cells (64). These results taken together would all be consistent with a "multiple factor" hypothesis.[3] Furthermore, although recent in vivo studies implicate systemically administered glucagon as a stimulatory hormone (20), in contrast to other reports (19) as well as the results described here, the in vivo action of glucagon and thyroid hormones also could be one of indirectly stimulating insulin release (68–71). In this regard, recent in vivo studies support the concept that *both* insulin and glucagon are involved with hepatocellular growth (72) in a manner consistent with the results described in this in vitro system (Fig. 5).

The possibility is suggested, therefore, that alterations of the intrahepatic ratio, or balance, of insulin to glucagon (I:G) may play a crucial, regulatory role in hepatocellular growth processes. The "reversal" of a low I:G, such as that obtained by the administration of insulin to alloxan-diabetic rats (64) or of amino acids to protein-starved rats (73, 74), could set into motion additional endocrine and hepatic changes (75–78) required to initiate DNA synthesis in the liver.

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REFERENCES

1. LEFFERT, H., and D. PAUL. 1972. *J. Cell Biol.* **52**:559.
2. PAUL, D., H. LEFFERT, G. SATO, and R. W. HOLLEY. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:374.
3. LEFFERT, H., and D. PAUL. 1973. *J. Cell. Physiol.* **81**:113.
4. LEFFERT, H., and S. SELL. 1974. *J. Cell Biol.* **61**:823.
5. LEFFERT, H. 1974. *J. Cell Biol.* **62**:767.
6. KOCH, K., and H. L. LEFFERT. 1974. *J. Cell Biol.* **62**:780.
7. JOHN, D. W., and L. L. MILLER. 1969. *J. Biol. Chem.* **244**:6134.
8. MILLER, L. L., and E. E. GRIFFIN. 1971. Insulin Action. Academic Press, Inc., New York. 487.
9. JEFFERSON, L. S., and A. KORNER. 1967. *Biochem. J.* **104**:826.
10. MILLER, L. L. 1965. *Fed. Proc.* **24**:737.
11. SALMON, W. D., JR., and W. H. DAUGHADAY. 1957. *J. Lab. Clin. Med.* **49**:825.
12. DAUGHADAY, W. H., K. HALL, M. S. RABEN, W. D. SALMON, J. L. VAN DEN BRANDE, and J. J. VAN WYK. 1972. *Nature (Lond.).* **235**:107.
13. VAN WYK, J. J., K. HALL, J. L. VAN DEN BRANDE, and R. P. WEAVER. 1971. *J. Clin. Endocrinol. Metab.* **32**:389.
14. DULAK, N. C., and H. TEMIN. 1973. *J. Cell. Physiol.* **81**:153.
15. VAN WYK, J. J., L. E. UNDERWOOD, R. L. HINTZ, D. R. CLEMMONS, S. VOINA, and R. P. WEAVER. 1974. *Rec. Prog. Horm. Res.* **30**: In press.
16. CANZANELLI, A., D. REPPORT, and R. BUILD. 1949. *Am. J. Physiol.* **157**:225.
17. EPPINGER, H. 1937. Die Leberkrankheitem. Springer Verlag, Vienna.
18. YOUNGER, L. R., J. KING, and D. F. STEINER. 1966. *Cancer Res.* **26**:1408.
19. PRICE, J. B., K. TAKESHIGE, M. H. MAX, and A. B. VOORHEES. 1972. *Surgery (St. Louis).* **72**:74.
20. SHORT, J., R. F. BROWN, A. HUSAKOVA, J. R. GILBERTSON, R. ZEMEL, and I. LIEBERMAN. 1972. *J. Biol. Chem.* **247**:1757.
21. D. GOSPODAROWICZ. 1974. *J. Biol. Chem.* In press.
22. MOUGEY, E. H., and J. W. MASON. 1963. *Anal. Biochem.* **6**:223.
23. ALEXANDER, N., and J. F. JENNINGS. 1974. *Clin. Chem.* **20**:553.
24. TODARO, G. J., G. K. LAZAR, and H. GREEN. 1963. *J. Cell Comp. Physiol.* **66**:325.
25. TEMIN, H. 1971. *J. Cell. Physiol.* **78**:161.
26. VAN WYK, J. J., K. HALL, J. L. VAN DEN BRANDE, R. P. WEAVER, K. UTHNE, R. L. HINTZ, J. H. HARRISON, and P. MATHEWSON. 1971. *Proc. Second Internatl. Symp. Excerpta Med. Int. Congr. Ser. No. 244.* 155.
27. VAHERI, A., E. RUOSLAHTI, T. HOVI, and S. NORDLING. 1973. *J. Cell. Physiol.* **81**:355.
28. HENDERSON, I. C., R. E. FISHEL, and J. N. LOEB. 1971. *Endocrinology.* **88**:1471.
29. HADDEN, J. W., E. M. HADDEN, M. K. HADDOX, and N. D. GOLDBERG. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3024.
30. STEINER, A. L., A. S. PAGLIAVA, L. R. CHASE, and D. M. KIPNIS. 1972. *J. Biol. Chem.* **247**:1114.
31. KRAM, R., and G. M. TOMKINS. 1973. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1659.

[3] Similar conclusions have been proposed from studies on tail regeneration in adult newts (65–67).

32. SAMUELS, H., J. S. TSAI, and R. CINTRON. 1973. *Science* (*Wash. D. C.*). **181**:1253.
33. SAMUELS, H., and J. S. TSAI. 1973. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3488.
34. MARLISS, E. B., T. T. AOKI, and G. F. CAHILL, JR. 1972. *In* Glucagon: Molecular Physiology, Clinical and Therapeutic Implications. P. J. Lefebvre and R. H. Unger, editors. Pergamon Press, Ltd., Oxford. 123.
35. JEFFERSON, L. C., J. H. EXTON, R. W. BUTCHER, E. W. SUTHERLAND, and C. R. PARK. 1968. *J. Biol. Chem.* **243**:1031.
36. LAKSHMANAN, M. R., C. NEPOKROEFF, and J. W. PORTER. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3516.
37. NEPOKROEFF, C. M., M. R. LAKSHMANAN, G. C. NESS, R. E. DUGAN, and J. W. PORTER. 1974. *Arch. Biochem. Biophys.* **160**:387.
38. ROZENGURT, E., and A. B. PARDEE. 1972. *J. Cell. Physiol.* **80**:273.
39. HAUSCHKA, P. V., L. P. EVERHART, and R. W. RUBIN. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3542.
40. BUTCHER, R. W., G. A. ROBISON, and E. W. SUTHERLAND. 1972. *In* Biochemical Actions of Hormones. G. Litwack, editor. Academic Press, Inc., New York. **2**:21.
41. PARK, C. R., and J. H. EXTON. 1972. *In* Glucagon: Molecular Physiology Clinical and Therapeutic Implications. P. J. Lefebvre and R. H. Unger, editors. Pergamon Press, Ltd., Oxford. 77.
42. KRAHL, M. E. 1974. *Annu. Rev. Physiol.* **36**:331.
43. ADAMSON, L. F., A. C. HERINGTON, and J. BORNSTEIN. 1972. *Biochim. Biophys. Acta.* **279**:146.
44. TEPPERMAN, J., and H. TEPPERMAN. 1960. *Pharmacol. Rev.* **12**:301.
45. KRAHL, M. E. 1961. The Action of Insulin on Cells. Academic Press, Inc., New York.
46. MACDONALD, R. I., and A. KORNER. 1971. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **13**:62.
47. TOMKINS, G. M., T. D. GELEHRTER, D. GRANNER, D. MARTIN, JR., H. H. SAMUELS, and E. B. THOMPSON. 1969. *Science* (*Wash. D.C.*). **116**:1474.
48. LITWACK, G., and S. SINGER. 1970. *In* Biochemical Actions of Hormones. G. Litwack, editor. Academic Press, Inc., New York. II:114.
49. TATA, J. R. 1970. *In* Biochemical Actions of Hormones G. Litwack, editor. Academic Press, Inc., New York. I:89.
50. OPPENHEIMER, J. H. 1973. *Mt. Sinai J. Med.* **40**:491.
51. CUATRECASAS, P. 1969. *Proc. Natl. Acad. Sci. U. S. A.* **63**:450.
52. FREYCHET, P., J. ROTH, and D. M. NEVILLE. 1971. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1833.
53. SNODGRASS, P. J. 1972. *Arch. Fr. Mal. App. Dig.* **61**:405*c*.
54. NESS, G. C., R. E. DUGAN, M. R. LAKSHMANAN, C. M. NEPOKROEFF, and J. W. PORTER. 1973. *Proc. Natl. Acad. Sci. U.S.A.* **70**:3839.
55. BROWN, M. S., S. E. DANA, and J. L. GOLDSTEIN. 1974. *J. Biol. Chem.* **249**:789.
56. INBAR, M., M. SHINITZKY, and L. SACHS. 1974. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **38**:268.
57. BRETSCHER, M. S. 1973. *Science* (*Wash. D. C.*). **181**:622.
58. PAPAHADJOPOULOS, D. 1974. *J. Theor. Biol.* **43**:329.
59. HOLLEY, R. W. 1974. *Proc. Natl. Acad. Sci. U. S. A.* In press.
60. DULBECCO, R. 1970. *Nature* (*Lond.*). **227**:802.
61. RIXON, R. H., and J. F. WHITEFIELD. 1972. *Proc. Soc. Exp. Biol. Med.* **141**:93.
62. RABES, H. M., and H. BRANDLE. 1969. *Cancer Res.* **29**:817.
63. BUCHER, N. L. R., and R. A. MALT. 1971. *N. Engl. J. Med. Prog. Ser.*
64. YOUNGER, L. R., J. KING, and D. F. STEINER. 1966. *Cancer Res.* **26**:1408.
65. VETHAMANY-GLOBUS, S., and R. A. LIVERSAGE. 1973. *J. Embryol. Exp. Morphol.* **30**:397.
66. VETHAMANY-GLOBUS, S., and R. A. LIVERSAGE. 1973. *J. Embryol. Exp. Morphol.* **30**:415.
67. VETHAMANY-GLOBUS, S., and R. A. LIVERSAGE. 1973. *J. Embryol. Exp. Morphol.* **30**:427.
68. SAMOLS, E., J. M. TYLER, and V. MARKS. 1972. *In* Glucagon: Molecular Physiology, Clinical and Therapeutic Implications. P. J. Lefebvre and R. H. Unger, editors. Pergamon Press, Ltd., Oxford. 151.
69. UNGER, R. H., and P. J. LEFEBVRE. 1972. *In* Glucagon: Molecular Physiology Clinical and Therapeutic Implications. P. J. Lefebvre and R. H. Unger, editors. Pergamon Press, Ltd., Oxford. 213.
70. MARLISS, E. B., C. B. WOLLHEIM, B. BLONDEL, L. ORCI, A. E. LAMBERT, W. STAUFFACHER, A. A. LIKE, and A. E. RENOLD. 1973. *Eur. J. Clin. Invest.* **3**:16.
71. ZUNZ, E., and J. L. BARRE. 1935. *Arch. Int. Physiol. Biochim.* **42**:1.
72. STARZL, T. E., A. FRANCAVILLA, C. G. HALGRIMSON, F. R. FRANCAVILLA, K. A. PORTER, T. BROWN, and C. W. PUTNAM. 1973. *Surg. Gynecol. Obst.* **137**:179.
73. SHORT, J., N. B. ARMSTRONG, R. ZEMEL, and I. LIEBERMAN. 1973. *Biochem. Biophys. Res. Commun.* **50**:430.
74. ANTHONY, L. E., and G. FALOONA. 1974. *Metab.* (*Clin. Exp.*) **23**:303.
75. TAVILL, A. S., A. G. EAST, E. G. BLACK, D. NADKARNI, and R. HOFFENBERG. 1973. *In* Protein Turnover. CIBA Foundation Symposium 9 (new series)., G. E. W. Wolstenholme and M. O'Connor, editors. Elsevier, Excerpta Medica, and North Holland; Amsterdam, London, and New York. 155.
76. GREENE, H. L., O. D. TAUNTON, F. B. STIFEL, and R. H. HERMAN. 1974. *J. Clin. Invest.* **53**:44.
77. MACMANUS, J. P., D. J. FRANKS, T. YONDALE, and B. M. BRACELAND. 1972. *Biochem. Biophys. Res. Commun.* **49**:1201.
78. ILLIANO, G., G. P. E. TELL, M. I. SIEGEL, and P. CUATRECASAS. 1973. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2443.