The asialoglycoprotein receptor (AsGR) is characteristic of fully differentiated hepatocytes. AsGR expression in confluent cultures of HepG2 cells grown in minimal essential medium (MEM) requires a 300-350-dalton dialyzable fraction of fetal bovine serum (FBS).

Addition of dialyzed FBS (DFBS) of $10^{-7}$ M biotin or biocytin $\text{(M, 372)}$ permitted full expression of AsGR by HepG2. Affinity chromatography of FBS on streptavidin-Sepharose abolished its ability to support AsGR production. The bound material, when released by heat denaturation and resolved by thin layer chromatography, yielded three cinnamaldehyde-positive components, of which the major detectable one migrates with authentic biocytin and reconstitutes DFBS.

Sera from several species, which do not support AsGR production by HepG2, contain less than 10% biotin found in FBS as determined by direct enzyme-linked immunosorbent assay. These results indicate that biotin or a derivative is the low molecular weight serum factor of FBS required for expression of AsGR. Isolation of messenger RNA from HepG2 revealed no difference in AsGR transcripts when cells were grown in MEM-10% FBS or MEM-10% DFBS. Thus a biotin-dependent post-transcriptional event permits the ultimate expression of the AsGR by HepG2 cells.

*b* This work was supported by National Institutes of Health Grants DK-29297, DK-17702, and the Emil L. Zuckerman Foundation for Research in Childhood Liver Disease. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Liver Research Center, U-517, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

** Present address: Dept. of Physiology and Biophysics, Mt. Sinai Medical School, New York, NY 10029.

1 The abbreviations used are: AsGR, asialoglycoprotein receptors; MEM, minimal essential medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DFBS, dialyzed FBS.

by chemical carcinogenesis in rats, AsGR activity (3) and lectin protein (4) are progressively lost. In hepatic regeneration induced by two-thirds hepatectomy, AsGR activity is selectively lost from the cell surface, whereas normal intracellular concentrations are retained (5).

The relationship to hepatocellular maturation is also apparent in cultured cells. The human hepatoblastoma line HepG2 expresses maximum receptor activity only in confluent cultures (6). HepG2 cells grown to confluence in minimal essential medium (MEM) made 10% with respect to fetal bovine serum (FBS) demonstrate an AsGR with ligand-binding characteristics and molecular mass comparable to the receptor purified from human liver. However, dialysis or ultrafiltration, with removal of the low molecular weight fraction of FBS, dramatically reduces expression of AsGR, under conditions where protein synthesis and total cellular protein content are comparable to control cells (7). Since reconstitution of dialyzed FBS (DFBS) with a 300–350-dalton ultrafiltrate of FBS restores full expression of AsGR, we analyzed this fraction to discover the nature of the bioactive factors.

MATERIALS AND METHODS

(--)-Biotin, biocytin, egg white avidin, and monomeric streptavidin-agarose were purchased from Sigma; biotinylated horseradish peroxidase was obtained from Zymed Laboratories, San Francisco, CA. The cell culture system, preparation of FBS fractions, assays of AsGR activity, antibody preparation and immunodetection procedures have been described previously (7). Cells were plated at a density of 10^6 cells/35 mm^2 dish in MEM-10% FBS for 24 h, and achieved confluence in 4–6 days in MEM supplemented with DFBS, biotin, and various sera. In later experiments cells were grown in Nu Serum (NS) or MEM-10% NS made $10^{-6}$ M in biotin.

Recombinant plasminogen used as probes included a full-length rat AsGR cDNA probe (pRHL-4) provided by Dr. K. Drickamer, Columbia University, a chicken $\beta$-actin probe (8), and HHL-1, isolated from a gt11 human liver cDNA expression library (CloneTech Laboratories, Inc., Palo Alto, CA) using the pRHL-4 probe. The isolated human clone HHL-1 was subcloned into a riboprobe pSP64 cloning vector (Promega Biotech) using standard techniques (9).

RNA Isolation and Northern Blotting—RNA was extracted with guanidine thiocyanate (10) from approximately 10^6 HepG2 cells grown to confluence in MEM-10% FBS or MEM-10% DFBS; then passed over an oligo(dT)-cellulose column to enrich for poly(A)* RNA (11). RNA samples were then electrophoresed on horizontal denaturing formaldehyde-agarose gels and transferred to nitrocellulose membranes which were hybridized to $^{32}$P-nick-translated DNA (12), washed twice with 2 X SSC at 22 °C for 10 min (low stringency) and exposed to Kodak XAR-5 x-ray film using image-intensifying screens. For cell lysate mRNA dot-blot assay, cells were harvested in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, an aliquot was removed for protein determination using the Bradford reagent (Bio-Rad), and the lysate was made 0.25% Nonidet P-40. Following centrifugation at 11,000 rpm for 2 min, the supernatant was made 7% SSC-formaldehyde and heated at 80 °C for 15 min. Equal aliquots,
based on protein equivalents, were applied to a Nytran membrane and baked at 80°C in a vacuum oven for 1 h, until dry (13) before hybridization with 32P-nick-translated DNA. Blots were washed twice with 2 × SSC at 22°C for 5 min, then 2 × SSC containing 0.5% SDS at 65°C for 30 min, 0.1 × SSC at 22°C for 30 min, and finally 0.2 × SSC, made 0.1% SDS and 0.1% sodium pyrophosphate at 55°C for 15 min (high stringency). After developing blots probed with the cDNA for rat or human AsGR, membranes were washed and reprobed with chicken β-actin cDNA.

Thin Layer Chromatography (TLC)—Samples were concentrated by lyophilization, redisolved, and applied to Avicel precoated TLC plates (2.5 × 20 cm scored, Analtech, Inc., Newark, DE) and ascending chromatography performed with 1-butanol/pyridine/acetic acid/water (32:5:25:5:20) solvent system. After development, lanes were spray with ninhydrin or 4-dimethylaminozincaldehyde and heated at 80°C to develop colored product.

ELISA—Titretek (Linbro) wells were coated with egg white avidin (10 μg/well) in diluting buffer of 85 mM NaCO3, pH 9.6, incubated at 37°C for 60 min, followed by addition of 200 μl of 5% BSA in diluting buffer, further incubation at 37°C for 60 min and six washes with 50 mM PBS, pH 7.2, containing 0.5% Tween 20. Samples to be tested for biotin content were added to an equal volume of biotinylated horseradish peroxidase in PBS (1:40,000), and 100 μl placed in each well for incubation at 37°C for 60 min. After six washes with PBS-Tween 20, 100 μl of 0.1 M citrate buffer, pH 5.0, containing 0.03% H2O2 and o-phenylenediamine (Sigma) (1 tablet/12 ml) was added to each well. Following development of yellow reaction product at 22°C for approximately 15 min, an equal volume of 1 N NaOH was added and A405 measured in a Titretek plate reader.

RESULTS

The addition of biotin or biocytin to medium supplemented with dialyzed FBS (Fig. 1) during logarithmic growth supported normal expression of the AsGR by confluent HepG2 cells. Biotin was most effective at or above a final concentration of 10−8 M. Attempts to deplete FBS of biotin by adding avidin directly to MEM-10% FBS were unsuccessful, probably due to cellular uptake of the avidin-biotin complex (14); however, passage of FBS or serum ultrafiltrates containing the low molecular weight bioactive factor (Amicon YM-2 and YC-05 filtrates) over streptavidin-Sepharose did deplete them of the active factor.

To identify the active species in FBS retained by streptavidin-Sepharose, the gel was boiled in 10 mM NaOH for 20 min to release the heat-denatured product, as the active factor was previously shown to be alkali- and heat-stable (100°C for 1 h) (7). This concentrated supernatant was then subjected to TLC. Biocytin (RF 0.43) and two unidentified cinnamaldehyde-positive derivatives (with RF 0.68 and 0.98) were detectable, whereas biotin (RF 0.87) was not. Authentic biotin was detectable at 10−7 M. Elution from the cellulose of four bands corresponding to these RF values into MEM-dFBS, followed by addition of the filter-sterilized material to cultured HepG2 cells, resulted in full reconstitution and expression of the AsGR (Fig. 2). As a control, streptavidin-Sepharose did not previously incubated with FBS liberated no detectable bioactive species by these techniques.

Sera from other sources which were shown not to support AsGR production by HepG2 cells were analyzed by direct ELISA for their content of biotin (Table I). These sera contained <10% biotin content of FBS (2 × 10−7 M). However goat serum is enriched in biotin and comparable to FBS in its ability to support AsGR production, as measured by immuno-

![Fig. 1. Effect of growing HepG2 cells in biotin-depleted or -enriched medium on steady-state concentration of AsGR (45 kDa). Control HepG2 cells (C) were grown to confluence in 10% dFBS-MEM (2 × 10−8 M biotin final concentration), dialyzed (D) were grown in 10% dFBS (<10−10 M), and other cells were grown in 10% dFBS-MEM to which biotin had been added with final concentrations of 10−8−10−9 M. Lysate equivalent to 1 × 106 cells was applied to each lane and the protein resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, then incubated with a 1:100 dilution of monospecific anti-human AsGR antiserum followed by 125I-labeled protein A.](image1)

![Fig. 2. Immunodetectable AsGR in HepG2 cells following reconstitution of dFBS with biotin derivatives removed from serum with streptavidin-Sepharose. TLC fractions were eluted into 10% dFBS-MEM, assuming 50% yield from the original FBS, and HepG2 cells were grown, harvested, and analyzed as described in the legend to Fig. 1.](image2)

![Fig. 3. AsGR and actin transcripts in HepG2 cells grown in FBS- or dFBS-enriched medium. Poly(A)+ RNA was prepared from control cells (MEM-10% FBS (lane 1)), cells grown in MEM-10% dFBS (lane 2), rat liver (lane 3), and angler fish pancreas (lane 4), electrophoresed, transferred to nitrocellulose, and probed as described under "Materials and Methods" with (A) rat AsGR cDNA probe, and (B) chicken β-actin cDNA probe.](image3)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Determination of biotin content of sera by direct ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Source</td>
<td>Biotin (×10−8 M)</td>
</tr>
<tr>
<td>Cow</td>
<td>20</td>
</tr>
<tr>
<td>Fetal (3)</td>
<td>20</td>
</tr>
<tr>
<td>Newborn (1)</td>
<td>ND</td>
</tr>
<tr>
<td>Adult (2)</td>
<td>ND</td>
</tr>
<tr>
<td>Rat (3)</td>
<td>1.8</td>
</tr>
<tr>
<td>Rabbit (3)</td>
<td>2</td>
</tr>
<tr>
<td>Goat (2)</td>
<td>18</td>
</tr>
<tr>
<td>Horse (2)</td>
<td>2</td>
</tr>
<tr>
<td>Human (6)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*The normal steady-state level of AsGR in confluent HepG2 cells grown in the presence of various sera was compared with control cells, by immunoblot, as described in the legend to Fig. 1. 
*ND, not detected. The limits of detection of the ELISA are 8 × 10−8 M.
under "Materials and Methods" was fractionated on a 1% agarose gel containing formaldehyde, transferred to nitrocellulose, and probed with the nick-translated cDNA clone HHL-1. Hybridization was at 43 °C for 48 h. Kb, kilobase.

DISCUSSION

Previously we have shown that HepG2 cells require a low molecular weight (300–350 Da) serum factor for expression of AsGR. That this factor is biotin has now been demonstrated by (a) direct addition of biotin and its derivative, biocytin, to cells cultured in dialyzed FBS-MEM, (b) reconstitution of dialyzed FBS with avidin-bound material having identical RF values on TLC as authentic biotin and biocytin, and (c) correlation by ELISA of their biotin content with the ability of various sera to support the normal steady-state concentration of AsGR.

The precise requirement of HepG2 cells for biotin has not been determined because this cofactor is highly conserved intracellularly, and depletion of sera by exhaustive dialysis is incomplete due to biotinylatation of a high-affinity 43-kDa serum carrier protein (14). In addition, the ELISA utilized may underestimate the actual content of biotin derivatives, such as biocytin, which is the predominant (90%) species in serum, due to their lower binding affinity. These other biotin derivatives adsorbed from serum by streptavidin-Sepharose supported normal AsGR expression (RF values 0.98 and 0.68) and as little as 10^-10 M added biotin (Fig. 1) was associated with detectable AsGR. The biotin content of FBS obtained by the ELISA is in general agreement with published values obtained by bioassay, approximately 2 × 10^-8 M (14, 15) and sera from other sources, which did not support AsGR production, have an order of magnitude lower biotin content. The effective biotin concentration in 10% FBS-MEM is comparable to the concentration of biotin shown to give maximal enhancement of guanylate cyclase (16) and to saturate the biotin receptor of mouse liver plasma membranes (17).

Down-regulation of AsGR expression in various models of altered hepatocellular physiology has been shown to be mediated at both transcriptional and translational levels. Huber et al. (18) describe transcriptional regulation in primary rat hepatocytes cultured for 24 h and in Morris hepatoma 7777 cells, whereas post-translational regulation is likely in primary development, regeneration, preneoplastic, and neoplastic rat liver, where AsGR specific messenger RNA levels do not differ from control. Biotin-depleted HepG2 cells contain little biosynthetically labeled AsGR, whereas mRNA levels are preserved (Fig. 5). It would appear that the effects of biotin on the expression of AsGR are at some post-transcriptional level. Whether expression of both AsGR polypeptides is affected by biotin is difficult to determine due to the low level of the minor species (H2) present in HepG2 cells (19). Examples in which biotin has been implicated as a regulatory cofactor of specific protein biosynthesis are limited. The incorporation of amino acids into a subset of cellular proteins (90–110 kDa) is selectively decreased in biotin-deficient HeLa cells, as is the proportion of active ribosomes (20). Rous sarcoma virus-transformed baby hamster kidney cells cultured in medium deficient in biotin are arrested in G1. The addition of biotin to the minimal medium has been shown to stimulate the production of "autocrine factors" required for the protein synthesis necessary for the cells to enter S phase and multiply (21 S.C.).

A striking parallel exists between our observations and those of Spence and Koudelka (21), who found that biotin
induced the activity of glucokinase and the intracellular levels of cGMP in primary cultures of adult rat hepatocytes. The effects of biotin upon enzyme activity could be mimicked by the addition of 8-bromo-cGMP and were not additive, suggesting that the effect of biotin may be mediated through changes in the cGMP level via induction of guanylate cyclase (17). Using an *in vitro* translation assay, Spence (22) was able to demonstrate that biotin and 8-bromo-cGMP increased the amount of translatable glucokinase mRNA, proposing a shift from a nontranslating to a translating pool of mRNA to explain induction of the enzyme. This is a plausible explanation for the observed effects of biotin and recently has been suggested as the regulatory mechanism for other inducible proteins (23–27). If the transition of mRNAs from nontranslatable to translatable pool underlies the biotin effect, this post-transcriptional event could represent a fundamental control mechanism for cellular differentiation.

Acknowledgments—We wish to thank Sun-jin Park and Ann Marie DelliPizzi for their excellent technical assistance.

REFERENCES