Expression of the asialoglycoprotein receptor by the human hepatocellular carcinoma cell line HuH-7 in response to intracellular cGMP concentrations was previously shown to be regulated at the translational level. In a cell-free system, initiation of asialoglycoprotein receptor mRNA translation was dependent on the presence of the 7-methylguanylate cap site and was independent of 8-bromo-cGMP levels in which the cells were grown prior to RNA isolation. Stable transfection of COS-7 cells with deletion constructs of the asialoglycoprotein receptor H2b subunit localized the cGMP-responsive cis-acting element to the mRNA 5' untranslated region (UTR). Addition of biotin (an activator of guanylate cyclase) induced the expression of β-galactosidase present as a chimeric plasmid containing the H2b 187-nucleotide 5'-UTR. An RNA gel retardation assay identified a 37-nucleotide cognate sequence within this 187-nucleotide UTR. An RNA gel retardation assay defined the cis-acting element as a chimeric plasmid containing the H2b 187-nucleotide 5'-UTR. An RNA gel retardation assay defined the cis-acting element to the mRNA 5'-untranslated region (UTR). Addition of biotin (an activator of guanylate cyclase) induced the expression of β-galactosidase present as a chimeric plasmid containing the H2b 187-nucleotide 5'-UTR. An RNA gel retardation assay identified a 37-nucleotide cognate sequence within this 187-nucleotide region. Titration of the 5'-UTR with a cytosolic fraction isolated from HuH-7 grown in the presence or absence of 8-bromo-cGMP or biotin provided direct evidence for an RNA-binding protein responsive to intracellular levels of cGMP. Based on these findings, it seems reasonable to propose that reduction of intracellular levels of cGMP by biotin deprivation results in a negative trans-acting factor associating with the 5'-UTR of asialoglycoprotein receptor mRNAs, thereby inhibiting translation.

Regulated expression of cell-surface lectins has been implicated in such diverse processes as endocytosis, bacterial and viral infection, regulation of cell proliferation, homing of lymphocytes, and metastasis of cancer cells (1). The asialoglycoprotein receptor (ASGR) is the hepatocellular prototype of a cell-surface lectin responsive to the differentiated state of the liver cell (for review, see Ref. 2). In addition to being a model of receptor-mediated endocytosis (3), the presence of ASGR on hepatocytes provides a membrane-bound active site for cell-to-cell interactions (4, 5), has made possible the selective targeting of chemotherapeutic agents (6) and foreign genes (7), and has also been implicated as a site that mediates hepatitis B virus uptake (8).

A human hepatoma cell line (HepG2) has provided a convenient model to investigate ASGR biosynthesis. When HepG2 cells were grown to confluence in a minimal essential medium or in a chemically defined medium containing a variety of hormones and growth factors supplemented with dialyzed fetal bovine serum, expression of ASGR was reduced by 60–70% (9). The low molecular weight factor required for the restoration of ASGR expression was isolated, purified, and identified as biotin (10). Similar results were obtained with a second hepatocellular carcinoma cell line, HuH-7 (11), indicating that the effect was not cell line-specific. Though usually not considered as part of an induction pathway, the effects of biotin upon the steady state expression of ASGR could be mimicked by the addition of the second messenger 8-bromo-cGMP (8-Br-cGMP), and these additions were not additive (11, 12). This suggested that the effect of biotin may have been mediated through changes in the cGMP level via biotin activation of the membrane-associated guanylate cyclase (13).

Estimates of the steady state level of ASGR mRNA suggested that cGMP-regulated expression of ASGR was at the posttranscriptional level (11, 12). Polysome analysis of ASGR subunits H1 and H2 mRNAs indicated that the addition of 8-Br-cGMP caused a shift of ASGR mRNA from the ribonucleoprotein fraction into a translationally active membrane-associated polysomal pool. Although the biochemical mechanisms have not been determined, cGMP has been suggested to regulate the expression of other proteins at a translational level (13, 14) and has been shown to increase total protein synthesis in isolated hepatocytes (15). In mammalian cells, translation of most mRNA species appears to occur by the association of the preinitiation complex at or near the 7-methylguanylate cap structure at the 5'-untranslated region (UTR) of mRNA and scanning downstream to the site of protein synthesis initiation (16). The 60 S ribosomal subunit is subsequently recruited to the complex, and translation begins. Recovery of the ASGR mRNA in the ribonucleoprotein fraction during biotin deprivation suggested that intracellular levels of cGMP may play a significant role in modulating the initiation phase of ASGR mRNA translation. The bimodal polysomal distribution of ASGR mRNA was characteristic of a class of mRNAs that were inefficiently translated (17). Current evidence suggests that mRNAs in these functionally distinct fractions differ structurally or through the proteins they interact with (18). Within this group of mRNAs, most interactions between RNA and cytosolic proteins were defined by motives localized to the 5'-UTR (19).

In the present study, the potential role of the 5'-UTR as the cis-acting element governing the cGMP-modulated expression of ASGR was established. In vitro transcription coupled with an RNA gel retardation assay defined the cis-acting element within a 37-nucleotide region. In addition, the effective concen-
COS-7 cells (20) resistant to 400 μM MEM supplemented with 10% fetal bovine serum (FBS) or dialyzed HuH-7 cells were cultured in Eagle’s minimum essential medium (MEM). Total RNA was isolated from cells grown in (1) FBS, (2) dFBS control cells, or cells treated with (3) 500 μM 8-Br-cGMP, (4) ANF, or (5) SNP as above. Northern blot analysis was performed using 10 μg of total RNA/lane, and the transferred RNA was sequentially hybridized with probes for H1 and H2 ASGR subunits. The blot was stained with methylene blue to confirm that equal amounts of RNA had been transferred.

**Experimental Procedures**

**DNA Constructs**—The 5’ and 3’-UTR regions of the H2b cDNA of ASGR were deleted using polymerase chain reaction to introduce unique restriction sites (XbaI, 6 nucleotides upstream of ATG translation start site or BamHI, 9 nucleotides downstream of the translation stop site). The resulting constructs were subcloned into either pcDNA3 for selection of stable transfectants in COS-7 or pGEM-4Z for selection of stable transfectants in HuH-7 or transfected COS-7. For Northern blot analysis, regardless of whether cells were maintained in cultured or isolated hepatocytes (25–29), HuH-7 cells were homogenized, the cytosol (S-100) was prepared by centrifugation at 100,000 × g, and the aliquots were stored at −135 °C. RNA was precipitated by addition of 75% ethanol to 4°C. RNA was reconstituted in water and digested with RNase-free DNase. RNA concentrations were determined using absorbance at 260 nm.

**Western Blot and Immunoprecipitation Protocols**—Cells were incubated with 200 μCi/ml [35S]Met/Cys (Promega) for 1 h before pulse labeling for 1 h with 1 μCi/ml [35S]Met/Cys, 200 μCi/ml Cells were harvested following a 2-h chase in medium supplemented with 0.1 mM methionine/cysteine. ASGR immunoprecipitated from aliquots of cell lysate containing equal amounts of radiolabeled protein was resolved on a 10% SDS-PAGE, and the resulting fluorograms were quantitated by densitometric scanning. The mean and standard deviation from three independent experiments as a percent of control are shown. B, effect of 8-Br-cGMP, ANF, and SNP on the steady state concentration of the H1 and H2 subunit-related mRNA. Total RNA was isolated from cells grown in (1) FBS, (2) dFBS control cells, or cells treated with (3) 500 μM 8-Br-cGMP, (4) ANF, or (5) SNP as above. Northern blot analysis was performed using 10 μg of total RNA/lane, and the transferred RNA was sequentially hybridized with probes for H1 and H2 ASGR subunits. The blot was stained with methylene blue to confirm that equal amounts of RNA had been transferred.

**RESULTS**

**Short Term cGMP-regulated Expression of ASGR**—Based on our previous findings that biotin was required for expression of ASGR by HepG2 and HuH-7 cell lines, we proposed that the mechanism of biotin regulation was mediated by maintaining the intracellular level of cGMP via the activation of guanylate cyclase (29). HuH-7 cells were grown to near-confluence in MEM supplemented with 10% FBS or 10% dFBS to which 10 to 1000 μM 8-Br-cGMP, 10 nM ANF, or 100 μM sodium nitroprusside (SNP) (shown to produce nitric oxide (25), an activator of soluble guanylate cyclase (29)) were added (Fig. 1A). Within 1 h of the addition of 500 μM 8-Br-cGMP and activators of both the particulate (ANF) and soluble (SNP) guanylate cyclases (25–29), the biosynthetic rate of ASGR was increased by 6.7-, 8.3-, and 4.2-fold, respectively, when compared with untreated cells in dFBS alone. No difference in the abundance of specific mRNAs was detected by Northern blot analysis, regardless of whether cells were main-
performed using 10 mM G418. The cell lines were grown to near-confluence in MEM supplemented with 10% dialyzed FBS. Cells were harvested and replated at a 1:3 ratio in the same medium with or without 10^-8 M biotin supplementation. When the cells reached near-confluence (72 h postplating), the levels of β-galactosidase activity in cell lysates were determined.

Table I

| Plasmid                  | Biotin |
|-------------------------|--------|
| pSV-β-gal               |        |
| + 5'-UTR sense orientation | 68 ± 11 | 32 ± 4 |
| + 5'-UTR antisense orientation | 83 ± 14 | 102 ± 17 |

Values shown are means ± S.D. of three independent transfections normalized to lysate protein and are expressed as a percent of the pSV-β-gal plasmid and grown in the presence of biotin.

Fig. 2. Cap-dependent translatability of ASGR H2 subunit mRNA isolated from biotin-deprived HepG2. Cells were maintained in MEM supplemented with 10% FBS or dFBS with or without 500 μM 8-Br-cGMP for 24 h before the isolation of mRNA. Equal amounts of mRNA (2 μg) were added to the translation mixture plus or minus m^7GpppG. ASGR H2 translation product was recovered by immunoprecipitation using a subunit-specific antibody, as described previously (49). The resulting fluorogram was quantified by densitometry.

Fig. 3. Expression of the H2 subunit of ASGR in stably transfected COS-7 cells. The full-length (H2 FL) and the 5’ (H2 5D) or 3’ (H2 3D) deleted UTR sequences of the H2b of ASGR cDNAs were subcloned into pcDNA3 vector and stable transfectants selected with 400 μg/ml of G418. The cell lines were grown to near-confluence in MEM supplemented with 10% FBS or dFBS with or without 1.0 mM cGMP. Cells were metabolically labeled with [35S]Met/Cys, and the extent of protein synthesis was described as described in Fig. 1. The failure of growing cells in dFBS to inhibit ASGR expression by the H2 5D line strongly indicates that the cGMP cognate sequence was located in the 5’-UTR of H2b mRNA. The film was exposed for 72 h for H2 3D and H2 FL and for 18 h for the H2 5D cell line. Northern blot analysis was performed using 10 μg total RNA/lane, and the transfer was hybridized with an H2-specific probe. The blot was stained with methylene blue to confirm that equal amounts of RNA had been transferred.

In Vivo Translation of ASGR—In mammalian cells, translation of most mRNAs appears to occur by association of a preinitiation complex at a 7-methylguanylate cap site and subsequent scanning to the translation initiation site (16). To establish the cap site status of ASGR mRNA in cells grown in FBS as compared with dFBS and dFBS supplemented with 500 μM cGMP, the extent of cap-dependent in vitro translation was determined. Total mRNA was isolated from HuH-7 cells (5 x 10^7) and translated in a rabbit reticulocyte lysate in the presence or absence of m^7GpppG, an inhibitor of cap-dependent initiation (30). The labeled ASGR translation product was recovered by immunoprecipitation using a polyclonal antibody to

affinity-purified human receptor. Resolution on 10% SDS-PAGE and subsequent fluorography indicated that inclusion of m^7GpppG reduced translation of ASGR mRNA isolated from both control and biotin-deprived cells with or without 8-Br-cGMP to an equal extent (>90%) (Fig. 2). These results indicated that initiation of ASGR mRNA translation was cap-dependent and that addition of a 7-methylguanylated cap to ASGR mRNA was independent of biotin deprivation.

Localization of the Cis-acting H2 mRNA Cognate Sequence—To localize the cis-acting element, mutated H2 cDNAs from which the entire 5’ or 3’-UTR was deleted were constructed by polymerase chain reaction amplification. These constructs, along with the full-length H2 cDNA, were cloned into the eukaryotic expression vector pcDNA3 carrying a neomycin resistance gene. Stable transfectants of COS-7 cells were selected with 400 μg/ml G418. As shown in Fig. 3, deletion of the 5’-UTR resulted in loss of the cGMP requirement for H2 expression. In contrast, deletion of the 3’-UTR was without effect. These results indicated that the cGMP-responsive element was located in the 5’-UTR of the H2 mRNA. Northern blot analysis indicated that there was no significant difference in mRNA levels to account for this differential response to biotin deprivation or supplementation with 8-Br-cGMP (Fig. 3), supporting translational regulation in the transfected COS-7 cell lines.

Transient transfection of HuH-7 with the chimeric plasmid confirmed that the putative cis-acting element was localized within the 5’-UTR (Table I). Addition of biotin to the culture medium resulted in a two-fold increase in β-galactosidase activity. Interestingly, the presence of the 5’-UTR in the plasmid reduced β-galactosidase expression when compared with the original or a chimeric plasmid in which the 5’-UTR was inserted in the antisense orientation by almost 35%, even when biotin was added. This finding was consistent with the reduced level of H2b translation when compared with the H1 ASGR subunit under normal physiologic conditions (2).

RNA Gel Retardation Assay—The 5’-UTR (187-base pair) cDNA fragment of the H2b of ASGR was directionally cloned into pGEM-4Z vector for the generation of a nested set of 5’-UTR mRNA fragments by in vitro transcription for an RNA-protein binding assay (Fig. 4). RNA fragments of the 5’-UTR were added in 100-fold molar excess prior to the addition of the full-length 187-nucleotide-labeled RNA probe and resolution on 4% PAGE. As illustrated in Fig. 5, the failure of the Sp6-FokI transcript to inhibit the band shift assay indicated that a cognate sequence lies between 70 and 110 nucleotides relative to the Sp6 promoter. Since translational regulation due to protein-protein interactions between two regions of a tran-
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DISCUSSION

In mammalian cells, translation of most mRNA species appears to occur by the association of a preinitiation complex at a 7-methylguanylate cap site and subsequent scanning downstream to the site of protein synthesis initiation (16, 31). Our studies showed that cap site addition to ASGR mRNA was independent of biotin deprivation (Fig. 2). However, the recovery of the ASGR message in the ribonucleoprotein fraction during biotin deprivation (11, 12) suggested that intracellular levels of cGMP play a significant role in modulating the initiation phase of translation (31). Perhaps the best-defined example of translational regulation is that of ferritin synthesis in iron-deficient cells (16, 30). Analysis of the cis-acting mRNA sequences led to the definition of the iron-responsive element with a putative stem-loop structure in the 5′-UTR (30). Modeling of the ASGR H2b 5′-UTR indicated the presence of two potential regions of secondary structure. The free energy levels (−7.4 and −8.5 kcal/mol) of these two stem-looped regions was far below that usually considered necessary to prevent recognition of a cap site (−50 kcal/mol). However, they might provide the loop structure necessary for specific recognition by exposing the RNA backbone and bases to interactions with protein groups (32, 33).

In the absence of a highly ordered 5′-UTR stem-loop structure, translation may be regulated by a short linear sequence (16). A highly conserved CCAUCNN sequence localized within the 5′-UTR of both ASGR subunit mRNAs isolated from either human or rat has been identified as a conserved RNA-binding cognate sequence within the 5′-UTR of ornithine decarboxylase (34). The presence of this conserved sequence within the putative cis-acting element as indicated by gel retardation assay (Fig. 5) supports the possibility that it may serve as a recognition motif for the cGMP responsive trans-acting factor.

One plausible explanation for cGMP-regulated expression of ASGR would be modulation of a trans-acting factor phosphorylation status. Although the modulation of ASGR by cGMP was not liver-specific (Fig. 6), it should be viewed in the context of the original finding in HepG2 cells (11, 12). Since there was little, if any, cGMP-dependent protein kinase detected in hepatocytes (35, 36), the classic cGMP signal transduction pathways mediated by cGMP-dependent protein kinase was presumed to be absent in liver cells (36). Therefore, if a phosphorylation/depot phosphorylation signal transduction pathway was involved in translational regulation of ASGR expression, one of the cGMP-binding phosphodiesterases (PDEs) would be the most likely effector target. As opposed to cGMP-dependent protein kinase, the various cGMP PDEs are regulated allosterically by the binding of cGMP to noncatalytic binding sites (37). Modulation of cGMP levels can either inhibit or stimulate PDE hydrolytic activity, increasing or decreasing intracellular
cGMP itself or cAMP (38). Indeed, a number of recent studies have suggested that cGMP-stimulated PDE may play a central role in regulating the intracellular concentrations of cAMP (39, 40). Based on our previous findings that increased levels of cAMP resulted in the down-regulation of ASGR (41), induction of a cGMP-stimulated PDE resulting in a protein dephosphorylation via reduction of cAMP is a reasonable mechanism for cGMP-regulated expression of ASGR.

Presently, it may be premature to speculate that changes in the phosphorylation state of any protein via PDE mediates the effects of cGMP on translation of ASGR, especially in the light of the recent discovery of new types of cyclic nucleotide receptors that include other non-catalytic sites (37). Our understanding of the potential cGMP cascade in liver is still in its infancy, and there may yet be other schemes to account for cGMP action, such as Ca\(^{2+}\) ion flux or induction of inositol triphosphate (42, 43). Whatever the biochemical mechanism of the cGMP action may be, it is reasonable to speculate that in the absence of cGMP, a negative trans-acting factor associates with the 5'-UTR of the ASGR mRNA, thereby inhibiting translation. Purification of the ASGR mRNA-binding protein should provide new insight into the physiologic affect and molecular target of cGMP in the hepatocyte.

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