Activation of LDL receptor gene expression in HepG2 cells by hepatocyte growth factor

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Abstract The effect of recombinant human hepatocyte growth factor (HGF) on low density lipoprotein (LDL) receptor gene expression was studied in the human hepatoma cell line HepG2. HepG2 cells were incubated with serum-free media in the presence and absence of HGF for various times and ^{125}I-labeled LDL specific binding at 4°C, uptake at 37°C, and the levels of LDL receptor mRNA were measured. Incubation with HGF produced time- and concentration-dependent increases in ^{125}I-labeled LDL binding (2-fold), uptake (2.5-fold), and LDL receptor mRNA (6-fold). HGF increased the rate of LDL receptor gene transcription 4- to 5-fold relative to that of several "house-keeping" genes as measured by nuclear run-on transcription. The half-life of LDL receptor mRNA, measured with actinomycin D, was not increased in HGF-treated cells. The stimulation of LDL receptor expression occurred independently of changes in cellular cholesterol or DNA biosynthesis or total cell protein. HepG2 cells were transiently transfected with plasmids bearing either three copies of repeats 2 and 3 (pLDLR(23)$_3$LUC) or one copy of the LDL receptor promoter from -556 to +53 (pLDLR600LUC) linked to firefly luciferase. Incubation of pLDLR(23)$_3$LUC- or pLDLR600LUC-transfected cells with HGF for 4 or 24 h at 37°C produced a concentration-dependent increase in luciferase activity. A maximal stimulation of 3- to 6-fold was achieved for each construct at an HGF concentration of 100 ng/ml. In contrast, HGF had little or no effect on reporter activity in HepG2 cells transfected with a luciferase reporter plasmid bearing the HMG-CoA reductase promoter extending from -325 to +22. Thus, when compared to the native LDL receptor promoter, multiple copies of repeats 2 and 3 of the LDL receptor promoter can fully support activation of the luciferase reporter gene by HGF, demonstrating that the effect of HGF is mediated through the SRE-1. The lack of HGF effects mediated through the HMG-CoA reductase sterol regulatory element suggests, however, that sterol depletion may not be responsible for the induction of the LDL receptor promoter by growth factors. The signalling pathways or effectors responsible for activation of the LDL receptor and HMG-CoA reductase genes thus differ in their response to HGF. These data suggest that the level of SREBP's reaching the nucleus may be determined by as yet unidentified second messengers as well as by sterols.—**Pak, Y. K., M. P. Kanuck, D. Berrios, M. R. Briggs, A. D. Cooper, and J. L. Ellsworth.** Activation of LDL receptor gene expression in HepG2 cells by hepatocyte growth factor. *J. Lipid Res.* 1996. **37:** 985–998.

Supplementary key words growth factors • gene transcription • liver regeneration

Low density lipoprotein (LDL) receptors play a central role in cholesterol homeostasis at the whole body level by regulating the clearance of LDL from the circulation (1). Cholesterol balance at the cellular level is also achieved by LDL receptor-mediated uptake and degradation of plasma LDL (1). The LDL receptor thus plays a pivotal role in maintaining both plasma and cellular sterol balance by regulating the catabolism of LDL.

The primary mode of regulation of LDL receptor activity demonstrated to date is by feedback repression of LDL receptor gene transcription. When cells are deprived of sterols, transcription of the LDL receptor gene increases, and conversely, when sterols accumulate within cells, transcription of the LDL receptor gene decreases (2, 3). As for many genes transcribed by RNA polymerase II, recent studies have shown that LDL receptor gene transcription is mediated by sequence elements within the 5′-flanking region of the LDL receptor gene. High level transcription of the LDL receptor gene appears to be mediated by three imperfect direct repeats, designated 1–3, within the LDL receptor promoter (4–7). Repeats 1 and 3 appear to bind Sp 1 or another member of the GC-box transcription factor family and appear to be constitutively positive elements (6). Sterol-mediated repression and induction of the LDL receptor promoter is mediated by a ten nucleotide element with the core sequence, 5′-CACCCCAC-3′ termed the sterol regulatory element-1 or SRE-1 (7),

Abbreviations: LDL, low density lipoprotein; SRE-1, sterol response element-1; SREBP, SRE binding protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; LPDS, lipoprotein-deficient serum; HGF, hepatocyte growth factor.

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embedded within repeat 2. This element has recently been shown to bind basic-helix-loop-helix leucine zipper nuclear proteins called SREBP-1 and SREBP-2 (8–10). By single nucleotide mutagenesis and gel mobility shift analyses, a direct correlation between mutations that disrupted sterol repression and those that abolished binding of SREBP to the SRE-1 was found suggesting that the SREBP mediated the response to sterols.

Elegant studies have now established that there is a unique form of regulation of these transcription factors (10–12). They are each synthesized as a precursor form that is embedded in the endoplasmic reticulum and nuclear envelope. In the absence of sterols, one or more proteases are induced to cleave the amino terminal transcription regulatory domains of the SREBP from its carboxy terminal membrane spanning domain. This allows translocation of the truncated SREBP to the nucleus where it activates transcription of at least two SRE-governed genes, the LDL receptor and HMG-CoA synthase genes. The activity of the truncated SREBP can be rapidly terminated by an ALLN-sensitive calpain I protease in the nucleus. Thus, transcription can be rapidly increased or decreased by the supply of SREBP which, in turn, depends on the activity of the sterol-responsive cytosolic protease(s). The mechanism for activation of the LDL receptor promoter by the SREBP is, however, not yet clear. Recent in vivo genomic footprinting experiments have demonstrated that the proximal promoter region encompassing repeats 1–3 of the LDL receptor gene is occupied by multiple proteins in vivo and these proteins are poised to activate transcription with minimal changes in the underlying DNA contacts (13). Thus, the SREBP or related proteins are likely to be involved in novel protein–protein interactions over this region that serve to activate transcription.

Although repeats 1–3 of the LDL receptor promoter are required for basal and sterol-mediated repression of transcription, it is not clear whether these elements mediate all forms of regulation. Recent evidence from a number of laboratories, including ours, suggests that activation of the LDL receptor gene in HepG2 cells and lymphocytes by serum growth factors can occur in the absence of demonstrable changes in cell sterol metabolism (14–18). A physiologic correlate of these cell culture studies may be the regenerating rat liver. Within 2–4 h after 70% partial hepatectomy, the levels of LDL receptor mRNA and protein in the liver remnant rise severalfold (19). This induction of LDL receptor gene expression occurs during a period of active lipid deposition in the liver (20, 21) suggesting that the mitogenic stimulus for regeneration may override sterol-mediated repression of LDL receptor gene transcription. A number of serum factors have been implicated in the regenerative response of the liver after injury (22); one of these humoral factors is hepatocyte growth factor/scatter factor or HGF (23, 24). HGF accumulates rapidly in the serum of partially hepatectomized rats and is a potent mitogen for the liver during regeneration in vivo and for normal hepatocytes in cell culture (23, 24). This effect appears to be mediated through the cell surface HGF receptor that has been identified as the product of the c-Met proto-oncogene, a membrane-spanning tyrosine kinase receptor (25). To investigate whether HGF can regulate LDL receptor gene expression in liver-derived cells, the effects of HGF on LDL receptor gene expression in HepG2 cells were investigated.

MATERIALS AND METHODS

Materials

Cytidine 5′-[α-^{32}P]triphosphate (~3,000 Ci/mmol), adenosine 5′-[γ^{32}P]triphosphate (~6,000 Ci/mmol), and deoxyadenosine 5′-α-[^{35}S]thiotriphosphate (>1,000 Ci/mmol) (all triethyl ammonium salts) were obtained from Amersham (Arlington Heights, IL). Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from Pharmacia-LKB (Piscataway, NJ). RNA transcription kits, RNase-Block II, Nuc Trap Push Columns, Epicurian Coli XL 1-Blue competent cells, and pBluescript II KS (+/-) were obtained from Stratagene (La Jolla, CA). RPA II kits were obtained from Ambion

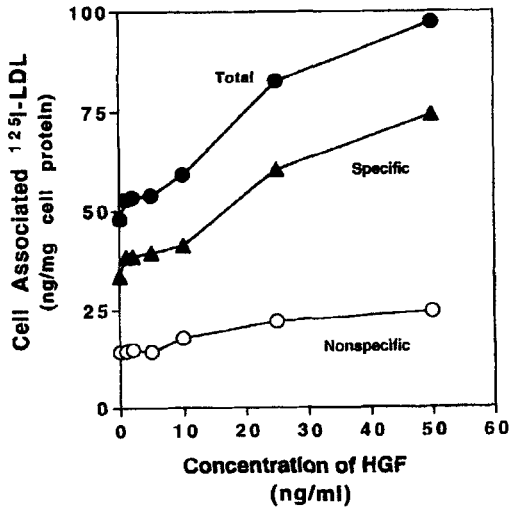

Fig. 1. Effect of HGF on uptake of ^{125}I-labeled LDL by HepG2 cells. Each dish of cells was incubated with the indicated concentration of HGF protein in a final volume of 1.0 ml of serum-free MEM for 5.0 h at 37°C. The media were removed and each dish was incubated with 10.0 μg of protein/ml of ^{125}I-labeled LDL in the presence and absence of 200 μg protein/ml of unlabeled LDL in a final volume of 0.4 ml of medium B. After incubation for 2.0 h at 37°C, cell-associated ^{125}I-labeled LDL was determined as described under Methods. Each point represents the average of two separate experiments performed in duplicate.

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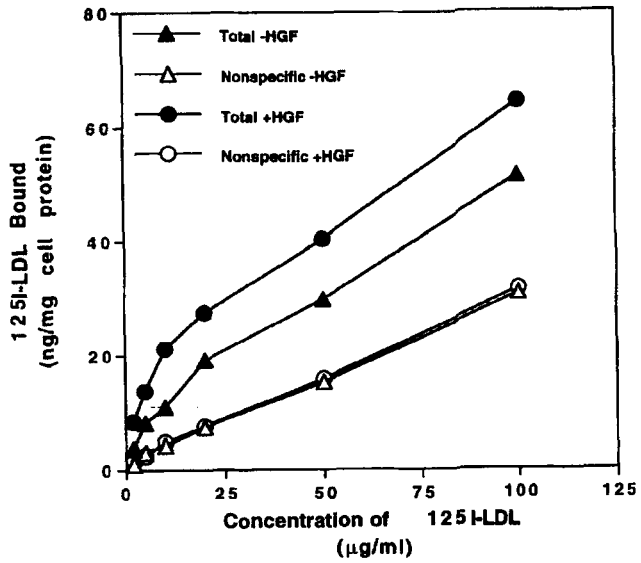

Fig. 2. Effect of HGF on cell surface binding of ^{125}I-labeled LDL to HepG2 cells. Each dish of cells was incubated in the presence or absence of 50 ng of HGF protein/ml in a final volume of 1.0 ml of serum-free MEM. After 5.0 h at 37°C, the media were removed and each dish was incubated with the indicated concentration of ^{125}I-labeled LDL in the presence or absence of 500 µg protein/ml of unlabeled LDL in a final volume of 0.4 ml of medium B. After incubation for 4.5 h at 4°C, the binding of ^{125}I-labeled LDL was determined as described under Methods. Each point represents the average of two separate experiments on single dishes of cells.

(Austin, TX). Proteinase K, MMLVH⁻ reverse transcriptase, and all tissue culture supplies were obtained from GIBCO-BRL (Grand Island, NY). The plasmids pLDLR3 and pHcGAP were obtained from the American Type Culture Collection (Rockville, MD), pXT and pHbAPr-1-neo-β-gal were kindly provided by Dr. Todd Leff [Warner Lambert Parke-Davis Laboratories, (Ann Arbor, MI)] and Dr. John Leavitt [Research Institute, Palo Alto Medical Foundation, (Palo Alto, CA)], respectively and pGL2 basic was obtained from Promega (Madison, WI). Purified recombinant human HGF was a generous gift from Dr. Paul Godowski [Genentech, Inc., (South San Francisco, CA)] or was purchased from Collaborative Biomedical Products (Bedford, MA). Mevinolin was a generous gift from Merck & Co. Inc. (Rahway, NJ). NitroPlus 2000 nitrocellulose membranes were purchased from Micron Separation Inc., (Westborough, MA). All other chemicals were molecular biology grade and were purchased from Sigma Chemical Co. (St. Louis, MO) or as described previously (18).

Plasmid construction

pLDLR600LUC was constructed by PCR-mediated subcloning of -556 to +53 of the human LDL receptor promoter into the polylinker of pGL2-basic. pLDLR(23)$_3$ was constructed by multimerizing repeat 2 + 3 of the LDL receptor promoter (8) and cloning into the Sal I site of pGL2 basic a fragment of 65 bp containing an Adenovirus E1b TATA sequence. A linker was cloned into the Hind III site. The TATA-proximal repeat 3 has incurred a three bp deletion of the sequence, CCT, representing nucleotides 12 to 14 of repeat 3 (AAACTCCTCCC**CCT**GC, deletion is in bold type). This has no effect on any sterol-mediated or other regulatory processes of the plasmid construct (M. R. Briggs, unpublished observations). The human HMG-CoA reductase promoter sequence from -325 to +22 was subcloned into the luciferase reporter plasmid pGL2-basic immediately adjacent to the luciferase start site.

A series of oligonucleotides encompassing repeats 2 and 3 of the LDL receptor promoter and containing Xba I restriction sites at both ends were synthesized and purified by denaturing polyacrylamide gel electrophoresis according to standard procedures. The sequences of oligonucleotides with the sequences corresponding to repeats 2 and 3 underlined are: R23 (repeat 2 and 3), CTAGTTGAAAATCACCCCACTGCAAACTCCTCC CCCTGCTA. The coding and complementary oligonucleotides were hybridized, subcloned into the Xba I site of the vector pXT (26), and used to transform *E. coli* strain RR1. The resulting clones were screened by PCR using a primer on the CAT gene and either the sense or the anti-sense oligonucleotide as the second primer. The identities and orientation of the final subclones were verified by dideoxynucleotide sequencing using Sequenase 2.0. The plasmids pEB-LDLR KS(+) containing

TABLE 1. Effect of HGF on cell protein and [^{14}C]acetate incorporation into cellular cholesterol

Incubation	Experiment 1		Experiment 2	
	[^{14}C]Acetate Incorporation	Cell Protein	[^{14}C]Acetate Incorporation	Cell Protein
	dpm/mg protein ($\times 10^4$)	*mg/well*	*dpm/mg protein* ($\times 10^4$)	*mg/well*
MEM	8.4 ± 1.9	0.69 ± 0.05	6.4 ± 3.0	0.74 ± 0.01
MEM + HGF	8.7 ± 1.5	0.74 ± 0.06	9.9 ± 2.8	0.75 ± 0.09

HepG2 cells were incubated in the presence and absence of 50 ng of HGF/ml and the incorporation of [^{14}C]acetate into cellular cholesterol and the total cellular protein were determined as described under Methods. The data represent the mean ± SD for four flasks of cells in each experiment.

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a 0.98 kb EcoR I-Bgl II fragment of the LDL receptor cDNA, pHX-G3PD KS(+) containing a 0.55 kb Hind III-Xba I fragment of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA, and pPX-G6PD KS(+) containing a 0.62 kb Pst I-Xho I fragment of glucose-6-phosphate dehydrogenase (G6PDH) cDNA all in pBluescript II KS(+) were prepared as described previously (3).

Cell culture and transfection

HepG2 cells and human skin fibroblasts were cultured in medium A (Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum, 292 μg glutamine/ml, 100 units of penicillin/ml, and 100 μg of streptomycin/ml) as described previously (18). A 10.0-μg sample of each luciferase reporter construct was mixed with 5.0 μg of a CMV-promoter driven β-galactosidase plasmid, and 5.0 μg of pGEM4 in a final volume of 1.0 ml calcium phosphate co-precipitation as described above. A 0.1-ml sample was added to the appropriate well of a 96-well microtiter plate seeded with 1.3 × 10^4 HepG2 cells the day before in medium A. After 6.0 h at 37°C, the cells were washed and refed with fresh medium A. The compounds were then added to the appropriate wells as described in the legends to the figures.

The day before transfection, HepG2 cells were subcultured by trypsinization and seeded into flasks at a density of approximately 8.7 × 10^4 cells/cm^2. Cells (10–20% confluent) were transfected with 10 μg of pHbAPr-1-neo-β-gal and 30 μg of the pXT-R23 construct by calcium phosphate co-precipitation (27). After incubation for 16–24 h at 37°C, the cell monolayers were washed 3 times with phosphate-buffered saline (PBS) and refed with media A containing 800 μg of G418/ml. After approximately 2 months of selection by growth in G418-containing medium, resistant colonies (20–50 colonies) were pooled and expanded in mass culture. In each of the experiments described below, HepG2 cells were used at 90–95% confluency. After incubation with growth factors or drugs as described in the figure legends, the cells were harvested, and CAT and β-galactosidase activities (27) and the levels of CAT and LDL receptor mRNA were measured as described below. The level of CAT activity in each sample was quantified as [^{14}C]acetyl chloramphenicol/total [^{14}C]chloramphenicol and normalized to that of β-galactosidase.

RNA preparation, Northern blot, RNase protection, and nuclear run-on transcription analyses

Total RNA was isolated by the guanidinium-isothiocyanate procedure as described previously (18). For Northern blotting, 20 μg of total RNA was electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose membranes with 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) as transfer buffer, and cross-linked by UV irradiation. The membrane was pre-hybridized at 42°C in 6× SSC, 2× Denhardt's solution, 50% formamide, 0.1% SDS, and 100 μg/ml salmon sperm DNA. The membranes were probed sequentially with a 0.98 kb EcoR I-Bgl II fragment of pLDLR3 and a 0.5 kb Xba I-Hind III fragment of pHcGAP ^{32}P-labeled by random priming. The membranes were hybridized in fresh pre-hybridization solution at 42°C for 16–24 h, washed with 1× SSC/0.1% SDS at room temperature for 15 min twice, followed by two

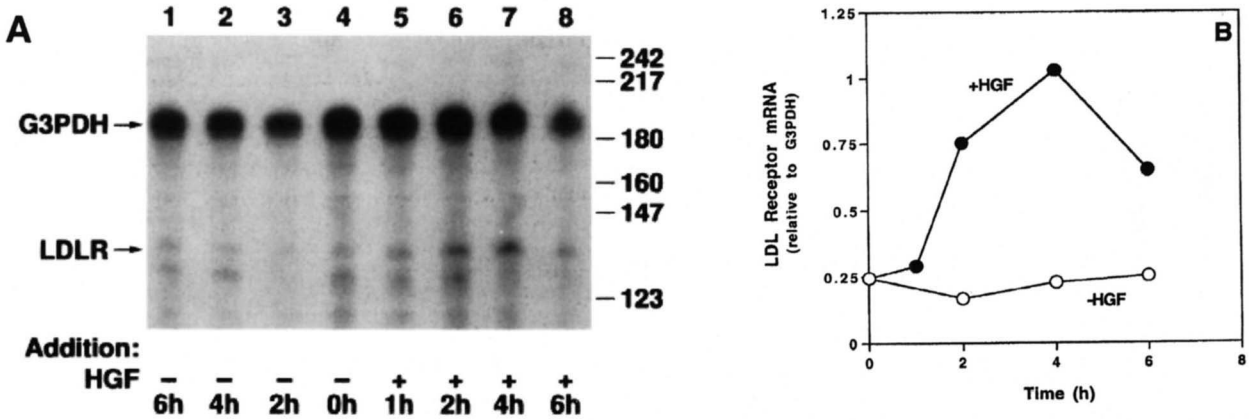

Fig. 3. Time course of induction of LDL receptor mRNA by HGF. A: Each dish of HepG2 cells was incubated in the presence or absence of 50 ng HGF protein/ml in a final volume of 4.0 ml of serum-free MEM for the indicated time at 37°C. Total cellular RNA was isolated and 10.0 μg of RNA was used to measure LDL receptor (LDLR) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNAs by ribonuclease protection as described under Methods. An autoradiogram is shown. The numbers on the right represent the mobility of ^{32}P-labeled Hpa II digested pBR322 size markers in nucleotides. B: The relative levels of LDLR and G3PDH mRNA on the autoradiogram shown in panel A were quantitated by scanning densitometry. The figure shown is representative of three separate experiments.

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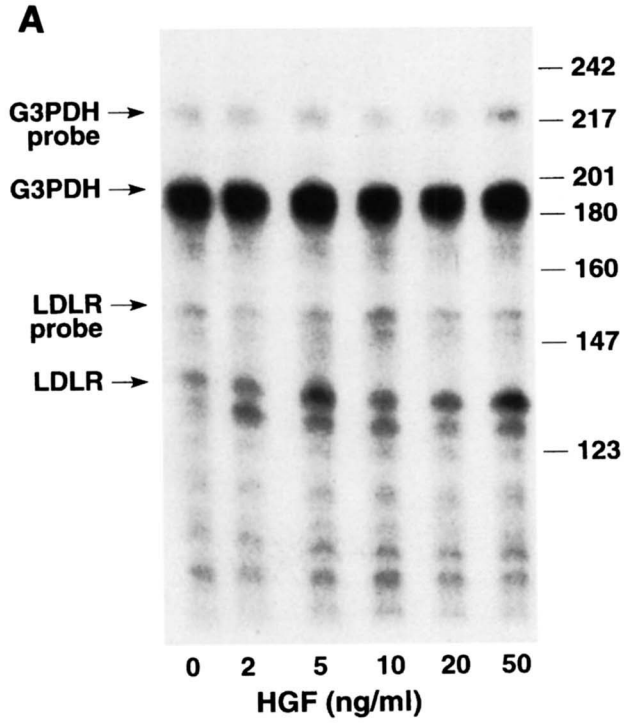

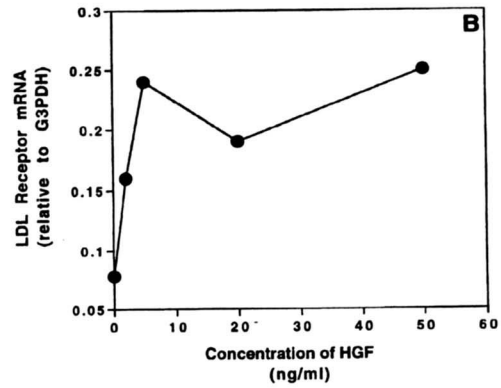

Fig. 4. Concentration-dependent induction of LDL receptor mRNA by HGF. A: Each dish of HepG2 cells was incubated with the indicated concentration of HGF protein in a final volume of 4.0 ml of serum-free MEM for 4.0 h at 37°C. Total cellular RNA was isolated and the levels of LDL receptor and G3PDH mRNAs were measured as described in the legend to Fig. 3. B: The relative levels of LDLR and G3PDH mRNA on the autoradiogram shown in panel A were quantitated by scanning densitometry. The figure shown is representative of two separate experiments.

washes of 15 min each in 0.25× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 60°C. The membranes were air dried, exposed to pre-flashed Kodak X-OMAT AR film, and the relative level of LDL receptor mRNA was quantitated by densitometric scanning of the autoradiograms. LDL receptor and G3PDH riboprobes were prepared from PCR-generated templates and RNase protection was performed exactly as described previously (3) using 10.0 μg of total RNA per sample. The sizes of the riboprobes were 167 nt and 244 nt for the LDL receptor and G3PDH genes, respectively. The specific activities were approximately 8×10^7 and 3.5×10^7 cpm/μg for the LDL receptor and G3PDH probes, respectively. Isolation of HepG2 nuclei by Np-40 lysis and nuclear run-on transcription analysis was performed as described (3) using pEB-LDLR KS(+), pHX-G3PD KS(+), pPX-G6PD KS(+), and pBluescript II KS(+) linearized by digestion with EcoR I, Hind III, Pst I, and Hind III, respectively to detect newly synthesized mRNAs.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Complementary DNAs were synthesized from 5 μg of total RNA in 10 μl reaction mixture containing 1× RT buffer, random primer, 10 mM DTT, 1 mM of dNTPs, 10 U RNasin (Promega Co.), and 100 U MMLVH⁻ reverse transcriptase at 42°C for 1 h. A 1.0-μl sample of the reverse transcriptase reaction mixture was added into 50 μl PCR reaction mixture containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.2 mM of dNTPs, 2 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase, and the CAT primers (5′-TCACTGGATATACCACCGTTG-3′ and 5′-CCGGCGAATTTCTGCCATTCA-3′), which generate a 734 bp PCR product. After 12 cycles (94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min), fresh reagents and the G3PDH primers (5′-CATCACCATCTTCCAGGAGCG-3′ and 5′-CCACCACTGACACGTTGGCAG-3′), which generate a 511 bp PCR product, were added and the amplification was continued for another 18 cycles. An aliquot was then removed for measurement of CAT and G3PDH mRNAs. Preliminary experiments demonstrated that these conditions were within the linear range of amplification of both gene fragments. The PCR reaction mixture (20 μl) was loaded onto a 1% agarose gel and transferred onto nitrocellulose paper. A 0.52 kb Hind III-Msc I fragment of CAT cDNA and a 0.5 kb Xba I-Hind III fragment of pHcGAP were used as ^{32}P-labeled probes. The filters were pre-hybridized, hybridized, and washed as described above for Northern blotting analysis. The levels of CAT and G3PDH mRNA were quantified by scanning densitometry of the autoradiograms and the 734 bp CAT RT-PCR product was normalized to the 511 bp G3PDH RT-PCR product.

Preparation of lipoproteins and the lipoprotein-free fraction of serum

Plasma from normolipemic individuals was collected into 0.15% EDTA, the erythrocytes were removed, and

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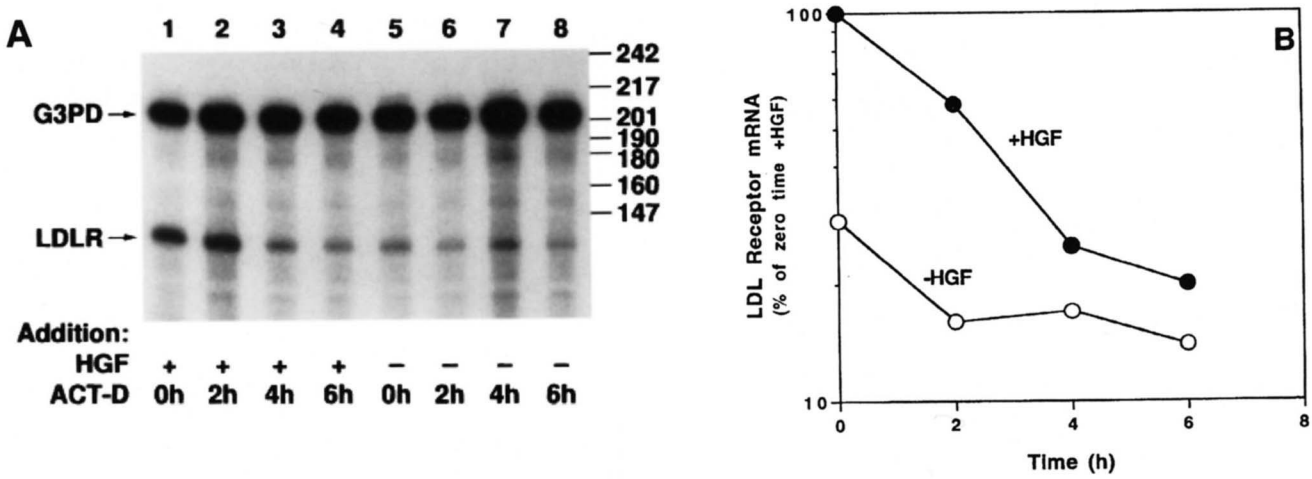

Fig. 5. Effect of HGF on the turnover of LDL receptor mRNA. A: Each dish of HepG2 cells was incubated in the presence or absence of 50.0 ng HGF protein/ml in a final volume of 3.0 ml of serum-free MEM for 4.0 h at 37°C. The media were removed, the cells were washed, and incubated for the indicated time with 10.0 µg of actinomycin D/ml dissolved in ethanol (final ethanol concentration = 0.8%). Total cellular RNA was isolated and the levels of LDL receptor and G3PDH mRNAs were measured as described in the legend to Fig. 3 using 30.0 µg of RNA per sample. B: The relative levels of LDL receptor and G3PDH mRNAs were quantitated by scanning densitometry of the autoradiograms. The level of LDL receptor mRNA in the HGF-treated cells at the zero time point was set at 100%. Each point represents the mean value from two separate experiments.

the LDL (1.019 < d < 1.063) were isolated by ultracentrifugation in KBr, processed, and stored as described (19). The lipoprotein-free (d > 1.210 or LPDS) fraction of calf serum was isolated as described previously (18). LDL were radiolabeled with ^{125}I by the Iodogen procedure to an average specific activity of 94 cpm/ng protein.

Binding and uptake of ^{125}I-labeled LDL

HepG2 cells were cultured in 12-well plates and the binding of ^{125}I-labeled LDL at 4°C and uptake at 37°C were measured using the indicated concentration of ^{125}I-labeled LDL in 0.4 ml of medium B as described previously (18). Specific binding and uptake were determined in the presence or absence of 500 µg protein/ml of unlabeled LDL. The radioactivity was normalized per mg of cell protein, which was determined on an aliquot of the cell suspension after neutralizing with HCl.

[^{14}C]acetate incorporation into cholesterol and [^{14}C]thymidine incorporation into DNA

HepG2 cells were seeded into 6-well plates at a density of 2×10^5 cells/cm^2 (day 0) and were grown in medium A as described above. On day 3 or 4, the media were removed and the cells were washed with 2.0 ml of serum-free MEM. The wash was discarded and to each dish was added 10 µCi of [^{14}C]acetate in a final volume of 1.0 ml of serum-free MEM and the samples were returned to the incubator for 1.0 h at 37°C. HGF was added at the indicated concentrations and the cells were incubated for an additional 4.0 h at 37°C. The cellular lipids were extracted, saponified, and separated by thin-layer chromatography as described previously (18).

To measure [^{14}C]thymidine incorporation into DNA, HepG2 cells were subcultured in 6-well plates as described above. On day 4, the media were removed and the cells were washed and incubated with 2.0 ml of serum-free MEM with or without 50.0 ng HGF/ml. After 3.0 h at 37°C, each well received 1.0 µCi of [^{14}C]thymidine and the incubation was continued for 1.0 h at 37°C. The cells were placed on ice and washed with 2.0 ml of ice-cold PBS containing 50.0 µg of thymidine/ml and then twice with PBS alone. The washes were discarded and each well sequentially received 2.0 ml of ice-cold methanol for 5.0 min at 4°C, 10% TCA for 15 min at 4°C, and were washed with 2.0 ml of ethanol–ether 3:1 (v/v). The cells were solubilized with 1.0 ml of 1.0 N NaOH for 10 min at room temperature. Cell extracts were neutralized with HCl and samples were removed for determination of cell protein and scintillation counting.

Other methods

A 100-ng sample of Hpa II-digested pBR322 was end-labeled to a specific activity of 4×10^7 cpm/µg and used as a size marker for the RNase protection analyses. Equilibrium binding analysis was carried out using the computer program LIGAND and statistical analyses were performed using an unpaired Student's *t*-test or a Mann-Whitney test. Protein was measured by the Bio-Rad protein assay kit using bovine serum albumin as standard.

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RESULTS

Effects of HGF on ^{125}I-labeled LDL binding and uptake in HepG2 cells

After partial hepatectomy in rats, the level of HGF in blood rises within minutes (28) and HGF becomes a hepatic mitogen after a brief lag period (29). LDL receptor activity in the residual liver tissue rises within hours (19). Thus, it is possible that HGF contributes to the early induction of LDL receptor gene expression. To assess whether HGF rapidly regulates LDL receptor activity in liver-derived cells, HepG2 cells were incubated with serum-free media in the presence and absence of various concentrations of HGF for 4.0 h at 37°C and the uptake of ^{125}I-labeled LDL was determined. Incubation with HGF produced a concentration-dependent increase in the specific uptake of ^{125}I-labeled LDL that reached a maximum of 2.2-fold at an HGF concentration of 50 ng/ml (**Fig. 1**). Nonspecific uptake, determined in the presence of a large excess of unlabeled LDL, was not affected. To evaluate whether the HGF-mediated induction of ^{125}I-labeled LDL uptake was due to an increase in the number of cell surface LDL receptors, HepG2 cells were incubated with 50 ng of HGF/ml and saturation binding of ^{125}I-labeled LDL to these cells was determined at 4°C. Compared to cells incubated in serum-free media alone, the total and specific (total - nonspecific) binding of ^{125}I-labeled LDL were increased in cells incubated with HGF at all concentrations of ^{125}I-labeled LDL tested (**Fig. 2**). HGF had no apparent effects on nonspecific binding of ^{125}I-labeled LDL. Equilibrium binding analysis revealed that HGF increased the high affinity binding for ^{125}I-labeled LDL from 0.6×10^{-11} mol/mg cell protein to 1.1×10^{-11} mol/mg cell protein for cells incubated in the absence or presence of HGF, respectively. The dissociation constant or K_d of ^{125}I-labeled LDL binding was 2.0×10^{-9} M for cells incubated in the absence and 1.2×10^{-9} M for cells incubated in the presence of HGF. Over a 4.0-h period, HGF had no significant effects on the mass of total cellular protein or on [^{14}C]acetate incorporation into total nonsaponifiable cellular cholesterol (**Table 1**). In addition, HGF has no significant effects on [^{14}C]thymidine incorporation into DNA over this period (data not shown). This is consistent with the report by Shiota et al. (30) where HGF inhibited the growth of hepatocellular carcinoma cells but is in contrast to serum repletion where DNA synthesis is increased (31). These data thus demonstrate that HGF increases the binding and uptake of ^{125}I-labeled LDL in HepG2 cells in a manner independent of cholesterol synthesis or cell growth.

Effect of HGF on the level of LDL receptor mRNA

To assess whether the HGF-mediated increase in LDL receptor activity was due to an increase in the level of LDL receptor mRNA, HepG2 cells were incubated with various concentrations of HGF. After various time periods at 37°C, total cellular RNA was isolated, and the level of LDL receptor mRNA was measured by ribonuclease protection. In the experiments described below, the levels of LDL receptor mRNA were expressed relative to that of the "house-keeping" gene G3PDH. After a lag of approximately 1.0 h, HGF produced a transient time-dependent increase in the level of LDL receptor mRNA that rose 3.0-fold within 2.0 h and reached a

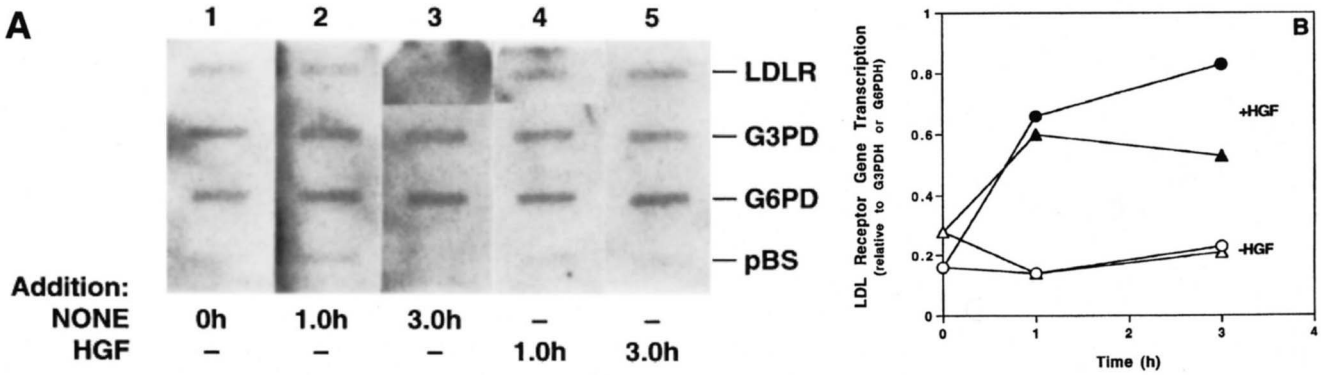

Fig. 6. HGF increases the rate of transcription of the LDL receptor gene in HepG2 cells. Each dish of cells was incubated in the presence or absence of 50.0 ng of HGF protein/ml in a final volume of 15 ml of serum-free MEM. After the indicated time at 37°C, nuclei were isolated and run-on transcription analyses were performed as described under Methods. Each strip was hybridized with an equal amount of each ^{32}P-labeled RNA sample (~5.7×10^6 cpm). A: Representative autoradiogram. B: The LDL receptor, G3PDH, and G6PDH transcription rates were measured by densitometric scanning of the autoradiograms. The level of LDL receptor (LDLR) gene transcription was expressed relative to either G3PDH (circles) or G6PDH (triangles). pBS represents control (no insert) pBluescript II KS(+) DNA. The figure shown is representative of two separate experiments.

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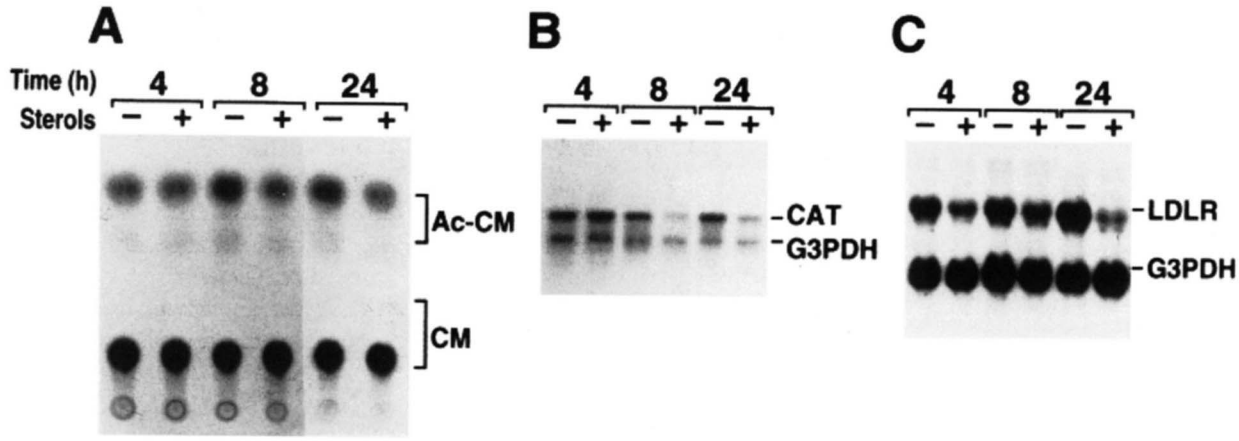

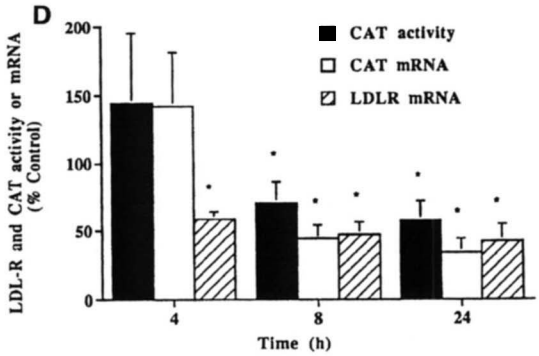

Fig. 7. Sterol-mediated repression of CAT and LDL receptor gene transcription in HepG2 cells transfected with pXT-R23. HepG2 cells were stably transfected with pXT-R23 and pools of transfected cells were subcultured as described under Methods and refed with fresh medium A 24 h prior to the beginning of each experiment. Cells were incubated in the absence or presence of 10 μg 25-hydroxycholesterol/ml for the indicated time at 37°C. The cells were then harvested for measurement of CAT and β-galactosidase activities and for RNA analysis as described under Methods. The acetylated forms of [^{14}C]chloramphenicol were separated from unreacted [^{14}C]chloramphenicol by thin-layer chromatography and detected by autoradiography. The ratios of acetylated forms of chloramphenicol to total chloramphenicol were calculated and the values were normalized to the β-galactosidase activity measured in each extract. Representative autoradiograms are shown of CAT activity (panel A), CAT mRNA determined by RT-PCR (panel B), and endogenous LDL receptor mRNA determined by Northern blotting (panel C). The level of endogenous G3PDH mRNA was used as a control for RT-PCR and Northern blotting. The relative levels of CAT, LDL receptor, and G3PDH mRNA were measured by scanning densitometry of the autoradiograms and the results are presented in panel D. The values are expressed as a percent of the ethanol control for each time point. Each bar represents the mean ± SE of 3–4 independent experiments. *Significant difference ($P < 0.05$) from control.

maximum of 4.2-fold within 4.0 h (**Fig. 3,** panels A and B). The level of LDL receptor mRNA declined thereafter. Over this time course, HGF had no effects on the level of G3PDH mRNA. HGF did not affect the level of LDL receptor mRNA in cultures of human skin fibroblasts over this time course (data not shown) suggesting that the effect requires specific HGF receptors. Similar to the induction of ^{125}I-labeled LDL uptake by HGF, the induction of LDL receptor mRNA was dependent on the concentration of HGF used. The effect was near maximal at 5.0 ng/ml HGF (**Fig. 4,** panels A and B). Thus, incubation with HGF increases both ^{125}I-labeled LDL uptake and LDL receptor mRNA in HepG2 cells.

Effects of HGF on turnover of LDL receptor mRNA and on the relative rate of LDL receptor gene transcription

As the accumulation of a specific mRNA can be regulated by either an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of these processes, the relative rates of LDL receptor gene transcription and mRNA turnover in HGF-stimulated cells were determined. To determine whether LDL receptor mRNA stability is altered by HGF, HepG2 cells were incubated in the presence or absence of HGF for 4.0 h at 37°C, actinomycin D was added to block further transcription, and incubation was continued for up to 6.0 h. Incubation with HGF produced a 3.7-fold induction of LDL receptor mRNA relative to incubation in serum-free media alone (**Fig. 5,** panel A, compare lanes 1 and 5). Blockade of transcription with actinomycin D reduced the level of LDL receptor mRNA in a time-dependent manner for cells incubated in the presence or absence of HGF (Fig. 5, panel A lanes 1–4 and lanes 5–8, respectively). The level of G3PDH mRNA remained relatively stable over this time course. These data were quantitated by scanning densitometry and are presented in Fig. 5, panel B. The half-times for turnover of LDL receptor mRNA were approximately 2.5 h and 4.0

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h for cells incubated in the presence or absence of HGF, respectively. These data demonstrate that the induction of LDL receptor mRNA in HepG2 cells by HGF is not due to increased mRNA stability.

To determine whether the rate of LDL receptor gene transcription is altered in HGF-treated cells, HepG2 cells were incubated with HGF and, after various times at 37°C, nuclei were isolated for use in nuclear run-on transcription assays. Incubation in serum-free media alone for 1.0 or 3.0 h produced little change in LDL receptor gene transcription relative to transcription of either the G3PDH or G6PDH genes (**Fig. 6,** panel A, lanes 1, 2, and 4). Transcription of the LDL receptor gene was increased at each time point, in contrast, in cells incubated with serum-free media containing 50 ng HGF/ml (Fig. 6, panel A, lanes 3 and 5). Scanning densitometry revealed that HGF increased transcription of the LDL receptor gene 4.1-fold within 1.0 h and 5.2-fold at 3.0 h relative to that of G3PDH (Fig. 6, panel B). Similar results were noted when the data were normalized to that of G6PDH. The induction of LDL receptor gene transcription thus precedes and closely parallels the elevation of LDL receptor mRNA.

Effects of oxysterols or HGF on transcription of the CAT gene under control of repeats 2 and 3 of the LDL receptor promoter

To examine the mechanism by which HGF induces LDL receptor gene transcription in greater detail, HepG2 cells were stably transfected with plasmids bearing repeats 2 and 3 of the LDL receptor promoter. Our initial studies utilized the plasmids pHβAPr-1-neo-β-gal and either pXT or pXT-R23 with CAT as the reporter. Repeats 2 and 3 appear to be the minimum sequences necessary to confer sterol repression on a heterologous promoter (6) and thus should confer regulation of CAT mRNA and activity in response to changes in cell cholesterol metabolism. Transcription of the endogenous LDL receptor gene could then be compared to that of the CAT gene in growth factor-treated cells.

Cellular CAT activity in HepG2 cells transfected with pXT-R23 was elevated 300- to 500-fold relative to cells transfected with the enhancerless pXT vector alone (data not shown). To assess whether the SRE-1 in pXT-R23 would promote CAT gene transcription in a sterol-dependent manner, HepG2 cells transfected with pXT-R23 were incubated in the presence and absence of 25-hydroxycholesterol for various times at 37°C. The cells were harvested, and the levels of LDL receptor and CAT mRNA and activity were measured. Incubation with 25-hydroxycholesterol produced a time-dependent reduction in the level of LDL receptor mRNA (**Fig. 7,** panel C) to 42% of control at 24 h. The levels of CAT activity and mRNA were also reduced by oxysterols; however, their reduction was delayed compared to that of the endogenous LDL receptor mRNA (Fig. 7, panels A and B). The lack of reduction of CAT activity or mRNA by oxysterols at early (4.0 h) time points was probably related to differences in mRNA stability as the half-lives of CAT and LDL receptor mRNA measured in the presence of actinomycin D in other experiments were 8.0 h and 4.0 h, respectively. Consistent with studies in other cell types (4–7), these data confirm that the SRE-1 can confer repression of CAT gene transcription by oxygenated sterols in HepG2 cells transfected

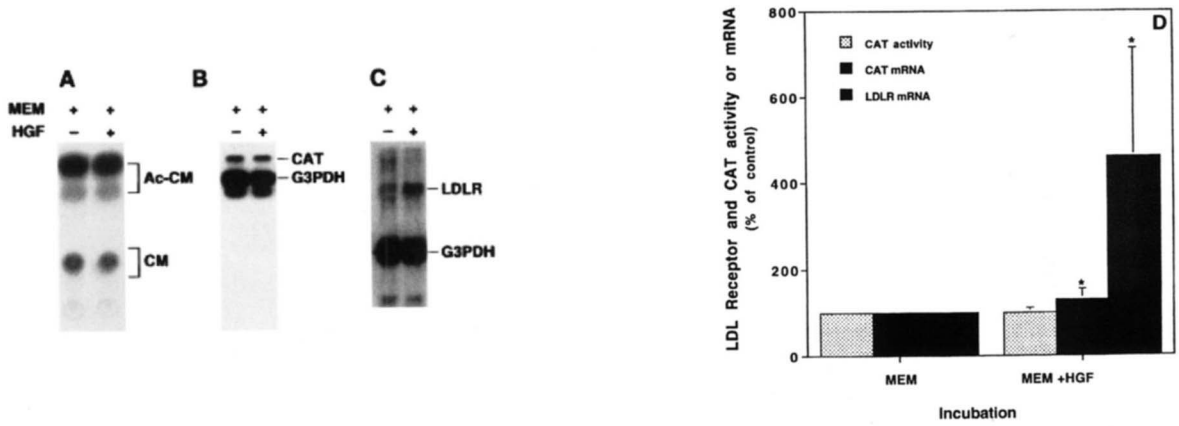

Fig. 8. Effect of HGF on CAT and LDL receptor gene expression in pXT-R23 transfected HepG2 cells. Transfected cells were subcultured as described in the legend to Fig. 7. On day 4, the media were removed, the cells were washed, and to each flask was added either MEM alone or MEM containing 50 ng of HGF protein/ml. After 4 h incubation at 37°C, the cells were harvested for measurement of CAT and β-galactosidase activities and total RNA was isolated for measurement of CAT mRNA and endogenous LDL receptor and G3PDH mRNAs as described in the legend to Fig. 7. Autoradiograms of a representative experiment are shown in panels A, B, and C for CAT activity, CAT mRNA, and endogenous LDL receptor mRNA, respectively. The autoradiograms from five separate experiments were quantitated as described in the legend to Fig. 7 and the results are shown in panel D. Each bar represents the mean ± SE. *Significant difference ($P < 0.05$) from control.

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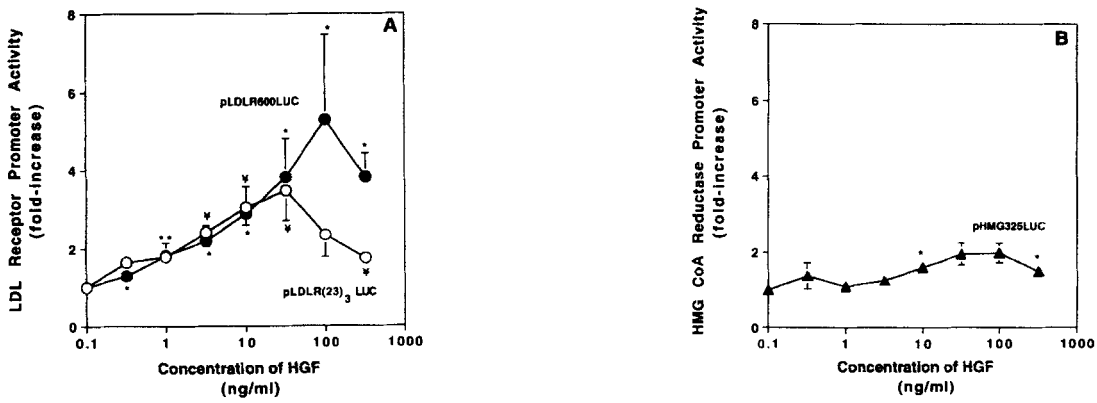

Fig. 9. Effect of a 5.0-h incubation with HGF on luciferase gene expression in pLDLR600LUC, pLDLR(23)3LUC, or pHMG325LUC transfected HepG2 cells. HepG2 cells were transfected with the indicated reporter for 6.0 h, washed, and refed with media containing 10% FBS. After overnight incubation, the cells were washed and media containing 10% LPDS alone or with varying concentrations of HGF were added. After 5.0 h at 37°C, the cells were lysed for luciferase and β-galactosidase measurements. Each point represents the mean ± SEM for 6 (panel A) or 3 (panel B) transfections. The data were normalized to the level of luciferase activity seen at 0.1 ng/ml HGF. pLDLR600LUC is the native LDL receptor promoter, pLDLR(23)3 is the multimerized repeats 2 + 3 from the LDL receptor promoter, and pHMG325 is the HMG-CoA reductase promoter driving luciferase as described under Methods. Differences significant compared to 0.1 ng/ml HGF: $*P < 0.01$, $**P < 0.05$, $?P < 0.01$.

with pXT-R23. However, the sluggish response of the CAT gene to oxysterol-mediated repression demonstrates a limitation for using CAT as a reporter.

To determine whether HGF could stimulate CAT gene expression in the pXT-R23 transfected cells, HepG2 cells transfected with pXT-R23 were incubated in serum-free media in the presence or absence of HGF. After various times at 37°C, the cells were harvested and the levels of LDL receptor and CAT mRNA and activity were determined as described above. Relative to incubation for 4.0 h with serum-free media alone, the level of LDL receptor mRNA was increased nearly 5-fold in cells incubated with HGF (**Fig. 8**). The level of CAT mRNA was increased much less, about 30% in the HGF-treated cells, but this was statistically significant. No changes in the level of CAT activity were observed in extracts from these cells. Thus, relative to changes in LDL receptor mRNA, a single copy of repeat 2 and 3 drives CAT gene expression in response to HGF, but not to the same extent as it drives endogenous LDL receptor gene expression.

Effect of HGF on transcription of the luciferase gene under control of repeats 2 and 3 of the LDL receptor promoter

Because of the sluggish and weak response of the CAT reporter seen in the studies described above, plasmids were constructed that used firefly luciferase as the reporter gene. In these constructs, the luciferase gene was placed under control of either the native LDL receptor promoter extending from -556 to +53 (pLDLR600LUC) or three copies of repeats 2 and 3 in tandem (pLDLR(23)3LUC). HepG2 cells were transiently transfected with these plasmids and the effect of HGF on luciferase activity was determined. In the experiments described below, the transfected cells were incubated in the presence and absence of HGF in media containing 10% LPDS, rather than under the serum-free conditions described above. These conditions were used due to the poor cell viability observed when cells were placed in serum-free media immediately after transfection. Incubation for 5 h at 37°C with increasing amounts of HGF increased luciferase activity in pLDLR600LUC- and pLDLR(23)3LUC-transfected cells (**Fig. 9,** panel A) to a maximum of about 3- to 6-fold, over control, at HGF concentrations of 32–100 ng/ml. For comparison, incubation of the pLDLR(23)3LUC-transfected cells with 10^{-5} M mevinolin for 5.0 h increased luciferase activity 3.1-fold. No significant differences in the luciferase activity produced from either construct were observed except at the highest concentration of HGF tested. The reason for the decline in luciferase activity with pLDLR(23)3LUC at this concentration of HGF is not clear.

To learn whether the induction of luciferase activity by HGF was in response to sterol deprivation, parallel cultures of HepG2 cells were also transfected with plasmids bearing a fragment of the HMG-CoA reductase promoter extending from -325 to +22, a region that encompasses the reductase sterol regulatory element (2). Incubation of these cells with HGF produced little or no consistent effects on luciferase activity (Fig. 9, panel B). Similar results were observed when the incubations with HGF were extended to 24 h (**Fig. 10**):

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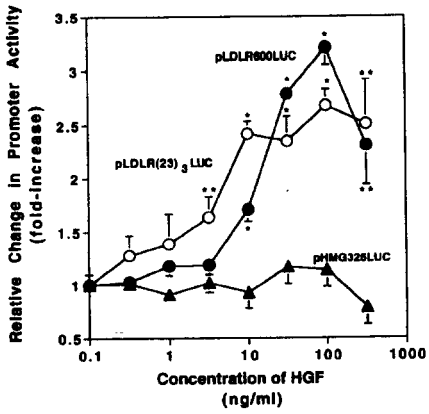

Fig. 10. Effect of a 24 h incubation with HGF on luciferase gene expression in pLDLR600LUC, pLDLR(23)$_3$LUC, or pHMG325LUC transfected HepG2 cells. After a 6.0 h calcium phosphate transfection and overnight incubation in media containing 10% FBS, cells were treated with the indicated concentration of HGF for 24 h in media containing 10% LPDS. Data are mean ± SEM for triplicate transfections. Differences significant from incubation with 0.1 ng/ml HGF: $*P < 0.005$, $**P < 0.05$.

incubation with HGF produced a dose-dependent induction of luciferase activity from pLDLR600LUC- and pLDLR(23)$_3$LUC-transfected cells whereas no changes in reporter gene activity were found in cells transfected with pHMG325LUC. The pHMG325LUC construct was sterol-sensitive as incubation of the transfected cells with 10^{-5} M mevinolin for 5.0 h increased luciferase activity nearly 2-fold. These data thus demonstrate that when compared to the native LDL receptor promoter, multiple copies of repeats 2 and 3 of the LDL receptor promoter can fully support activation of the luciferase gene by HGF. The lack of effects mediated through the reductase sterol regulatory element suggests, however, that gross sterol depletion is not responsible for these changes.

DISCUSSION

The present investigation has established that incubation of HepG2 cells with recombinant human HGF stimulates the expression of the LDL receptor gene. Induction of receptor activity and mRNA occurred with as little as 2–5 ng of HGF protein/ml and was apparent within 2.0 h of incubation with this agent. As measured in nuclear run-on transcription analysis and in mRNA turnover studies, the HGF-mediated elevation of LDL receptor mRNA was due primarily to induction of LDL receptor gene transcription. These effects of HGF occurred within the range of reported serum concentrations of HGF in humans (32, 33). Although HGF is likely to have diverse effects on liver-derived cells, these finding are compatible with the hypothesis that HGF may be a physiologically important regulator of LDL receptor expression in the liver and could account for the rapid induction of LDL receptor transcription after partial hepatectomy.

Initially isolated and characterized from the serum of partially hepatectomized rats, rat platelets, or plasma from humans with acute liver failure, HGF is a ubiquitous serum growth factor that has affects on a variety of biological processes. It exhibits mitogenic, angiogenic, and/or motogenic properties on its target tissues (23, 24). On a molar basis, HGF is the most potent mitogen known for normal hepatocytes both in vitro and in vivo and is likely to be the most important stimulus for liver regeneration following experimentally induced liver damage (23, 24). However, HGF is not a simple mitogen for hepatocytes as it inhibits the growth of hepatoma cells (30) and, in the present study, increased LDL receptor expression in the absence of changes in cell growth. The difference in response of normal hepatocytes and hepatomas could be related to differences in cell origin or alterations in the HGF receptor or its signalling pathways. In vivo HGF only becomes a hepatic mitogen after the normal liver is primed by partial hepatectomy (29). Thus, HGF may have pleiotrophic effects in this, as in other cell types. Comparison of the kinetics of induction of the serum HGF concentration (28) with that of LDL receptor protein and mRNA (19) after partial hepatectomy suggests that HGF could potentially regulate LDL receptor gene expression in vivo. Further, the action of HGF as a rapidly acting, short-term inducer of LDL receptor expression is consistent with our previous work documenting the induction of LDL receptor activity in HepG2 cells by a serum protein factor (18, 28).

A number of serum factors and hormones have been reported to regulate LDL receptor expression in liver-derived cells. Tumor necrosis factor-α (TNF-α) (34), interleukin-1β (IL-1β) (34), oncostatin-M (16), epidermal growth factor (EGF) (16, 17), insulin (35), estrogen (36), and whole serum (31) or a fraction of serum (18) stimulated LDL receptor expression in cultured rat and human cells. In addition to these in vitro studies, recent reports have shown that growth hormone (37, 38), glucagon (39), insulin (40), and estrogen (41) also act as positive regulators of LDL receptor activity in vivo. The mechanism(s) by which such a diverse group of factors regulate LDL receptor expression is not yet clear.

The best-studied example of LDL receptor gene regulation to date is the sterol-mediated feedback repression of transcription (2). If this were the common event, induction of LDL receptors by growth factors, cytokines, and hormones would be initiated by a change in a critical regulatory pool of sterol that would then alter the amounts or activities of nuclear proteins, such as SREBP, that activate transcription of the LDL receptor

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promoter. The results of several recent studies, however, have suggested alternate mechanisms for sterol-independent control of hepatic LDL receptor activity. Signalling by specific serum growth factors or hormones may override sterol-dependent transcriptional repression in liver-derived cells. For example, part of the resistance to suppression of hepatic LDL receptor gene transcription in rats may be due to the presence of growth hormone (42). Stimulation of LDL receptor gene transcription by oncostatin M (16), insulin (35, 43), whole (31) or fractionated serum (18) occurred in the absence of demonstrable changes in cell cholesterol metabolism and in sterol-loaded cells. Similarly, Graham and Russell (44) recently reported that EGF increased LDL receptor activity in HepG2 cells in the absence of changes in cholesterol synthesis or cell proliferation. The stimulus for liver regeneration in vivo, in addition, appears capable of over-riding sterol-dependent repression as the induction of LDL receptors after partial hepatectomy occurred over a time of active lipid deposition in the residual liver (19–21). The results of the present study also demonstrated that the induction of LDL receptor gene transcription by HGF occurred in the absence of changes in cell cholesterol metabolism. HGF had no significant effects on the incorporation of [^{14}C]acetate into radiolabeled cholesterol and, in preliminary experiments, incubation of HepG2 cells with 25-hydroxycholesterol did not diminish the induction of LDL receptor mRNA by HGF (A. J. Carlstrom and J. L. Ellsworth, unpublished observations).

To further test this, HepG2 cells were transfected with a reporter plasmid bearing genetic elements believed to be responsible for sterol-mediated regulation of the LDL receptor gene. When the experiments were performed in transient transfections using luciferase as the reporter, there was induction of reporter activity driven by repeats 2 and 3 by both HGF and mevinolin in experiments of 4–5 h. The rapid induction of LDL receptor gene expression by sterol depletion was studied by Sudhof et al. (5) using cells transfected with a large fragment of the LDL receptor promoter extending to -1563. CAT gene transcription mediated by this element was rapidly induced (within 2 h) after removal of sterols from the media. The time course of induction of CAT activity under control of smaller fragments of the LDL receptor promoter, however, has not been reported. The sluggish response of CAT expression to either oxysterols or HGF observed in the present study suggests that there is a discrepancy between CAT and luciferase in their responsiveness. This is partly due to a slow induction of CAT transcription as CAT mRNA levels had barely risen at 4.0 h of incubation with HGF and to a prolonged half-life of the CAT mRNA. These data suggest that there are limitations to the use of CAT as a reporter for studies of rapid LDL receptor gene regulation. In addition, with transient transfection, different conditions are required to maintain cells than are required for the stably transfected cells. Thus, studies utilizing promotor reporter constructs to study physiologic responses may require several complimentary approaches to arrive at valid conclusions.

The results of the present study suggest that other sequence elements are not required to mediate rapid induction of transcription in the absence of sterols or in response to growth factors. Unlike the HMG-CoA reductase gene where there is a hormone response element in addition to the SRE, hormonal effects on the LDL receptor are likely to be evoked by changes in the amount or activity of SREBP. This could be mediated by changes in a small regulatory pool of sterol induced by HGF, which in turn affects the protease that regulates the release of SREBP from the endoplasmic reticulum. Arguing against this is the result with the HMG-CoA reductase promoter construct. Had there been a change in a regulatory sterol pool, there should have been induction of the reporter driven by this promoter.

This suggests an alternative whereby nonsterol factors are capable of increasing the rate of release of SREBP from the endoplasmic reticulum or a decrease in the rapid degradation of SREBP bound to its cognate DNA. There are a number of ways this latter possibility could be mediated. The HGF receptor is a membrane spanning protein tyrosine kinase that becomes autophosphorylated upon HGF binding (45) and, depending on the cell type, associates with a number of cytoplasmic signal transducers. In A549 lung carcinoma cells, HGF binding to its receptor stimulates the Ras-guanine nucleotide exchanger (46) and induces the association of phosphatidylinositol-3-kinase with the HGF receptor (47). As Ras stimulation of mitogen-activated protein kinase (MAP-kinase) mediates transduction of a number of growth factor signals, HGF induction of LDL receptor gene expression could be through the MAP-kinase cascade. One of these kinases could alter the phosphorylation state of the SREBP cleaving enzyme or SREBP protease and, thus, affect their activity. The inquiry into this and other possible mechanisms (12) of sterol-independent regulation of LDL receptor expression could eventually help explain why there are different serum levels of LDL amongst individuals with apparently similar sterol balance.

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