C-myc is required for the G_0/G_1–S transition of primary hepatocytes stimulated with a deleted form of hepatocyte growth factor

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Primary rat hepatocytes stimulated *in vitro* with the addition of a deleted form of hepatocyte growth factor (dHGF) enter the S-phase 48 h after addition of the growth factor. The c-*myc* gene is believed to play a role in a variety of cellular stages, such as proliferation, differentiation and cell death. In primary hepatocytes c-*myc* was expressed constitutively at both mRNA and protein levels, independently of the growth conditions. dHGF induced significant c-*myc* expression at times correlated with the long-lasting pre-S phase, and no induction was observed at the G_0/G_1 traverse compared with the unstimulated hepatocytes. An antisense construct coding for all three exons of c-*myc* was imported into hepatocytes by using the transferrin receptor-mediated endocytosis methodology (transferrinfection). Expression of the antisense construct inhibited the biosynthesis of the c-Myc protein, however it did not interfere with the expression of c-*met*, encoding the receptor for HGF/dHGF. Continuous expression of the antisense construct inhibited entry of the hepatocytes into the S-phase. Regulated induction of the antisense c-*myc* by dexamethasone for up to 6 h in culture, did not interfere with the entry of hepatocytes into the S-phase. c-*myc* expression was shown to be required between 6 and 12 h in dHGF-stimulated hepatocytes, and inhbition of its expression during this time by the antisense *myc* construct did not allow these cells to enter the S-phase. Inhibition of c-*myc* biosynthesis between 24 and 48 h hours slightly affected the DNA synthetic response. It is proposed that the expression of c-Myc protein interferes with the 'priming' of hepatocytes to become responsive to growth-factor stimuli, or in the absence of such stimuli it interferes with the maintenance of a non-proliferating phenotype and subsequent *in vitro* de-differentiation.

INTRODUCTION

Primary hepatocytes of rodent or human origin do not proliferate under culture conditions *in vitro* unless stimulated with growth factors such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) [1–4]. Among the known growth factors specific for hepatocyte proliferation the recently discovered hepatocyte growth factor (HGF) was shown to be distinct from the other known factors [5–8]. Proliferation of primary hepatocytes in culture was associated with changes in the expression of the c-*myc*, c-*fos* and Ha-*ras* genes [9–10]. A role in liver development and in liver-cell proliferation has been assigned to both the c-*myc* and c-*jun* genes [11,12]. EGF and TGF-α were found to induce increased expression of c-*myc* mRNA [13,14], although it has also been observed that c-*myc* is constitutively expressed in cultured hepatocytes and that its product, the c-Myc protein, possesses a half-life that is much longer than that observed in cells bearing an amplified or altered c-*myc* gene [15–17]. The c-Myc protein, which is a transcription factor containing basic region-helix-loop-helix and leucine-zipper structural motifs, forms heterodimers with another bHLH-Zip protein called Max [18,19]. Microinjected c-Myc protein in quiescent cells was shown to function, at least in part, as a stimulus for the cells to enter the S-phase [20]. c-Myc protein seemed to be required for cells requiring a growth factor for growth rather than for cells constitutively expressing c-*myc*, which were shown to have reduced growth factor requirements [21]. c-Myc was also observed to be a potent inducer of apoptosis in cells where a proliferation block was imposed, or in cells already growth-arrested but with c-*myc* still activated [22]. In primary hepatocytes, exogenous expression of a c-*myc* construct did not seem to reduce their growth factor requirement, although it co-operated with EGF in super-inducing DNA synthetic activity [23].

We investigated the expression of the c-Myc protein in unstimulated primary hepatocyte cultures and also in those stimulated with dHGF, a potent growth inducer, which is a variant of HGF bearing a five amino acid deletion [24]. Our data have shown that c-Myc protein is constitutively expressed in resting primary hepatocytes and its synthesis is up-regulated in response to dHGF. An exogenous antisense c-*myc* construct containing all three exons of the gene was able to block the biosynthesis of hepatocyte c-Myc protein(s), and by that to inhibit the traverse of these cells to the S-phase. It is also shown that inhibition of c-Myc protein biosynthesis in the early G_1-phase significantly reduces the dHGF-induced DNA synthetic response. This cell cycle-related inhibition was not due to transcriptional or translational block of the c-*met* gene which encodes the HGF/dHGF receptor. From our data, it is revealed that the constitutive expression of c-*myc* in hepatocytes may contribute to the maintenance of a non-proliferating phenotype of these cells *in vitro*, however, the inhibition of c-Myc protein synthesis between 6 and 12 h after growth factor-stimulation blocks the traverse of these cells towards the S-phase.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma Chemical Co. and radiochemicals were from Amersham, U.K. Cytomegalovirus β-

Abbreviations used: CMVβ-gal, cytomegalovirus early promoter-driven β-galactosidase; HGF, hepatocyte growth factor; dHGF, a variant of HGF bearing a five amino acid deletion; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; MMTV, murine mammary tumour virus; DMEM, Dulbecco's modified Eagle's medium; MEM, Eagle's minimum essential medium; ECL, enhanced chemiluminescence; [^{3}H]TdR, thymidine; PCNA, anti-proliferating cell nuclear antigen.

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galactosidase (CMVβ-gal) gene was from Promega Biochemicals, dHGF was a gift from Dr. N. Shima (Snow Brand Milk, Tochigi, Japan) and pBS01*myc* was provided by Dr. R. Eisenman (Seattle, U.S.A.).

Methods

Isolation and culture of primary rat hepatocytes

Hepatocytes from young adult rats (120–140 g) were prepared using the two-step collagenase perfusion technique [25]. The purified hepatocytes were plated onto collagen-coated 60 mm-diam. dishes (unless otherwise stated) at a density of 5×10^5 cells/3 ml of Dulbecco's modified Eagle's medium (DMEM) with additions reported elsewhere [23]. Hepatocytes were allowed to attach and were then fed with fresh medium containing insulin at 0.5 μg/ml and 50 ng of dHGF/dish, where appropriate. The cells were incubated in a humidified 5% CO_2/95% air atmosphere and the medium was thereafter changed every 24 h. Hepatocytes used for transfections by employment of the calcium phosphate precipitation technique were incubated overnight in insulin-supplemented DMEM, and addition of various factors (dHGF and/or dexamethasone) was carried out after washing off the transfection mixture.

Transfection of primary hepatocytes

Primary hepatocytes incubated overnight in 3 ml of DMEM supplemented with insulin, were transfected using the calcium phosphate precipitation technique with the pMMTVasmyc01 (MMTV, murine mammary tumour virus) and/or the pCMVβ-gal constructs. The transfection mixture consisted of 6 μg of DNA (pMMTVasmyc01 and/or pCMVβ-gal; 5:1) in 250 μl of 0.25 M $CaCl_2$ mixed with an equal volume of 2 × BBS (280 mM NaCl, 50 mM BES and 1.5 mM Na_2HPO_4, pH 6.95) (where BES is *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid) [26]. Calcium chloride and 2 × BBS were mixed gently (by inverting the tube twice) in a 1.5 ml Eppendorf tube and incubated at room temperature for 20 min, allowing the precipitate to form. The mixture, after brief spinning (2000 rev./min) at room temperature, was pipetted directly into the dishes. The hepatocytes were washed twice with fresh DMEM 10–12 h after transfection and then fed with DMEM containing various additions (see Figure legends for details).

For transferrinfection of the primary hepatocytes, the original protocol was followed [27,28]. Attached cells were transferrinfected with 5×10^{11} particles/ml (10–30 μl) of inactivated biotinylated human E1A-defective adenovirus (a gift from Dr. E. Wagner, Institute of Molecular Pathology and Bender Ltd., Vienna, Austria) in HBS (150 mM NaCl/10 mM Hepes, pH 7.3) modified with streptavidin-polylysine (600 ng) and then allowed to form a complex with plasmid DNA [6 μg total, 1:5 pCMVβ-gal: antisense construct]. Subsequently, transferrin-polylysine (6 μg) was added, and after a final incubation for 30 min at room temperature, the transferrinfection mixture (0.5 ml) was added to 1.5 ml of DMEM (supplemented with insulin).The transferrinfection complex was added to the cells and incubated for 4 h. Afterwards, the transfection mixture was replaced by ordinary DMEM and various additions were made (see Figure legends for details).

DNA synthesis in hepatocyte cultures

[methyl-^{3}H]Thymidine [^{3}H]TdR (5 μCi/dish; specific radioactivity > 85 Ci/mmol) was added to the cultures for 2 h. DNA and radioactivity were estimated as previously described [3,23].

Histochemistry

Hepatocyte monolayers were washed three times with PBS, fixed for 5 min at room temperature with 0.5% (v/v) glutaraldehyde in PBS and then stained for between 30 min and 12 h at 37 °C by using 5-bromo-4-chloro-3-indolyl-β-galactopyranoside as a substrate for β-galactosidase (EC 3.2.1.23). The cultures were then washed once in PBS followed by distilled water, 96% ethanol and examined microscopically [29].

Plasmid construction

For the construction of the antisense c-*myc* expression plasmid, the starting plasmid was pMMTV, which contains an MMTV promoter upstream of a polylinker cloning site. Downstream splice and polyadenylation sites are derived from Simian virus-40 early region. A 1.8 kb *Eco*RI cDNA fragment of pBS01, containing all three exons of c-*myc* [17], was cloned into the unique *EcoRI* site of the polylinker. cDNA orientations in the MMTV vector were confirmed by restriction enzyme mapping and their expression was verified by Northern (RNA) blot analysis of RNAs extracted from dexamethasone-treated or untreated cells.

RNA isolation and hybridization conditions

Total RNA from cultured or freshly isolated hepatocytes was isolated by using the guanidinium thiocyanate/phenol method [30]. RNA was electrophoresed on a 1%-agarose/1 × Mops gel and transferred onto Hybond-N membrane (Amersham International). [α-^{32}P]CTP-labelled *Eco*RI fragment (1.8 kb) was used and the membranes were hybridized for 18 h at 68 °C and subsequently washed with 0.2% (v/v) SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS. The membranes were then exposed to Kodak XAR-2 at −70 °C using two intensifying screens. Equal loading controls were performed by either ethidium bromide staining of the gels or by re-probing the membranes with a β-actin probe.

Western blotting analysis of hepatocyte proteins

Total hepatocyte protein lysates were made by resuspending the cells (from 2–3 dishes) in lysis buffer containing 20 mM Hepes, 5 mM KCl, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 30 μl/ml aprotinin (Sigma), 100 μM sodium orthovanadate and 1 mM PMSF. Hepatocyte proteins (25 μg/lane unless otherwise stated) were separated on 15% SDS/PAGE and then transferred onto Immobilon membrane using a BioRad protein-transfer apparatus. Membranes were blocked with PBS/0.1% (v/v) Tween 20 containing 5% BSA (fraction V) for 30 min, washed three times (7 min each) with PBS/0.1% (v/v) Tween 20 and further incubated for 1 h with the monoclonal antibodies CT14.GT3 and 1-9E10 (anti-*myc*, American Type Culture Collection), the anti-proliferating cell nuclear antigen (PCNA; Dianova), or anti-*met* (a gift from Dr. G. F. Vande Wounde, NIH, Bethesda, MD, U.S.A.) antibodies. Anti-mouse (for *myc* and PCNA antibodies) or rabbit (for *met* antibodies)-peroxidase-labelled secondary antibody (Dianova) was then added (35 min), and after washing with PBS/0.1% (v/v) Tween 20 as above, the reactive species were revealed using a commercially available enhanced chemiluminescence kit (ECL; Amersham International).

Immunoprecipitation of hepatocyte proteins and SDS/PAGE analysis

Cultured hepatocytes were pulse-labelled with 150 μCi/dish of a [^{35}S]Met/Cys mixture in Met/Cys-free minimal essential medium (Sigma) for 2 h. The culture supernatants were then collected and

the respective antibodies were added (anti-mouse albumin, anti-mouse α-fetoprotein, anti-human transferrin and anti-human α_2-macroglobulin; ICN) and rocked at room temperature (100 rev./min) for 2 h. Afterwards Pansorbin (50 %) (Calbiochem) was added and the incubation proceeded for an additional hour under the conditions described above. The immunocomplexes were washed three times and subsequently analysed as previously described [23].

RESULTS

Effects of dHGF on c-*myc* expression in primary hepatocytes

We investigated the expression of the c-*myc* gene at the mRNA and the protein level in hepatocyte cultures treated with or without dHGF, a variant form of HGF which, like HGF itself, is a potent stimulator of hepatocyte proliferation [24]. The expression of the c-*myc* gene in primary hepatocytes treated with or without dHGF was analysed by Northern blotting and is shown in Figure 1(A). During the culture time (4 days) c-*myc* was constitutively expressed, however in dHGF-supplemented hepatocyte cultures the level of its expression was approximately 4-fold that of the control at 24, 48 and 72 h post-addition of dHGF. At later times in culture (96 h), the expression of c-*myc* was returned to baseline levels (results not shown).

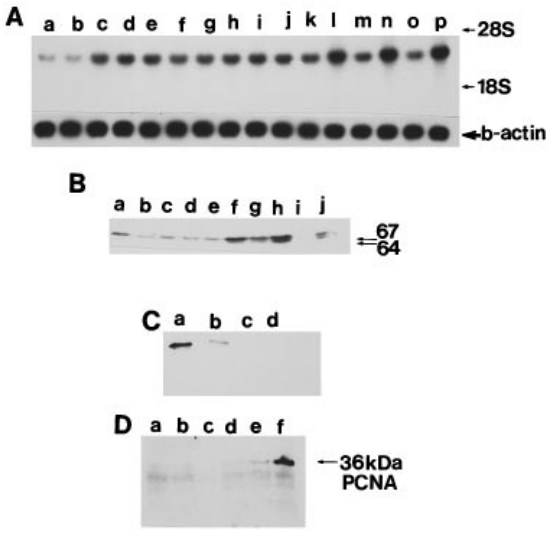

Figure 1 c-*myc* expression in hepatocytes stimulated with dHGF

(**A**) Northern blot analysis of hepatocyte total RNA (20 μg) hybridized with a cDNA for c-*myc* (upper panel) and for β-actin (lower panel) as probes. Lanes a and b, RNA from freshly isolated hepatocytes from two independent experiments; lanes c, e, g, i, k, m and o, RNA from unstimulated hepatocytes cultured for 3, 6, 9, 12, 24, 48 and 72 h respectively; lanes d, f, h, j, l, n and p, RNA from dHGF-stimulated hepatocytes isolated at identical time-points. (**B**) Western blot/ECL analysis of hepatocyte c-Myc protein(s). Lanes a, c, e, g and i, hepatocyte proteins from unstimulated cultures were subjected to SDS/PAGE/Western blot analysis and c-Myc-reacting proteins were revealed by ECL; lanes b, d, f, h and j, hepatocyte *myc*-reacting proteins under dHGF-stimulated conditions. Culture times: lanes a and b, 3 h; lanes c and d, 12 h; lanes e and f, 24 h; lanes g and h, 48 h; and lanes i and j, 72 h. Arrows indicate the molecular masses of the hepatocyte *myc* species in kDa. (**C**) Competition assay of the myc-specific peptide, CT14.GT3 monoclonal antibody, reacted at 1:2000 (v/v) (lane a) or 1:10000 (v/v) (lane b) dilution with hepatocyte proteins separated by SDS/PAGE and transferred onto Immobilon membranes. Lanes c and d, identical experiment as for lanes a and b but the c-Myc antibody was pre-incubated with the immunogenic peptide. (**D**) Detection of PCNA in dHGF-stimulated hepatocytes. Lanes a–c, hepatocyte proteins were analysed with SDS/PAGE/Western blot and PCNA-reacting proteins were revealed as in (**B**). Lanes a–f, reacting proteins from hepatocytes stimulated for 3, 6, 12, 24, 48 and 72 h respectively.

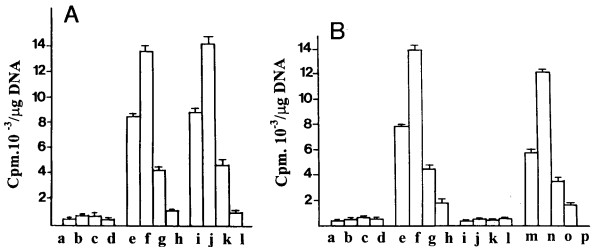

Figure 2 DNA synthesis in normal and in pMMTV-transferrinfected hepatocytes in the presence of dHGF and/or dexamethasone

(**A**) Hepatocytes were cultured in the presence of insulin (lanes a–d), insulin + 20 ng of dHGF/dish (lanes e–h) or insulin + 50 ng of dHGF/dish (lanes i–l). DNA synthesis was estimated at different time-points after a 2 h pulse-labelling with 5 μCi of [^{3}H]TdR and was expressed as the mean of triplicate values ± S.E.M. from at least three independent experiments. Culture times: lanes a, e and i, 48 h; lanes b, f and j, 72 h; lanes c, g and k, 96 h; and lanes d, h and l, 120 h. (**B**) After transferrinfection of the hepatocytes with the pMMTV vector in the presence of insulin (lanes a–d), DNA synthesis was estimated as described in the Methods section. DNA synthesis in pMMTV-transferrinfected hepatocytes at different time-points was estimated in the presence of insulin + dHGF (50 ng/dish) (lanes e–h), in the presence of dexamethasone (10^{-6} M) (lanes i–l), or in the presence of dexamethasone and dHGF (lanes m–p). Culture times: lanes a, e, i and m, 48 h; lanes b, f, j and n, 72 h; lanes c, g, k and o, 96 h; and lanes d, h, l and p, 120 h. Values represent means ± S.E.M of triplicates from two or more independent experiments.

c-Myc protein expression was constitutive throughout the culture time and dHGF markedly induced the synthesis of c-Myc protein at 24 and 48 h in culture (Figure 1B). This induction of c-Myc protein expression coincided with the increased c-*myc* mRNA levels observed in the Northern analysis, although c-*myc* mRNA was also increased at 72 h. In most blots, c-Myc protein appeared as a 64–67 kDa doublet, the 67 kDa predominating, as was also shown in studies involving material from cell lines expressing amplified or altered *myc* genes [17]. In hepatocytes, previous studies have revealed c-*myc*-reacting proteins migrating to 55 kDa, although this phenomenon may be attributed to the significant proteolysis involved in the methodologies used (immunoprecipitation) [23,31]. Throughout our studies, two monoclonal antibodies have been used: CT14.GT3 and 1-9E10 raised against the c-Myc C-terminal peptide [32], and also a commercially available MYC polyclonal antibody (CRB, U.K.). A competition assay of the *myc*-specific peptide is shown using 1:2000 (w/v) (normally used) and 1:10000 (v/v) dilution of the CT14.GT3 monoclonal antibody (Figure 1C).

Stimulation of hepatocyte DNA synthesis by dHGF

In hepatocyte bioassays, both HGF and dHGF have shown similar dose–response curves for concentrations up to 10 ng/ml, thus implying that both forms share the same receptor and that they bind to it with the same affinity [33]. Dose–response curves for hepatocyte DNA synthesis were similar for up to 10 ng/ml growth factor, although dHGF, compared with HGF, was shown to produce a significantly more intense response at concentrations above 30 ng/ml [24]. We therefore used dHGF at 50 ng/dish, which was able to induce hepatocyte DNA synthesis at significant levels at times beyond 48 h in culture (Figure 2A). At 72 h, the DNA synthetic activity was maximal and declined to approx. control levels at 120 h.

PCNA, which is a 36 kDa non-histone nuclear protein, is required for DNA synthesis and repair. PCNA is detected in cells in the S-phase of the cell cycle and beyond, because of the long half-life of the protein [34]. In hepatocytes, PCNA became

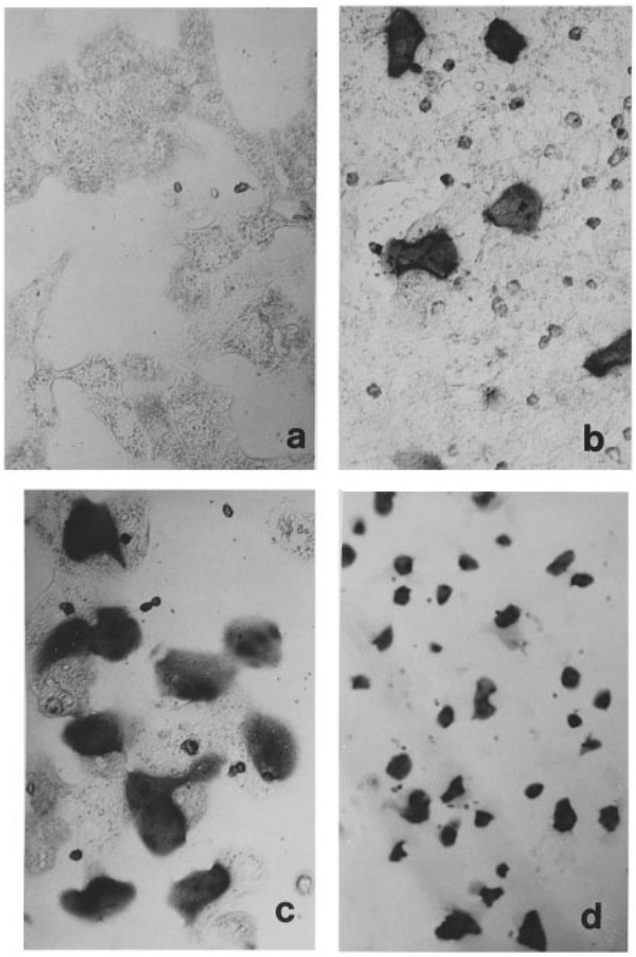

Figure 3 Analysis of β-galactosidase expression in primary hepatocytes after incubation with modified adenovirus complexes (transferrinfection) or transfected using the calcium phosphate methodology (magnification: a–c, $\times$120; d, $\times$60)

(**a**) Primary hepatocytes incubated with complexes lacking pCMVβ-gal DNA. (**b**) Hepatocytes transfected with calcium phosphate methodology; the transfection mixture contained both the antisense *myc* construct and the pCMVβ-gal (5:1). (**c**) Hepatocytes transferrinfected with complexes containing DNA constructs as in (**b**). (**d**) Lower magnification of (**c**).

significantly detectable at 72 h after dHGF stimulation (Figure 1D).

Effects of the transferrinfected antisense *myc* construct on Myc protein synthesis and hepatocyte proliferation

Cell surface receptors have been used as natural internalization sites for targeting DNA to various cells. The introduction of foreign DNA constructs via receptor-mediated endocytosis by employing the transferrin receptor has been used with success in a number of cell lines [27,28,35]. In this study, transferrinfection of primary hepatocytes is reported for the first time, using an antisense *myc* expression plasmid (pMMTVasmyc01) together with the pCMVβ-galactosidase plasmid. After removal of the transferrinfection mixture (4 h) the cells were fed with DMEM, and after overnight incubation were processed for histochemical detection of β-galactosidase (Figure 3). We have carried out two sets of experiments, one performing transferrinfection after overnight incubation of attached hepatocytes and another one

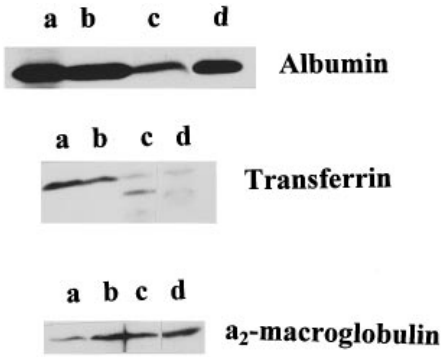

Figure 4 Hepatocyte differentiation markers in dHGF-stimulated/transferrinfected hepatocyte cultures

Immunoprecipitation analysis of [^{35}S]Met/Cys-labelled albumin, transferrin and α_2-macroglobulin secreted by hepatocytes stimulated with dexamethasone (10^{-6} M) at 3 days in culture (a), from hepatocytes in the presence of dHGF (50 ng/ml) and dexamethasone (10^{-6} M) (b) and from antisense *myc*-transferrinfected cultures in the presence of dHGF and dexamethasone at concentrations as above (c). Control transferrinfections using the pMMTV vector are shown in (d).

using hepatocytes immediately after their attachment (4 h after perfusion). In preparations with the cell viability approaching 90%, as confirmed by the Trypan Blue exclusion test, the efficiency of the transferrinfection was not different using the two differentially incubated hepatocytes. However, if viability of the initial cell population was below 82%, the freshly attached hepatocytes were judged as not suitable for transferrinfection.

No blue cells appeared when hepatocytes were transferrinfected with a mixture lacking DNA. Using the transferrinfection complexes containing 10–30 μl of 5×10^{11} particles/ml of modified adenovirus, the percentage of the cells expressing β-galactosidase was as high as 50% (Figure 3C). Using the same constructs, but employing the calcium phosphate precipitation methodology, the percentage of blue hepatocytes was below 10% (Figure 3B). Another advantage of the transferrinfection, compared with the ordinarily used calcium phosphate precipitation technique, was that the viability of the hepatocytes remained unaffected throughout the culture time. In our hands, hepatocytes transfected with the calcium phosphate precipitation technique showed a decreasing viability 48 h after transfection. In experiments where the hepatocytes were exposed to the transfection mixture (calcium phosphate/BBS alone or containing expression plasmids) for up to 5 h, no improvement in cell viability was observed at later stages in culture (results not shown). In all experiments involving determination of [^{3}H]TdR incorporation into hepatocyte DNA, the transferrinfection technique was employed on freshly attached hepatocytes, modifying neither the culture times, compared with the control non-transfected cells, nor the timing of the addition of the factors.

In experiments with hepatocytes transferrinfected with either the pCMVβ-galactosidase plasmid together with the antisense *myc* construct, or with mixtures lacking DNA, immunoprecipitation analysis for the detection of albumin, transferrin, α-fetoprotein and α_2-macroglobulin was carried out. Albumin, transferrin and α_2-macroglobulin, known markers of the hepatocyte differentiated phenotype, were continuously expressed, although a significant decrease in the amount of the excreted markers was observed in transferrinfected hepatocyte cultures (Figure 4). We have been unable to detect α-fetoprotein activity throughout the culture time (4–5 days; results not shown). These

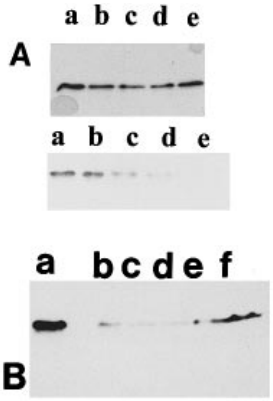

Figure 5 Effects of antisense *myc* construct on hepatocyte Myc protein expression

(**A**) Antisense c-*myc* was imported in hepatocytes by transferrinfection and dHGF (50 ng/dish) and dexamethasone (10^{-6} M) were added to the cultures. Lanes a–e (upper panel), dHGF/Dex-stimulated hepatocytes transferrinfected with the pMMTV vector at 0, 6, 24, 34 and 48 h after the addition of dexamethasone. Lanes a–e (lower panel), dHGF/Dex-stimulated hepatocytes, transferrinfected with the antisense *myc* construct at times identical with those shown in the upper panel. Lysates were separated by SDS/PAGE and then the immunoblotted *myc*-reacting species were revealed by ECL. (**B**) Effects of transient induction of the antisense *myc* expression on hepatocyte Myc protein expression. In hepatocytes transferrinfected with the antisense *myc* construct, dexamethasone was added for the first 24 h after removal of the complexes, while dHGF was present in culture. Dexamethasone was then removed and the hepatocytes were supplemented with 100 ng/ml dHGF, and c-Myc protein expression was followed as described in the Methods section. Lane a, hepatocyte c-*myc* species from 24 h dHGF-stimulated hepatocytes, transferrinfected with pMMTV (vector). Lanes b–f, hepatocyte c-Myc protein species 3, 6, 24, 48 and 96 h after dexamethasone removal and in the presence of dHGF alone at 100 ng/ml.

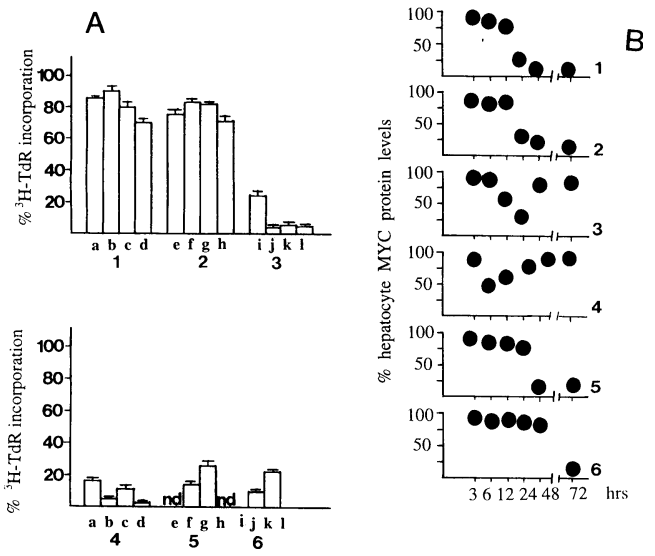

Figure 6 DNA synthesis and *myc* levels in antisense *myc*-transferrinfected hepatocytes

Antisense *myc* construct was imported by transferrinfection in hepatocytes as described in the Methods section. After transferrinfection, dexamethasone (10^{-6} M) was added to dHGF-stimulated hepatocytes for different times, and [^{3}H]TdR incorporation into DNA and *myc* levels were determined. (**A**) Percentage of [^{3}H]TdR incorporation compared with control incorporation (vector-transferrinfected hepatocytes). Dexamethasone and dHGF were present continuously (a–d, upper graph), for 12 h (e–h, upper graph), for 6 h (i–l upper graph) or for 3 h (a–d lower graph) of [^{3}H]TdR incorporation. dHGF was present continuously and dexamethasone was added at 24 h (lower graph) or at 48 h (i–l, lower graph). [^{3}H]TdR incorporation: bars a, e and i, 48 h; bars b, f and j, 72 h; bars c, g and k, 96 h; and bars d, h and l, 120 h. nd, not determined. Letters i and l in the lower graph also represent not determined (nd) incorporations. Bars represent means ± S.E.M. of triplicate values of at least two independent experiments. (**B**) Percentage of *myc* levels compared with their vector-transferrinfected counterparts. dHGF was present continuously and dexamethasone was also added continuously (1), for 12 h (2), for 6 h (3) or for 3 h (4). Dexamethasone was added at 24 (5) or 48 h (6) in cultures continuously stimulated with dHGF. Dots represent values from densitometric scanning of the autoradiographs from more than one experiment.

data are supportive of the fact that transferrinfection and the expression of the antisense c-*myc* construct (confirmed by the almost complete inhibition of Myc biosynthesis) did not promote alterations of the hepatocyte phenotype under short-term culture conditions.

Primary hepatocytes at 5×10^5 cells/dish were transferrinfected with pMMTVasmyc01 following seeding of the cultures. After 4 h, dexamethasone was added (10^{-6} M), and at the same time dHGF was introduced and the expression of c-Myc protein was followed for the next 48 h (Figure 5A, lower panel). Dexamethasone and the transferrinfection procedure itself did not alter the global protein synthesis nor its pattern in the cultured hepatocytes at all time-points studied (results not shown). A gradual inhibition of c-Myc protein synthesis was detected, and, in fact, at 48 h the levels of the hepatocyte c-Myc protein seemed to be inhibited at approximately 95% (as assessed by densitometric scanning of the autoradiographs), compared with the normal non-transfected hepatocytes treated with both dexamethasone and dHGF (Figure 5A, upper panel).

We then studied whether the addition of dHGF was able to restore the c-Myc protein(s) in hepatocytes transfected with the pMMTVasmyc01 construct. Hepatocyte cultures transfected with the antisense *myc* construct were treated with dexamethasone (10^{-6} M) for the first 24 h and supplemented thereafter with 100 ng/ml of dHGF. The expression of the c-Myc protein was followed at 3, 6, 24, 48 and 96 h after removal of dexamethasone. dHGF, when present alone (after 24 h) in culture, only partially restored hepatocyte c-Myc protein levels at 48 and 96 h after its initial addition (Figure 5B, lanes e and f). Therefore, although dHGF seemed to induce the synthesis of c-Myc protein(s) in growth-stimulated primary hepatocytes (non-transfected), this activity seemed to require the existence of a basal level of expression of the c-*myc* gene. A different explanation might be that the introduced antisense *myc* construct possesses a long-lasting turnover, thus still being active in inhibiting translation of newly synthesized hepatocyte c-*myc* mRNA. It should also be noted that the induction of antisense c-*myc* expression after transferrinfection, resulted in a gradual inhibition of hepatocyte c-Myc protein synthesis identical with that observed by employing the calcium phosphate methodology (results not shown).

Using the transferrinfection technique we explored whether the regulated expression of the antisense c-*myc* was able to modify the dHGF-induced DNA synthetic response (Figure 6).

The induction of the antisense c-*myc* expression by addition of dexamethasone (10^{-6} M) continuously and for 12 h in cultured hepatocytes, resulted in an approx. 90 and 83.4% inhibition respectively, in [^{3}H]TdR incorporation observed at 72 h. Induction of antisense c-*myc* expression for up to 6 h did not affect the [^{3}H]TdR incorporation into hepatocyte DNA estimated at 72 and 96 h (Figure 6A, bars b, f and j, and bars c, g and k respectively). Control values of [^{3}H]TdR incorporation into hepatocytes transferrinfected with the vector (pMMTV) in the presence of dHGF and/or dexamethasone are shown in Figure 2(B). Transferrinfection of hepatocytes with the vector did not affect significantly the DNA synthetic response elicited by dHGF,

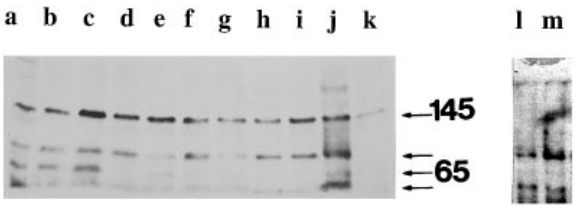

Figure 7 Hepatocyte c-Met proteins in dHGF-stimulated and antisense c-*myc* transferrinfected hepatocytes

Hepatocytes were transferrinfected with an antisense *myc* construct and dHGF was added. Hepatocyte proteins were analysed by SDS/PAGE/Western blot as in Figure 1(B) and *met*-reacting species were revealed by ECL. Lane a, hepatocyte *met* species from non-transferrinfected (normal) hepatocytes stimulated with dHGF for 24 h. Lanes b–f, hepatocyte *met* species from normal hepatocytes in the presence of dHGF and dexamethasone, and lanes g–k, from hepatocytes transferrinfected with antisense *myc* in the presence of dHGF (50 ng/dish) and dexamethasone (10^{-6} M). Culture times: lanes b and g, 3 h; lanes, c and h, 6 h; lanes d and i, 12 h; lanes e and j, 24 h; and lanes f and k, 48 h. Molecular mass values are given on the right in kDa. Right-hand panel: species revealed from NIH 3T3 cells (l) or from NIH 3T3 cells transfected with an *myc* construct (m).

and this was slightly modified in the presence of dexamethasone. In antisense *myc*-transferrinfected hepatocytes, dHGF was added without the presence of dexamethasone. At 24 h, dexamethasone was added and DNA synthesis was estimated at 48 and 72 h (Figure 6A). There was an approx. 13 and 24% decrease respectively, in [^{3}H]TdR incorporation into hepatocyte DNA, compared with the vector-transfected hepatocyte cultures. We also studied the effects of antisense *myc* expression in hepatocyte cultures treated for 48 h with dHGF and with dexamethasone added at the 48th hour until the fifth day in culture. For DNA synthesis estimated at 72 and 96 h and in hepatocytes where the antisense construct was induced, a less than 5 and 22% (approx.) decrease respectively was observed compared with control values of vector-transfected cells at the same time-point (Figures 6 and 2B).

We have carried out other experiments to determine the hepatocyte c-Myc levels under the different conditions induced after transferrinfection with the antisense-*myc* construct. These data are shown in Figure 6(B). Continuous treatment of the transferrinfected hepatocytes with dexamethasone resulted in a decline in c-Myc levels 12 h after addition of the factor. A similar decline in c-Myc levels was observed by treating the transferrinfected hepatocytes with dexamethasone for 12 h. Under both conditions at 72 h, no c-Myc reactivity was detectable (Figure 6B, graphs 1 and 2). With dexamethesone present only for 6 h in the antisense *myc*-transferrinfected hepatocyte cultures, the c-Myc levels were decreased down to 17.3% of the control levels (vector-transferrinfected) by 24 h, and were restored to about 83% of the control levels by 72 h (Figure 6B, graph 3). The presence of dexamethasone for 3 h in culture caused a decline in hepatocyte c-Myc levels at approx. 50% of the control levels for up to 12 h, and c-Myc expression seemed to be restored to approx. the control levels (87.2%) by 72 h (Figure 6B, graph 4). Addition of dexamethasone in the transferrinfected hepatocyte cultures after 24 or 48 h caused a rapid decline in c-Myc levels observed at 48 and 72 h respectively. These data comply with the results presented on [^{3}H]TdR incorporation (Figure 6A), thus suggesting that the rapid decrease in c-Myc levels observed between 6 and 12 h renders the hepatocytes unable to enter the S-phase at later times in culture. However, the decreased c-Myc levels occurring between 0 and 6 h and after 12 h (after induction with dexamethasone) did not cause an inhibitory effect in hepatocyte [^{3}H]TdR incorporation observed at later times (Figure 6B, graphs 4–6).

HGF and its variant dHGF bind equally to the c-*met* receptor and this was revealed by an inhibition assay of ^{125}I-labelled HGF to the receptor (c-*met*) [36]. Therefore the 15 nucleotides deletion in the first kringle of HGF, generating dHGF, did not alter the affinity of HGF for the c-*met* receptor [36]. We have carried out an analysis of hepatocyte proteins reacting with specific c-*met* polyclonal antiserum (Figure 7). *Met*-reacting species ranging from 145 to 65, and 48 kDa, were revealed by Western blot/ECL analysis, and no significant differences were observed in the levels of hepatocyte c-*met* proteins (145 kDa) under conditions of induced or non-induced antisense c-*myc* construct. We have been unable to confirm that the 65 and 48 kDa-reacting species possess any structural similarities to the known *met* species (α- and β-subunits), therefore we believe that these polypeptides represent species cross-reacting with the anti-*met* polyclonal antibody. In control experiments shown in Figure 7 (right-hand panel) using fibroblastic cell lines known to lack *met* receptor (NIH3T3 and *myc*-transfected NIH 3T3), the immunoblotted lysates failed to show any reactivity in the range of the 145 kDa species, although the 65 and 48 kDa species, and also some minor protein species ranging around 40 and 14 kDa (not shown), were still detectable by all *met* antisera used (see Materials section). These data show that the reduced DNA synthetic response of the transferrinfected hepatocytes was not due to down-regulation of the c-*met* gene.

DISCUSSION

We have investigated whether the addition of a potent growth factor for primary hepatocytes can induce changes in the expression of the c-*myc* gene which is known to be regulated in a cell cycle-dependent manner in mitogen-stimulated cells. It has been shown that HGF induced c-*fos* and c-*myc* expression in a time-dependent manner in a maximum time of 30 min for c-*fos* and 8 h for c-*myc* in rat fetal hepatocytes [37]. In this study, however, a deleted variant of HGF, i.e. dHGF, was able to induce significant changes in the expression of c-*myc* in adult hepatocytes at both the mRNA and the protein level, at time-points beyond those observed using fetal hepatocytes [37]. Cultured hepatocytes undergo DNA synthesis usually 48 h after the addition of a potent growth stimulus under *in vitro* conditions (EGF, TGF-α, HGF) [2–5]. Under the conditions used in the present studies, dHGF induced a biphasic DNA synthetic response between 48 and 72 h in culture. We observed that the steady-state levels of the hepatocyte c-Myc protein(s) were significantly high independently of the growth conditions. In relation to this fact, it has been reported that c-*myc* in hepatocytes is constitutively expressed and the half-life of the protein is unusually long compared with other mitogen-stimulated cells [31,32,38]. Expression of c-*myc* was increased 24 h after the addition of dHGF and could still be observed at 48 and 72 h. Another system in which a marked increase in the expression of c-*myc* has been reported is that of the oocytes, where c-Myc protein reaches a level many times higher than that found in proliferating somatic cells [39]. This was attributed to a large RNA pool and/or an unusual stability of the protein product compared with that observed in mitogen-stimulated somatic cells [39]. The constitutive expression of c-*myc* in mature hepatocytes implied that its functions seemed to be uncoupled from the initiation of cell proliferation. It has been reported that over-expression of c-*myc* in other cellular models can induce initiation of responses other than cell proliferation, such as apoptosis and some forms of differentiation and hypertrophic cell growth [19].

Cultured hepatocytes, under mitogen-stimulated or unstimulated conditions, after 1–2 weeks in culture, experience significant detachment, although maintenance of these cells in culture for several weeks in the presence of growth factors and DMSO has been reported [40]. This might imply that these cells experience apoptosis, which is characterized by increased levels of expression of c-*myc* and proliferation arrest. This possibility clearly requires further investigation using a long-term culture, with the hepatocytes maintaining their differentiated functions. Hepatocytes isolated after tissue dispersion and cultured under low or high cell-densities respond differentially to growth factor stimuli and show striking variability in the expression of c-*myc* [41]. Therefore the peak-shaped mitogen-induced c-*myc* expression seemed to be correlated with the ability of the hepatocyte to respond to growth factors and to progress towards the S-phase. It has been shown previously that over-expression of exogenously imported c-*myc* in hepatocytes cannot initiate a proliferating response but acts in co-operation with a growth factor (EGF) in super-inducing DNA synthesis [23,42]. When fetal hepatocytes were incubated with HGF, c-*myc* mRNA levels were increased 10-fold 30 min after the addition of the growth factor [43]. This up-regulation persisted for 24 h and returned to the baseline levels by 48 h [43]. Affinity cross-linking of the HGF receptor with ^{125}I-labelled HGF confirmed the increased expression of the HGF receptor in hepatocytes cultured at low cell densities, although Northern analysis did not reveal differences in c-*met* expression under conditions of low or high cell density [44]. These data supported the idea that c-*met* is either regulated post-transcriptionally or that the addition of a growth signal (HGF) is accompanied by receptor translocation from an intracellular pool [44]. Therefore, the responsiveness of cultured hepatocytes to stimulation by dHGF is mostly dependent upon the ability of the hepatocytes to exit G_0, an event accompanied by elevated levels of c-*myc* and entirely regulated by the cell density [41,45]. Under the present conditions of transferrinfected antisense *myc* hepatocytes, no significant difference in the affinity cross-linking of *met* with ^{125}I-labelled dHGF was observed (results not shown). We have been unable to detect significant variations in c-Met protein levels in antisense *myc*-transferrinfected hepatocytes, clearly showing that the receptor expression and availability did not interfere with dHGF-induced DNA synthesis.

Our data also showed that inhibition of c-Myc protein synthesis at times preceding the DNA synthetic response *in vitro* and during the S-phase (24–48 h) did not significantly affect the magnitude of the DNA synthetic response elicited after dHGF stimulation. However, inhibition of c-Myc protein synthesis between 6 and 12 h resulted in complete blockage of hepatocyte DNA synthesis observed at later times in culture. This suggests that c-*myc* is a required component of the 'priming' process of the hepatocytes, which renders these cells responsive to growth factors [46]. The 'priming' process is accompanied by the exit from the G_0 to the G_1 phase of the cell cycle. c-*myc* antisense oligonucleotides were reported to inhibit mitogen-induced c-Myc protein expression in human T-lymphocytes and to prevent S-phase transition.These antisense oligonucleotides were shown not to inhibit G_0–G_1 traverse, however this was assessed by morphological criteria [47]. By inhibiting the constitutively expressed c-*myc* between 6 and 12 h, hepatocytes may face a gap in their 'priming' process, therefore becoming unable to exit G_0. Exit of hepatocytes from G_0 is a prerequisite for these cells to proceed to the S-phase, although exit from quiescence can also lead to the development of other non-proliferating phenotypes. It should also be considered that inhibition of c-Myc protein expression in unstimulated hepatocytes did not result in cell death or alterations in the morphology of the cells in short-term culture (results not shown). In experiments reported by others, it was demonstrated that various cells 'protected' by the antisense c-*myc* display the morphological characteristics of normal cells [48].

The transcriptional activity of c-*myc* is regulated by its ability to form heterodimers with partners such as *max*. Once hepatocytes isolated from experimental animals are placed in culture this can lead to the induction of c-*myc* expression; this might also be sufficient to influence the expression of c-*myc*-associated partners, such as *max*. The cultured hepatocytes showing stable expression of c-*myc*, may be diverted towards the development of two potential phenotypes: one elicited in the presence of a mitogen which induces c-*myc* expression in a time-regulated and peak-shaped manner and drives the cells towards proliferation (by eventually favouring the formation of *myc*:*max* heterodimers rather than *max*:*max* homodimers). In the absence of a growth stimulus, the level of constitutively expressed c-*myc* may not be sufficient to shift the transcriptional repressor complex (*max*:*max*) to the transcriptionally active *myc*:*max*, this event leading, *in vitro*, to a conserved quiescent phenotype.

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