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Differential induction of 'metabolic genes' after mitogen stimulation and during normal cell cycle progression

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SUMMARY

Mitogenic stimulation of quiescent cells not only triggers the cell division cycle but also induces an increase in cell volume, associated with an activation of cellular metabolism. It is therefore likely that genes encoding enzymes and other proteins involved in energy metabolism and biosynthetic pathways represent a major class of mitogen-induced genes. In the present study, we investigated in the non-established human fibroblast line WI-38 the induction by mitogens of 17 genes whose products play a role in different metabolic processes. We show that these genes fall into 4 different categories, i.e. non-induced genes, immediate early (IE) primary genes, delayed early (DE) secondary genes and late genes reaching peak levels in S-phase. In addition, we have analysed the regulation of these genes during normal cell cycle progression, using HL-60 cells separated by counterflow elutriation. A clear cell cycle regulation was seen with those genes that are induced in S-phase, i.e. thymidine kinase, thymidylate synthase and dihydrofolate reductase. In addition, two DE genes showed a cell cycle dependent expression. Ornithine decarboxylase mRNA increased around mid-G_1, reaching maximum levels in S/G_2, while hexokinase mRNA expression was highest in early G_1. In contrast, the expression of other DE and IE genes did not fluctuate during the cell cycle, a result that was confirmed with elutriated WI-38 and serum-stimulated HL-60 cells. These observations suggest that $G_0 \rightarrow S$ and $G_1 \rightarrow S$ transition are distinct processes, exhibiting characteristic programmes of gene regulation, and merging around S-phase entry.

Key words: mitogens, growth factors, immediate early genes, delayed early genes, cell cycle, gene regulation, cell metabolism, DNA synthesis

INTRODUCTION

After exiting from mitosis, mammalian cells have the option to either start a new round of cell division or to leave the cell cycle and enter a resting state, usually referred to as quiescence or G_0 (for reviews see Baserga, 1985; Müller et al., 1993). A major parameter for the cell's decision to choose one way or the other is the concentration of mitogens in the surrounding medium. Once a cell has entered G_0, it can stay in this state until, in response to an increased level of mitogens, the cell will then re-enter the division cycle at some point before S-phase and resume proliferation. Obviously, a cell has to double its volume during the cell cycle to compensate for the loss in volume after mitosis (Baserga, 1981, 1984). Therefore, the cell division cycle, which comprises the actual replication of the cellular genome and subsequently the mitotic segregation of the daughter cells, is superimposed with another cycle, the growth cycle (for reviews see Baserga, 1981, 1985; Müller et al., 1993). The requirement for growth in size is even more important if a stimulated quiescent cell re-enters the cell cycle because of the smaller volume of G_0 cells relative to their post-mitotic G_1 counterparts. Cell growth has to be controlled as closely as the division cycle to ensure a proper progression of the cell cycle. This is for instance suggested by the observation that a critical cell size is required for a cell to be able to enter S-phase (Baserga, 1981, 1984). In addition, many metabolic inhibitors arrest the cell in specific phases, including G_1, indicating once again that the $G_0/G_1 \rightarrow S$ progression is not only dependent on mitogen-mediated signals stimulating the division cycle, but also on conditions that maintain cellular metabolism and cell growth (for reviews see Baserga, 1981, 1985; Müller et al., 1993). Recent observations suggesting a role for the tumor suppressor gene p53 at a metabolic check point emphasise the significance of these mechanisms (Livingstone et al., 1992; Yin et al., 1992).

Stimulation of cells with mitogens ultimately leads to the induction of a vast number of genes which are distinguished not only by the functions of their products, but also by their mode of expression (for reviews, see Nathans et al., 1988, 1991; Herschman, 1989; Bravo, 1990; Hershman, 1991; Müller et al., 1993). These genes thus differ in their basal level and induced transcription, in the timing and stability of induction and in the requirement for protein synthesis prior to the onset of transcription. By now, we know more than hundred genes that are regulated by mitogens, many of them being induced within minutes after stimulation and in the

absence of protein synthesis. These genes, which are usually referred to as immediate early (IE) genes, often encode transcription factors themselves, thus contributing to spreading the mitogenic signal throughout the genome. The IE response is followed by the induction of the secondary delayed early (DE) genes. Many of these genes presumably represent the targets for the transcription factors encoded by the IE genes, and their induction is therefore dependent on protein synthesis. Apart from the DNA-binding proteins and transcriptional regulators, an array of genes fulfilling other functions in very diverse cellular processes has been identified. Among these are genes encoding (i) secreted products, such as colony-stimulating factor-1 (CSF-1), proliferin and monocyte-derived neutrophil chemotactic factor (MONAP); (ii) structural proteins, including fibronectin, tenascin, collagen and actin; (iii) secreted proteases, such as collagenase and urokinase; (iv) enzymes involved in nucleotide and DNA synthesis; (v) proteins involved in signal transduction, such as calmodulin and c-Ha-ras; and (vi) cell cycle genes of the cdk and cyclin families (for reviews see Nathans et al., 1988, 1991; Herschman, 1989, 1991; Bravo, 1990; Müller et al., 1993). The fact that mitogenic stimulation also triggers cell growth (i.e. an increase in volume) associated with an activated metabolism (Schneider et al., 1978) makes it likely that genes encoding enzymes and other proteins involved in energy metabolism and biosynthetic pathways (for reviews see Baserga, 1984, 1985; Müller et al., 1993) represent another major class of mitogen-induced genes. This notion is supported by a few cases where such genes have been shown to be subject to regulation by mitogens, such as the glucose transporter (Hiraki et al., 1988), the mitochondrial chaperonin *hsp*-60 (Wick et al., 1994, accompanying paper), ornithine decarboxylase (e.g. Haddox et al., 1980; Katz and Kahana, 1987) and enzymes in nucleotide and DNA synthesis (e.g. Johnson et al., 1978; Hendrickson et al., 1980; Navalgund et al., 1980; Kaufman and Sharp, 1983; Liu et al., 1985; Coppock and Pardee, 1987). We have therefore decided to investigate this question in more detail and have analysed the inducibility of a total of 17 different genes whose products figure in different metabolic processes. Our data demonstrate that these genes fall into four different categories, i.e. non-induced genes, IE genes, DE genes and late genes induced after S-phase entry. In addition, we have analysed the regulation of these genes during normal cell cycle progression and show that only some DE gene and the late genes, induced around S-phase, are subject to a clear cell cycle regulation, thus emphasising the fact that $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progression are distinct processes exhibiting characteristic patterns of gene regulation.

MATERIALS AND METHODS

Cell culture, elutriation and FACS analyses

WI-38 cells (Hayflick, 1965), obtained from the American Type Culture Collection (ATCC), were cultured in a 1:1 mixture of Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) and MCDB110 medium, supplemented with 10% fetal calf serum (FCS). WI-38 cells were seeded at a density of 10^4 cells/cm^2 24 hours prior to starvation in serum-free medium for 72 hours and stimulated with 10% FCS for the indicated times. For stimulation of quiescent cells with pure mitogens, a mixture of 10 ng/ml of platelet-derived growth factor (PDGF; Gibco-BRL), 10 ng/ml of epidermal growth factor (EGF; Sigma) and 1.4×10^{-7} M dexamethasone (Merck) was used. In this case, the medium was supplemented according to Bettger et al. (1981). This treatment was as efficient as stimulation with 10% FCS with respect to the induction of DNA synthesis (data not shown). In some experiments protein synthesis was inhibited by the addition of cycloheximide (10 μg/ml; Sigma) or puromycin (20 μg/ml; Sigma) to the medium. HL-60 cells were grown in RPMI-1640 medium supplemented with 5% FCS plus 5% newborn calf serum. Counterflow elutriation of HL-60 and WI-38 cells is described in the accompanying paper by Wick et al. (1994). FACS anaylses of Hoechst 33258 stained cells were performed as previously described (Sewing et al., 1993).

Determination of HMGR activity in cell extracts

HMGR activity was determined as described (Larsson et al., 1989) with the exception that substrate ([^{14}C]HMG-CoA) and reaction product ([^{14}C]mevalonolactone) were separated by thin-layer chromatography. A 50 μl sample of the reaction mixture was applied to a Polygram Sil G (Macherey-Nagel) thin-layer plate, which was developed in ethyl acetate/acetone (4:1, v/v) (Cavenee et al., 1981). Radioactivity was quantitated by β-scanning of the chromatogramme using a Molecular Dynamics PhosphorImager.

RNA isolation and analysis by PCR

RNA was isolated according to Chomczynski and Sacchi (1987). Reverse transcription of RNA into cDNA and quantitative PCR analysis were performed as described (Mumberg et al., 1991; Sewing et al., 1993). The number of cycles was chosen as to obtain a linear amplification of the PCR products. Quantitation was performed by β-scanning of the gels using a Molecular Dynamics PhosphorImager. Data were corrected using the L7 signal as the standard. The following primer pairs were used for PCR amplification:

HMGR (Luskey and Stevens, 1985):
(5′-primer): 5′-ACTGAATTCAATGCATGGCCTCTTTGTGG-3′ (71 to 90)
(3′-primer): 5′-ACTGAATTCTGACATGCAGCCAAAGCAGC-3′ (631 to 650)
LDL-receptor (Yamamoto et al., 1984):
(5′-primer): 5′-ATTGGATCCTGCTTGTCTGTCACCTGCAA-3′ (200 to 219)
(3′-primer): 5′-ATTGGATCCGCCAACTTCATCGCTCATGT-3′ (810 to 820)
ODC (Hickok et al., 1987):
(5′-primer): 5′-ACTGGATCCTCATGAACAACTTTGGTAAT-3′ (86 to 105)
(3′-primer): 5′-ACTGAATTCTGAAAGCTGATGCAACATAG-3′ (921 to 940)
GT (Mueckler et al., 1985):
(5′-primer): 5′-ATTGGATCCCTGACGGGTCGCCTCATGCT-3′ (201 to 220)
(3′-primer): 5′-ATTGGATCCCGGAACAGCTCCAGGATGGT-3′ (951 to 970)
HK (Nishi et al., 1988):
(5′-primer): 5′-ATTGGATCCCTTCACGGAGCTGAAGGATG-3′ (111 to 130)
(3′-primer): 5′-ATTGGATCCCTCGTCTCCTTCCACCAGAT-3′ (818 to 837)
TPI (Brown et al., 1985):
(5′-primer): 5′-ATTGGATCCAGCTAGATCCCAAGATTGCT-3′ (531 to 550)
(3′-primer): 5′-ATTGGATCCAAGGAAGCCATCCACATCAG-3′ (1041 to 1060)
LDH (Chung et al., 1985):
(5′-primer): 5′-ACTGAATTCATGGCAACTCTAAAGGATCA-3′ (327 to 346, exon 1)

(3′-primer): 5′-ACTGAATTCCTGGATTTGAAACAATAAGC-3′ (296 to 315, exon 3)
PRPS (Iizasa et al., 1989):
(5′-primer): 5′-ACTGAATTCTCGCATCAGGACCTATCCCA-3′ (91 to 110)
(3′-primer): 5′-ACTGAATTCTCTGGAAATAGCTGGTCCAG-3′ (821 to 840)
TK (Bradshaw and Deininger, 1984):
(5′-primer): 5′-AGTACAAGTGCCTGGTGATC-3′ (197 to 216)
(3′-primer): 5′-CAGGGCTGCATTGCAGAATC-3′ (735 to 754)
DHFR (Chen et al., 1984):
(5′-primer): 5′-ATTGGATCCTTCGCTAAACTGCATCGTCG-3′ (1331 to 1350, exon 1)
(3′-primer): 5′-ATTGGATCCCCTTTCTCCTCCTGGACATC-3′ (61 to 80, exon 5)
TYS (Takeishi et al., 1985):
(5′-primer): 5′-GATGAATTCCCTCTGCTGACAACCAAACG-3′ (310 to 338)
(3′-primer): 5′-ACTGAATTCAGGTCTGGGTTCTCGCTGAA-3′ (911 to 930)
PFK (Sharma et al., 1989):
(5′-primer): 5′-ACTGAATTCGCTGTCTTAACCTCTGGTGG-3′ (131 to 150)
(3′-primer): 5′-ACTGAATTCGTCGTCATCTGGTGGACATT-3′ (771 to 790)
GAPDH (Arcari et al., 1984):
(5′-primer): 5′-CGTCTTCACCACCATGGAGA-3′ (360 to 379)
(3′-primer): 5′-CGGCCATCACGCCACAGTTT-3′ (640 to 659)
G6PDH (Takizawa et al., 1986):
(5′-primer): 5′-ACTGAATTCATCGTGGAGAAGCCCTTCGG-3′ (51 to 70)
(3′-primer): 5′-ACTGAATTCCGCACGAAGTGCATCTGGCT-3′ (891 to 910)
PYDH (Ho et al., 1989):
(5′-primer): 5′-ACTGAATTCGAAGATGCTCGCCGCCGTCT-3′ (49 to 68)
(3′-primer): 5′-ACTGAATTCTGCGCTCCCACGATGCCATT-3′ (533 to 552)
SUO (Kita et al., 1990):
(5′-primer): 5′-ACTGAATTCCTGGAGACAAACCTCATATG-3′ (101 to 120)
(3′-primer): 5′-ACTGAATTCTCCTTGTGCAGTTCATGATG-3′ (681 to 700)
ANT-2 (Ku et al., 1990):
(5′-primer): 5′-ATTGGATCCACAGATGCCGCTGTGTCCTT-3′ (2007 to 2026, exon 1)
(3′-primer): 5′-ATTGGATCCGGTAGATGATAATACCCTGC-3′ (3555 to 3574, exon 2)
cdc2 (Lee and Nurse, 1987):
(5′-primer): 5′-GGAGAAGGTACCTATGGAGTTGTG-3′ (171 to 194)
(3′-primer): 5′-AGTCTCTGTGAAGAACTCTTCTAGAGT-3′ (499 to 525)
L7 (Herzog et al., 1990):
(5′-primer): 5′-TAGGATCCCATGGAGGGTGTAGAAGAGAAG-3′ (20 to 39)
(3′-primer): 5′-TTGAGAATTCAATCATGGTAGACACCTTAG-3′ (765 to 784)

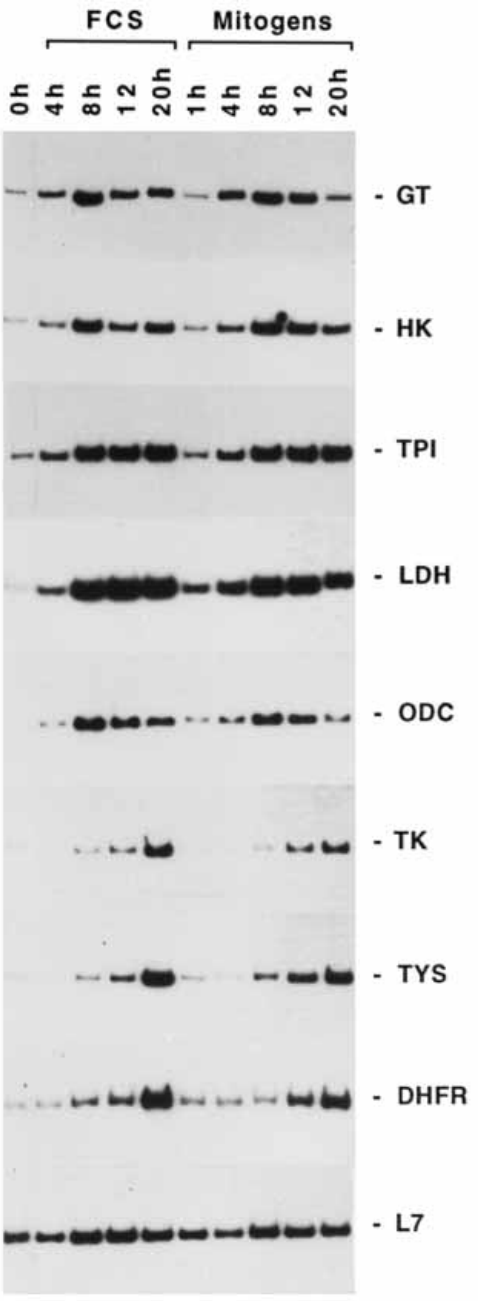


Fig. 1. RNA expression analysis of 9 different genes following stimulation of quiescent WI-38 cells with either 10% fetal calf serum (FCS; left panel) or a mixture of pure mitogens (right panel; 10 ng PDGF/ml, 10 ng EGF/ml; 1.4×10^{-7} M dexamethasone). RNA were reverse transcribed and the cDNA amplified by PCR in the presence of radioactively labelled dCTP. The PCR products were separated on acryl amide gels and visualised by exposure to X-ray films.

RESULTS

Identification of mitogen-induced genes encoding metabolic enzymes

WI-38 cells are non-established diploid fibroblasts that enter a state of replicative quiescence (G_0) under serum starvation. Stimulation of such cells with fetal calf serum (FCS) or with specific mitogens (PDGF + EGF + dexamethasone) induces DNA synthesis after a lag of 12-18 hours. A detailed analysis of $G_0 \rightarrow S \rightarrow G_2$ progression after serum stimulation of quiescent WI-38 cells has been published by Sewing et al. (1993). One goal of the present study was to identify mitogen-inducible genes encoding proteins that are associated with metabolic

processes (hereafter referred to as 'metabolic genes'). Towards this end, we analysed the expression of 17 genes whose products figure in diverse biochemical pathways, most notably glycolysis, cholesterol biosynthesis and nucleotide synthesis (see Table 1), at different times after stimulation of quiescent WI-38 cells with serum or defined mitogens. This study was carried out by quantitative PCR analysis of reverse transcribed RNA as previously described (Mumberg et al., 1991; Sewing et al., 1993). We also determined the expression of L7, whose product is a component of the large ribosomal subunit. L7 expression is constant in quiescent, stimulated and growing cells, and can thus be used as an internal control (Rittling et al., 1986). The data and quantitations presented in Figs 1 and 2 and summarised in Table 1 show that 9 of the genes analysed were induced by both serum and by defined mitogens. These were the genes encoding the glucose transporter (GT), hexokinase (HK), triosephosphate isomerase (TPI), lactate dehydrogenase (LDH), phosphoribosyl-pyrophosphate synthase (PRPS), ornithine decarboxylase (ODC), thymidine kinase (TK), thymidylate synthase (TYS) and dihydrofolate reductase (DHFR). In addition, the genes encoding 3-hydroxymethyl 3-glutaryl CoA reductase (HMGR) and the low density lipoprotein receptor (LDLR) were induced by serum (Fig. 3A,B), but surprisingly not by the mixture of pure mitogens (Fig. 3A,C), although both treatments were similarly efficient at the induction of DNA synthesis (data not shown). The expression of another group of genes did not significantly (<2-fold) change after serum or mitogen stimulation, including glyceraldehyde-phosphate dehydrogenase (GAPDH), glucose 6-phosphate dehydrogenase (G6PDH), phosphofructokinase (PFK), pyruvate dehydrogenase (PYDH), adenine nucleotide transporter (ANT-2) and succinate ubiquinone oxidoreductase (SUO), a mitochondrial gene.

Table 1. Serum-induced and non-induced metabolic genes in the human diploid fibroblast cell line WI-38

(A)	Non-induced genes
	Glyceraldehyde-phosphate dehydrogenase (GAPDH)
	Glucose-6-phosphate dehydrogenase (G6PDH)
	Phosphofructokinase (PFK)
	Pyruvate dehydrogenase (PYDH)
	Adenine nucleotide transporter (ANT-2)
	Succinate ubiquinone oxidoreductase (SUO; mitochondrial gene)
(B)	Immediate-early (primary) genes
	Low density lipoprotein receptor (LDLR)
	3-Hydroxymethyl 3-glutaryl CoA reductase (HMGR)
(C)	Delayed-early genes
	Glucose transporter (GT)
	Hexokinase (HK)
	Triosephosphate isomerase (TPI)
	Lactate dehydrogenase (LDH)
	Phosphoribosyl-pyrophosphate synthase (PRPS)
	Ornithine decarboxylase (ODC)
(D)	Late genes
	Thymidine kinase (TK)
	Thymidylate synthase (TYS)
	Dihydrofolate reductase (DHFR)

The inducible genes fell into three categories: genes reaching peak levels after ≤2 hours (HMGR, LDLR; see Fig.

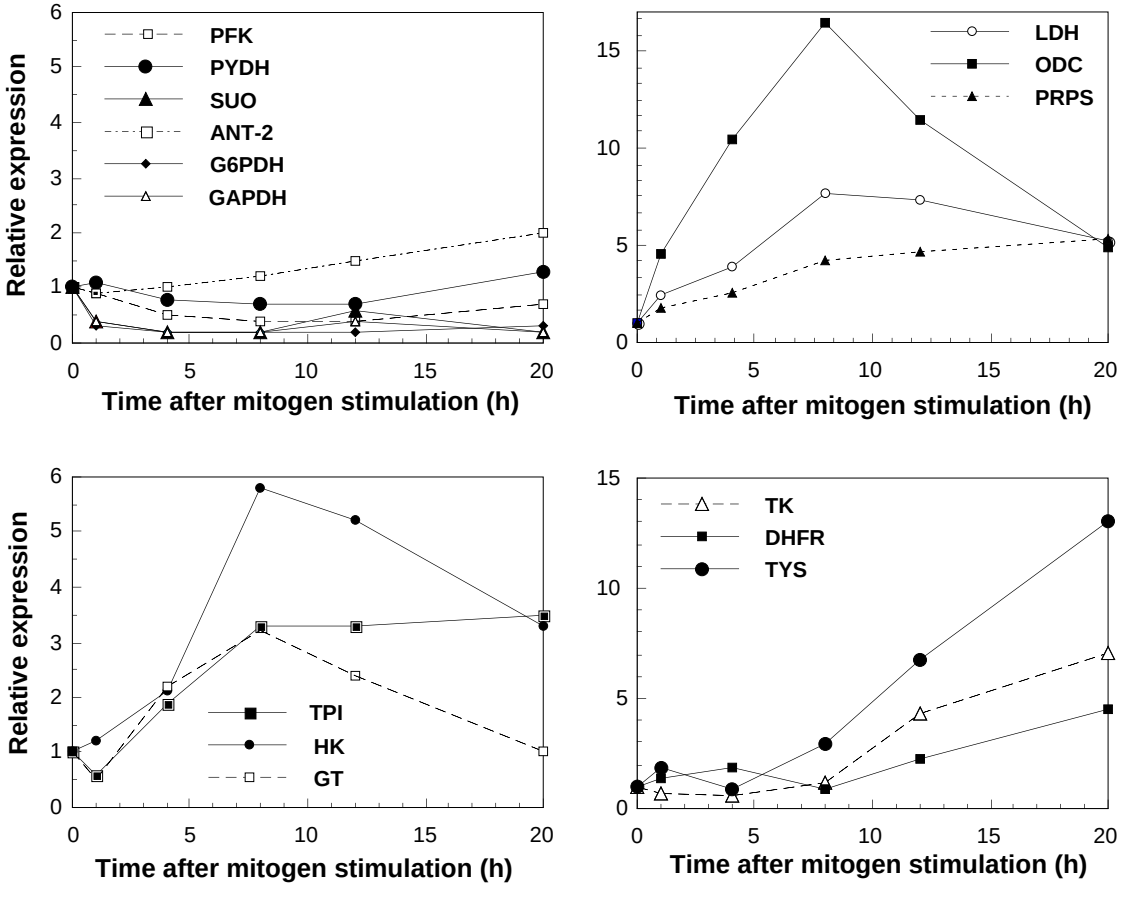


Fig. 2. Quantitative analysis of RNA expression of 15 different metabolic genes following stimulation of quiescent WI-38 cells with a mixture of pure mitogens (see legend to Fig. 1). Samples were analysed as in Fig. 1 and the gels were quantitatively evaluated by β-radiation scanning using a Molecular Dynamics PhosphorImager.

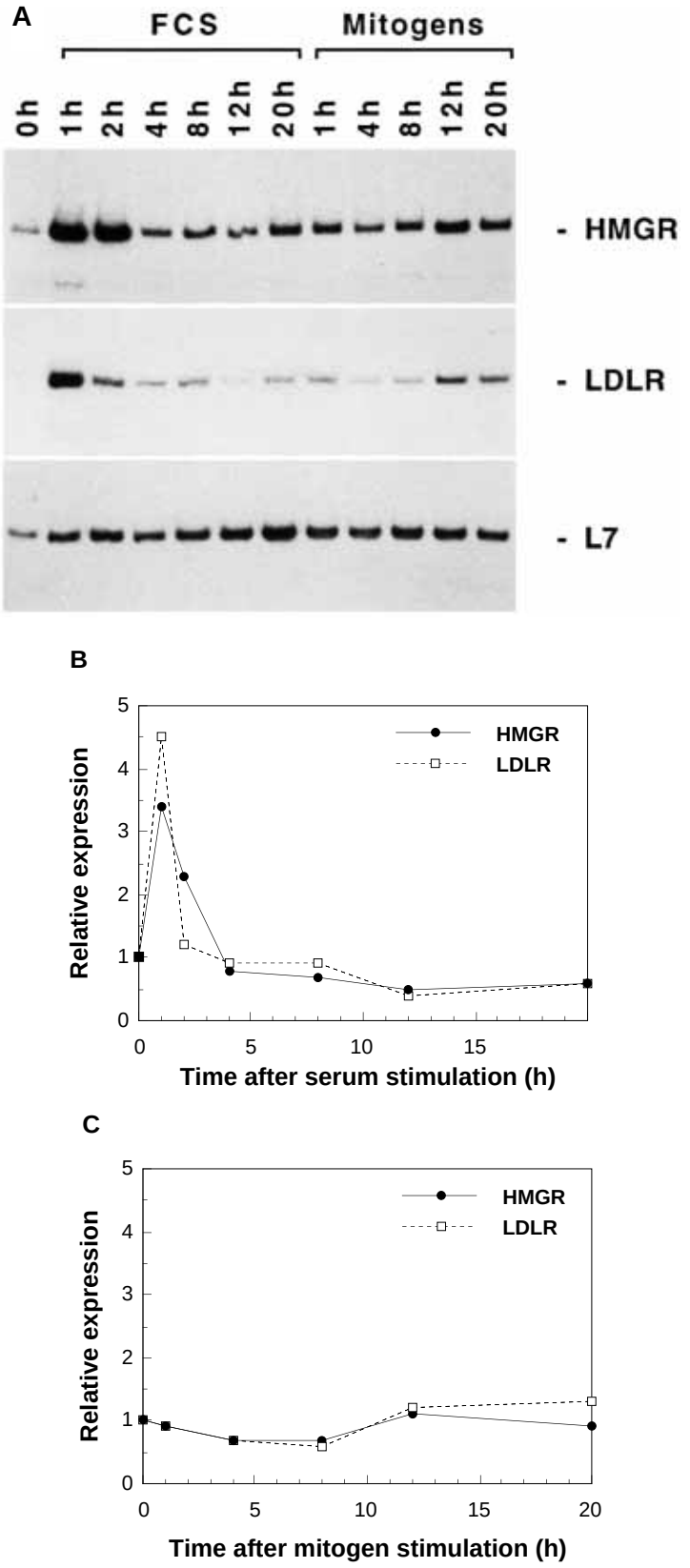


Fig. 3. Expression of HMGR and LDLR mRNA after stimulation of quiescent WI-38 cells with either 10% FCS (A,B) or a mixture of pure mitogens (A,C). Samples were analysed as in Fig. 1 and the gels were quantitatively evaluated by β-radiation scanning.

3B), genes showing a delayed induction with a peak at about 8 hours (GT, HK, TPI, LDH, PRPS, ODC) and genes showing maximum induction in S/G_2 (≥20 hours; TK, TYS, DHFR; see Fig. 2). On the basis of these kinetics one would classify the genes as IE, DE and late genes. This classification is supported by the experiments described below, analysing the effect of protein synthesis inhibition on the induction. The genes also differed in the level of induction, the maximum expression values varying between 3- and 17-fold. The strongest induction was seen with ODC and TYS (13- to 17-fold; Fig. 2), followed by LDH, HK, TK and LDLR (5- to 7-fold; Figs 2 and 3B).

Induction of HMGR activity by mitogens

As described above, stimulation of quiescent WI-38 cells with FCS resulted in an approximately 4-fold induction of HMGR mRNA levels, but no induction was seen with mitogen-stimulated cells. Since the mevalonate pathway plays a pivotal role in cell proliferation, e.g. by providing essential compounds such as cholesterol or farnesyl and geranyl geranyl pyrophosphate for protein prenylation (as in Ras), we reasoned that a second, perhaps post-translational, mechanism may exist that could induce the enzymatic activity of HMGR. We therefore prepared extracts from quiescent and stimulated WI-38 cells at different times after serum or mitogen treatment and assayed the HMGR activity in these extracts by measuring the conversion of ^{14}C-labelled HMG-CoA to mevalonolactone. The results of this experiment are shown in Fig. 4A and B and quantitations of the data are depicted in Fig. 4C and D. Stimulation by FCS (Fig. 4C) as well as by defined mitogens (Fig. 4D) resulted in a clear (10- to 12-fold) increase in the activity of HMGR, with peak levels seen at 4-5 hours. In the case of stimulation by mitogens, a second peak was reproducibly found at around 12 h, a finding that points to differences in the regulation of HMGR by serum or by defined mitogens not only at the level of mRNA expression, as discussed above, but also at the post-translational level.

Sensitivity of mitogen-induced gene expression to inhibitors of protein synthesis

To distinguish primary and secondary genes, we analysed the effects of the protein synthesis inhibitors cycloheximide or puromycin on the induction of the genes identified above. Quiescent WI-38 cells were stimulated with 10% FCS for 3 or 8 hours in either the presence or absence of the inhibitors. The results of this study (Fig. 5) clearly show that the induction of PRPS, HK, LDH, TPI and GT was inhibited and often even abolished by puromycin. In the case of TPI and GT, cycloheximide alone was able to induce these genes at least as efficiently as FCS, so that the results are difficult to interpret (see also Edwards and Mahadevan, 1992). This also applies to the expression of HMGR and LDLR mRNAs. It has been shown that in some instances cycloheximide can induce certain signal transduction pathway(s), which may complicate the interpretation of results obtained with this inhibitor. In the case of PRPS, HK and LDH, however, the results were clear and in agreement with the data obtained by treatment with puromycin. On the basis of these observations, we would classify the DE genes PRPS, HK, LDH, TPI and GT as secondary genes whose induction is dependent on de novo protein synthesis. In contrast, induction of the HMGR and LDLR was not inhibited by puromycin, indicating that these genes, identified above as

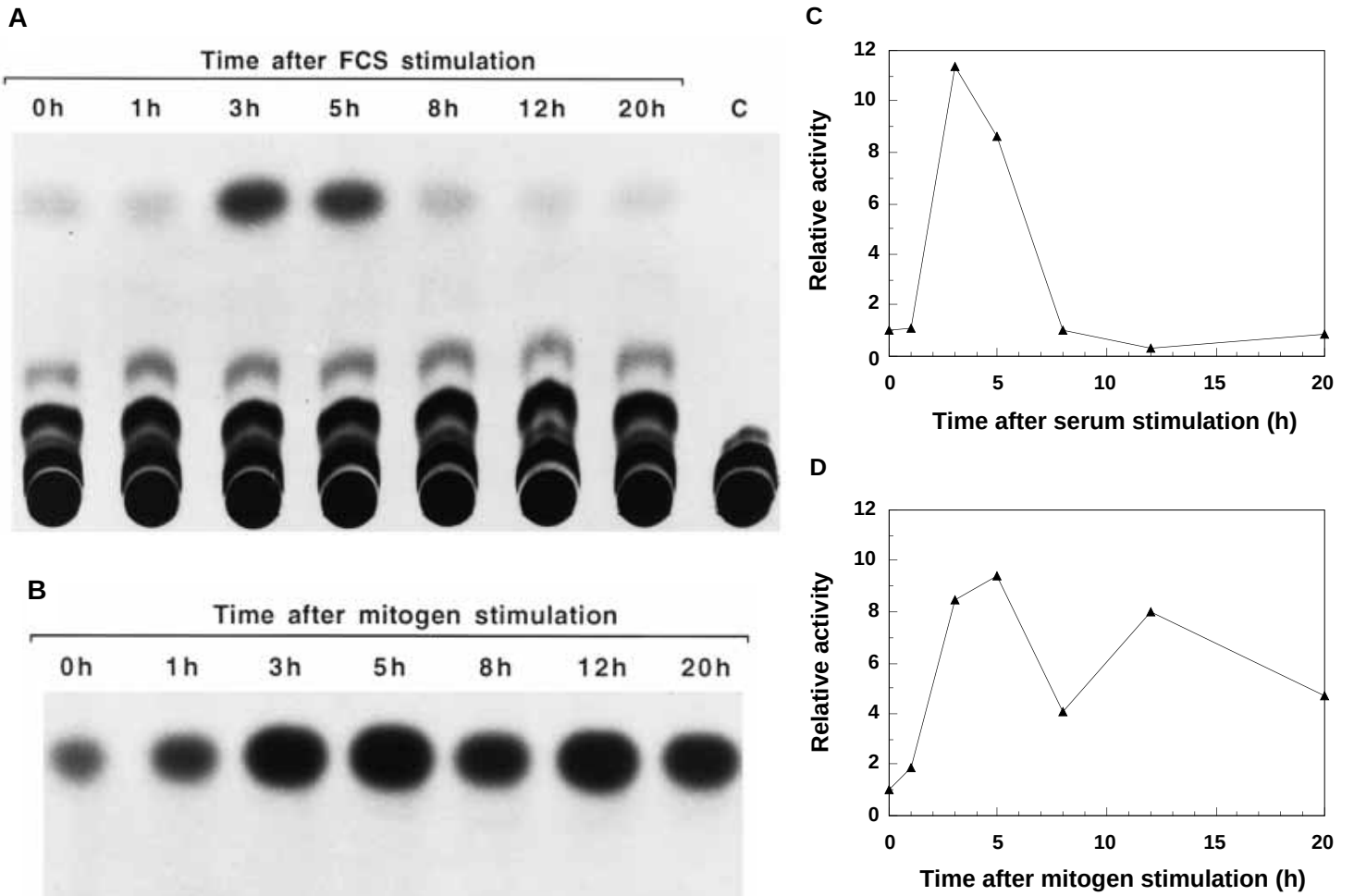


Fig. 4. Activity of HMGR in WI-38 cells after stimulation with either 10% FCS (A,C) or a mixture of pure mitogens (B,D). ^{14}C-labelled 3-hydroxy 3-methylglutaryl CoA was incubated with cell extracts and the product, mevalonolactone, was separated by thin-layer chromatography (spots at the top of A,B) from the substrate (bottom spots). The chromatography plates were quantitatively evaluated by β-radiation scanning.

IE genes, are indeed primary genes. The results obtained for ODC are not as clear, because the induction was diminished but not abolished by puromycin, which might suggest that the ODC gene is induced by both primary and secondary signals, i.e. by the post-translational activation of transcription factors and the transcriptional induction of their genes.

Expression of mitogen-induced genes in normally cycling cells

Since $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progression may substantially differ in their patterns of gene regulation, we were interested to analyse the expression of the inducible metabolic genes in normally cycling cells after separation by counterflow elutriation according to the stage in the cell cycle. As WI-38 cells are not ideally suited for this technique owing to their heterogeneity in size we started this analyses by using the human promyelocytic cell line HL-60. Fig. 6A shows the distribution of cells according to their DNA content in different fractions obtained after elutriation of normally cycling HL-60 cells. The data demonstrate that fractions F1 to F3 contain >90% cells in G_1, that the majority of S-phase cells is found in fractions F5-F7 and that fractions F8-F9 contain mostly G_2 cells. RNA isolated from these elutriated cells were used to analyse the expression of the inducible genes identified above. Fig. 6B shows the results of this experiment, standardised to the values measured for L7 whose expression is constant during the cell cycle. As expected, expression of the cdc2 cell cycle gene showed a steep increase in fractions 5-7, i.e. in S/early G_2, and remained high in fractions 8 and 9, i.e. in G_2. A similar pattern of expression of S/G_2 expression was also seen with those genes whose products are associated with nucleotide biosynthesis, i.e. TK, DHFR and TYS, but their induction was visible earlier as that of the cdc2 gene, namely in mid-late G_1. ODC expression also peaked in S-phase, but half-maximal induction

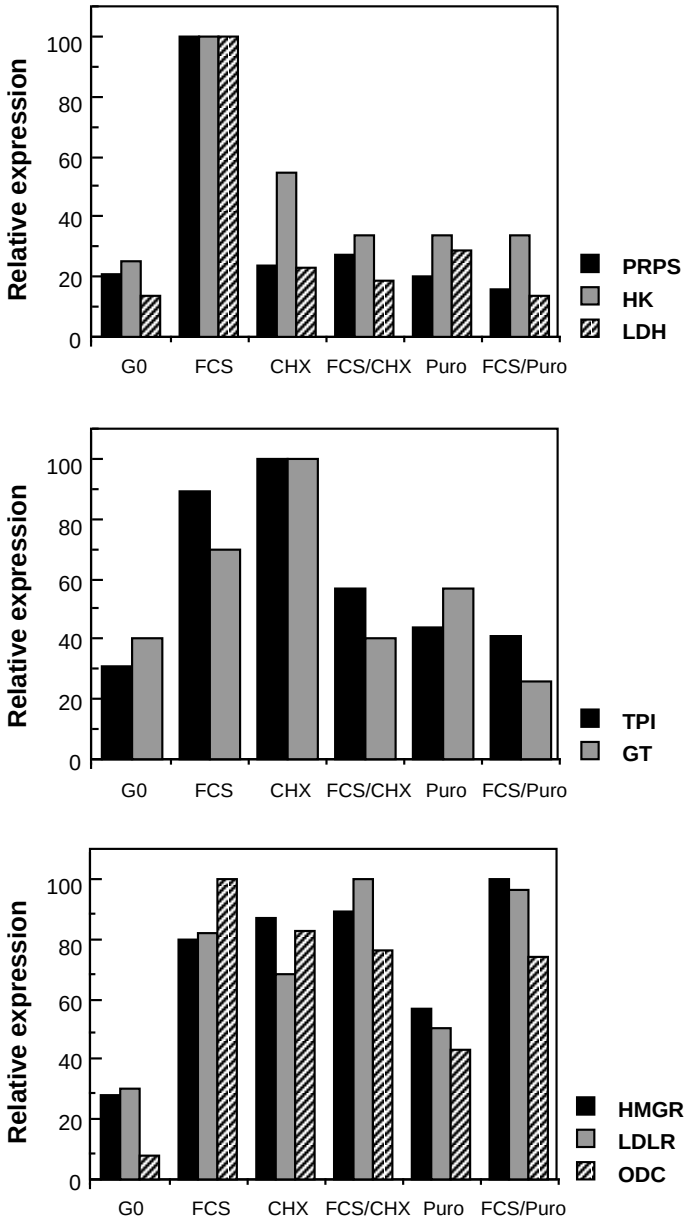


Fig. 5. Induction of mitogen-induced metabolic genes in the presence or absence of protein synthesis inhibitors. G_0, quiescent cells; FCS, stimulation with FCS; CHX, treatment with cycloheximide; FCS/CHX, stimulation with FCS in the presence of cycloheximide; Puro, treatment with puromycin; FCS/Puro, stimulation with FCS in the presence of puromycin. Each treatment was for 8 hours, except for HMGR and LDLR, where treatment was for 3 hours. RNAs were analysed by quantitative PCR and the data quantitatively evaluated by β-radiation scanning as in Fig. 2.

was reached prior to S-phase entry. The expression of ODC therefore precedes that of TK, DHFR and TYS. HK showed a markedly different cell cycle dependent RNA expression pattern. The highest level was found in fraction F1 representing cells in early-mid G_1. HK RNA levels continuously dropped in the following fractions, reaching the lowest level in early-mid G_2 and apparently increasing again in fraction 9, i.e. late G_2. This pattern was obtained in two independent experiments (see Fig. 6B, bottom left panel). The expression of four other genes, PRPS, LDH, LDLR and HMGR did not show any significant fluctuations in the 9 fractions of elutriated cells, indicating that these genes are not subject to cell cycle regulation. The same was found with the genes described above (see Table 1) not be induced by mitogenic stimulation (data not shown).

Since it could not be ruled out completely that the observed discrepancies among the gene expression patterns seen with stimulated and elutriated cells might be due to the fact that different cell types were used in both analyses, we decided to analyse the expression of the IE and DE early genes also in elutriated WI-38 cells. In spite of their heterogeneity, we were able to establish the conditions so as to give a satisfactory distribution of G_1 and $S+G_2/M$ cells. Fig. 7 shows that the fraction of $S+G_2/M$ cells in the 5 collected fractions progressively increased from 3% to 51%. In agreement with these results, we found the expected induction of cdc2 expression in fractions 4-6, and no significant fluctuations in the case of PFK. In keeping with the results obtained with elutriated HL-60 cells (Fig. 6), we found no cell cycle regulation of TPI, PRPS, LDH, LDLR and HMGR in the elutriated WI-38 cells (Fig. 7). The observed fluctuations were below 30% and occurred at random as in the case of HL-60 cells. In contrast to HL-60 cells, we did not find any significant fluctuation of HK mRNA levels. HK regulation was however also relatively weak in HL-60 cells and may therefore not have been detectable in the less well separated WI-38 cells.

The results described above suggest that the induction of IE and DE genes seen in Figs 1-3 is not mirrored by a similar regulation during normal cell cycle progression. To obtain further evidence in support of this conclusion we analysed in HL-60 cells the inducibility of the identified IE and DE genes. The results shown in Fig. 8 clearly support the conclusion that the patterns of gene expression found after mitogen stimulation and during the normal cell cycle are different. Five out of the six genes analysed (HK, LDH, PRPS, GT, TPI) were clearly inducible by serum stimulation (Fig. 8) in spite of the fact that their expression did not fluctuate to a similar extent (HK) or to any significant extent at all (all others) in normally cycling cells of the same type (see Fig. 6b). The sixth gene, ODC, was also induced in stimulated HL-60 cells, as found above with stimulated WI-38 and elutriated HL-60 cells.

DISCUSSION

The mitogen-stimulated growth of quiescent cells is associated with an activation of cellular metabolism which is mirrored by an increased activity of many RNA polymerase II-dependent genes, an accumulation of ribosomal RNA, an augmented synthesis of proteins and a stimulation of metabolic pathways such as glycolysis and nucleotide biosynthesis (for reviews see Baserga, 1981, 1984, 1985; Nathans et al., 1988, 1991; Herschman, 1989, 1991; Bravo, 1990; Müller et al., 1993). It is therefore likely that genes whose products are associated with metabolic processes represent a major target for mitogenic compounds. This hypothesis is substantiated by the fact that for some genes, most notably those whose products are engaged in nucleotide and DNA synthesis plus a few others such as ornithine decarboxylase (ODC), an induction by

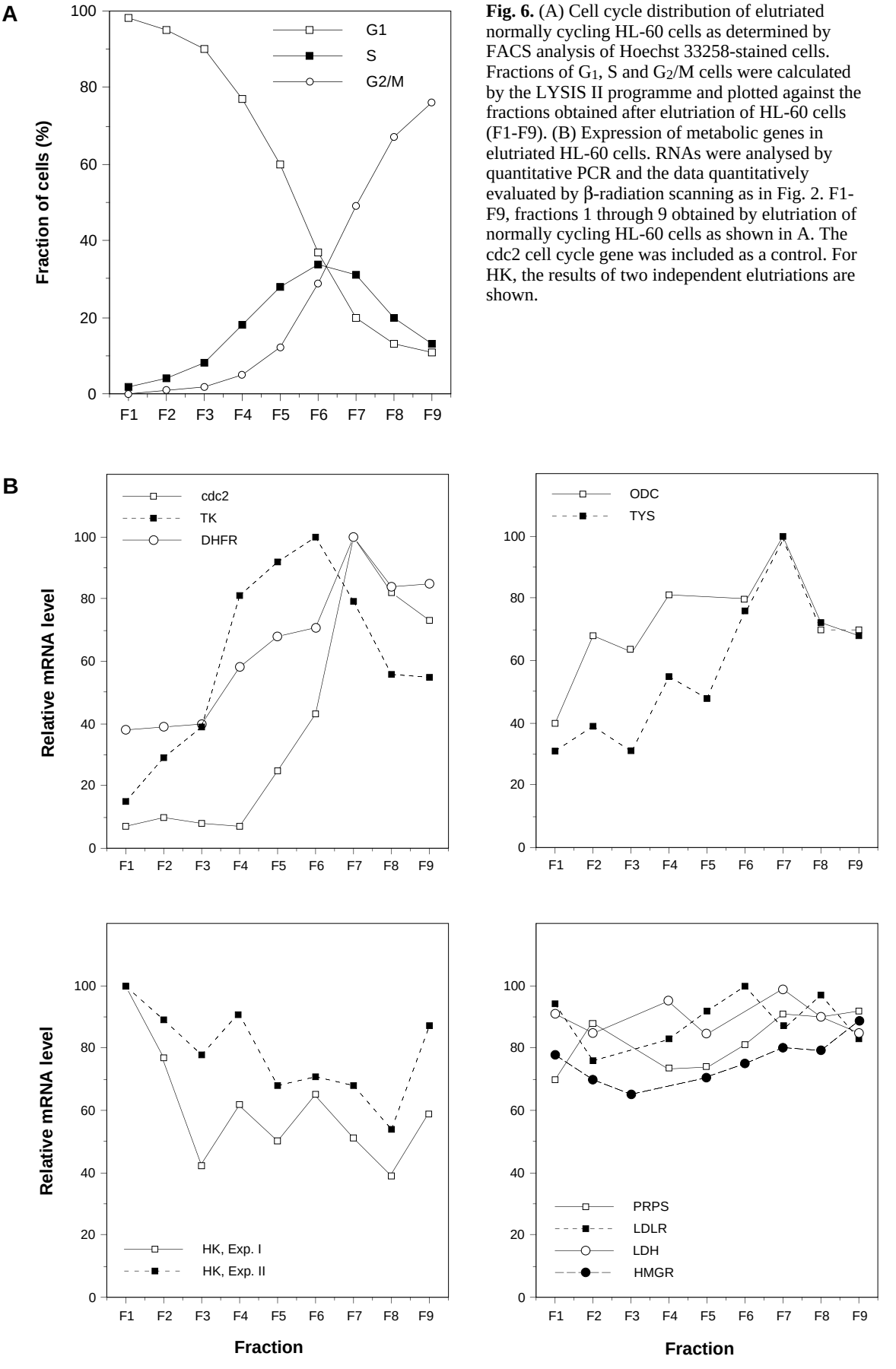
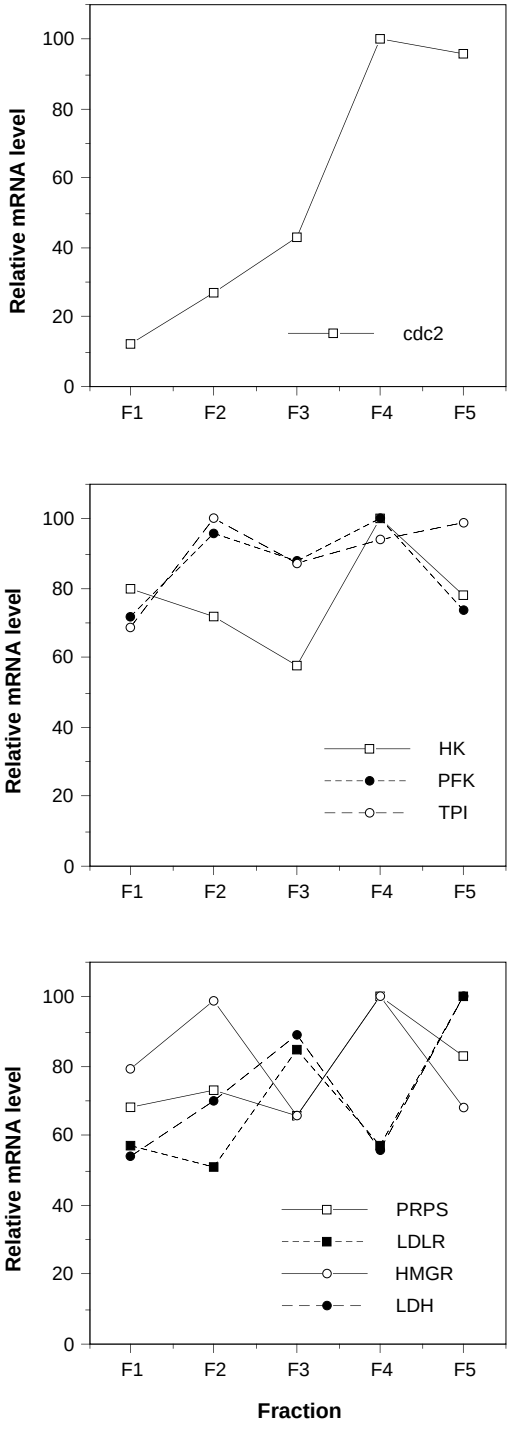


Fig. 6. (A) Cell cycle distribution of elutriated normally cycling HL-60 cells as determined by FACS analysis of Hoechst 33258-stained cells. Fractions of G_1, S and G_2/M cells were calculated by the LYSIS II programme and plotted against the fractions obtained after elutriation of HL-60 cells (F1-F9). (B) Expression of metabolic genes in elutriated HL-60 cells. RNAs were analysed by quantitative PCR and the data quantitatively evaluated by β-radiation scanning as in Fig. 2. F1-F9, fractions 1 through 9 obtained by elutriation of normally cycling HL-60 cells as shown in A. The cdc2 cell cycle gene was included as a control. For HK, the results of two independent elutriations are shown.



	F1	F2	F3	F4	F5
G1	97	94	80	63	49
S+G2/M	4	4	20	37	51

Fig. 7. Expression of metabolic genes in elutriated WI-38 cells. F1-F5, fractions 1 through 5 obtained by elutriation of normally cycling WI-38 cells. See legend to Fig. 6 for details. The cell cycle distribution shown at the bottom of the figure was determined as in Fig. 6A.

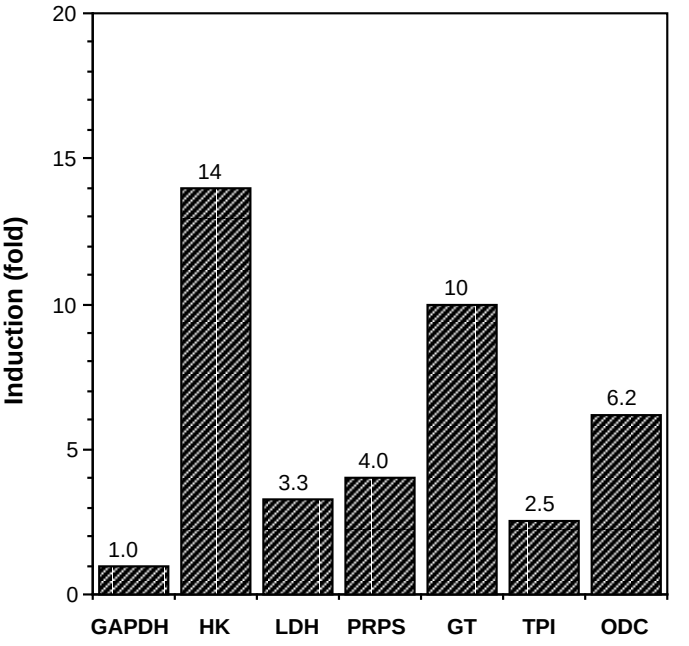


Fig. 8. Induction in HL-60 cells of IE and DE genes identified in serum-stimulated WI-38 cells. HL-60 cells were serum-deprived for 72 hours, RNA was analysed by reverse PCR at the indicated times post-stimulation, and the signals were quantitated as in Fig. 2. The values represent the maximum induction values seen after the following times post-stimulation: HK, 8-16 hours; LDH, 8-16 hours; PRPS, 8-20 hours; GT, 12-20 hours; TPI, 12-16 hours; ODC, 12-16 hours.

mitogens has been reported (Johnson et al., 1978; Haddox et al., 1980; Hendrickson et al., 1980; Navalgund et al., 1980; Kaufman and Sharp, 1983; Liu et al., 1985; Coppock and Pardee, 1987; Katz and Kahana, 1987). In the present study, we sought to investigate this question in further detail by analysing the regulation of 17 different metabolic genes in mitogen-stimulated WI-38 human fibroblasts.

Eleven out of the 17 genes analysed were found to be induced after stimulation of WI-38 cells with FCS. With respect to the kinetics of induction and its sensitivity to inhibitors of protein synthesis, the induced genes can be classified in four groups. (i) The genes encoding glyceraldehyde-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (GPDH), phosphofructokinase (PFK), pyruvate dehydrogenase (PYDH), adenine nucleotide transporter (ANT-2) and succinate ubiquinone oxidoreductase (SUO) were not induced. (ii) The genes coding for hydroxymethylglutaryl-CoA reductase (HMGR) and the low density lipoprotein receptor (LDLR) were induced rapidly and transiently, regardless of the presence of the protein synthesis inhibitor puromycin. These two genes can therefore be considered primary IE genes. (iii) The genes for the glucose transporter (GT), hexokinase (HK), triosephosphate isomerase (TPI), lactate dehydrogenase (LDH), phosphoribosyl-pyrophosphate synthase (PRPS) and ornithine decarboxylase (ODC) showed a delayed induction, but reached peak mRNA levels before DNA synthesis. Their induction was inhibited by puromycin, consistent with their classification as secondary DE genes. (iv) The late genes, i.e. the genes coding for thymidine kinase (TK), thymidylate

synthase (TYS) and dihydrofolate reductase (DHFR) were induced late during G_0→progression with increasing mRNA levels in S-phase. The induction of 5 of these genes, i.e. GT, ODC, TK, TYS and DHFR has also been described by others (Johnson et al., 1978; Haddox et al., 1980; Hendrickson et al., 1980; Navalgund et al., 1980; Kaufman and Sharp, 1983; Liu et al., 1985; Coppock and Pardee, 1987; Katz and Kahana, 1987; Hiraki et al., 1988). At the moment it is very difficult to address the question why certain metabolic genes are induced by mitogenic stimulation while others whose products play a role in the same biochemical pathways are not (e.g. HK and LDH versus GAPDH and PFK). It is possible that in the case of the latter post-translational mechanisms such as allosteric regulation in response to mitogenic stimulation play a key role. Such mechanisms have been described for at least two of the enzymes analysed in the present study, namely PFK (Schneider et al., 1978) and ODC (Atmar and Kuehn, 1981), and apparently also act on two other gene products, HMGR and LDLR, as described in the present study.

Since FCS is a rather undefined mixture of mitogens and other growth supporting compounds, we also asked the question whether the genes identified above as serum-inducible would also be induced by pure mitogens. This study showed that 9 of the 11 FCS-induced genes were also induced by a mixture of pure hormones, i.e. PDGF, EGF and dexamethasone. The only exceptions were HMGR and LDLR. We do not know what the reason for this observation is, since the only regulatory mechanism acting on these two genes, that has been investigated in detail, is the repression by compounds of the mevalonate pathway (e.g. cholesterol) via sterol responsive elements in their promoter sequences (for a review see Goldstein and Brown, 1990). This mechanism, however, cannot be responsible for the effect seen in this study, since the addition of FCS to the medium obviously does not lead to a decrease in the concentration of lipids. We therefore postulate that the HMGR and LDLR genes are actively induced by an unidentified stimulatory factor present in FCS. Another question raised by these results concerns the relevance of the induction by serum, since the defined mitogens, which are also able to trigger DNA synthesis, were unable to induce the two genes. At least for HMGR we could solve this potential problem by showing that its enzymatic activity is strongly activated by either FCS or the mixture of pure mitogens, i.e. irrespective of a transcriptional activation of the genes. Mitogenic stimulation of WI-38 cells can therefore act at at least two different levels on HMGR and LDLR, on their expression by inducing gene expression and by a post-translational mechanism. A regulation of HMGR activity by a variety of compounds has been described (Goldstein and Brown, 1990), but the mechanism responsible for the effect seen in this study remains to be identified.

Another interesting question relates to the mechanisms leading to the different kinetics of induction after mitogenic stimulation, i.e. the IE, the DE and the late response. For some of the late genes, such as TK and DHFR, an involvement of the transcription factor E2F/DRTF and protein complexes containing the retinoblastoma gene product (pRB) together with a cdc2-related protein kinase has been reported (Blake and Azizkhan, 1989; Dou et al., 1991, 1992; Wade et al., 1992; Means et al., 1992; for a review see Nevins, 1992). This suggests that different events have to take place to achieve an induction of these genes, i.e. the activation of E2F, the transcriptional induction of (a) cdk gene(s) and presumably the corresponding cyclin(s) and probably the phosphorylation of pRB. In the case of the IE genes, HMGR and LDLR, the mechanism of induction is not clear, but appears to involve only the activation of pre-existing transcription factors, since *de novo* protein synthesis is not required for their induction. This situation is, with regard to the mechanistic principle, thus similar to the induction of the c-*fos* and *fos*B genes. With these genes, a transcriptionally active complex binding to a serum response element (SRE) is formed from pre-existing serum response factor (SRF) and a ternary complex factor (TCF) upon serum stimulation (Marais et al., 1993; Zinck et al., 1993). It is unlikely that the same mechanism applies to the HMGR and LDLR genes, because no SREs have been found in their promoter sequences, but the activation of pre-existing transcriptional regulators must be involved. To elucidate the underlying mechanisms, the performance of a detailed structure-function analysis will be necessary. For the DE genes described in this study, the mechanisms leading to their induction are also unclear, but are likely to involve the products of some of the IE genes. A large number of IE genes encoding transcription factors or at least coding for proteins containing motifs reminiscent of transcriptional regulators has been identified by now (for a review see Nathans et al., 1991). Future studies will have to address the interplay of genes and gene products within a regulatory network controlled by signals elicited by mitogenic stimuli.

Finally, we studied the regulation of the mitogen- or serum-induced metabolic genes during normal cell cycle progression in HL-60 and WI-38 cells. One goal of this study was to compare the patterns of gene regulation during G_0→S and G_1→S progression and to obtain some hints as to where in the cell cycle the two processes might converge. Our results obtained with stimulated quiescent WI-38 and elutriated HL-60 cells yielded two conclusions. (i) Most of G_0→S and G_1→S progression is fundamentally different and (ii) after passing a point close to S-phase entry both processes seem to merge into a common pathway. Since these conclusion rest on observations made with two different cell lines we performed two additional sets of experiments to exclude the possibility that the observed differences are due to cell type specific characteristics. First, we analysed elutriated WI-38 cells. This study yielded very similar results as the analysis of separated HL-60 cells, although the fractionation of G_1, S and G_2/M cells worked considerably better with HL-60 cells owing to the heterogeneity of the WI-38 cells. It can therefore not be ruled out that a not very pronounced G_1-specific expression pattern might be obscured by the relatively high number of G_1 cells in the S+G_2/M fractions. We therefore also investigated the induction of the same genes in HL-60 cells. All IE and DE genes analysed were also induced by serum in mitogen-deprived HL-60 cells, thus supporting our conclusion that mitogen stimulation and cell cycle progression are associated with different patterns of gene expression. In additon, since HL-60 cells cannot enter a G_0 state, our findings suggest that the induction of genes by mitogens analysed in this study is due to stimulated signal transduction pathways rather than a consequence of cell cycle progression.

Certainly the number of genes analysed is still too low to be able to draw a precise picture, but the principle implied by the

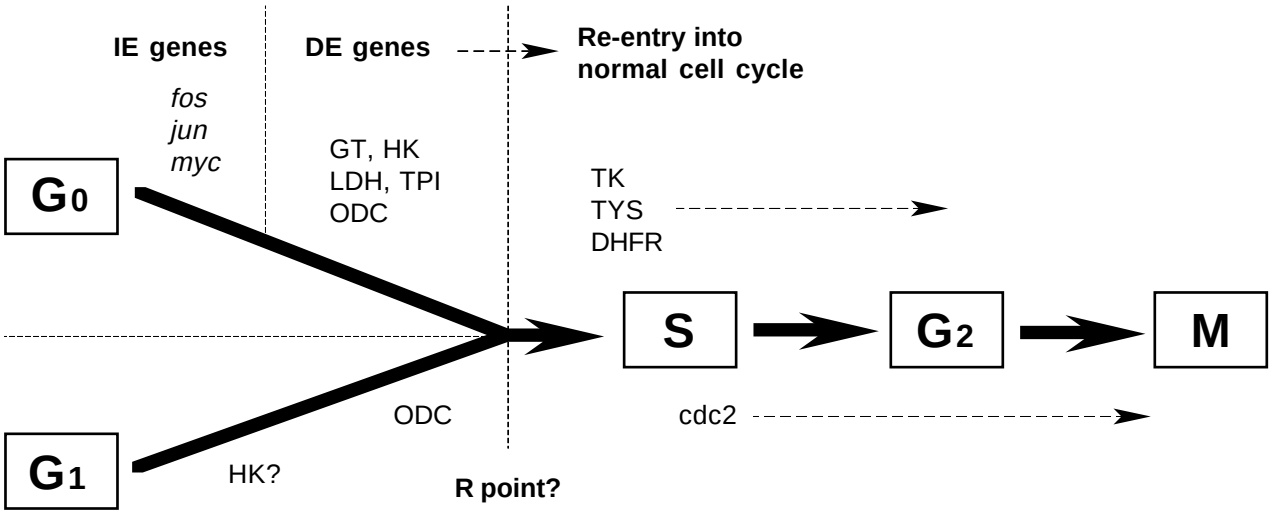


Fig. 9. Model of the relationship between $G_0 \rightarrow S$ transition and normal cell cycle progression based on the induction of metabolic genes as described in the present study.

hypothesis put forward above is supported by our data. A simplified model incorporating the results obtained in the present study is shown in Fig. 9. We and others have shown that the induction of a number of IE genes, including *fos* and *myc*, is specific for the $G_0 \rightarrow S$ transition (Hann et al., 1985; Thompson et al., 1985; Bravo et al., 1986), as is the activation of certain DE genes, such as LDH, GT, TPI and PRPS (this study). At least one DE gene, ODC, stimulated at about the same point during $G_0 \rightarrow S$ progression is also induced in the course of G_1 progression in normally cycling cells. However, at least three points argue against the conclusions that at this time point both $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progressions have converged. (i) At the same time after stimulation, other DE genes are induced but not activated in G_1 (see above), (ii) the timing of induction during $G_1 \rightarrow S$ progression is different, and (iii) ODC mRNA expression declines during S-phase in stimulated cells, but increases in asychronously growing cells. We would therefore rather conclude that the induction of ODC (and perhaps HK) is part of both $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progressions and both processes merge at a later time. Thus, the genes induced late after mitogenic stimulation (TK, TYS and DHFR) are upregulated at a similar time in normally cycling cells, i.e. in late G_1/S, and all three genes followed this pattern. One might therefore speculate that $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progressions merge at a point around S-phase entry. Further work is necessary to test this hypothesis and to define the point of convergence more precisely.

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