Journal of Cell Science 107, 227-239 (1994)
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JCS8402

Identification of serum-inducible genes: different patterns of gene regulation during $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progression

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SUMMARY

We have identified, by differential cDNA library screening, 15 serum inducible genes in the human diploid fibroblast cell line WI-38. The genes fall into two classes that are distinguished by their dependence on protein synthesis for the induction by serum, i.e., primary and secondary genes. While 11 of these genes encode known proteins, 4 other genes have not been described to date. The former genes encode proteins of diverse functions, including the monocyte-derived neutrophil chemotactic factor (MONAP), calmodulin, tropomyosin, tenascin, collagenase, plasminogen activator inhibitor-2a, the 'sperm-specific' cleavage signal-1 protein, metallothionein IIa and the mitochondrial chaperonin *hsp*-60. Interestingly, one of the unknown genes contains a large open reading frame for a polypeptide that is highly homologous to a previously unidentified long open reading frame in the opposite strand of the gene coding for the transcription factor HTF-4. We also studied the regulation of these serum-induced genes during cell cycle progression in normally cycling WI-38 and HL-60 cells separated by counterflow elutriation as well as in serum-stimulated HL-60 cells. Our results clearly show that, in contrast to the prevailing opinion, the expression of most genes induced after mitogen stimulation is not subject to a significant regulation in normally proliferating cells. This supports the hypothesis that the progression into S from either G_0 or G_1 are distinct processes with specific patterns of gene expression.

Key words: mitogen, growth factor, immediate early gene, delayed early gene, cell cycle, gene regulation

INTRODUCTION

Upon mitogen or growth factor stimulation of quiescent cells, a vast number of genes is transcriptionally activated (Cochran et al., 1983; Lau and Nathans, 1985; Almendral et al., 1988; Zipfel et al., 1989; Lanahan et al., 1992; Vincent et al., 1993; for reviews see Bravo, 1990; Hershman, 1991; Nathans et al., 1991; Williams et al., 1992; Müller et al., 1993). Many of these genes are induced rapidly and their induction is independent of de novo protein synthesis. These genes, which are induced as a consequence of the mitogen-mediated activation of pre-existing transcription factors, have been termed immediate early (IE) genes or primary genes. This first burst of gene expression is followed by the induction of a second class of genes, the delayed early (DE) genes, whose transcriptional activation in many cases is sensitive to inhibitors of protein synthesis. The diversity of the genomic response to mitogen stimulation is underscored by the fact that genes whose products are associated with many different cellular processes are activated, including secreted factors, proteins involved in building or degrading the extracellular matrix, metabolic enzymes and transport proteins, proteins involved in signal transduction, DNA-binding proteins and transcription factors, and the cdk/cyclin protein kinase subunits directly involved in cell cycle control (for reviews see Bravo, 1990; Hershman, 1991; Nathans et al., 1991, Williams et al., 1992; Müller et al., 1993).

Many genes of the IE response encode transcription factors belonging to the AP-1, NFκB, SRF and Myc families and in some cases the functional significance of their induction has been investigated (for reviews see Nathans et al., 1991; Müller et al., 1993). This applies primarily to the Fos family members c-Fos, FosB, Fra-1 and Fra-2, whose function in $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progression has been analysed by loss-of-function approaches. The most convincing study along these lines is probably that by Bravo and coworkers, who produced antibodies that discriminate between the different known members of the Fos and Jun families and used these for microinjection into serum-stimulated NIH3T3 cells (Kovary and Bravo, 1991, 1992). These authors showed that the inhibition of c-Fos alone has no significant effect on $G_0 \rightarrow S$ progression, while S-phase entry was clearly inhibited after injection of Jun-specific antibodies. In addition, DNA synthesis could be efficiently blocked by coinjecting antibodies against different members of the Fos family, suggesting that Fos family members may be functionally redundant with respect to the induction of S-phase entry after mitogen stimulation of quiescent cells. An important role for AP-1 in $G_0 \rightarrow S$ progression is also suggested by the fact that the microinjection of TPA responsive element (TRE) containing competitor oligonucleotides leads to the

inhibition of DNA synthesis (Riabowol et al., 1992). A crucial function in the control of $G_0 \rightarrow S$ transition has also been shown for c-*myc*. The c-*myc* gene was the first proto-oncogene reported to be induced by mitogens (Kelly et al., 1983; Müller et al., 1984), and direct role for its product, c-Myc, in $G_0 \rightarrow S$ progression was suggested relatively early by two independent studies. Using either inducible *myc* expression vectors in transfection experiments or recombinant Myc protein for microinjection into 3T3 cells it could be shown that c-Myc can substitute for a competence factor (like platelet-derived growth factor; PDGF) in the progression of cells from G_0 into S-phase (Armelin et al., 1984; Kaczmarek et al., 1985), and was therefore classified as a competence factor itself. Subsequently it was also shown, that antisense oligonucleotides against c-*myc* inhibit DNA synthesis (Heikkila et al., 1987; Prochownik et al., 1988; Wickstrom et al., 1988).

Much less is known about the functional significance of the induction of many other genes after mitogenic stimulation of quiescent cells, even though for many of these genes the biochemical function of their products is known or structural similarities with proteins of known function have been identified (see for example Kelly et al., 1983; Chafouleas et al., 1984; Greenberg and Ziff, 1984; Müller et al., 1984; Coppock and Pardee, 1985; Calabretta et al., 1986; Hazel et al., 1988; Hiraki et al., 1988; Masibay et al., 1988; Milbrandt, 1988; Ryder et al., 1988; Ryder and Nathans, 1988; Wahl et al., 1988; Ryseck et al., 1988, 1989; Oquendo et al., 1989; Tirone and Shooter, 1989; McDonnell et al., 1990; Borus et al., 1990; Davis et al., 1991; Yang and Lau, 1991; Yang and Tonk, 1991; Won et al., 1992; Sewing et al., 1993). In addition, many mitogen-induced genes identified to date are primary genes (see for example, Almendral et al., 1988; Zipfel et al., 1989), so that the number of known secondary genes is still comparatively low. The identification of secondary genes is, however, a highly relevant task because they are likely to be the ultimate mediators of the mitogenic response. In addition, the secondary genes are the targets for the primary gene products, and their identification and analysis are therefore paramount in elucidating the function of the transcription factors encoded by the primary genes. We have therefore initiated a study pertaining to the identification of genes that are induced relatively late after serum stimulation, but prior to S-phase entry. As a model system for this study we chose the human diploid, non-established lung fibroblast cell line WI-38, representing a normal cell as closely as possible. In this paper, we report the identification of 15 genes whose expression is transcriptionally induced by mitogenic stimulation of quiescent cells. While 11 of these genes encode known proteins, 4 other genes have not been described to date. The induction of 5 of these genes occurs 4-6 hours after stimulation and is blocked by protein synthesis inhibitors, both hallmarks of secondary genes. We also addressed another so far rather neglected issue, namely the regulation of mitogen-induced genes during cell cycle progression in normally cycling cells. Our results obtained with elutriated and serum-stimulated WI-38 and HL-60 cells indicate that, in contrast to the prevailing opinion, most genes induced after stimulation of mitogen-deprived cells are not regulated to a significant extent in normally proliferating cells. This supports the hypothesis that the transition into S from G_0 and G_1 are distinct processes with specific patterns of transcriptional regulation.

MATERIALS AND METHODS

Cell culture

WI-38 cells (Hayflick, 1965), obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 0.5% glucose. 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. In some experiments protein synthesis or transcription was inhibited by the addition of puromycin (20 μg/ml; Sigma), emetine (10 μg/ml; Sigma) or actinomycin D (5 μg/ml; Sigma) to the medium. HL-60 cells were grown in RPMI-1640 supplemented with 5% FCS plus 5% newborn calf serum.

Counterflow elutriation of HL-60 and WI-38 cells

Prior to elutriation the cells were incubated for 15 minutes at 37°C with 5 μg of actinomycin D/ml of medium. This treatment was carried out because the stress exerted by the elutriation procedure was found to be sufficient to induce certain IE genes, such as c-*fos*. If the cells were pretreated with actinomycin D as described above, this stress response was suppressed (our unpublished observations). Cells were separated by elutriation, using a Beckman JE-5.0 large capacity rotor and ice-cold PBS/1% newborn calf serum as the medium. Approximately 5×10^8 cells were loaded at a rotor speed of 1600 revs/minute and a flow rate of 20 ml/minute. With HL-60 cells, a series of nine 800 ml fractions was collected at increasing flow rates ranging from 43 to 85 ml/minute. In the case of WI-38 cells, 6 fractions were collected at flow rates of 60 to 105 ml/minute.

FACS analyses

HL-60 and trypsinized WI-38 cells were washed once with PBS and fixed in ice-cold 75% ethanol overnight at 4°C. After washing once with PBS, the fixed cells were stained for 15 minutes in Hoechst 33258 staining buffer (100 mM Tris, pH 7.4, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (v/v) Nonidet-P40, 0.2% (w/v) BSA, 2 μg/ml Hoechst 33258). Stained cells were analysed in a FACS-STAR Plus (Becton Dickinson) using an UV-laser excitation at 325 nm. The fluorescence was amplified linearly. Cell cycle distributions were calculated using the LYSIS II programme (Becton Dickinson).

RNA isolation and analysis by Northern blotting and PCR

RNA was isolated according to Chomczynski and Sacchi (1987) and analysed by Northern blotting as described (Müller et al., 1983). Reverse transcription of RNA into cDNA and quantitative PCR analysis were performed as described (Sewing et al., 1993). The number of cycles was chosen so 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:

Calmodulin (SenGupta et al., 1987)
(5′-primer): 5′-GACAAAGATGGTGATGGCAC-3′ (113-132)
(3′-primer): 5′-CCATCACCATCAATATCTGC-3′ (437-456)

CS-1 (Javed and Naz, 1992)
(5′-primer): 5′-GCACTCATGGGAATGTGTGG-3′ (197-216)
(3′-primer): 3′-CCAAATCTGGAGGTGTCTGC-3′ (559-578)

*hsp*60 (Venner et al., 1990)
(5′-primer): 5′-CGAGCCTTAATGCTTCAAGG-3′ (133-152)
(3′-primer): 3′-CCTCGATCAAACTTCATGCC-3′ (670-689)

PAI-2a (Antalis et al., 1988)
(5′-primer): 5′-GCTCTGCAATCAATGCATCC-3′ (380-399)
(3′-primer): 5′-CATCATCTGTACAGGTGTGC-3′ (734-753)

Collagenase (Whitham et al., 1986)
(5′-primer): 5′-GGAAACCAGATGCTGAAACC-3′ (296-315)
(3′-primer): 3′-CCAAGAGAATGGCCGAGTTC-3′ (721-740)

Tenascin (Siri et al., 1991)
(5′-primer): 5′-CCGCAGCCCCTGATGTTAAGG-3′ (389-409)
(3′-primer): 3′-CTGGGCAGATTTCACGGCTGC-3′ (785-805)

β-Actin (Ponte et al., 1984)
(5′-primer): 5′-GATGATGATATCGCCGCGCT-3′ (1160-1179, Exon 2)
(3′-primer): 5′-CTTCTCGCGGTTGGCCTTGG-3′ (1625-1644, Exon 3)

Tropomyosin (Lin and Leavitt, 1988)
(5′-primer): 5′-GCTCTCAAAGATGCCCAGGAG-3′ (472-493)
(3′-primer): 5′-CGAGTACTTATCCTCTGCAGC-3′ (910-931)

cdc2 (Lee and Nurse, 1987)
(5′-primer): 5′-GGAGAAGGTACCTATGGAGTTGTG-3′ (171-194)
(3′-primer): 5′-AGTCTCTGTGAAGAACTCTTCTAGAGT-3′ (499-525)

L7 (Herzog et al., 1990)
(5′-primer): 5′-TAGGATCCCATGGAGGGTGTAGAAGAGAAG-3′ (20-39)
(3′-primer): 5′-TTGAGAATTCAATCATGGTAGACACCTTAG-3′ (765-784)

cDNA library construction and screening

Poly(A)$^+$ RNAs were isolated from WI-38 cells 4, 6 and 9 hours after stimulation of quiescent cells with 10% FCS. The pooled RNAs were used for construction of a cDNA library with the ZAP-cDNA synthesis kit (Stratagene). Approximately 17,000 plaques were analysed by differential screening of replica filters using ^{32}P-labelled cDNA representing quiescent or stimulated (pooled as above) cells as the probes synthesised by reverse transcription of 1 μg of poly(A)$^+$ RNA with 200 U of Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL) in the presence of 10 μCi of [α-^{32}P]dCTP. Hybridisations were carried out for 48 hours at 42°C in a buffer containing 50% formamide as described for Northern blotting (Müller et al., 1983). Filters were washed at 60°C in decreasing concentrations of SSC/0.1%SDS (2×SSC→0.2×SSC).

RESULTS

Identification of serum induced genes by differential cDNA library screening

One of the goals of our study was the identification of genes in human diploid fibroblasts whose induction by mitogens or serum is dependent on protein synthesis and follows the activation of the transcription factors of the immediate early (IE) response. Such secondary genes are of great interest because they may represent the targets for gene products encoded by the IE genes. In addition, they may also play a role in normal cell cycle (G_1→S) progression, as opposed to the IE genes whose transcriptional activation is often part of the signal transduction cascade triggered by the mitogen(s) rather than being directly connected to the cell cycle. Towards this end, we constructed an oligo(dT)-primed cDNA library from WI-38 cells that had been serum deprived and restimulated for 4-9 hours. At this time the induction of many IE genes (like c-*fos*) is not detectable any more and the cells are approximately in the middle of the G_0→S transition, i.e. well before reaching the R point (DNA synthesis starts at 12-14 hours; see Sewing et al., 1993). Poly(A)$^+$ RNA was isolated from cells stimulated for 4, 6 and 9 hours and pooled for cDNA synthesis and construction of a library in the λZap phage vector. This library was screened with labelled cDNA probes from quiescent and serum stimulated (pooled 4, 6 and 9 hours) cells. The screening of 17,000 plaques yielded 152 plaques showing differential hybridisation after several rescreenings. After further analysis

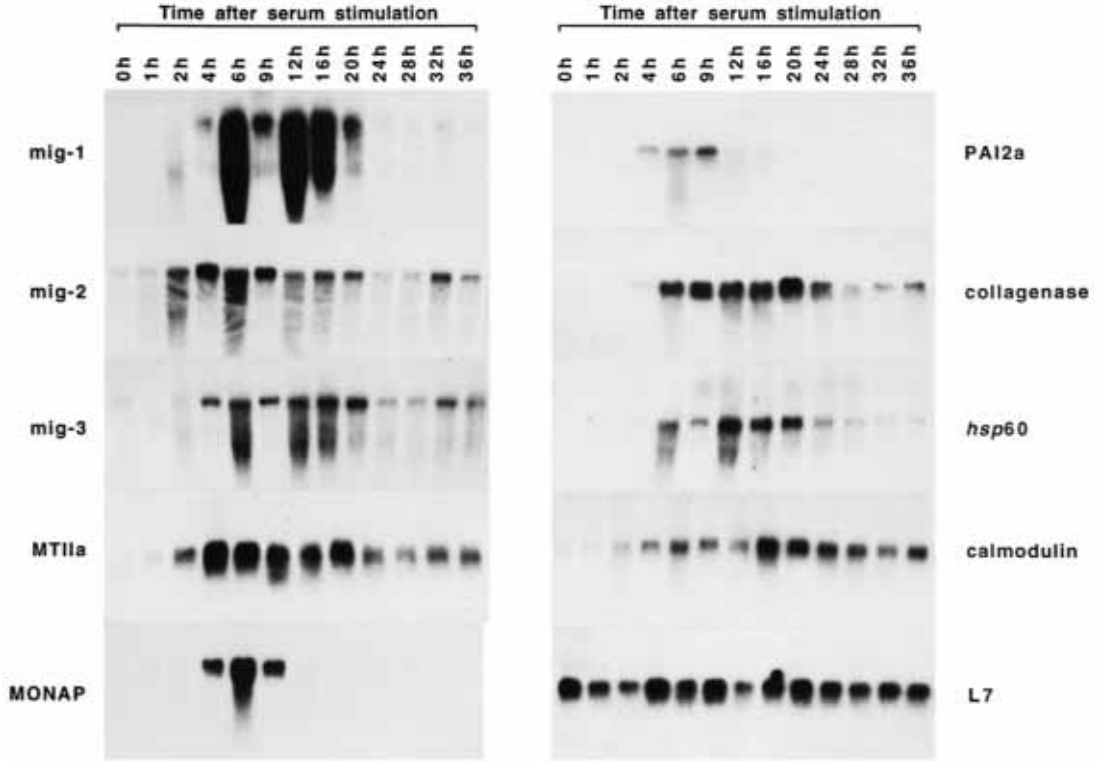


Fig. 1. RNA expression analysis of 10 different genes following stimulation of quiescent WI-38 cells with 10% fetal calf serum (FCS). Total RNA was separated on agarose gels and analysed by northern blotting. The 9 hour samples gave too low a signal with some probes presumably due to partial RNA degradation.

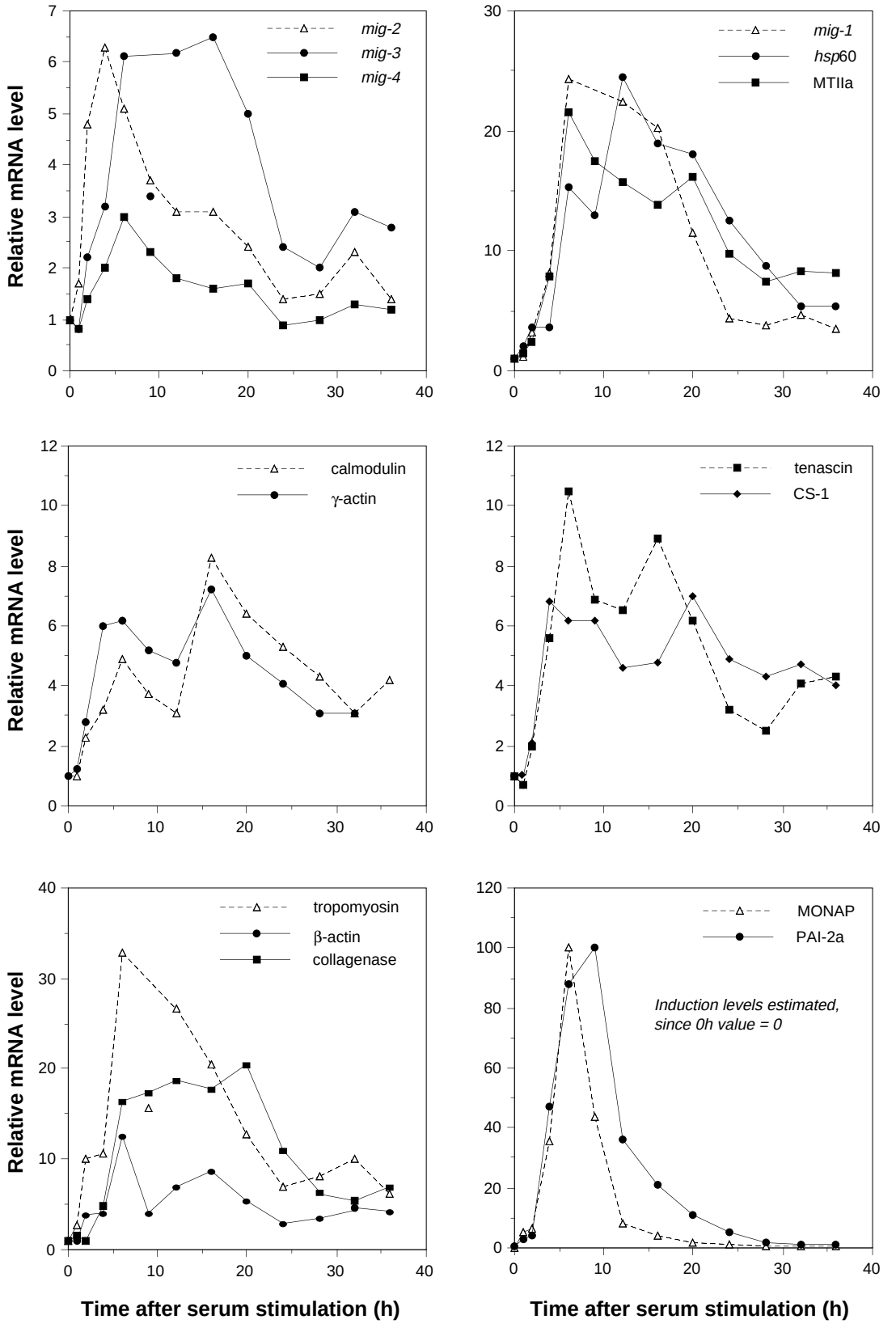


Fig. 2. Quantitative analysis of RNA expression of 15 different genes following stimulation of quiescent WI-38 cells with 10% FCS. Northern blots were quantitatively evaluated by β-radiation scanning using a Molecular Dynamics PhosphorImager.

of these phage by dot blot analysis, reverse Southern blotting and finally Northern blotting (using the same RNAs as above) 21 clones remained that showed a clear induction after serum stimulation.

All cDNA inserts were partially sequenced and compared to the GenBank and EMBL databases. These analyses showed that the clones represented 15 different genes (see Table 1), of which 4 have not been described to date. The 11 known genes

Table 1. Serum-induced genes in human diploid fibroblasts (WI-38)

Gene	Class*
A. Genes encoding regulatory proteins	
Monocyte-derived neutrophil chemotactic factor (MONAP)	1°
Calmodulin	1°
B. Genes encoding structural proteins and degrading enzymes	
Tropomyosin	1°
β-Actin	1°
γ-Actin	1°
Tenascin	1°
Collagenase	2°
Plasminogen activator inhibitor-2a (PAI-2a)	2°
Cleavage signal-1 protein (CS-1)	?
C. Genes encoding proteins involved in cellular metabolism	
Mitochondrial chaperonin *hsp*60	(2°)
Metallothionein IIa	2°
D. Unknown genes	
mig-1 (mRNA >6 kb)	2°
mig-2 (mRNA ~4 kb)†	1°
mig-3 (mRNA ~4 kb)	?
mig-4 (mRNA >5 kb)	1°

*Classification as primary or secondary gene based on the inducibility in the presence of the protein synthesis inhibitors puromycin or emetine: 1°, primary gene, independent of protein synthesis; 2°, secondary gene, dependent on protein synthesis; ( ) or ?, classification uncertain or impossible because the protein synthesis inhibitors caused strong induction themselves or gave contradictory results. See also Fig. 4 for original data.

†*mig*-2 is homologous to the opposite strand of HTF-4.

code for the monocyte-derived neutrophil chemotactic factor (MONAP), calmodulin, tropomyosin, β-actin, γ-actin, tenascin, collagenase, plasminogen activator inhibitor-2a (PAI-2a), the 'sperm-specific' cleavage signal-1 protein (CS-1), metallothionein IIa (MTIIa) and the mitochondrial chaperonin *hsp*-60. The isolated tropomyosin cDNA seems to represent a new isoform with differences scattered in both the coding and non-coding regions (see sequence submitted to the EMBL sequence data library; accession number Z24727). The 4 unknown genes were designated *mig*-1 through *mig*-4 (**mi**togen **i**nducible **g**ene). The induction kinetics for all genes are shown in Figs 1 and 2. In addition, a probe for L7, encoding a protein of the large ribosomal subunit, was included in these analyses as a non-inducible, cell cycle independent gene (Rittling et al., 1986). The data show that the observed induction patterns differed in a number of aspects, including (i) the maximum level of expression (3-fold for *mig*-4; >25-fold for *mig*-1, hsp-60, tropomyosin, MONAP and PAI-2a; 7- to 20-fold for most of the others), (ii) the time after stimulation when maximum induction is reached (4 hours for CS-1 and *mig*-2; 9 hours for PAI-2a; 12 hours for *hsp*60; 16 hours for calmodulin and γ-actin; 6 hours for all the others), and (iii) the duration of induction (≤8 hours for *mig*-2, MONAP and PAI-2a; >15 hours for most of the others; practically stable for CS-1). These data are in good agreement with the initial goal to identify genes that are induced approximately in the middle of the $G_0 \rightarrow S$ transition. In addition, the identification of genes for which an induction by mitogens has been shown previously in other systems (like MONAP, actin or calmodulin) beside the identification of novel mitogen-regulated genes (such as *hsp*-60, CS-1, PAI-2a, tenascin and the *mig* genes) shows the suitability of the approach taken in the present study. The DNA sequences determined for the 4 *mig* cDNAs have been deposited with the EMBL sequence data library (accession numbers Z24724, Z24725, Z24726, Z24740, Z24749).

A large region of *mig*-2 is homologous to the non-coding strand of the HTF-4 gene

The alignment of *mig*-2 with the GenBank and EMBL data base sequences gave a surprising result. *mig*-2 and the gene encoding the transcription factor HTF-4 (Zhang et al., 1991) showed a very high degree of homology, but this alignment was possible only with the non-coding strand of the HTF-4 gene. A closer inspection of the published HTF-4 sequences revealed a previously unidentified long open reading frame (ORF) of 299 amino acids with an ATG start codon in the HTF-4 non-coding strand (nucleotide positions 899 to 3). Using synthetic strand-specific oligonucleotides we could show that both strands of the HTF-4 locus are expressed in HeLa cells. In contrast, other cell types showed a strand-specific expression. Thus, WI-38 cells express only the HTF-4 mRNA at a detectable level, while in the human keratinocyte cell line HACAT the opposite strand is expressed at higher levels (data not shown). These observations suggest that both strands of the HTF-4 locus indeed express different products.

The Mig-2 polypeptide sequence derived from the cDNA clone and an alignment with the putative protein encoded by the HTF-4 opposite strand are shown in Fig. 4. The ORF frame identified in the *mig*-2 cDNA is 720 amino acids long, but at present the precise N-terminal start is unclear. It is possible that an ATG codon inside the sequenced region is used (e.g., Met-41 in the sequence in Fig. 3) or the sequence extends further N-terminally beyond the end of the isolated cDNA clone. For the purpose of the present study, this question is however of minor importance. The alignment of the two sequences shows a striking homology (63.2% identity; 90.5% similarity) over a region of approximately 290 amino acids (Fig. 3). This homology region represents about 40% of the Mig-2 sequence, the remaining 60% are unique to Mig-2 (Fig. 3).

Sensitivity of serum-induced gene expression to inhibitors of protein synthesis and transcription

We next investigated the role of protein synthesis in the induction of all genes identified in this study. Quiescent WI-38 cells were stimulated for 4 or 9 hours in either the presence or absence of the protein synthesis inhibitors puromycin or emetine. The results obtained with 9 genes are depicted in Fig. 4 and all results are corroborated in Table 1. The data clearly show that the genes fall into two classes, i.e., protein synthesis inhibitor insensitive primary genes and sensitive secondary genes. The latter category of genes, which with respect to the goals of our study is of particular interest, comprises *mig*-1, collagenase, PAI-2a, MTIIa and presumably *hsp*60. The classification of CS-1 and *mig*-3 proved impossible because the inhibitors caused a strong induction of the two genes themselves, so that an effect on serum induction could not be determined.

We then addressed the question as to whether the induction of the genes identified by the differential cDNA library screening is dependent on transcription as opposed to mRNA stabilisation. For this purpose, cells were exposed to the RNA polymerase inhibitor actinomycin D 1 hour after serum stimulation and expression of all secondary genes, plus tropomyosin

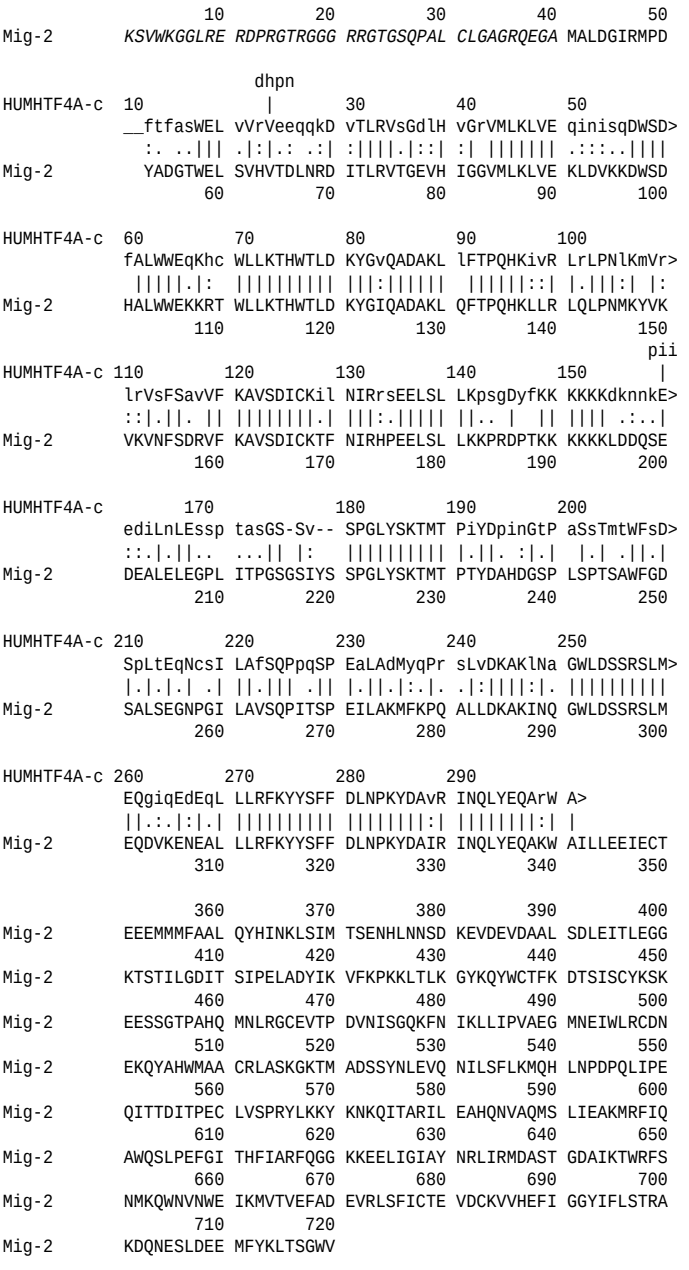


Fig. 3. Amino acid sequence of Mig-2 derived from the cDNA sequence. The precise N-terminal start is not known. It is possible that an ATG codon inside the sequenced region is used (e.g., Met41 in the deduced sequence above) or the sequence extends further N-terminally beyond the end of the isolated cDNA clone. The Mig-2 sequence was aligned to an ORF in the opposite strand of the HTF-4 gene (nucleotide positions 899 to 3). Identical amino acids are shown as vertical lines, similarities as dots.

and tenascin as examples of primary genes, was analysed 9 hours post stimulation either in the presence or absence of the inhibitor. The results in Fig. 5 clearly show that the actinomycin D treatment led to a complete block in the induction of all genes analysed, i.e. *mig*-1, collagenase, tropomyosin, tenascin, PAI-2a, MTIIa and *hsp*60. This is evident by the fact that expression at 9 hours in the presence of actinomycin D was as low as in quiescent cells. We therefore conclude that transcription plays a crucial role in the induction of these genes following mitogenic stimulation.

Expression of mitogen-induced genes in normally cycling cells

A relatively unexplored question concerns the differences in gene regulation during the $G_0 \rightarrow S$ transition after mitogenic stimulation of quiescent cells and the $G_1 \rightarrow S$ progression in normally cycling cells. We therefore decided to address this issue using the genes identified in this study to probe RNA isolated from cells in different phases of the cell cycle, separated by counterflow elutriation. As WI-38 cells are not ideal for elutriation due to the heterogeneity in cell size, we included the promyelocytic cell line HL-60 in this study. HL-60 cells are homogeneous with respect to both their karyotype (with very few tetraploid cells) and cell size and grow in suspension, which make them very suitable for separation by elutriation. Typical elutriation data showing the distribution of DNA content/cell in the different fractions obtained after elutriation of HL-60 and WI-38 cells are depicted in Figs 6A and 7. The fractions of G_1, S and G_2/M cells were calculated using the LYSIS II programme and are also indicated in the figures. It is clear from these data that, in the case of the elutriated HL-60 cells, fractions F1-F5 contain mostly G_1 cells with increasing amounts of S-phase cells, that the highest number of S-phase cells is contained in fractions F6 and F7, and that the majority of cells in fractions F8 and F9 is in G_2. WI-38 cells could also be separated by elutriation, although the resolution was lower as in the case of HL-60 cells for the reasons given above. With WI-38 cells, 5 fractions were collected which showed a progressive decline in the number of G_1 cells and a concomitant increase in the number of cells in S+G_2/M (3% in fraction 1; 51% in fraction 5).

RNA from all fractions was isolated, reverse transcribed into cDNA and analysed by quantitative PCR as described. Included in this experiment were two genes serving as controls: L7, encoding a ribosomal protein expressed in a cell cycle independent fashion, and cdc2, whose expression increases in S/G2. L7 was used as an internal standard, i.e. the levels determined for all other mRNAs were standardised to the corresponding L7 signal. As shown in the top left panels of Figs 6B and 7, expression of the cdc2 gene in the elutriated cells was as expected (Dalton, 1992), in that expression increased more than 10-fold in S and G_2. Analysis in the same experiment of the 7 serum-inducible genes, that were expressed and thus could be analysed in HL-60 cells, gave a rather surprising result. None of these genes, with the exception of PAI-2a, showed fluctuations of more than 30% in both cell lines, which occurred at random as shown in repetitions of the experiment and are therefore not significant (Figs 6B and 7). These genes included *mig*-1, *mig*-3, hsp60, CS-1, calmodulin and β-actin (Figs 6B and 7; data not shown). In addition, the expression of 3 genes not expressed in HL-60 cells was analysed in elutriated WI-38 cells with very similar results: no significant regulation was seen with *mig*-2, collagenase and tropomyosin (Fig. 7). With PAI-2a we observed however a clear albeit modest fluctuation in expression levels during the cell cycle in both HL-60 and WI-38 cells. In HL-60 cells, the level of PAI-2a decreased during G_1, reached

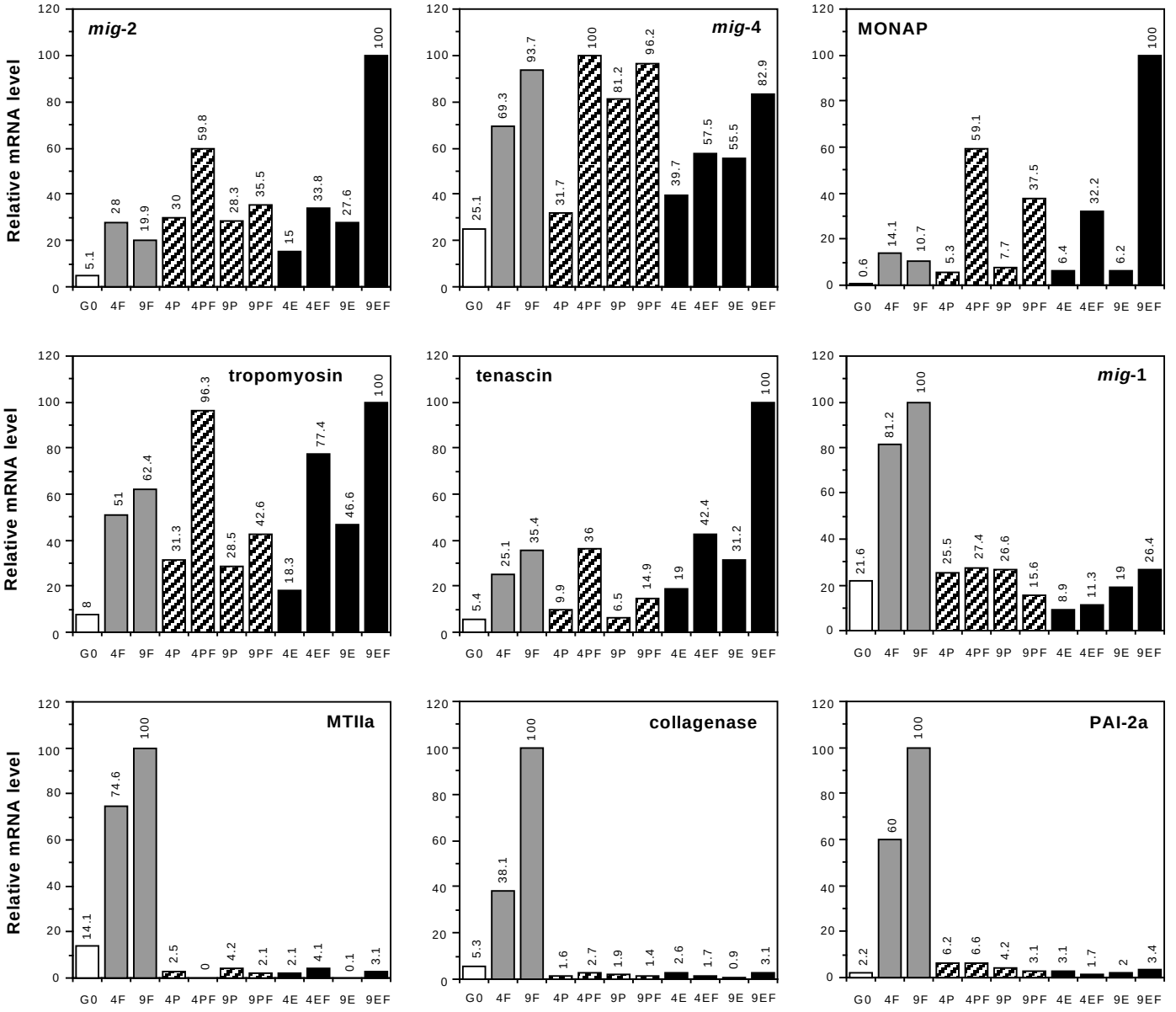


Fig. 4. Induction of serum regulated genes in the presence or absence of protein synthesis inhibitors. G_0, quiescent cells; 4F, 4 hour stimulation with FCS; 9F, 9 hour stimulation with FCS; 4P, 4 hour treatment with puromycin; 4PF, 4 hour stimulation with FCS in the presence of puromycin; 9P, 9 hour treatment with puromycin; 9PF, 9 hour stimulation with FCS in the presence of puromycin; 4E, 4 hour treatment with emetine; 4EF, 4 hour stimulation with FCS in the presence of emetine; 9E, 9 hour treatment with emetine; 9EF, 9 hour stimulation with FCS in the presence of emetine. RNAs were analysed by Northern blotting and the data quantitatively evaluated as in Fig. 2.

minimum levels at G_1/S and then increased again to peak levels in G_2. The difference between expression at G_1/S and in G_2 was approximately 2-fold and was seen reproducibly in 2 independent experiments (Fig. 7 and data not shown). In WI-38 cells, a similar increase during S/G_2 was observed, but the decrease in G_1 was not found in this case. In both cell lines, however, the level and timing of induction during the cell cycle are in contrast to the results obtained after serum stimulation of quiescent WI-38 cells, which gave a >30-fold induction before S-phase entry. Taken together this analysis leads to the conclusion that $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progression substantially differ in their genetic programmes.

The separation of WI-38 cells by counterflow elutriation yielded an almost 20-fold enrichment in G_1 cells (fraction F1 in Fig. 7), but the separation of S+G_2/M cells was comparatively poor. This means that the quality of the elutriated cells is clearly high enough to be able to detect G_2-specific or strongly G_1-specific genes, but weaker G_1-specific expression patterns would not be detectable. We therefore decided to analyse the inducibility of the identified IE and DE genes in HL-60 cells, in order to obtain further evidence that the patterns of gene expression seen after mitogen stimulation and during the normal cell cycle are different. The results shown in Fig. 8 clearly support this conclusion. All 6 genes analysed were clearly inducible by serum stimulation in spite of the fact that their expression did not change to any significant extent

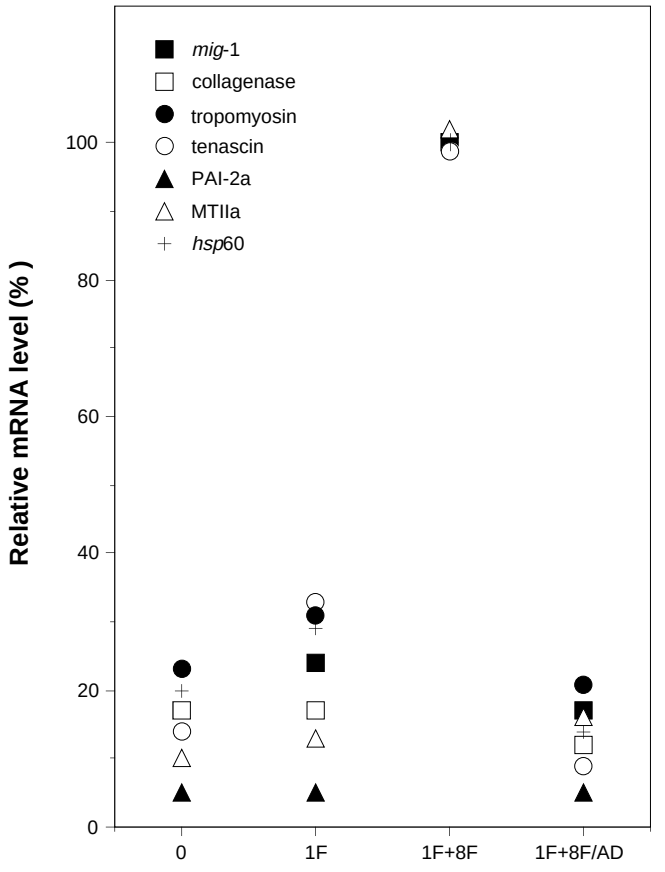


Fig. 5. Induction of serum regulated genes in the presence or absence of the RNA synthesis inhibitor actinomycin D. 0, quiescent cells; 1F, 1 hour stimulation with FCS; 1F+8F, 9 hour stimulation with FCS (normalised to 100% for all genes); 1F+8F/AD, 9 hour stimulation with FCS, 8 hour in the presence of actinomycin D. RNAs were analysed by Northern blotting and the data quantitatively evaluated as in Fig. 2.

in normally cycling cells of the same type (compare Figs 6B and 8).

DISCUSSION

The present study was undertaken to identify genes whose expression is induced during the $G_0 \rightarrow S$ progression in human fibroblasts. The cell line WI-38 is a non-established fibroblast line derived from human fetal lung that has retained many of the characteristics of normal fibroblasts in vivo, including a diploid karyotype and the ability to enter replicative senescence (Hayflick, 1965). Mitogenic stimulation of quiescent WI-38 cells should therefore reflect, at least to some extent, the stimulation of connective tissue cells as it occurs, for instance, in tissue repair and wound healing. To date, all screenings for mitogen-inducible genes have been performed with established rodent fibroblasts (e.g. Cochran et al., 1983; Almendral et al., 1988; Lanahan et al., 1992; Vincent et al., 1993) or with other cell types, such as lymphocytes (Zipfel et al., 1989). We therefore decided to use WI-38 cells for the identification of serum-inducible genes with two major goals in mind. First, most genes identified in other systems belong to the class of IE genes. To understand the functions of the IE gene products, especially those of the transcription factors, it is of paramount importance to identify their target genes, i.e., the secondary and DE genes induced by mitogenic stimuli. It was therefore our intention to design an experimental set-up that would allow for the isolation of cDNAs representing secondary genes. Second, we intended to use the identified genes to compare the genetic programme that is triggered when a quiescent cell is stimulated to enter DNA synthesis to that seen during normal $G_1 \rightarrow S$ progression.

Eleven out of the 15 genes identified in this study encode known proteins. For some of these genes a regulation by mitogenic compounds, such as serum growth factors or phorbol esters has been shown in other systems. This applies to the induction of MONAP (Kowalski and Denhardt, 1989), calmodulin (Chafouleas et al., 1984), actin (Greenberg and Ziff, 1984; Masibay et al., 1988; Vincent et al., 1993), tropomyosin (Ryseck et al., 1989), collagenase (Angel et al., 1987a) and MTIIa (Karin et al., 1984). For the other genes, i.e. tenascin, PAI-2a, CS-1 and *hsp*60, an induction during $G_0 \rightarrow S$ progression to our knowledge has not been described. Interestingly, in contrast to most of the screenings performed with the aim to identify IE genes, none of the 11 known genes identified here encodes a nuclear protein. This is a reflection of our experimental strategy (i.e. stimulation for 4-9 hours), since most mitogen-inducible transcription factor genes are IE genes with short-lived transcripts. Instead, the products encoded by the genes identified in this study are located in the cytoplasm, associated with the cytoplasmic membrane, secreted into the medium or are constituents of mitochondria (see Table 1 for a summary). What is the significance of their induction by mitogens? Of particular interest are those genes whose products are directly linked to the control of cell proliferation. This class of genes is exemplified by calmodulin, whose induction is likely to increase the cell's responsiveness to mitogenic stimulation (for a review see Rasmussen and Means, 1989). Another group of genes, whose expression might be more indirectly coupled to the cell cycle, are those whose products are involved in energy production and other metabolic pathways. It is conceivable that a stimulated cell induces a number of metabolic enzymes not only post-translationally but also at the level of transcription to ensure the rapid synthesis of all metabolites needed for progression into DNA synthesis (Baserga, 1985). In addition, mitogen-deprived cells are smaller than their normally cycling counterparts. Rapid growth of such cells after receiving a mitogenic signal may be achieved by stimulating cellular metabolism to its maximum. In this context, induction of the MTIIa gene might play a role. Of particular interest may be the serum-induced expression of the gene encoding the mitochondrial chaperonin *hsp*60, since the augmented synthesis of its product may facilitate the transport of proteins into the mitochondria, thus supporting an increased energy metabolism. The potential importance of mitochondrial proteins in growth control is also indicated by the induction of the mitochondrial proton/phosphate symporter, an energy-linked transport protein (Vincent et al., 1993). In addition, the Fos effector protein *fte*-1 has been shown to be homologous to a yeast protein involved in mitochondrial protein import (Kho and Zarbl, 1992). In the accompanying paper by Bürger et al. (1994), we show that genes

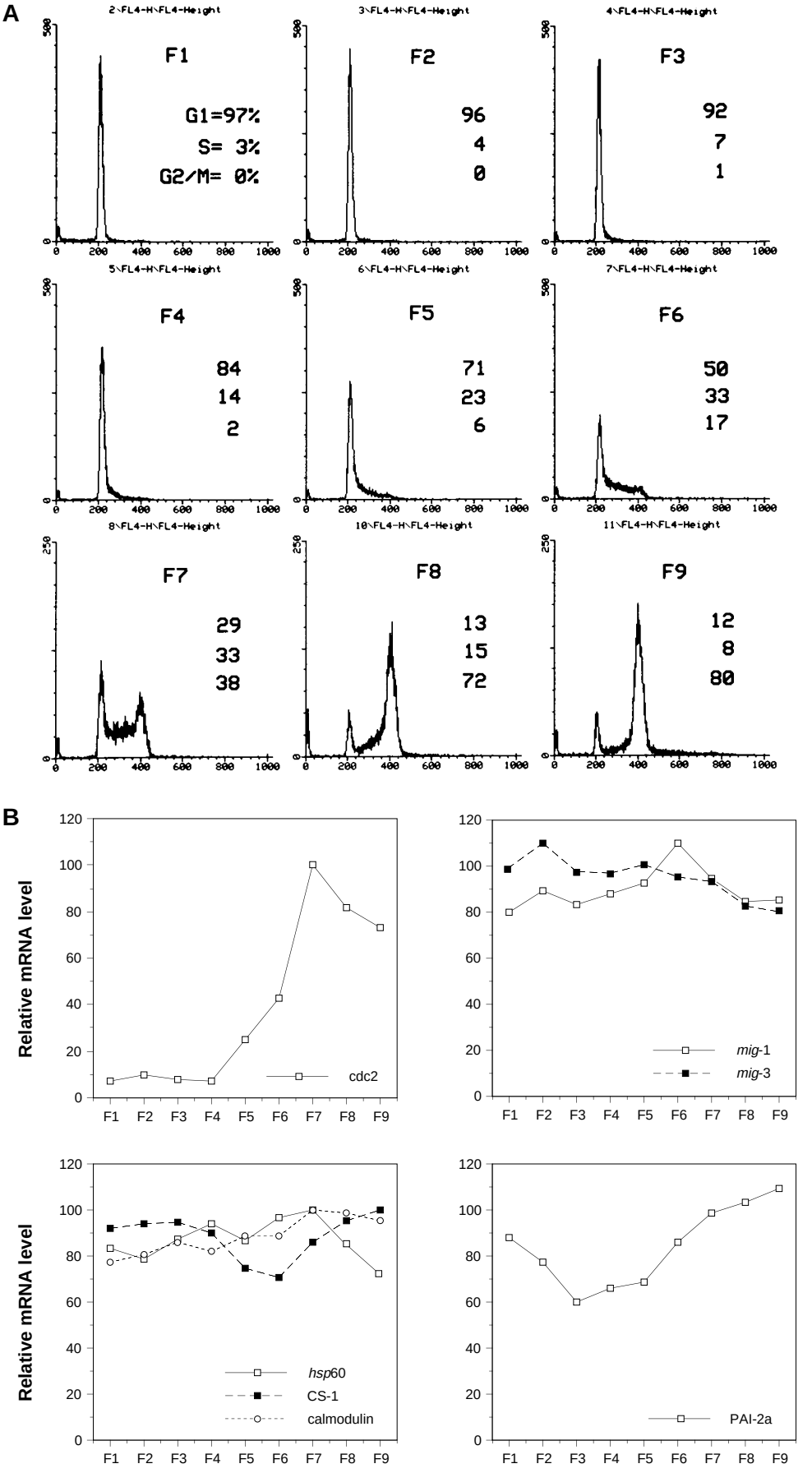
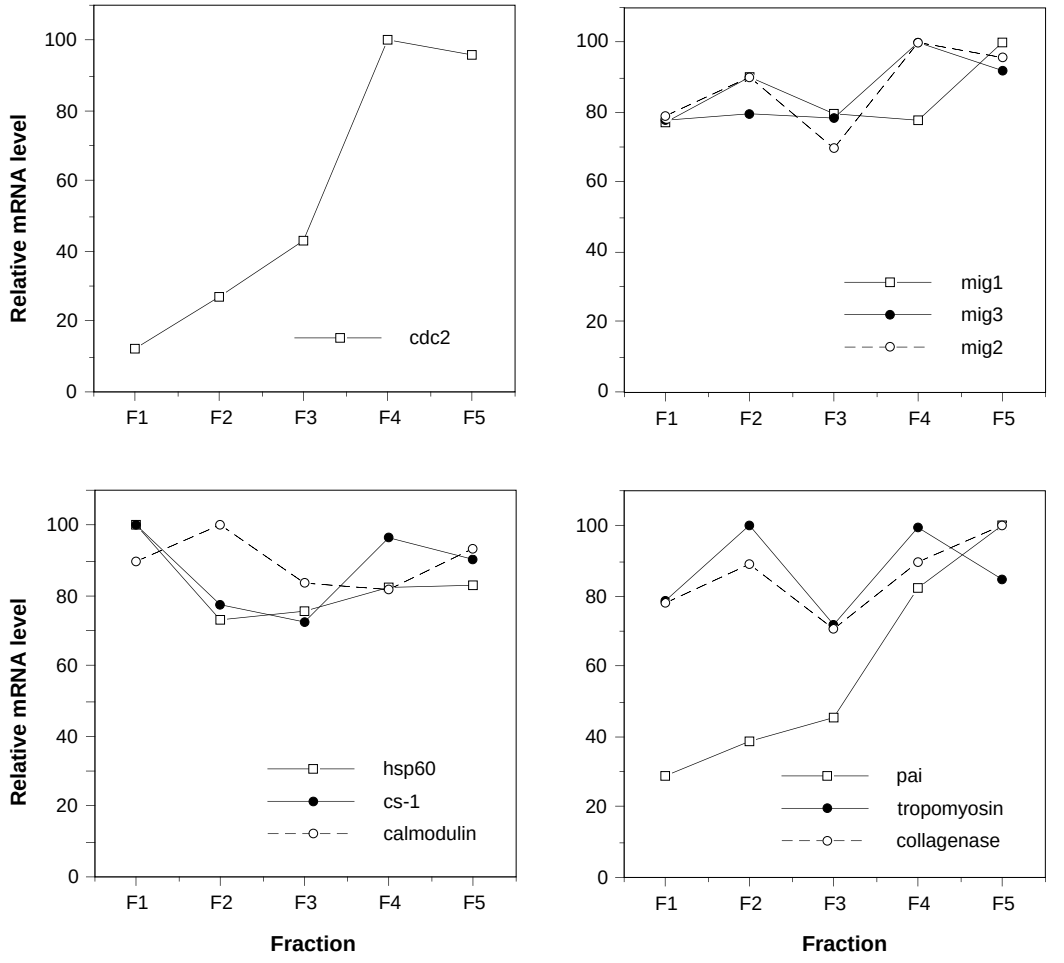


Fig. 6. (A) Cell cycle distribution of elutriated HL-60 cells as determined by FACS analysis of cells stained with Hoechst 33258. Fractions of G_1, S and G_2/M cells were calculated by the LYSIS II programme. F1-F9, fraction 1 through 9 obtained by elutriation of normally cycling HL-60 cells. The ordinate in each graph represents the number of particles (i.e., stained nuclei), the abscissa the range of fluorescence intensity (channels). (B) RNA expression in elutriated HL-60 cells. RNAs were reverse transcribed into cDNA and analysed by quantitative PCR. The data were quantitatively evaluated as in Fig. 2. F1-F9, fractions 1 through 9 obtained by elutriation of normally cycling HL-60 cells as shown in A.



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

Fig. 7. RNA expression in elutriated WI-38 cells. F1-F5, fraction 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.

encoding metabolic enzymes indeed represent a major group of genes induced during $G_0 \rightarrow S$ progression.

Another group of genes induced by mitogenic stimulation encodes products that are associated with connective tissue repair. In this context, proteases, protease inhibitors and extracellular matrix components that are produced by the stimulated fibroblasts may play an important role in the process of tissue regeneration. Three of the genes identified here might belong into this group, i.e., collagenase, PAI-2a and tenascin. In addition, induction of PAI-1 has previously been described by others (Vincent et al., 1993). The role of collagenase and PAI-2a might be directly associated with an reorganisation of the connective tissue and extracellular matrix. The induction of tenascin might serve a functionally different purpose, in that it may trigger the differentiation and proliferation of the surrounding epithelium (Mackie et al., 1988). Also factors like MONAP may play an indirect role in wound healing by contributing to an immune response (inflammation), which might be necessary to prevent infection of the damaged tissue (Antalis et al., 1988). Tissue repair is also associated with an increased cell locomotion. Apart from the proteases and protease inhibitors, such as collagenase and PAI-2a, which act on the extracellular environment, the intracellular microfilaments play a major role in this process. This may explain the induction of β-actin, γ-actin and the actin fibre associated protein tropomyosin during $G_0 \rightarrow S$ progression. The only serum-induced gene identified in this study whose function remains enigmatic is CS-1 (Javed and Naz, 1992). This gene encodes a protein identified by virtue of its association with the plasma membrane of spermatocytes, where its function is however unclear. Induction of the CS-1 gene in WI-38 cells results in two mRNAs, one of which seems to be fibroblast-specific (data not shown), pointing to different mechanisms of regulation and perhaps distinct functions in both cell types.

Apart from the known genes discussed above we have identified 4 new genes, *mig*-1 through *mig*-4, whose expression is induced during the $G_0 \rightarrow S$ progression. At present, we do not know what the functions of these genes might be, but the

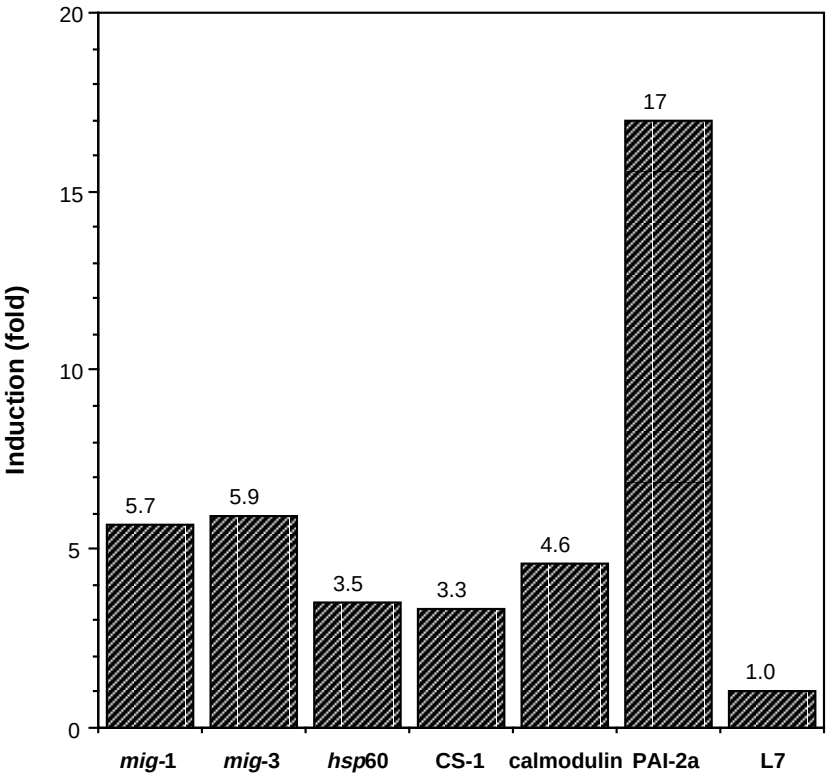


Fig. 8. Induction in HL-60 cells of IE and DE genes identified in serum-stimulated WI-38 cells. HL-60 cells were serum-deprived for 72 hours, RNA was analysed by reverse PCR at the indicated times post-stimulation, and the signals were quantitated as in Fig. 2. The values represent the maximum induction values seen after the following times post-stimulation: *mig*-1, 8-12 hours; *mig*-3, 8-16 hours; *hsp*60, 12-20 hours; CS-1, 8-16 hours; calmodulin, 8-20 hours; PAI-2a, 8-12 hours.

homology of the Mig-2 protein with an open reading frame discovered in the opposite strand of the HTF-4 gene (Zhang et al., 1991) is striking. The alignment of the two sequences shows a highly significant homology (63.2% identity; 90.5% similarity) over a region of approximately 290 amino acids (Fig. 3). This homology region represents about 40% of the Mig-2 sequence, the remainder being unique to Mig-2, suggesting both common and unique functions for Mig-2 and the putative protein derived from the opposite strand of the HTF-4 gene. The expression of both strands of the HTF-4 locus and the homology with another genomic locus, *mig*-2, raises intriguing new questions regarding the organisation and evolution of these loci and the function of their gene products.

At least 5 of the genes identified in the present study are secondary genes, as suggested by the abrogation of their induction by the protein synthesis inhibitors puromycin and emetine. These genes, collagenase, PAI-2a, *hsp*60, MTIIa and *mig*-1, are potential candidates for a regulation by products of the IE genes. For two of them, collagenase and MTIIa, functional AP-1 binding sites (TREs) have been identified in their promoter sequences (Angel et al., 1987b; Lee et al., 1987), and the tenascin gene promoter contains a TRE consensus sequence (Jones et al., 1990). It is therefore very likely that their induction is at least in part mediated by the prior activation of the *fos* and *jun* genes and their encoded products. The role of the IE proteins in the regulation of the secondary genes listed above can now be tested using loss-of-function and gain-of-function approaches. In this context, the recently described c-*fos* 'knock-out' mice (Johnson et al., 1992; Wang et al., 1992) will be particularly useful.

Another important question asked in the present study concerned the similarities and differences, respectively, in the gene expression patterns elicited by mitogenic stimulation of G_0 cells on the one hand and during $G_1 \rightarrow S$ progression on the other. We decided to separate cells from a normally proliferating population by counterflow elutriation to obtain cells in specific phases of the cell cycle. Since WI-38 cells are not ideal for this technique (see RESULTS), we included the human promyelocytic cell line HL-60 in this experiment. The results obtained in this study were surprising, since none of the 8 genes analysed showed a clear cell cycle dependent expression in either cell line that would have been similar to the induction pattern seen with stimulated G_0 cells. The cdc2 cell cycle gene, however, did give the expected results (Dalton, 1992) and showed clear increase in expression in S/G_2. While 7 genes showed no detectable regulation at all, expression of the PAI-2a gene increased about 2-fold during S and G_2. It may be speculated that the biological significance of this induction is associated with changes in morphology, adhesion and motility that occur during the cell's preparation for mitosis. However, both the level and the timing of induction were markedly different from the pattern seen after serum stimulation. In addition, we analysed the inducibility of the same genes discussed above in HL-60 cells. Surprisingly, all IE and DE genes analysed were also induced by serum in mitogen-deprived HL-60 cells, although these cells cannot enter a quiescent state. This finding supports the conclusion that most mitogen-inducible genes are not cell cycle regulated. Rather than being directly linked to cell cycle progression, the induction of many genes by mitogens seems to be a consequence of stimulated signal transduction pathways, an event that can occur throughout or at most stages of the cell cycle.

Our results lead to the conclusion that many of the genes induced during $G_0 \rightarrow S$ and $G_1 \rightarrow S$ progression are different, thus reflecting the fact that the two processes are functionally distinct events. This point is also strengthened by previous

observations which showed that the expression of the IE genes c-*fos* and c-*myc* does not fluctuate during the cell cycle (Hann et al., 1985; Thompson et al., 1985; Bravo et al., 1986). A major difference between G_0 and G_1 cells is the greatly reduced biosynthetic and genetic programme in the former. After stimulation, quiescent cells may therefore require a dramatic increase in the expression of many genes to achieve a rapid re-entry into the cell cycle, resulting in peak levels during the $G_0 \rightarrow S$ period and decreasing later to basal levels. In contrast, normally cycling cells may need the same gene products throughout the cell cycle, especially structural proteins or gene products associated with energy metabolism. It is therefore conceivable that such genes are not subject to a cell cycle dependent regulation. Interestingly, as shown in the accompanying paper by Bürger et al. (1994), several genes induced later after mitogenic stimulation (in S/G_2) are cell cycle regulated, presumably because both pathways merge into a common mechanism of cell cycle progression at a time around S-phase entry.

We are grateful to C. Schalk for performing the FACS analyses and to Dr M. Krause and S. Klingelhöfer for synthesis of oligonucleotides. This work was supported by the Deutsche Forschungsgemeinschaft (Mu601/5-2, Mu601/5-3 and Mu601/7-1) and the Dr Mildred Scheel-Stiftung für Krebsforschung. S. B. is the recipient of a fellowship from the Graduiertenkolleg 'Zell- und Tumorbiologie' at the Philipps-Universität Marburg.

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(Received 19 July 1993 - Accepted, in revised form, 11 November 1993)