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Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G_1 phase

(cell cycle/gene expression/B-*MYB* gene)

DAVID LIN*, MICHELLE T. SHIELDS*, STEPHEN J. ULLRICH†, ETTORE APPELLA†, AND W. EDWARD MERCER*‡

*Department of Microbiology and Immunology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107; and †Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Conditional expression of wild-type (wt) p53 protein in a glioblastoma tumor cell line has been shown to be growth inhibitory. We have now more precisely localized the position in the cell cycle where growth arrest occurs. We show that growth arrest occurs prior to or near the restriction point in late G_1 phase of the cell cycle. The effect of wt p53 protein on the expression of four immediate-early genes (c-*FOS*, c-*JUN*, *JUN*-B, and c-*MYC*), one delayed-early gene (ornithine decarboxylase), and two late-G_1/S-phase genes (B-*MYB* and DNA polymerase α) was also examined. Of this subset of growth response genes, only the expression of B-*MYB* and DNA polymerase α was significantly repressed. The possibility that decreased expression of B-*MYB* may be an important component of growth arrest mediated by wt p53 protein is discussed.**

The gene encoding the nuclear phosphoprotein p53 is the most frequently mutated gene identified in human cancers to date (for review see ref. 1). Although some of the biological properties of the wild-type (wt) p53 protein have been defined, such as the ability to suppress the growth of *in vitro* transformed rodent cells (2–4) or human tumor cells (5–7), the molecular basis of growth suppression remains unknown. The observation that wt p53 protein, but not mutant forms, can suppress cell growth suggests that wt p53 may play an important role in regulating cell proliferation (2–7).

Cell cycle controls that regulate the orderly flow of cells in and out of G_1 phase are the main determinant of the rate of postembryonic cell proliferation (8, 9). Two major control points have been defined in animal cells. One control point appears to operate in early G_1 phase and allows cells to exit the cell cycle and enter a nonproliferative state of arrest termed G_0 (8, 9). The second control point, the restriction point (R-point) occurs in late G_1 phase (8, 10). Time-course experiments indicate that protein synthesis is required throughout early G_1 phase for cells to pass the R-point and become committed to enter S phase and initiate DNA synthesis. The R-point is thus defined as the time after which inhibition of protein synthesis fails to inhibit entry into S phase (8, 10).

We have shown that induction of wt p53 protein expression in a human glioblastoma tumor cell line can inhibit cell cycle progression (5). In this model, growth inhibition was associated with a significant decrease in the steady-state mRNA levels of the replication-dependent histone H3 gene (5), which is known to be coordinately regulated with the onset of DNA replication (11), and the replication-independent proliferating cell nuclear antigen (PCNA) gene (12). On the contrary, the steady-state mRNA levels for the genes encoding endogenous p53, β_2-microglobulin, β-actin, and thymidine kinase were not found to be significantly affected (5, 12).

In the present communication, we have more precisely localized the position in the cell cycle where growth suppression occurs, and we have examined the effect of induction of wt-p53 protein on the expression of seven additional growth response genes. Evidence is presented that growth suppression mediated by wt p53 protein occurs prior to or near the R-point in late G_1 phase of the cell cycle and that only a subset of late-G_1/S-phase growth response genes are affected.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The parental human glioblastoma cell line T98G and the sublines GM47.23 and Mdel 4A were cultured in Earle's minimal essential medium containing 10% fetal calf serum (GIBCO) at 37°C as described (5, 12). The Mdel 4A subline was derived by transfection with the pMdel plasmid (4) as described (5). It contains an inducible human p53 cDNA with a genetically engineered 69-base-pair deletion between the *Bal* I restriction sites at nucleotides 627 and 696 resulting in an in-frame deletion that removes 23 amino acids from the p53 coding sequence. Mdel 4A cells express mutant mRNA and protein when induced with dexamethasone (DEX) (unpublished results).

Cell Cycle Studies. Cells were arrested in G_0 and serum-stimulated to reenter the cell cycle as previously described (5, 12). For R-point analysis, cycloheximide (Sigma) was added to the culture medium at a final concentration of 1 μg/ml.

RNA Isolation and Northern Blotting. Total RNA was extracted from cells by using the method described in ref. 13. RNA (10 μg per lane) was denatured with 6.3% (vol/vol) formaldehyde/50% (vol/vol) formamide and then size fractionated on a 1.2% agarose gel containing 6.6% formaldehyde. Blotting to nitrocellulose sheets was by standard procedures (ref. 14, p. 203). Prehybridization, hybridization, and posthybridization washes were as described (15).

Plasmids and cDNA Probes. Plasmid DNA was isolated by standard procedures (ref. 14, p. 86) and purified by Sepharose chromatography. The following plasmids and probes were used in this study: (*i*) p53H is a plasmid containing a partial-length wt human cDNA (15). The 1.8-kilobase (kb) *Xba* I cDNA fragment from this plasmid was used as a probe. (*ii*) The c-*FOS* plasmid containing a full-length human c-*FOS* cDNA (16) has been previously described (17). (*iii*) Plasmids containing full-length human c-*JUN* (18) and *JUN*-B (19) cDNAs were obtained from Bruno Calabretta (Thomas Jef-

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Abbreviations: wt, wild-type; PCNA, proliferating cell nuclear antigen; DEX, dexamethasone.

‡To whom reprint requests should be addressed at: Jefferson Cancer Institute, Thomas Jefferson University, 609 Bluemle Life Sciences Building, 233 South 10th Street, Philadelphia, PA 19107-5541.

ferson University). (*iv*) The c-*MYC* probe was a human cDNA obtained from Carlo Croce (20) (Thomas Jefferson University). (*v*) The pJODC plasmid contains a full-length human cDNA for ornithine decarboxylase obtained from a Jurkat T-cell cDNA library (unpublished data). The 1.8-kb *Hin*dIII cDNA fragment from this plasmid was used as a probe. (*vi*) The DNA polymerase α probe was isolated from a plasmid containing a partial-length cDNA for human DNA polymerase α as described (21). (*vii*) The B-*MYB* probe was isolated from a plasmid containing human B-*MYB* cDNA as described in ref. 22. All probes used for hybridization were excised from the plasmid vector by using the appropriate restriction enzymes, gel purified from plasmid vector sequences, and labeled to high specific activity as described (23).

Quantitation of Total Cellular RNA. The amount of total cellular RNA was quantitated in fixed cells stained with acridine orange (24, 25) by using a computer-assisted Zeiss microscope equipped with a photometer (Zeiss) and using UV excitation with a barrier filter ($F > 600$ nm). Red fluorescence was measured in arbitrary units of intensity and the values were normalized to control cells pretreated with RNase (26, 27).

RESULTS

Cellular RNA Content. Both cellular RNA and protein content increase dramatically when G_0-arrested cells are stimulated to proliferate (8, 9). To determine whether cellular RNA content also increases when cell cycle progression into S phase is blocked by the induction of wt p53 protein, total RNA per cell was measured in acridine-orange-stained cells by microfluorimetry (26, 27). For this study, three cell lines were employed: the parental T98G cell line, originally derived from a human glioblastoma, the GM47.23 subline, which contains an inducible wt p53, and the Mdel 4A subline, which contains an inducible genetically engineered deletion mutant of p53 (4). Cells were first arrested in G_0 and then stimulated with serum in the presence of [^{3}H]thymidine for 24 hr. The cells were then fixed and stained with acridine orange, and the relative RNA content per cell was measured. The cells were then processed for autoradiography and the percentage of labeled cells was determined. The results of a typical experiment are shown in Table 1. When G_0-arrested cells were stimulated to proliferate in the absence of DEX the mean amount of cellular RNA increased in all three cell lines examined from approximately 20 arbitrary units in G_0-arrested cells to 50 arbitrary units (i.e., more than double) in stimulated cells. The cells also entered S phase, as indicated by the thymidine labeling index, which increased from approximately 10% in G_0-arrested cells to approximately 80% in stimulated cells. The mean amount of cellular RNA also increased in all three cell lines when they were stimulated in the presence of DEX relative to that of G_0-arrested control cells. The thymidine labeling index indicated that parental T98G and Mdel 4A cells also entered S phase; however, the majority of GM47.23 cells did not enter S phase.

Table 1. Cellular RNA content and thymidine labeling index

Cell line	Condition	Fluorescence intensity,* arbitrary units: With DEX	Fluorescence intensity,* arbitrary units: Without DEX	Labeling index,† %: With DEX	Labeling index,† %: Without DEX
T98G	Quiescent	20 ± 5	ND	9 ± 6	8 ± 5
	Stimulated	49 ± 8	51 ± 2	80 ± 3	82 ± 6
GM47.23	Quiescent	15 ± 4	ND	10 ± 1	13 ± 7
	Stimulated	45 ± 6	57 ± 3	82 ± 9	16 ± 5
Mdel 4A	Quiescent	22 ± 6	ND	15 ± 7	18 ± 3
	Stimulated	47 ± 9	45 ± 7	81 ± 4	92 ± 2

*Average fluorescence intensity was measured in acridine-orange-stained cells by computer-assisted microfluorimetry. The numerical data represent the mean fluorescence intensity ($F > 600$ nm) in arbitrary units per cell ± SD. $n = 100$. ND, not done.

†The labeling index was determined by continuous labeling with [^{3}H]thymidine at 0.2 μCi/ml (1 Ci = 37 GBq) followed by autoradiography and light microscopic counting of cells. The numerical data represent the mean percentage of cells labeled in three experiments ± SD.

Restriction Point Analysis. For these experiments cells were maintained at high cell density in medium containing low serum to produce G_0 arrest or were released from G_0 arrest by replating at low cell density in growth medium containing DEX followed by incubation for 18–20 hr to produce the wt-p53-mediated block. The cells were then released from G_0 arrest by replating at low cell density in medium without DEX or were released from the wt-p53-induced block by replacing the DEX-containing medium with fresh medium without DEX (5, 12). Immediately after release [^{3}H]thymidine was added and at different times after release, beginning at 2 hr, cycloheximide was added. The percentage of cells capable of entering S phase was determined after continuous labeling with [^{3}H]thymidine for 24 hr followed by autoradiography. Fig. 1 shows the results of these experiments. Addition of cycloheximide up to 10 hr after release from G_0 arrest or after release from the block produced by induction of wt p53 protein inhibited the subsequent entry of cells into S phase. In contrast, addition of cycloheximide after 10 hr had very little effect on the ability of cells to enter S phase, regardless of whether they were released from G_0 arrest or from the block produced by induction of wt p53 protein. The results indicate that *de novo* protein synthesis is required for cells to enter S phase after release from the cell cycle block mediated by induction of wt p53 protein, sug-

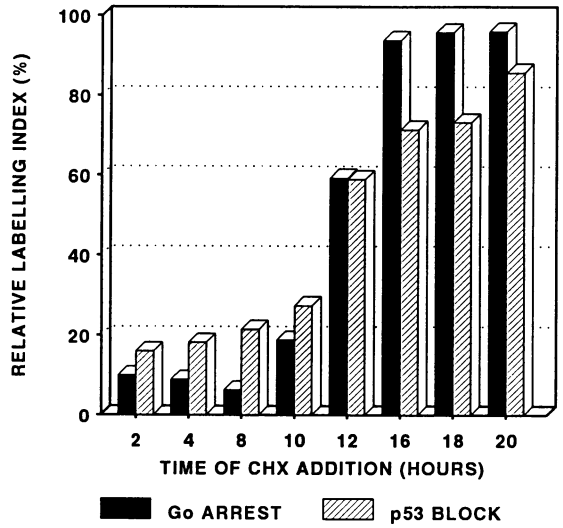

FIG. 1. R-point analysis of GM47.23 cells. Cells were growth-arrested in G_0 or at the wt-p53-mediated block and then stimulated from G_0 arrest or released from the wt-p53-mediated block as described in the text. [^{3}H]Thymidine was added at the time of stimulation from G_0 arrest or release from the wt-p53-mediated block. Under each condition the cells were labeled continuously for 24 hr and then processed for standard autoradiography. Cycloheximide (CHX) was added at 1 μg/ml at the times indicated on the abscissa. The percentage of labeled cells in CHX-treated cultures and untreated control cultures was determined by light microscope counting. The relative labeling index (%) is the ratio of labeled cells treated with CHX to untreated control cells released from G_0 arrest or the wt-p53-mediated block.

gesting that the block occurs prior to or near the R-point in late G_1 phase.

Expression of Growth Response Genes. Growth response genes are molecular markers of cell proliferation and are useful in dividing G_1 phase into substages (28, 29). We examined the expression of seven growth response genes to determine if their expression was altered in response to induction of wt p53 protein. These genes included four immediate-early genes (c-*FOS*, c-*JUN*, *JUN*-B and c-*MYC*), the delayed-early response gene (ornthine decarboxylase), and two late G_1/S-phase genes (B-*MYB* and DNA polymerase α). GM47.23 cells were first arrested in G_0 as previously described (5). wt p53 protein was induced in G_0-arrested cells by exposure to 1 μM DEX for 6 h prior to serum stimulation. Under these conditions wt p53 protein was detectable by immunoprecipitation with wt-specific monoclonal antibody PAb 1620 after metabolic labeling with [^{35}S]methionine (30). At 0.5 and 2 hr after serum stimulation RNA was isolated and Northern blots were prepared and sequentially probed for c-*JUN*, *JUN*-B, c-*FOS*, c-*MYC*, and p53 transcripts with the appropriate probes. The results are shown in Fig. 2. Induction of wt p53 protein had no effect on the expression of the mRNA transcripts for c-*JUN*, *JUN*-B, or c-*MYC*. An increase in c-*FOS* mRNA transcript was observed in cells induced to express wt p53 relative to uninduced control cells. In these experiments induction of wt p53 protein in G_0 cells was sufficient to inhibit serum-stimulated entry into S phase by 85% relative to uninduced control cells, as determined by [^{3}H]thymidine labeling followed by autoradiography (data not shown). To examine the effect of wt p53 on the expression of delayed-early and late-G_1/S-phase genes, cells were first arrested in G_0 and then stimulated in medium with and without DEX. At different times after stimulation RNA was isolated and Northern blots were prepared and sequentially probed for the mRNA transcripts corresponding to ornithine decarboxylase, B-*MYB* and DNA polymerase α. The results of these experiments are shown in Fig. 3. The steady-state levels of the mRNA transcripts for B-*MYB* and DNA polymerase α are significantly decreased in cells induced to express wt p53 protein relative to those observed in uninduced control cells. In contrast, no decrease in mRNA transcript levels is observed for the delayed-early response gene for ornithine decarboxylase on the same blot in cells induced to express wt p53 protein.

To rule out the possibility that the effect on B-*MYB* and DNA polymerase α mRNA expression was not simply due to the addition of the hormone-inducer DEX, parental T98G cells were arrested in G_0 and then stimulated to proliferate in medium containing DEX. At different times after stimulation RNA was isolated and Northern blots were prepared and sequentially probed for the steady-state levels of mRNAs for the two genes. The results for DNA polymerase α are shown in Fig. 4. The steady-state level of DNA polymerase α mRNA increases significantly in parental T98G cells treated with DEX at 18 and 24 hr. Shown also is one of many repeat experiments with the GM47.23 cell line in which DNA polymerase α mRNA levels do not increase in cells induced to express wt p53 protein. When the blot was stripped and reprobed for B-*MYB* an increase in B-*MYB* mRNA levels was also observed in parental T98G cells at 18 and 24 hr; however, the levels of B-*MYB* mRNA did not increase in GM47.23 cells (not shown). Table 2 summarizes the results of this study and previous studies (5, 12) on the effect of wt p53 protein expression on the steady-state mRNA levels for 12 different growth response genes. Taken together, the results clearly demonstrate that only a subset of endogenous growth response genes (late-G_1/S-phase genes) are affected when cell cycle progression is inhibited by induction of wt p53 protein.

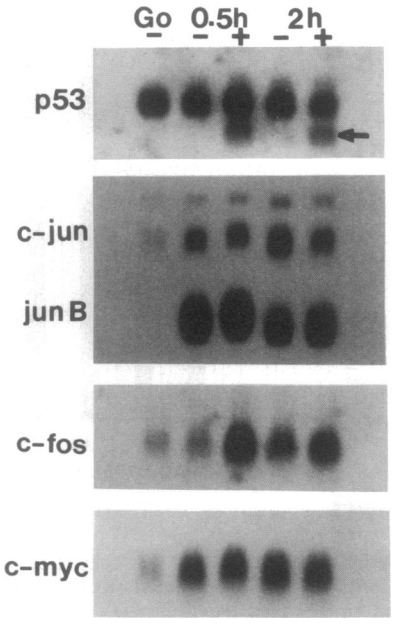

FIG. 2. Composite Northern blot of immediate-early gene expression in GM47.23 cells. RNA was isolated at the times indicated in hours (h) after serum stimulation. G_0-arrested cells were uninduced (−) or induced with DEX (+) to express wt p53 protein 6 hr prior to serum stimulation. The G_0 lane is RNA isolated from unstimulated and uninduced cells. The blot was sequentially hybridized with radiolabeled probes for c-*JUN*, *JUN*-B, c-*FOS*, c-*MYC*, and human p53. The arrow indicates the position of the inducible wt p53 2.0-kb transcript.

DISCUSSION

In the present study we have exploited the cell line GM47.23, which contains an inducible wt p53 protein (5, 12), to define the position in the cell cycle where growth suppression mediated by wt p53 protein occurs. Our results indicate that induction of wt p53 protein arrests cell cycle progression prior to or near the R-point in late G_1 phase. This is supported by experimental evidence which shows that cells arrested by induction of wt p53 protein have progressed out of G_0 into G_1

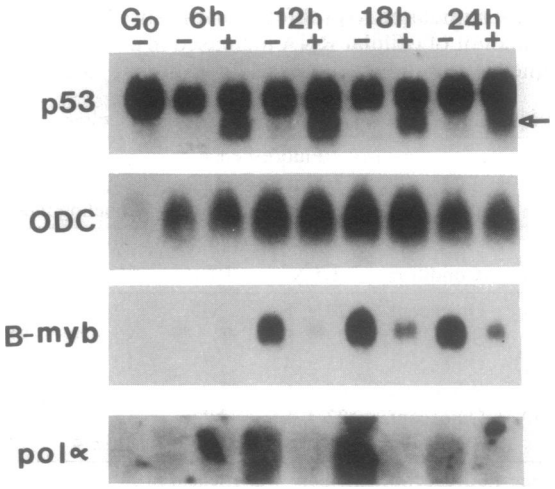

FIG. 3. Composite Northern blot of delayed-early and late-G_1/S-phase gene expression in GM47.23 cells. RNA was isolated at the times indicated in hours (h) from serum-stimulated uninduced (−) or induced (+) cells. The G_0 lane is as described in the legend of Fig. 2. The blot was sequentially hybridized with radiolabeled probes for human p53, ornithine decarboxylase (ODC), B-MYB, and DNA polymerase α (polα).

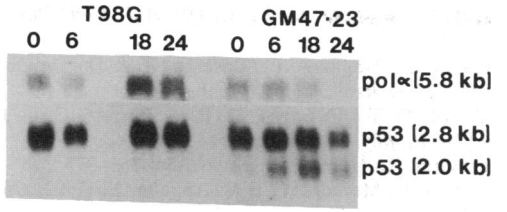

FIG. 4. Composite Northern blot of DNA polymerase α gene expression in T98G parental cells and GM47.23 cells. Cells were treated with DEX. The numbers at the top indicate the time in hr after stimulation from G_0 arrest. Time zero is RNA isolated from untreated G_0-arrested cells. The radiolabeled probes were for human p53 and DNA polymerase α (polα).

phase as indicated by an increase in total cellular RNA, most of which is ribosomal RNA (9), and an increase in the expression of the immediate-early growth response genes (c-*FOS*, c-*JUN*, *JUN*-B, and c-*MYC*) and the delayed-early response gene (ornithine decarboxylase). Evidence that cells have not passed the R-point and become committed to enter S phase is provided by the finding that *de novo* protein synthesis is required after release from the wt-p53-mediated block for the subsequent entry of cells into S phase and that an increase in the expression of the late-G_1/S-phase genes (B-*MYB* and DNA polymerase α) that normally occurs when G_0-arrested cells are stimulated to reenter the cell cycle does not occur when cell cycle progression is blocked by induction of wt p53 protein.

The finding that B-*MYB* expression does not increase when cell cycle progression is blocked by induction of wt p53 protein is especially important. B-*MYB* mRNA has been shown to be highly expressed in a variety of nonhematopoietic cell types (22). Our data indicate that, like c-*MYB*, B-*MYB* is expressed as a late-G_1/S-phase gene. The B-*MYB* gene exhibits a high degree of sequence homology with c-*MYB* at the amino acid level in domain I, which is 161 amino acids long and well conserved in the *MYB* gene family (22). The c-MYB protein has been shown to have sequence-specific DNA-binding activity and to function as a transcriptional activator (31, 32). Recent studies have revealed similar DNA-binding and transcriptional activator functions for the human B-MYB protein (33). Although the importance of B-*MYB* expression in cell cycle progression has not yet been established, expression of the c-*MYB* gene has been shown to be essential for cell proliferation (34) and required for progression into S phase (35, 36). Further, there is already some evidence to suggest that a functional link may exist between c-*MYB* and the PCNA and DNA polymerase α genes. In mitogen-stimulated normal peripheral blood lymphocytes antisense oligodeoxynucleotides to c-*MYB* can inhibit the G_0-to-S-phase transition, and this inhibition is accompanied by down-regulation of the mRNAs for both PCNA and DNA polymerase α (37). Further, constitutive expression of c-*MYB* in the fibroblast cell line TK$^-$ts13, which is a G_1-phase-specific temperature-sensitive cell cycle mutant (38), can increase the steady-state levels of mRNA for both PCNA (39) and DNA polymerase α (37) even at the restrictive temperature where late-G_1/S-phase genes such as thymidine kinase, histone H3, PCNA, and DNA polymerase α are not normally expressed (40, 41). If B-*MYB* plays a role in cell cycle progression of nonhematopoietic cells similar to that which c-*MYB* plays in hematopoietic cells, the abrogation of its expression in GM47.23 cells induced to express wt p53 protein may be directly related to the observed effects on the expression of the PCNA and DNA polymerase α genes. It is possible that wt p53 may act upstream of B-*MYB* and that the decreased expression of the PCNA and DNA polymerase α genes may be due to the requirement for B-*MYB* to directly transactivate these genes. Alternatively, B-*MYB* may act indirectly by transcriptionally activating other genes whose products are required for efficient expression of the PCNA and DNA polymerase α genes. Further experiments will be required to investigate these interesting possibilities.

Finally, we have to signal a discrepancy between our results and those reported by others (42, 43) regarding the effect of wt p53 on c-*FOS* and c-*JUN* expression. Two other groups have reported that wt p53 represses the expression of c-*FOS* and c-*JUN* in transient cotransfection assays. In the present study, no significant decrease in the steady-state transcript levels of the endogenous c-*FOS* or c-*JUN* genes was observed. In fact, an increase in c-*FOS* mRNA is observed in cells induced to produce wt p53 protein. This does not appear to be an effect of the hormone-inducer DEX alone, since no increase in c-*FOS* transcript is observed in parental T98G cells treated with DEX (data not shown). The reason for this discrepancy is unclear at this moment, but it may be differences in the assays used or the cell lines employed. The results presented in this communication are consistent with the notion that wt p53 protein plays a role in modulating the expression of a subset of growth response genes that are critically important for G_1 progression into S phase (12, 42).

Table 2. Effect of p53 on growth response gene expression

Class	Gene	Relative mRNA level
Immediate-early	c-*FOS*	↑/=*
	c-*JUN*	=*
	JUN-B	=*
	c-*MYC*	=*
	Actin	=†
Delayed-early	Ornithine decarboxylase	=‡
	Endogenous p53	=‡
Late-G_1/S-phase	B-*MYB*	↓‡
	Thymidine kinase	=†
	PCNA	↓†
	DNA polymerase α	↓‡
	Histone H3	↓†

Steady-state mRNA levels in induced cells and uninduced control cells were quantitated by densitometry scanning of autoradiographs. =, No change; ↓, decrease; ↑, increase.
*Transcript levels measured at 0.5 and 2 hr after serum stimulation.
†See Mercer *et al.* (5, 12).
‡Transcript levels measured at 6, 12, 18, and 24 hr after serum stimulation.

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