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# A Growth Arrest-Specific (*gas*) Gene Codes for a Membrane Protein

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**A set of growth arrest-specific (*gas*) genes whose expression is negatively regulated by serum has recently been identified. We report on the detailed analysis of one of these genes (*gas3*). The kinetics of regulation by the presence and absence of serum were investigated, and it was found that this gene is regulated at the post-transcriptional level. The encoded protein deduced from the nucleotide sequence showed some similarity to a mitochondrial oxyreductase, and in vitro translation established that the protein product is a transmembrane glycoprotein.**

At least one control point in mammalian cell proliferation occurs in the $G_1$ phase, and growth arrest is accomplished at this point by depletion of growth factors or serum (3, 22, 28). Although many of the biochemical and the genetic changes upon entry into the $G_1$ phase after serum induction of growth-arrested cells have been delineated (2–4, 19, 20), the molecular machinery necessary for accumulating cells into the $G_0$ phase has not been elucidated. Since cell proliferation involves both positively and negatively acting signals (9), a shift of an integrated balance to the quiescent state will probably induce a series of metabolic changes necessary for survival under conditions that are inadequate for cell proliferation. In fact, transformed cells may not be able to reach growth arrest and as a consequence are susceptible to conditions favoring the stationary phase.

The potential importance of this line of research has recently been stressed by the finding of an interaction between oncogenes and anti-oncogenes (27). In contrast to the wealth of knowledge on the numerous genes that are induced after growth stimulation (19, 20), we have recently started to characterize the genetic and biological mechanisms involved in growth arrest (23, 24).

## MATERIALS AND METHODS

**Cell lines and cell culture conditions.** NIH 3T3 cells were kindly provided by R. Müller (Institut für Molekular Biologie und Tumorforschung, Marburg, Federal Republic of Germany). They were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), penicillin, and streptomycin (100U/100 μg/ml). For serum starvation, the medium was changed to 0.5% FCS when cells were subconfluent, and the cells were then left in the same medium for 48 h. Under these conditions, incubation with 50 μM bromo deoxyuridine (BUdR) for an additional 24 h resulted in labeling of less than 3% of the nuclei. For induction of DNA synthesis, fresh medium containing 20% FCS was added to the arrested cells. The cells were then harvested at various times for RNA isolation. After 24 h of BUdR incorporation, more than 90% of the nuclei scored positive. For density-dependent inhibition, cells were plated at $10^4/cm^2$ in 10% FCS. Twelve hours after plating (considered as the starting point for growing cells), the medium was changed every 2 days. After 4 days in culture, incubation with BUdR for 2 h resulted in less than 1% incorporation. When used, cycloheximide and actinomycin D concentrations were, respectively, 10 and 5 μg/ml.

**DNA synthesis assay.** Cells grown on cover slips (20 by 20 mm) in the same culture dishes from which RNA was prepared were incubated for 2 h in the presence of 50 μM BUdR. After this time they were fixed for 5 min in methanol at 4°C and then for 5 min in acetone at 4°C. DNA was then denatured by treatment with 1.5 N HCl for 10 min. The cover slips, after washing with phosphate-buffered saline (PBS), were incubated with mouse monoclonal antibody against BUdR (12) for 1 h at 37°C, washed three times in PBS, and then incubated for 45 min at 37°C with tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse immunoglobulin antibodies. Total nuclei were visualized with Hoechst stain 33342 (1 μg/ml). More than 500 nuclei were observed for each cover slip. The percentage of activation was calculated as the ratio between cells positive for TRITC and total cells (Hoechst 33342-stained nuclei).

**RNA preparation.** Total cellular RNA from cell cultures was prepared by washing the plates twice with PBS, followed by addition of lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% *N*-laurylsarcosine). RNA was then prepared as described previously (6). For poly(A) selection, mAP paper (Amersham Corp.) was used as instructed by the manufacturer.

**Northern (RNA) blot analysis.** Total cellular RNA (10 μg) was used for Northern analysis on 1% agarose gels containing 6.7% formaldehyde (21). Integrity and relative amounts of RNA were analyzed by ethidium bromide staining. Gels were transferred for 16 h to a Duralon-UV nylon membrane (Stratagene). RNA was cross-linked by exposure to UV light (Stratalinker; Stratagene). Hybridization was performed in 1 M NaCl–1% sodium dodecyl sulfate (SDS) at 65°C, using $5 \times 10^5$ cpm of probe per ml, prepared by random primer synthesis (Pharmacia).

**Full-length clone.** From the known sequence and orientation given by the presence of the poly(A) tail of the original clone, a suitable restriction site near the 5' end was used to screen a cDNA library generated from $G_0$ mRNA (15) and cloned by an orientation-specific strategy (11) in the lambda vector T7-T3/E-H (14).

**DNA sequencing and sequence analysis.** DNA fragments to be sequenced were subcloned in the Bluescript plasmid (Strategene). Double-stranded DNA and lambda DNA were

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isolated and sequenced (10), using the T7 sequencing kit (Pharmacia). Specific synthetic oligonucleotides were also used as primers for the sequencing reaction. The entire sequence was read on both strands. The sequence of each nucleotide was determined three times on the average. Nucleotide and amino acid sequences analyses were carried out by using the University of Wisconsin Genetics Computer Group sequence analysis software package (version 5.3).

**Run-on experiments.** Nuclei were isolated from NIH 3T3 cells as described previously (13). For the run-on transcription assay, 100 μl of the nuclear suspension was mixed with 100 μl of reaction buffer (10 mM Tris hydrochloride [pH 8], 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each ATP, CTP, and GTP, 150 μCi of [α-$^{32}$P]UTP [400 Ci/mmol; Amersham]) and incubated for 30 min at 30°C. The $^{32}$P-labeled RNA was then isolated by passage through a Sephadex G-50 (Pharmacia) spun column equilibrated in TLES (10 mM Tris hydrochloride [pH 7.5], 0.1 M LiCl, 0.1 mM EDTA, 0.05% SDS). The DNA was spotted onto a nylon membrane (Stratagene); 10 μg of previously denatured DNA (0.25 M NaOH for 20 min at room temperature, neutralized by addition of an equal volume of 0.25× SSC [SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) was applied per slot. Hybridization was performed in 1 M NaCl–1% SDS–1 mg of heparin per ml–100 μg of salmon sperm DNA per ml at 65°C for 36 h with a probe concentration of $2 \times 10^6$ cpm/ml. The filters were then washed twice with 2× SSC at room temperature for 10 min, with 2× SSC–1% SDS at 65°C for 15 min, and finally with 0.2× SSC–0.1% SDS at room temperature for 10 min.

**In vitro transcription and translation.** The Bluescript vector KS+, containing the full-length clone, was linearized downstream from the T7 promoter with *Hin*dIII and transcribed according to the protocol of the supplier (Stratagene). Typically, 1 μg of plasmid DNA was incubated for 60 min at 37°C in 40 mM Tris hydrochloride (pH 8)–8 mM $MgCl_2$–50 mM NaCl–1 mM ATP, CTP, and UTP–0.2 mM GTP–1 mM cap analog–10 mM dithiothreitol–35 U of RNasin–20 U of T7 polymerase (Stratagene) in a total volume of 20 μl. The DNA template was removed by adding 15 U of RNase-free DNase (Pharmacia), followed by incubation at 37°C for 15 min in a 40-μl reaction volume. RNA was then purified as described for the nuclear run-on assay. After extraction by phenol-chloroform, the RNA was precipitated with 2 volumes of ethanol. Translation reactions were performed by using rabbit reticulocyte lysate (Amersham) as instructed by the supplier. Approximately 1 μg of RNA was translated in a 25-μl reaction volume containing 50 μCi of [$^{35}$S]methionine (Amersham) at 30°C for 60 min. Translocation across membranes was studied by including 6 μl of dog pancreatic microsomes (DPM; Amersham) in the translation mixture. After incubation for 1 h at 30°C, the translation products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (18). For fluorography, the gels were fixed in 45% methanol–7% acetic acid for 1 h and then treated with En$^3$Hance (Dupont, NEN Research Products). The gels were then rinsed with water, dried, and exposed for autoradiography at −80°C for 1 to 3 days.

**Protease protection, endo H digestion, and TX-114 extraction.** To 10 μl of the translation mixture with microsomes, 1 μl of a 3-mg/ml proteinase K solution was added, and the mixture was incubated on ice for 1 h. A control containing 0.3% Nonidet P-40 was also included. The reactions were stopped by trichloroacetic acid (TCA) precipitation (5% TCA; 20 min at 4°C). For endoglycosidase H (endo H) digestion, 25 μl of the translation mixture was diluted with 200 μl of PBS containing $Ca^{2+}$ and $Mg^{2+}$ and centrifuged at 20,000 × *g* for 2 h. The pellet was resuspended in 200 μl of endo H buffer (50 mM sodium acetate [pH 5.5], 10 mM EDTA, 0.2% Nonidet P-40, 0.1 M 2-mercaptoethanol, 0.02% SDS) and divided into two samples. One sample was treated with 1 mU of endo H (Boehringer Mannheim Biochemicals), and the other (control) was untreated; both were then incubated at 37°C overnight.

To 20 μl of the translation mixture with microsomes, 200 μl of an ice-cold solution (10 mM Tris [pH 7.4], 100 mM NaCl, 0.5% Triton X-114 [TX-114]) was added, and the mixture was then incubated at 30°C for 3 min. After centrifugation at 800 × *g* for 3 min, the detergent phase was found as an oil droplet at the bottom of the tube (5). The detergent phase was further purified by dissolving it in 200 μl of 100 mM Tris (pH 7.4)–100 mM NaCl; the sample was incubated for 3 min on ice and 3 min at 30°C and centrifuged for 3 min at 800 × *g*. The final detergent phase was dissolved in SDS sample buffer. The supernatant of the first-phase separation was further extracted by adding 0.5% (final concentration) TX-114, incubated for 3 min on ice and 3 min at 30°C, and then centrifuged for 3 min at 800 × *g*. The final aqueous supernatant phase was precipitated by addition of 5% TCA and centrifuged for 10 min at 11,000 × *g*; the pellet was dissolved in SDS sample buffer.

## RESULTS

**Disappearance of *gas3* mRNA after serum induction.** The isolation for the growth arrest-specific gene *gas3* was previously reported (24). It is one of six genes specifically expressed at growth arrest in NIH 3T3 cells.

The expression of *gas3* mRNA during the growth cycle was investigated by Northern blot analysis by using total RNA prepared from serum-starved NIH 3T3 cells and at various times after synchronous induction into cell division by addition of 20% FCS. The RNA identified by the *gas3* probe (around 1.8 kilobases) was abundantly expressed at growth arrest (48 h in 0.5% FCS) and reached the lowest level of expression 6 h after serum addition (Fig. 1). A low level of expression was maintained throughout the growth cycle.

To normalize each RNA sample loaded on the Northern blot, the same filter was hybridized with the cDNA probe GAPDH, known to remain constant throughout the cell cycle (Fig. 1A). The percentage of cells entering S phase for each time indicated is shown in Fig. 1B.

**Kinetics of appearance of *gas3* mRNA after serum starvation and density-dependent inhibition.** The level of expression of *gas3* RNA was analyzed on Northern blots by using total RNA samples extracted at various times after serum deprivation of exponentially growing NIH 3T3 cells. NIH 3T3 cells were shifted to 0.5% FCS 12 h after seeding in 10% FCS.

*gas3* RNA was detectable after only 12 h in medium containing low serum, reaching the maximum level after 48 h (Fig. 2A). Normalization of RNA amounts was performed with the GAPDH probe on the same Northern blot.

The percentage of cells in the S phase was also measured by analyzing BUdR incorporation in cells grown on cover slips for each time point. Less than 15% of the cells remained in the S phase 24 h after serum starvation (Fig. 2B).

To assess the expression of *gas3* RNA during density-dependent growth inhibition, NIH 3T3 cells were seeded in 10% FCS, and every 2 days the medium was replaced with fresh 10% FCS. *gas3* RNA increased significantly as soon as 2 days after seeding and continued to increase steadily for 6

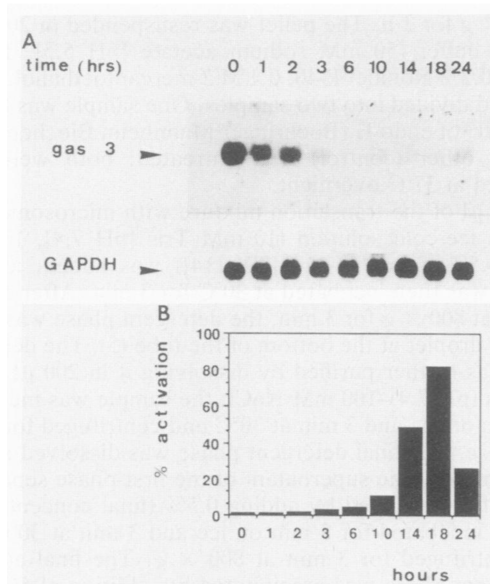


FIG. 1. Growth cycle regulation of *gas3* gene expression. (A) Northern blot. RNA was extracted from arrested NIH 3T3 cells (48 h in 0.5% FCS) and at the indicated times after addition of 20% FCS. Equal amounts (10 μg) of total RNA were analyzed by Northern blotting. The same blot was probed, as indicated, with *gas3* and GAPDH cDNA probes. (B) Analysis of DNA synthesis levels.

days from seeding, with little further increase at 8 days (Fig. 3A). The GAPDH control expression level did not change, and DNA synthesis analysis, performed on cells grown on cover slips in the same dishes used to extract RNA, showed a significant decrease as early as 2 days after seeding (Fig. 3B).

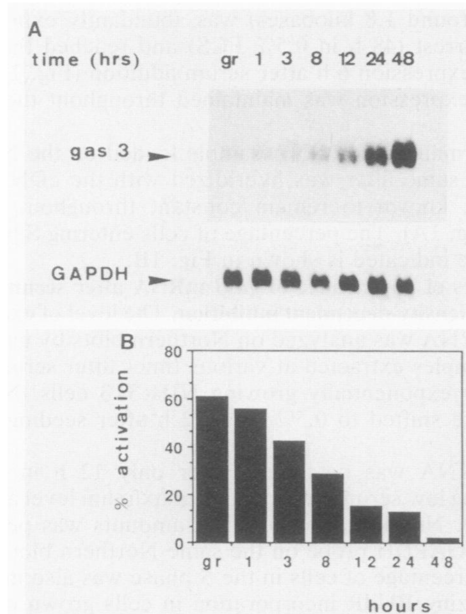


FIG. 2. Induction of *gas3* gene expression upon serum starvation. (A) Northern blot. RNA was isolated from actively growing NIH 3T3 (gr; 24 h after seeding in 10% FSC) and at the indicated times after serum starvation in 0.5% FCS. Equal amounts (10 μg) of total RNA were analyzed by Northern blotting. The same blot was probed, as indicated, with *gas3* and GAPDH cDNA probes. (B) Analysis of DNA synthesis levels.

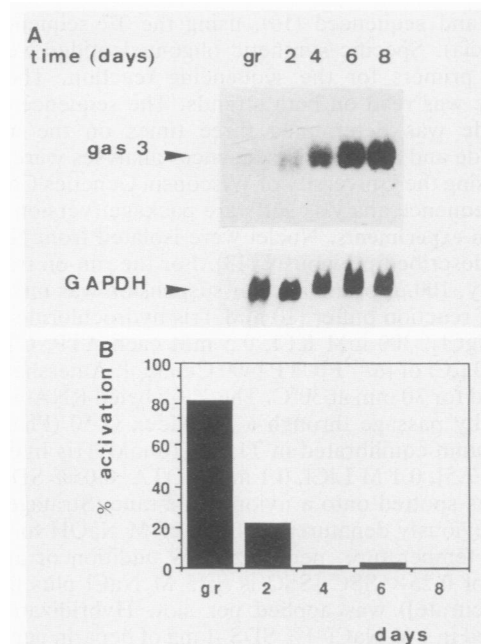


FIG. 3. Accumulation of *gas3* gene mRNA after density-dependent inhibition. (A) Northern blot. RNA was isolated from actively growing NIH 3T3 cells (gr; 12 h after seeding in 10% FCS) that were kept thereafter in the same dish with 10% FCS for different times. The culture medium (containing 10% FCS) was replaced every 2 days. Equal amounts (10 μg) of total RNA were analyzed by Northern blotting. The same blot was probed, as indicated, with *gas3* and GAPDH cDNA probes. (B) Analysis of DNA synthesis levels.

**Full-length cDNA sequence.** Figure 4 shows the DNA and deduced amino acid sequences of a full-length *gas3* cDNA clone having a total of 1,817 nucleotides. The open reading frame, presenting a typical consensus sequence for translation initiation (17), codes for only 144 amino acids. From hydropathy plots, it was deduced that the sequences from amino acids 2 to 31, 65 to 91, and 96 to 119 represent three potential membrane-spanning domains. One potential N-linked glycosylation site is present at residue 41.

**In vitro translation.** The protein product translated in vitro in a rabbit reticulocyte system programmed with in vitro-synthesized *gas3* RNA had an apparent molecular size of 18 kilodaltons on SDS-PAGE (Fig. 5A, lane a; lane e represents the control with no exogenous RNA added). When the translation was performed in the presence of DPM, the estimated size increased to 22 kilodaltons (lane b). The increased size was probably due to the addition of high-mannose oligosaccharide chains, since treatment with endo H shifted the apparent size back to that observed in the absence of microsomal membranes (lane c versus lane d), whereas the control incubated in the absence of endo H remained unaltered (lane d). These results also suggest that the signal sequence responsible for translocation into the endoplasmic reticulum is not cleaved, since the membrane-translocated, endo H-treated product (lane c) had the same apparent mobility as the product synthesized in the absence of microsomes (lane a). Treatment of the microsomally translocated *gas3* product with proteinase K did not change its apparent mobility (Fig. 5B, lane g) with respect to the untreated control (lane i). The only difference was the disappearance of the nontranslocated primary product due to its complete digestion (compare lane g with lane i). When

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         10                            30                            50
CCGGAGCCTCCCACTGCCCCCTTGCTTTGCGCGCGCGTGACCCGCAGCACAGCTGTCTTT
         70                            90                           110
GGGGACGCCAGCAACCCAGTGGACGCACCGGAGTTTGTGCCTGAGGCTAATCTGCTCTGA
        130                           150                           170
GATAGCTGTCCCTTTGAACTGAAACAGGCACCGCTCCTCTGATCCCGAGCCCAACTCCCA
        190                           210                           230
GCCACCATGCTCCTACTCTTGTTGGGGATCCTGTTCCTGCACATCGCGGTGCTAGTGTTG
        M  L  L  L  L  L  G  I  L  F  L  H  I  A  V  L  V  L
        250                           270                           290
CTCTTCGTCTCCACCATCGTCAGCCAATGGCTCGTGGGTAATGGACACACGACTGATCTC
 L  F  V  S  T  I  V  S  Q  W  L  V  G  N  G  H  T  T  D  L
        310                           330                           350
TGGCAGAACTGTACCACATCCGCCTTGGGAGCCGTCCAACACTGCTACTCCTCATCAGTG
 W  Q (N) C  T  T  S  A  L  G  A  V  Q  H  C  Y  S  S  S  V
        370                           390                           410
AGCGAATGGCTGCAGTCTGTCCAGGCCACCATGATCCTGTCTGTCATCTTCAGCGTCCTG
 S  E  W  L  Q  S  V  Q  A  T  M  I  L  S  V  I  F  S  V  L
        430                           450                           470
GCTCTGTTCCTGTTCTTCTGCCAGCTCTTCACTCTCACCAAAGGCGGCCGGTTTTACATC
 A  L  F  L  F  F  C  Q  L  F  T  L  T  K  G  G  R  F  Y  I
        490                           510                           530
ACTGGATTCTTCCAAATCCTTGCTGGTCTGTGCGTGATGAGTGCAGCGGCCATCTACACA
 T  G  F  F  Q  I  L  A  G  L  C  V  M  S  A  A  A  I  Y  T
        550                           570                           590
GTGAGGCACAGTGAGTGGCATGTCAACACTGACTACTCCTATGGCTTCGCTACATCCTGG
 V  R  H  S  E  W  H  V  N  T  D  Y  S  Y  G  F  A  T  S  W
        610                           630                           650
CCTGGGTGGCCTTTCCCCTAGCCCTCCTCAGTGGTATCATCTATGTGATCCTGCGGAAAC
 P  G  W  P  F  P  *
        670                           690                           710
GCGAATGAGGCGCCCGACGACGCACCGTCCGTCTAGGCTCTGAGCGCGCATAGGGTCCAC
        730                           750                           770
AGGGAGGGAGGAAGGAAACCAGAGAACAAAACCAACCAACCAAAAAAGAGCTAGCCCCAA
        790                           810                           830
ACCCAAACGCAAGCCAAACCAAACAGAACGCAGTTGAGTGGGGATTGCTGTTGATTGAAG
        850                           870                           890
ATGTATATAATATCTATGGTTTATAAAACCTATTTATAACACTTTTTACATATATGTACA
        910                           930                           950
TAGGATTGTTTTGCTTTTTATGTTGACCGTCAGCCTCGTGTTGAATCTTAAACAACTTTA
        970                           990                          1010
CATCCTAACACTATAACCAAGCTCAGTATCTTTGTTTTGTTTCGTTTTTTTTTTTAATCT
       1030                          1050                          1070
TTTTGTTTTGCTCAGACATAAAAACTCCACGTGGCCCCCTTTCATCTGAAAGCAGATACC
       1090                          1110                          1130
TCCCTCCCACTCAACCTCATAGGATAACCAAAGTGTGGGGACAAACCCCAGACAGTTGAA
       1150                          1170                          1190
GACCTTTACACTATGGGTGACCCAGTGCATTTAGCAGGAGTATCCACTGCCCGAATCCAT
       1210                          1230                          1250
GTGTGAAGCCCTAAGCACTCACAGACGAAAAGCCCTGACCGGAACCCTCTGCAAAAACAG
       1270                          1290                          1310
TAATAGCTGGTGGCTCCTGAACACTTGACCCTGTAGACGGAGTACTGGGGCCACACGTTT
       1330                          1350                          1370
AAATGAGAAGTCAGAGACAAGCAATCTGTGAAATGGTGCTATAGATTTACCATTCCTTGT
       1390                          1410                          1430
TATTACTAATCGTTTAAACCACTCACTGGAAACTCAATTAACAGTTTTATGCGATACAGC
       1450                          1470                          1490
AGAATGGAGACCCGATACAAACGGTTCATAACTGCTTTCATACCTAGCTAGGCTGTTGTT
       1510                          1530                          1550
ATTACTACAATAAATAAATCTCAAAGCCTTCGTCAGTCCCACAGTTTTCTCACGGTCGGA
       1570                          1590                          1610
GCATCAGGACGAGCATCTAGACCCTTGGGACTAGCGAGTTCCCTGGCTTTCTGGGTCTAG
       1630                          1650                          1670
AGTGTTCTGTGCCTCCAAGGACTGTCTGGCGATGACTTGTATTGGCCACCAACTGTAGAT
       1690                          1710                          1730
GTATATACGGTGTCCTTCTGATGCTAAGACTCCAGACCTTTCTTGTTTTTGCTTGCTTTC
       1750                          1770                          1790
TCTGATTTTATACCAACTGTGTGGACTAAGATGCATCAAAATAAACATCAGAGTAACTCA
       1810
AAAAAAAAAAAAAAAAA
```

FIG. 4. DNA and deduced protein sequence from a full-length cDNA clone of the *gas3* gene. The hydrophobic putative transmembrane domains are underlined; the 3′ untranslated sequence shows three putative instability motifs ATTTA (underlined) and two consensus signals AATAAA for poly(A) addition (underlined). A potential N-glycosylation at residue 41 is circled.

the proteinase K treatment was performed in the presence of detergent (lane h), both products were completely digested.

To assess whether the *gas3* protein is an integral membrane or a secretory protein, we performed TX-114 phase separation experiments (5). *gas3* product synthesized in vitro in the presence of DPM (lane i) was extracted with

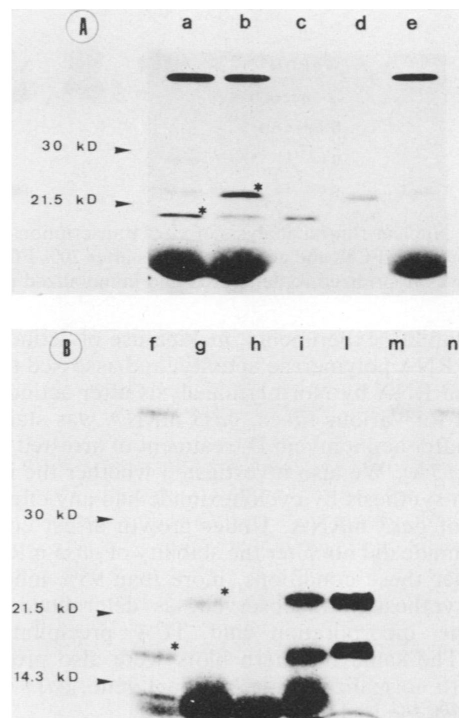


FIG. 5. SDS-PAGE analysis of in vitro transcription-translation products of *gas3* mRNA. (A) A 7 to 20% gradient SDS-PAGE analysis of synthetic *gas3* mRNA translated in a rabbit reticulocyte extract in the absence of DPM (lane a), in the presence of DPM (lane b), in the presence of DPM and after treatment with endo H (lane c), or in the presence of DPM and incubated as for lane c but without endo H (lane d). Lane e represents the mock translation product without *gas3* mRNA. In lanes c and d, the microsomal membranes were purified by centrifugation before endo H treatment. (B) A 17.5% SDS-PAGE analysis of *gas3* mRNA translated in a rabbit reticulocyte extract (lane f), in the presence of DPM and after treatment with protease K (lane g), as in lane g but in the presence of 0.3% Nonidet P-40 (lane h), and in the presence of DPM without further treatment (lane i). Also shown are the TX-114 detergent phase of the products in lane i (lane l), upper aqueous phase of the TX-114-extracted product (lane m), and mock translation in the presence of DPM without *gas3* mRNA (lane n).

TX-114. The *gas3* product was found only in the detergent phase (Fig. 5B, lane l); in the remaining aqueous phase, no *gas3* product was detectable (lane m). These results suggest that the *gas3* product is a bona fide integral membrane protein.

**Mechanism for the coordinate regulation of *gas3* expression.** A nuclear run-on experiment was performed to assess whether transcriptional regulation is responsible for the decreased expression of *gas3* RNA after serum addition in arrested NIH 3T3 cells. Nuclei collected at various times after FCS addition to $G_0$ NIH 3T3 cells synthesized *gas3* RNA at the same level after growth induction (Fig. 6). The GAPDH nuclear transcription remained constant throughout, albeit at a higher level of basal transcription than the *gas3* gene. γ-Actin RNA was positively regulated during growth induction, reaching maximum transcription 30 min after FCS addition and decreasing 3 h later. In contrast, another *gas* gene, *gas1*, shows a clear negative regulation at the transcriptional level. We conclude that the decreased expression of *gas3* mRNA is probably dependent on a posttranscriptional regulation. We therefore performed *gas3*

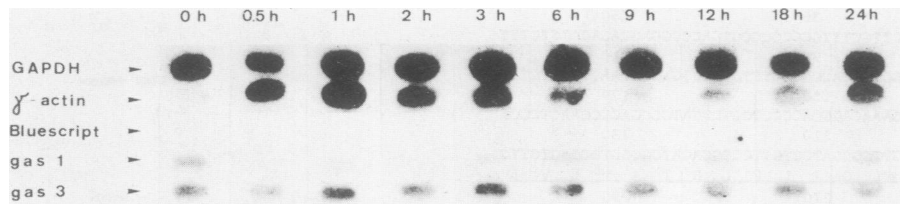


FIG. 6. Nuclear run-on analysis of *gas3* transcription during the growth cycle. Nuclei were isolated from NIH 3T3 cells at growth arrest (0 h; 48 h in 0.5% FCS) and at various times after 20% FCS addition. The nuclear preparations were allowed to incorporate [α-$^{32}$P]UTP, and the RNA was hybridized to denatured and immobilized plasmid DNA containing the indicated cDNA inserts.

mRNA stability experiments, making use of actinomycin D to inhibit RNA polymerase activity, and assessed the stability of *gas3* RNA by Northern analysis after actinomycin D treatment for various times. *gas3* mRNA was stable for at least 3 h after actinomycin D treatment of arrested NIH 3T3 cells (Fig. 7A). We also investigated whether the inhibition of protein synthesis by cycloheximide had any effect on the stability of *gas3* mRNA. Under growth arrest conditions, cycloheximide did not alter the stability of *gas3* mRNA (Fig. 7B). Under these conditions, more than 95% inhibition of protein synthesis was observed, as determined by [$^{35}$S] methionine incorporation and TCA precipitation (not shown). The same Northern blots were also probed with GAPDH to normalize for an invariant gene. *gas3* mRNA is therefore stable under growth arrest.

If actinomycin D was added together with serum, the expression of *gas3* remained constant, in contrast to the down regulation observed in the absence of the drug (Fig. 8A versus C). The GAPDH control showed, as expected, no difference with or without actinomycin D, whereas the increased expression of γ-actin mRNA after FCS activation was completely abolished by actinomycin D (Fig. 8A versus C).

These data suggest that the decreased stability of *gas3* mRNA after induction of growth by FCS may be influenced either directly or indirectly by de novo transcribed RNA(s) responsible for the *gas3* mRNA degradation. We therefore investigated whether such an RNA needs to be translated into an active protein(s) product. If cycloheximide was added together with 20% FCS, the usual down regulation of *gas3* mRNA was observed (Fig. 8B). Both GAPDH and γ-actin behaved normally, as in the control without cycloheximide (Fig. 8B versus C). These results suggest that if an RNA product is required for down regulation, it is not necessarily translated into a protein product.

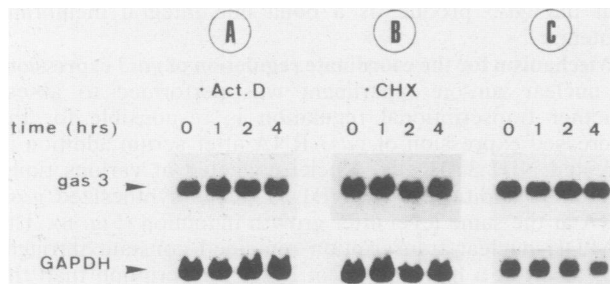


FIG. 7. Stability of *gas3* mRNA under growth arrest conditions. RNA was extracted from growth-arrested NIH 3T3 cells (0; 48 h in 0.5% FCS) after various times of treatment with actinomycin D (5 μg/ml) (A), with cycloheximide (10 μg/ml) (B), or without both drugs (C). Equal amounts (10 μg) of total RNA were analyzed on Northern blots. The same blot was probed, as indicated, with *gas3* and GAPDH cDNA probes.

## DISCUSSION

It is still an open question whether growth arrest or senescence of mammalian cells is mediated by specific genes or reflects a passive resting stage of the cell, requiring only induction signals for growth. Studies with cell hybrids demonstrate, however, that tumor cells become normalized when fused to resting or senescent cells (26), and the recent studies on the tumor suppressor genes (16) seem to indicate that specific genes are involved in counteracting the growth-inducing elements (27). The identification of *gas* cDNAs (24) has led us to characterize the structure and the regulation of their expression before assessing their relevance in growth arrest. The analysis of in vitro translation products of *gas3* mRNA suggests that the *gas3* product is a true integral membrane glycoprotein: it strongly associates with the detergent TX-114, and proteinase K protection experiments show that it is fully protected by the microsomes.

The deduced protein sequence suggests the presence of three putative transmembrane domains and confirms the existence of one N-glycosylation site. The data regarding the structure of the *gas3* protein could imply that the protein traverses the lipid bilayer three times, exposing the C terminus and the region containing the N-glycosylation site in the lumen of the endoplasmic reticulum. Possibly the N-terminal hydrophobic segment is fully embedded in the bilayer, and the linking peptide composed of four residues between the two remaining putative transmembrane segments is not exposed to interaction with the cytoplasmic side (Fig. 4).

In comparing the deduced protein sequence with sequences in the data banks, some similarities with the NADH ubiquinone oxyreductase chain 1 have been encountered.

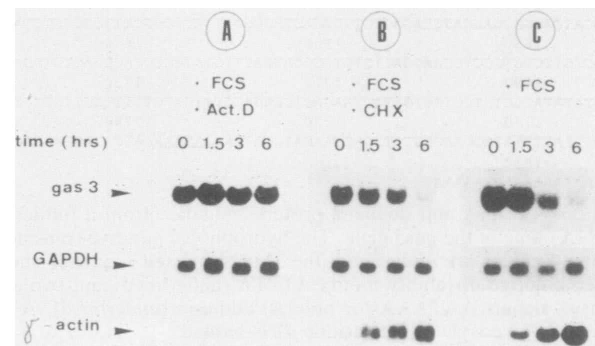


FIG. 8. Stability of *gas3* mRNA after growth induction with 20% FCS. RNA was extracted from arrested NIH 3T3 cells (48 h in 0.5% FCS) and at the indicated times after addition of 20% FCS (C) in the presence of actinomycin D (5 μg/ml) (A) or cycloheximide (10 μg/ml) (B). Equal amounts (10 μg) of total RNA were analyzed by Northern blotting. The same blot was probed, as indicated, with *gas3*, GAPDH, and γ-actin cDNA probes.

```
gas 3      2  L L L L G I L F L H I A V L V L L F V S T I V S Q W L V G  30
NADH-OX    1  M F M I N I L M L I I P I L L A V A F L T L V E R K V L G  29
              . . . + . * * . * . * . + * + + + . . . * + * . . . + + *

gas 3     58  V S E W L Q S V Q A T M I L S V I F S V L A L  80
NADH-OX   57  I K E P L R P A T S S A S M F I L A P I M A L  79
              + . * . * . . + . . + . . . . + + . . + . * *

gas 3    103  Q I L A G L C V M S A A A I Y T V R H S E W H V N T D Y S  131
NADH-OX  100  L G V L F M L A M S S L A V Y S I L W S G W A S N S K Y A  128
              . . + + . . . + * * . + * + * + + . + * . * . . * + . * .
```

FIG. 9. Amino acid sequence comparison between *gas3* protein and bovine NADH-ubiquinone oxydoreductase chain I (NADH-OX). Comparison was performed by using an alignment program (8) against release 17 of the NBRF protein sequence data base.

These similarities are, however, limited to the hydrophobic regions of the protein product (Fig. 4 and 9), and the sequences deviate considerably outside these regions. A functional relationship between the oxyreductase and the *gas3* protein is therefore unlikely and probably only indicates that the hydrophobic membrane-spanning regions happen to be located in similar positions in the two proteins.

The expression of *gas3* mRNA has been detected in several mouse fibroblast cell lines and in primary mouse embryo fibroblasts. In all cases, it shows the same regulation as reported for NIH 3T3 cells. It is also expressed in several mouse tissues, with highest abundance in the lung and lower intestine (not shown).

We cannot predict the function of the *gas3* protein in the context of growth arrest from the results presented here. However, the conclusions that can be drawn from study of the control of *gas3* mRNA expression suggest another potentially different physiological marker of growth arrest.

The nuclear run-on experiment clearly indicates that *gas3* is not regulated at the transcriptional level (Fig. 6); the serum-stimulated down regulation therefore probably occurs at the posttranscriptional level. In contrast to the long half-life of *gas3* mRNA under growth arrest conditions in the presence or absence of actinomycin D (Fig. 7), the serum-induced disappearance of *gas3* mRNA is prevented by actinomycin D (Fig. 8). Protein synthesis inhibition, as accomplished by cycloheximide, does not alter the stability of *gas3* mRNA under growth arrest conditions nor does it inhibit its down regulation induced by serum addition (Fig. 7 and 8).

These results may imply that the down regulation of *gas3* mRNA is mediated by an RNA that does not need to be translated. Alternative explanations are, of course, possible, since it has been shown (7) that actinomycin D could have effects other than inhibiting RNA synthesis. It is relevant that the 3′ untranslated region of *gas3* (like that of *gas2*) contains AUUUA sequence elements (underlined in Fig. 4). These elements are known (25) to control at least in part the stability of some short-lived mRNAs. If this AU motif is not present in the 3′ untranslated region of the genes that are transiently induced during serum stimulation, the stability of the corresponding mRNAs is enhanced (1). An RNA might in fact be required as part of the nuclease system involved in degradation of mRNAs containing an AU motif.

The machinery that is responsible for *gas3* mRNA destabilization may thus require the synthesis of an RNA that is induced by serum. Study of this mechanism will be informative in understanding posttranscriptional regulation in general.

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## ADDENDUM

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number M32240.

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