Regulation and Expression of a Growth Arrest-Specific Gene (gas5) during Growth, Differentiation, and Development

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The growth arrest-specific *gas5* gene was isolated from mouse genomic DNA and structurally characterized. The transcriptional unit is divided into 12 exons that span around 7 kb. An alternative splicing mechanism gives rise to two mature mRNAs which contain either 11 or 12 exons, and both are found in the cytoplasm of growth-arrested cells. In vivo, the *gas5* gene is ubiquitously expressed in mouse tissues during development and adult life. In Friend leukemia and NIH 3T3 cells, the levels of *gas5* gene mRNA were high in saturation density-arrested cells and almost undetectable in actively growing cells. Run-on experiments indicated that the *gas5* gene is transcribed at the same level in both growing and arrested cells. On the other hand, in dimethyl sulfoxide-induced differentiating cells a sharp decrease in the rate of transcription was observed shortly before the cells reached the postmitotic stage. These results indicate that in density-arrested cells accumulation of *gas5* mRNA is controlled at the posttranscriptional level while in differentiating cells expression is regulated transcriptionally.

The identification of genes preferentially expressed in quiescent or senescent cells has recently attracted interest (18, 31, 32, 40). Several groups have identified genes with high expression levels in nondividing cells, and reports on the mechanisms that regulate their expression and the proteins they encode are forthcoming (5, 6, 17, 20, 22, 29, 39). We have studied a group of genes termed growth arrest-specific genes (*gas1* to *gas6*) isolated from growth-arrested mouse fibroblasts (25, 35). The mRNAs of these genes accumulate when cells exit from the cell cycle (10, 25, 35). Expression of these genes is obviously controlled at the posttranscriptional level, except for *gas1*, which appears to be transcriptionally regulated on the basis of run-on and transfection experiments (10). Axon-regulated expression of *gas3* mRNA has been detected in Schwann cells (43, 50).

In this report, we present the molecular characterization of the *gas5* gene and two differentially spliced *gas5* mRNAs and the expression of the gene in embryo and adult mouse tissues. Regulation of expression of the *gas5* gene was also studied during growth and differentiation of Friend leukemia (FL) cells. FL cells are proerythroblasts which can undergo erythroid differentiation after treatment with chemical inducers (4, 8) and represent a useful system for study of regulation of gene expression in growing and differentiating cells (8, 15, 24). The expression of many genes, including genes associated with induction of cell proliferation, is known to be modulated following induction of differentiation in FL cells (2, 3, 7, 11, 13, 16, 49). We found that *gas5* mRNAs are not expressed in growing FL cells but accumulate in saturation density-arrested cells obviously under posttranscriptional regulation. Following induction of differentiation, however, the cells fail to express the *gas5* gene, probably owing to transcriptional regulation. In vivo analysis indicated that the *gas5* gene is ubiquitously expressed in mouse tissues during embryo development and in adult life.

MATERIALS AND METHODS

Library screening. Three cDNA libraries, two from NIH 3T3 cells and one from a 10-day old mouse embryo, kindly provided by R. Bravo and D. Duboule respectively, were used and screened with a probe for *gas5* previously described (35). A mouse genomic library (Stratagene) was used for isolation of the genomic clones. For screening, an aliquot of the library was plated at a density of 10^5 PFU/450-cm^2 dish. A total of 1.5×10^6 plaques were screened. Two lifts were made from each petri dish by using nylon membranes (Duralon; Stratagene). Plaques that specifically hybridized with the *gas5* probe were isolated and rescreened twice. Inserts from purified lambda bacteriophages were excised and subcloned into Bluescript pKS M13+ vectors (26).

Cell culture conditions. FL cells were routinely cultured in RPMI medium with 10% fetal calf serum (GIBCO) at 37°C. Cells were seeded at 2×10^5/ml and then either cultured in RPMI with 10% FCS or, to induce differentiation, in the same medium plus 1.5% dimethyl sulfoxide (DMSO), 5 mM *N,N'*-hexamethylene-bis-acetamide (HMBA), or 0.1 mM hemin. Cells were counted with a hemocytometer, and differentiation was determined by benzidine staining (4).

RNA preparation and Northern (RNA) blot analysis. Cells were washed twice with phosphate-buffered saline and lysed in guanidinium thiocyanate buffer, and RNA was isolated by CsCl gradient centrifugation as previously described (41). Total RNA (20 μg) was fractionated by denaturing agarose gel electrophoresis and transferred to nylon membranes by overnight blotting. Filters were hybridized overnight with 2×10^6 cpm of ^{32}P-labeled DNA probes per ml. DNA probes were labeled by random priming to an efficiency of around 10^9 cpm/μg. Filters were washed extensively with a final wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C and then autoradiographed. All of the probes used have been previously described (10).

Nuclear run-on transcription assay. Nuclei were isolated

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from either control or DMSO-treated cells after 24 or 96 h after induction of differentiation, and the newly synthesized RNA was labeled with [^{32}P]UTP and purified as previously described (27). Labeled RNA (2 × 10^6 cpm/ml) was hybridized against 3 μg of plasmid DNA immobilized on nitrocellulose membranes. Hybridizations were performed in a solution containing 50% formamide, 0.5% SDS, 5× SSC, and 5× Denhardt's solution at 42°C for 3 days. Filters were washed twice for 15 min in 0.1× SSC–0.1% SDS at room temperature and then further for 30 min at 65°C.

Primer extension. A ^{32}P-labeled oligonucleotide primer (10^5 cpm) was annealed to 50 μg of total RNA from FL cells in 40 mM PIPES [piperazine-*N*,*N*'-bis(ethanesulfonic acid)] (pH 6.4)–0.4 M NaCl–1 mM EDTA–80% formamide for 16 h at 37°C, precipitated with ethanol, and suspended in 20 μl of reverse transcription buffer. The annealed primer was extended at 42°C for 1 h with RNase H-mouse mammary tumor virus reverse transcriptase (BRL, Bethesda Md.). After RNase treatment, phenol-chloroform extraction, and ethanol precipitation, the reaction was analyzed on a 6% acrylamide–urea gel with a sequencing reaction as a marker.

In vitro transcription and translation. Full-length *gas5* cDNAs cloned into Bluescript pKS M13+ vectors were linearized and transcribed with T7 polymerase by using a Stratagene transcription kit. DNA was removed by digesting the reaction with RNase-free DNase, and RNA was purified on a Sephadex G50 column equilibrated with water. Approximately 1 μg of RNA was translated in a 25-μl reaction volume of wheat germ lysate (Promega) containing 30 μCi of [^{35}S]methionine (Amersham). The translation products were analyzed on an 18% SDS–polyacrylamide gel (26). The gel were fixed in 30% methanol–15% acetic acid, treated with En^{3}Hance (Dupont, NEN Research Products), dried, and exposed for autoradiography.

DNA sequencing. Double-stranded plasmid DNA was sequenced by following the dideoxynucleotide procedure of Sanger et al. (34) by using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio).

In situ hybridization. Embryos and fetuses were fixed after dissection in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C. Noon of the day the vaginal plug was found considered day 0.5 postcoitus (p.c.). Paraffin sections were cut and hybridized as previously described (14, 44). Slides were exposed for 3 weeks and developed with D19 developer (Kodak).

Nucleotide sequence accession numbers. The cDNA and genomic sequences reported here have been submitted to the EMBL-GenBank-DDBJ data bank and assigned accession numbers X59728 and X59729, respectively.

RESULTS

Expression of the *gas5* mRNA in growing and differentiated Friend erythroleukemia cells. By using a partial cDNA isolated in a differential library from growth-arrested NIH 3T3 cells (35), it was established that *gas5* mRNA is preferentially expressed in the quiescent phase of the cell cycle in NIH 3T3 cells and in other fibroblast cell lines (10, 35). Expression of the *gas5* gene was first studied during the growth and differentiation of FL cells. Total RNA was extracted at different days after cells were seeded with or without DMSO and analyzed by Northern blotting. Results presented in Fig. 1A suggest that expression of *gas5* mRNA is growth regulated in these cells, similar to what previously was observed in NIH 3T3 cells (10, 35). In fact, no *gas5* mRNA was detected in actively growing cells, while high
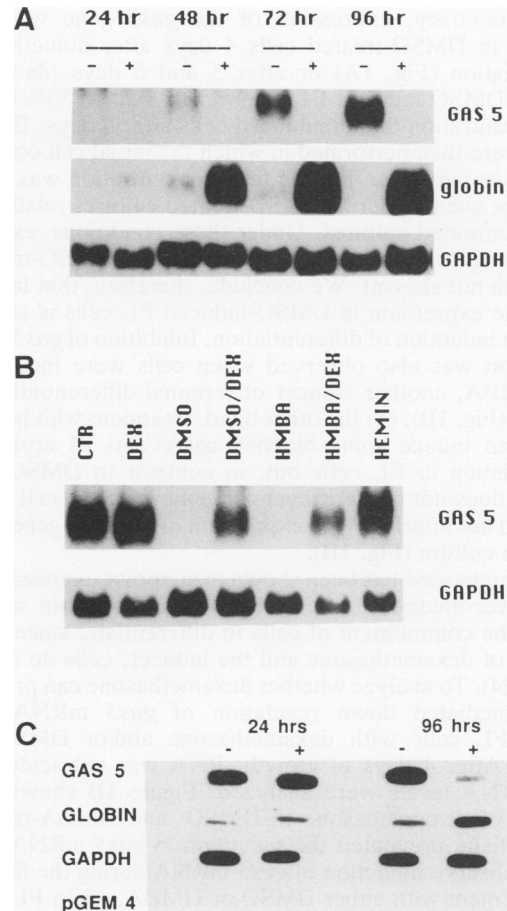

FIG. 1. (A) Northern blot analysis of expression of the *gas5* gene in FL cells at different days in untreated cells (−) or in cells induced to differentiate with 1.5% DMSO (+). Total cellular RNA (20 μg) was loaded in each lane, and the gel was blotted as described in Materials and Methods. The same blot was hybridized first with a *gas5* probe and subsequently with an α-globin probe, as a marker of differentiation, and then with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a control of RNA loading. (B) Expression of *gas5* mRNA after induction of differentiation with DMSO, HMBA, and hemin with or without dexamethasone (DEX). Cells were seeded at 2 × 10^5 per ml with or without DMSO (1.5%), HMBA (5 mM), dexamethasone (10^{-7} M), or hemin (0.1 mM). CTR refers to control cells. After 4 days, the cells were harvested, RNA was extracted, and the levels of *gas5* mRNA were analyzed by Northern blotting. (C) Run-on analysis of *gas5* transcription at 24 and 96 h in control cells (−) or cells induced to differentiate with 1.5% DMSO (+). Labeled RNA was extracted from purified nuclei and hybridized against 3 μg of plasmid DNA spotted with a dot blot apparatus on nitrocellulose paper. One representative of five experiments is shown.

levels were present in cells that had reached saturation density, i.e., after 3 to 4 days of culture. Cell growth was assessed by both cell counts and [^{3}H]thymidine incorporation: typically, after 4 days of culture the labeling index decreased to less than 10% and no significant further increase in cell number was observed (data not shown). Lack of expression of *gas5* mRNA after 4 days of culture was also observed when FL cells were suspended at the same concentration in fresh medium after 2 or 3 days in culture to verify that expression of *gas5* mRNA was not due to nutrient depletion or acidification of the medium (data not shown).

On the contrary, expression of the *gas5* gene was not detected in DMSO-treated cells 4 days after induction of differentiation (Fig. 1A) or after 5 and 6 days (data not shown). DMSO-induced FL cells reach a 20 to 30% lower cell concentration than uninduced cells after 4 days. Experiments were then performed in which the initial cell concentration was varied so that the final concentration was comparable or even higher in DMSO-treated cultures relative to that of untreated cultures. Under these conditions, expression of the *gas5* gene was still undetectable in DMSO-treated cells (data not shown). We conclude, therefore, that lack of *gas5* gene expression in DMSO-induced FL cells is associated with induction of differentiation. Inhibition of *gas5* gene expression was also observed when cells were incubated with HMBA, another inducer of terminal differentiation in FL cells (Fig. 1B). On the other hand, treatment with hemin, which can induce some biochemical events of erythroid differentiation in FL cells but, in contrast to DMSO and HMBA, does not cause irreversible exit from the cell cycle (4, 8), did not interfere with expression of the *gas5* gene after 4 days in culture (Fig. 1B).

Dexamethasone has been shown to suppress expression of the inducer-mediated differentiated phenotype but not to abolish the commitment of cells to differentiate, since after removal of dexamethasone and the inducer, cells do differentiate (24). To analyze whether dexamethasone can prevent inducer-mediated down regulation of *gas5* mRNA, we treated FL cells with dexamethasone and/or DMSO or HMBA. After 4 days of growth, RNA was extracted and *gas5* mRNA levels were analyzed. Figure 1B shows that addition of dexamethasone to DMSO- and HMBA-treated cells partially prevented the reduction in *gas5* mRNA. We did not observe induction of *gas5* mRNA during the first 24 h of treatment with either DMSO or HMBA, when FL cells may undergo a transient arrest in G_1 (data not shown). It appears, therefore, that in differentiated FL cells down regulation of *gas5* mRNA is initiated beyond commitment and is associated with induction of a completely differentiated phenotype and/or irreversible exit from the cell cycle.

Expression of *gas5* mRNA is regulated at the posttranscriptional level during growth and at the transcriptional level in differentiated cells. To investigate whether the changes in the level of *gas5* mRNA were transcriptionally regulated, run-on experiments were performed on actively growing, density-arrested, and differentiated cells. Figure 1C shows that the *gas5* gene is efficiently transcribed in both actively growing and density-arrested cells, suggesting that mRNA accumulation in density-arrested cells is regulated at the posttranscriptional level. On the other hand, in DMSO-induced cells a 10-fold lower rate of transcription was observed in comparison with actively growing or density-arrested FL cells, suggesting transcriptional control. Time course experiments showed that reduced transcription of the *gas5* gene first occurred on day 3 after induction of terminal cell differentiation (data not shown).

Analysis of *gas5* mRNA expression in vivo. To gain more information on *gas5* expression, RNA was prepared from mouse embryos at different days of gestation (Fig. 2A). The mRNA was detected from days 10 to 17, with a peak at days 15 to 16; no expression was detected in the placenta. *gas5* mRNA was abundant in all of the adult tissues tested, except liver and spleen (Fig. 2B), with the highest level in brain tissue.

The spatial and temporal distribution of *gas5* gene expression was also studied by in situ hybridization of mouse embryos at different stages of gestation (from days 8.0 to
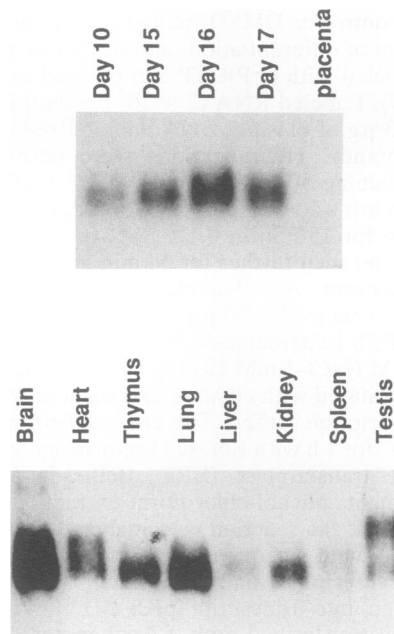

FIG. 2. Pattern of expression of the *gas5* gene in RNA from a whole mouse embryo at different days of gestation and in adult tissue (upper and lower panels, respectively).

13.5 p.c. Fig. 3). The *gas5* gene was found to be expressed in transverse embryo sections at days 8, 9.75, and 10.5 p.c., at which times all tissues appeared to be positively labeled (Fig. 3A, B, and D). Expression at day 10.5 p.c. was high in the neural tube and the limb buds (Fig. 3D). In Fig. 3E (sense probe) and F (antisense probe) parasagittal embryo sections at day 10.5 p.c. show the hybridization specificity. Again, the neural tube can be distinguished as well as the primitive gut and the lung buds (Fig. 3F). In Fig. 3H, a sagittal section on day 13.5 p.c. presents strong positive signals in the facial area, including the tongue, the internal ear, and the nasal pit. The splanchnic area, liver, gut, and lungs were all strongly positive, while the heart remained negative. Also note that the forebrain region and the hypothalamic region appeared to be significantly more labeled than other brain regions. In the dorsal part of the embryo, both the spinal ganglia and the prevertebral bodies were positive.

Identification of two differentially spliced *gas5* mRNAs. A total of 16 cDNAs were isolated by screening three different cDNA libraries to isolate full-length clones corresponding to the entire *gas5* mRNA. All of the clones had identical 3' ends, finishing with a poly(A) tail, and most of them presented a common 5' end. Sequence analysis indicated that five clones had identical sequences, although two of them appeared to be partial clones. The other 11 contained additional stretches of sequences inserted internally in the sequence common for the first 5 clones. This heterogeneity may have arisen from either cloning of intermediate splicing products or alternative splicing of mRNAs. It may also have originated from different but related genes.

Primer extention analysis was then performed by using an oligonucleotide complementary to bases 95 to 108 (see Fig. 6). As shown in Fig. 4, one major band of 108 bp was detected. This suggests that all of the *gas5* cDNAs originate at a unique transcription start site in FL cells (Fig. 4). The same results were also observed in NIH 3T3 cells (data not shown).
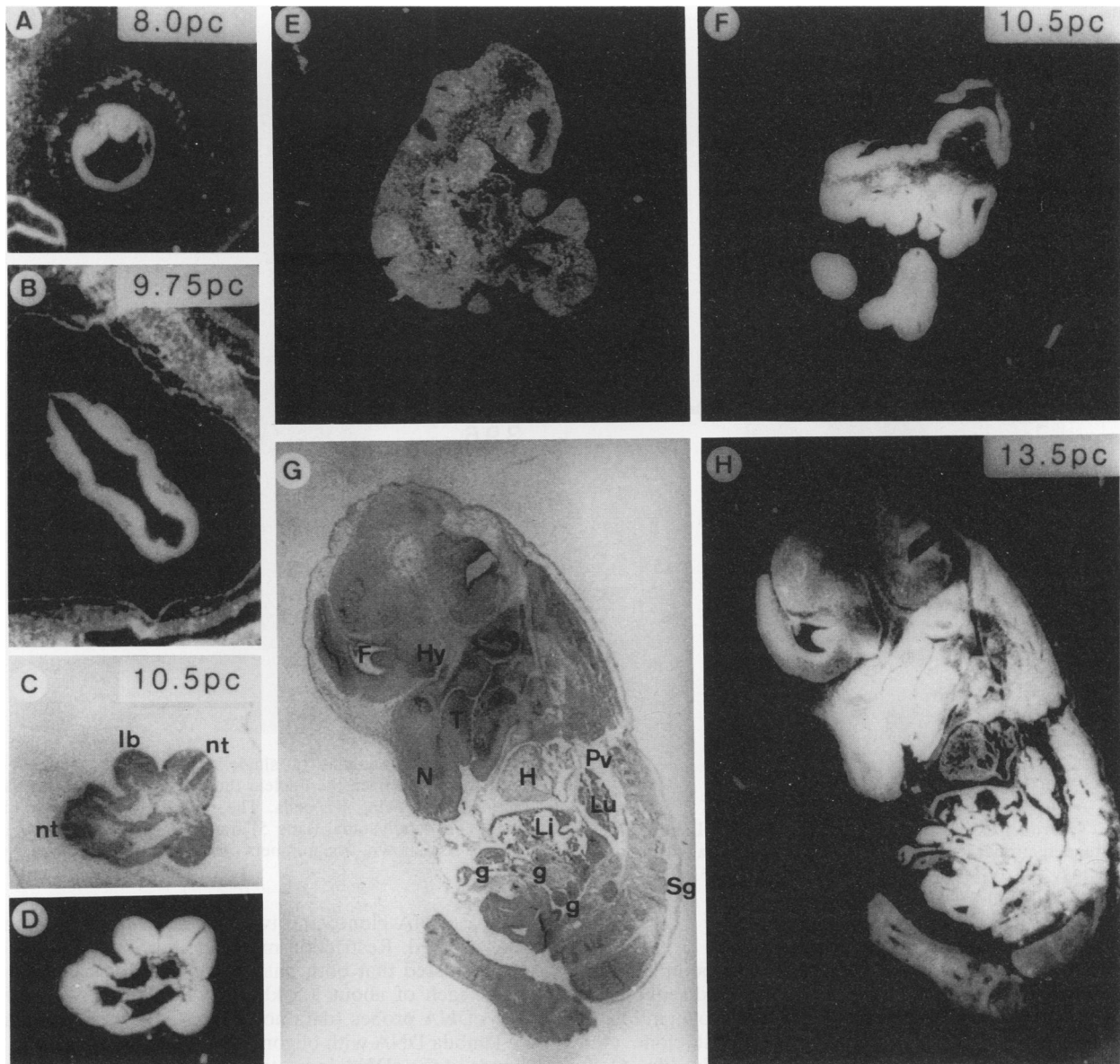
FIG. 3. In situ hybridization analysis of expression of the *gas5* gene in mouse embryos and fetuses at different stages of gestation (from days 8.0 to 13.5 p.c.). Panels A, B, and D show transverse section of embryos at days 8, 9.75, and 10.5 p.c. Panels E and F are parasagittal sections from an embryo at day 10.5 p.c. Panel H is a sagittal section of a day 13.5 p.c. embryo. Panel G is the corresponding bright-field image of panel H. Abbreviations: lb, limb buds; nt, neural tube; T, tongue; Ie, internal ear area; N, nasal pit; Hy, hypothalamic region; Li, liver; g, gut; Lu, lungs; H, heart; F, forebrain region; Sg, spinal ganglia; Pv, prevertebral bodies.

To distinguish whether any of the 16 cDNA clones originated from unprocessed nuclear transcripts or mature cytoplasmic mRNAs, two oligonucleotides were prepared from the 5' and 3' regions of the isolated *gas5* cDNAs (corresponding to bases 52 to 76 and 430 to 454, respectively, of the sequence shown in Fig. 6). These oligonucleotides were used as primers for polymerase chain reaction (PCR) analysis of RNA prepared from nuclei and cytoplasmic and polysomal fractions of saturation density-arrested FL cells. Figure 5 shows results of PCR product analysis with RNA preparations from both actively growing and growth-arrested FL cells. Large amounts of heterogeneously sized transcripts were observed in RNA extracted from nuclei of actively growing cells (Fig. 5, lane 1), while low or undetect-

able levels of the two mature bands of about 400 bp were found in cytoplasmic RNA from these cells (lane 2). The nuclear accumulation of larger and smaller RNAs was much less impressive in growth-arrested cells (lane 3). The two *gas5* bands were, on the other hand, more abundant in cytoplasmic RNA in density-arrested cells, with a small fraction in the polysomal region (lanes 4 and 5). The latter was eliminated after EDTA treatment of the polysomes. A similar pattern (data not shown) was observed when PCR was performed with oligonucleotides corresponding to the first 24 nucleotides (Fig. 6, bp 1 to 24) derived from sequencing of a genomic clone and an oligonucleotide corresponding to the most 3' sequence (Fig. 6, bp 475 to 507).

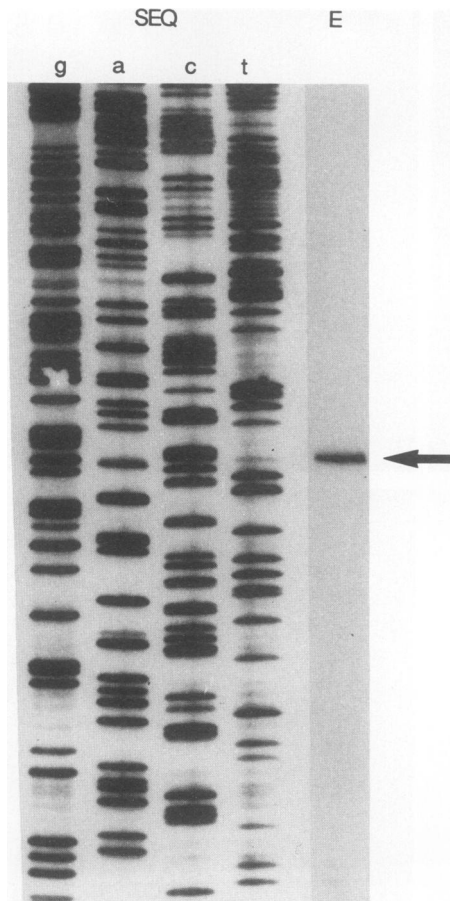The two bands expressed in the cytoplasm of growth-

FIG. 4. Primer extension analysis of *gas5* RNA. Lane E shows the extended primer (arrow). The reaction was loaded on a 6% sequencing gel. A known DNA sequence (SEQ) was used as molecular weight markers (lanes g, a, c, and t).
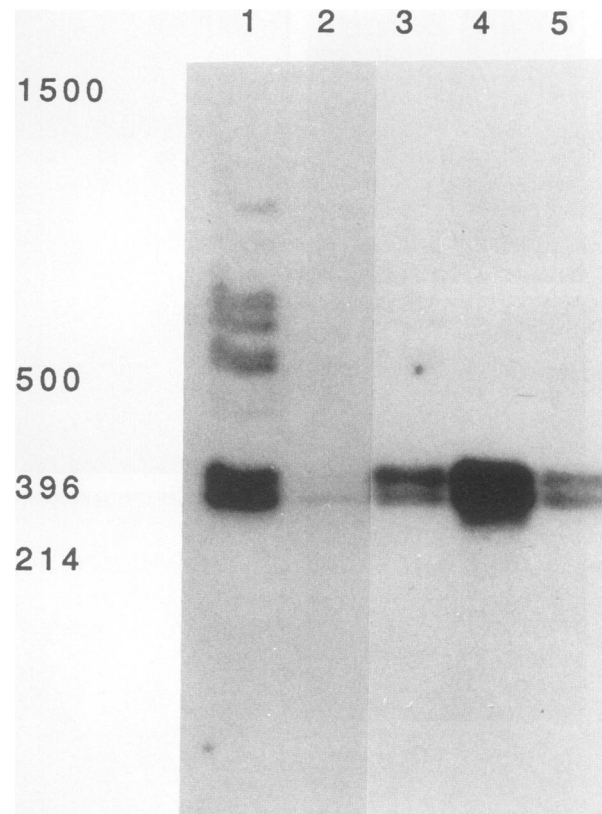

FIG. 5. PCR analysis of single-strand cDNA prepared from RNAs extracted from the nuclear (lane 1) and cytoplasmic (lane 2) fractions of growing FL cells. The nuclear (lane 3), cytoplasmic (lane 4), and polysomal (lane 5) fractions of density-arrested FL cells are also shown. The numbers on the left are sizes in base pairs.

arrested cells were isolated on low-melting-point agarose gels, cloned into a pBluescript KS plasmid, and sequenced. The sequence of the complete *gas5* cDNA is shown in Fig. 6. Sequence comparison showed that the small clone (466 nucleotides) lacks 41 nucleotides (in italics in Fig. 6) at positions 218 to 259 of the larger one (507 nucleotides) but the rest is identical.

When the sequences of these two mRNAs were examined for open reading frames (ORF), only short ones were identified. No stop codon precedes the larger ORF found in *gas5* mRNA; it starts at the ATG codon at position 102, is preceded by a GGAGG sequence, and is followed by an A at position +4 (23). This ATG is followed by an ORF of 123 nucleotides in the smaller clone and an ORF of 117 nucleotides in the larger one. The predicted differential splicing leads to a difference in nucleotide sequence allowing for three different amino acids at the C termini of the putative polypeptides encoded by the *gas5* mRNAs. In vitro translation using wheat germ cell extract and RNA transcribed from plasmids expressing the two *gas5* cDNAs yielded two proteins of the expected size (Fig. 7).

Cloning of a genomic clone corresponding to the *gas5* gene. To understand whether some cDNA clones corresponded to precursor mRNAs or originated from related genes, cDNA and genomic clones were compared. A total of 10⁶ recombinant phages from a murine genomic library were screened by

using a cDNA clone, and two recombinant lambda phages were isolated. Restriction mapping and Southern blot analysis indicated that both phages contained two *Eco*RI fragments each of about 3.5 kb, that hybridized with labeled *gas5* cDNA probes (data not shown). Direct sequencing of the lambda DNA with oligonucleotides from both the 5′ and 3′ ends of the cDNA indicated that the two clones contained the complete *gas5* genomic locus. The two *Eco*RI fragments, each around 3.5 kb long, in lambda 27.3 were subcloned into pBS M13+ plasmid vectors, and the genomic *gas5* clone was sequenced both by preparing deletion mutants by the Exonuclease III-mung bean nuclease protocol and by using specific oligonucleotides corresponding to sequences present in the cDNAs. This allowed identification of all of the boundary sequences between exons and introns in the *gas5* gene. The organization of the gene is shown in Fig. 8. Determination of the exon-intron junction revealed that the additional sequences present in the 11 cDNA clones, that differed from the two mRNAs found in the cytoplasm, correspond to introns. The nucleotide sequences at the exon-intron boundaries were obtained by comparing DNA sequences of cDNAs and genomic subclones. The 5′ donor and 3′ acceptor splice sites are in good agreement with the reported splice consensus sequences observed in many genes (30).

This suggests that incomplete or alternative splicing of a single common mRNA precursor can account for all of the cDNAs isolated. The two *gas5* cDNAs representing the

AGCCTTTCGGAGCTGTGCGGCATTCTGAGCAGGAATGGCAGTGTGGACCTCTGTGATGGG 60

ACATCTTGTGGGATCTCACAGCCAGTTCTGTGGCAAAGGAGGATGAAGGCTTACGAGGAC 120
                            M K A Y E D

TCGTCAGGAAGCTGGATAACAGAGCGAGCGCAATGTGCTAGAATAGAAGACCAGAAAATG 180
S S G S W I T E R A Q C A R I E D Q K M

AAATGGTGGAGTTTGAGGCTGGATAGACAGTTTGAAAG*TTAACTGGTTGCATGCTTGTTC* 240
K W W S L R L D R Q F E S

*ATTTGGCTGGCTTGCTTGGG*TACAAATAATGGTTTGAATAAAGAAAGGTATTA<u>CGC</u>ATGG 300

GTCACCTCAAGTGAAGGCACTGCAAACACAATGATTGGTCATTCTGAATTTCCGGTCCTT 360

CATTCTGAATTTCAAAGGCTCCTGTGACAAGTGGACATGCAGTGACTGCACCTTTGTTTC 420

TGAGGTGCCTGGATGGAGGCTCAAATAGAAGATGGTGTCAGATATATTGTGTTAAAATTT 480

TACC<u>ATTAAA</u>GTGTATTATAACATGAA 507

FIG. 6. Nucleotide sequence of full-length *gas5* cDNA. The nucleotide sequence numbering (residues 1 to 507) refers to the largest mRNA species cloned. The nucleotide sequence in italics from positions 218 to 259 is present only in the larger clone. Removal of these 41 nucleotides by splicing generates the smaller mRNA species, which is therefore 466 bases long. The poly(A) signal sequence is underlined. The underlined triplet at positions 294 to 296 was recovered in the cDNAs but was not observed in the genomic sequence. The putative ORF for the large mRNA is shown in the one-letter amino acid code. The small mRNA contains amino acids R, Y, and K at the C-terminal end instead of the terminating serine in the large mRNA.

mature products (Fig. 5) also originate from transcription of the *gas5* genomic locus. They are derived by elimination of exon 7 in the smaller mRNA, which therefore contains only 11 instead of 12 exons. We have identified three nucleotides, GCG, at positions 294 to 296 in the sequences of cDNA
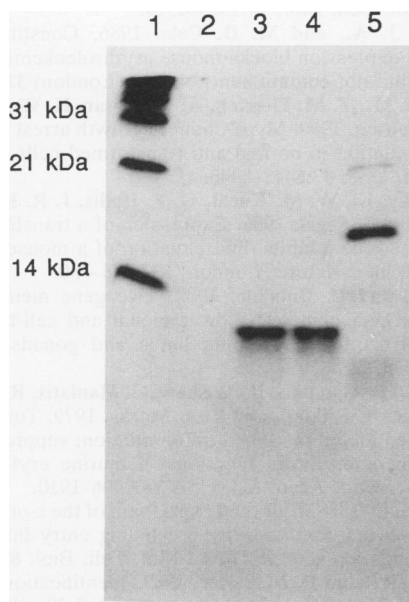

FIG. 7. In vitro translation of the two full-length *gas5* RNAs. Two products which ran with an apparent molecular mass of 8 kDa were generated when in vitro-translated mRNA from the larger or small *gas5* cDNA (lanes 3 and 4, respectively) was translated in a wheat germ system as described in Materials and Methods. Lanes: 1, Bio-Rad protein molecular weight markers (14,400, 21,400, 31,000, 42,699, 66,200, and 97,400); 2, a negative control in which water was loaded instead of mRNA; 5, an unrelated protein of 18 kDa synthesized in vitro and run as an additional molecular weight marker.
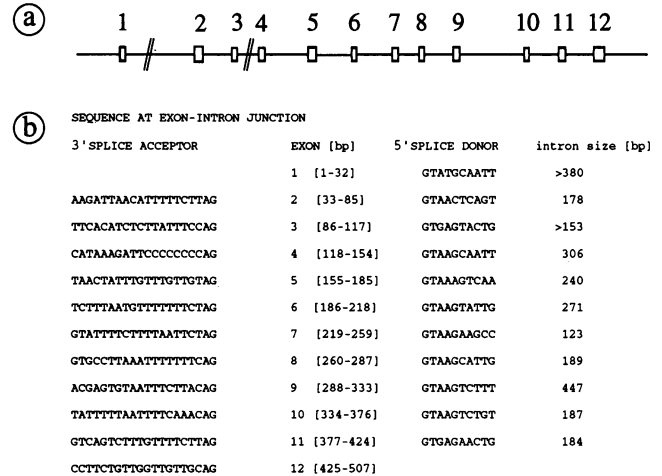

FIG. 8. (A) Map of the murine *gas5* gene. The gene is shown in the 5'-to-3' orientation and is drawn to scale. The exons are shown as numbered boxes. (B) Sequence arrangements around the acceptor and donor sites of the exons from the murine *gas5* gene.

SEQUENCE AT EXON-INTRON JUNCTION

3'SPLICE ACCEPTOR	EXON [bp]	5'SPLICE DONOR	intron size [bp]
	1 [1-32]	GTATGCAATT	>380
AAGATTAACATTTTTCTTAG	2 [33-85]	GTAACTCAGT	178
TTCACATCTCTTATTTCCAG	3 [86-117]	GTGAGTACTG	>153
CATAAAGATTCCCCCCCCAG	4 [118-154]	GTAAGCAATT	306
TAACTATTTGTTTGTTGTAG	5 [155-185]	GTAAAGTCAA	240
TCTTTAATGTTTTTTTCTAG	6 [186-218]	GTAAGTATTG	271
GTATTTTCTTTTAATTCTAG	7 [219-259]	GTAAGAAGCC	123
GTGCCTTAAATTTTTTTTCAG	8 [260-287]	GTAAGCATTG	189
ACGAGTGTAATTTCTTACAG	9 [288-333]	GTAAGTCTTT	447
TATTTTTAATTTTCAAACAG	10 [334-376]	GTAAGTCTGT	187
GTCAGTCTTTGTTTTCTTAG	11 [377-424]	GTGAGAACTG	184
CCTTCTGTTGGTTGTTGCAG	12 [425-507]		

clones and PCR products of the mRNA which we could not identify in the genomic sequence.

We also sequenced about 300 bp in the region upstream of the start of transcription. In this region of the *gas5* gene, a TTATATA sequence is present at position −27 although no CCAAT sequence was found. The rest of the sequence is relatively GC rich and contains a *cis* consensus region for transcription factor Sp1 at −84 and −264. A cyclic AMP response element-binding site is located at position −42.

DISCUSSION

This report describes the molecular characterization of the *gas5* gene, including genomic organization and analysis of its transcripts. Sequence analysis of the *gas5* genomic clone showed that the mature transcripts are derived from a complex splicing process which joins together 11 to 12 exons, giving rise to two mRNAs. PCR analysis revealed that both the precursor transcript and the mature mRNAs accumulate in the nuclei of growing cells, with low concentrations of mature forms present in the cytoplasm, while in growth-arrested cells almost only the mature mRNAs can be detected in both the nuclei and the cytoplasm.

Genomic Southern blot hybridization using a *gas5* cDNA revealed that a few related genes may exist in the mouse genome (data not shown). However, the isolated *gas5* genomic clone appears to contain all of the sequences recovered in 16 different cDNA clones from three different mouse cDNA libraries, indicating that they are all derived from the same gene. Expression of the other loci has not been established, and they may therefore be pseudogenes. The extra nucleotides present in the mRNA but not in the genomic clones imply that posttranscriptional modification occurs.

In vitro analysis indicated that the *gas5* mRNAs are expressed in a growth-regulated fashion in FL cells, being almost undetectable in actively growing cells and abundant in saturation density-arrested cells. Following terminal differentiation and concurrent irreversible exit from the cell cycle, FL cells failed to express the *gas5* gene. However, when cells were induced to differentiate with hemin they expressed part of the erythroid phenotype but retained the

potential to proliferate and express the *gas5* mRNA at growth arrest. This suggests that in FL cells, failure to express the *gas5* gene at saturation density requires full expression of the differentiated phenotype and/or irreversible exit from the cell cycle. The *gas5* gene is also widely expressed during mouse embryo development and in adult tissues.

Despite numerous attempts, the biological function of the *gas5* gene product remains unclear. A search of the data banks showed no homology with other known genes or proteins. We expressed sense and antisense RNAs for *gas5* in both FL and NIH 3T3 cells but observed no detectable alteration in growth rate or differentiation of the cells (data not shown). This negative result may, however, mean only that too low a level of antisense RNA was available to inhibit the sense mRNA. Attempts to detect in vivo the polypeptides that may be encoded by this gene have not been successful. Recently, with peptide antibodies, we have identified a candidate protein but it has not been established whether this protein is encoded in the *gas5* cDNA (21).

Expression of the *gas5* gene is under posttranscriptional regulation in cycling cells, while its transcription is shut off in terminally differentiated cells. It will be interesting to isolate the *cis* DNA sequences that regulate expression of this gene and to clarify the mechanisms that repress its expression in differentiating cells. Similarly, it remains to be verified whether the limited amount of *gas5* mRNA associated with polysomes reflects a translational control.

Ability to distinguish between differentiation-specific and growth-specific events represents an important step in understanding gene expression during terminal differentiation (1, 19, 33, 36, 38). Availability of probes for genes expressed preferentially in quiescent cells allows distinction between different growth conditions like arrest, quiescence, differentiation, and senescence (12, 18, 31, 42).

In this context, it is interesting to compare and contrast the expression pattern of the *gas5* gene with another well-studied quiescent specific marker, statin (38, 45–48). Statin was originally shown to be expressed in the nuclei of quiescent and senescent human diploid fibroblasts (45, 47). However, when studied in a differentiation model, like lens epithelial cells, statin was found only in cells committed to the postmitotic stage (i.e., differentiated and senescent cells) but not in either growing or quiescent lens cells (28). This indicates that irreversible loss of proliferative potential may result in the expression of statin, a pattern different from that of the *gas5* gene in FL cells. Whether this reflects a regulation difference between the two genes or a difference between cell lineages remains to be established. However, in analogy to the *gas5* gene in FL cells, down regulation of two other *gas* genes has been observed following induction of muscle differentiation in rat L6 cells (9).

It appears, therefore, that *gas5* expression is lost when cells enter a postmitotic stage. Extension of the analysis of expression of the *gas* genes in other differentiation models and in senescent cells will help to better define differences and similarities between reversible quiescent G_0/G_1 versus other conditions in which an irreversible growth arrest is observed, like senescence and differentiation (12, 37).

ACKNOWLEDGMENTS

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