Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1848–1852, March 1994
Cell Biology

Structure, function, and chromosome mapping of the growth-suppressing human homologue of the murine *gas1* gene

(membrane protein/oncosuppressor gene/microinjection/*in situ* hybridization)

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Communicated by Lennart Philipson, October 11, 1993

ABSTRACT **We describe the isolation, growth-suppressing activity, and chromosomal localization of the human homologue of the murine growth-arrest-specific gene *gas1*. Overexpression of h-*gas1* is able to block cell proliferation in the A549 lung carcinoma and the T24 bladder carcinoma cell lines. No effect was observed when h-*gas1* was introduced into the osteosarcoma cell line SAOS-2 and into the adenovirus-type-5 transformed cell line 293. This finding is related to our previous evidence that simian virus 40-transformed NIH 3T3 cells are also refractory to murine *gas1* overexpression, suggesting that the retinoblastoma and/or p53 gene products have an active role in mediating the growth-suppressing effect of *gas1*. We also show that h-*gas1* is on chromosome 9q21.3-22.1, in a region considered to be a fragile site. Altogether, the results raise the possibility that h-*gas1* may be a target for genetic alterations leading to its inactivation in tumor cells.**

Cell proliferation is governed by the active balance between growth-promoting factors (protooncogene products) and growth-suppressing factors (tumor suppressors). Genetic alterations that result either in a constitutive activation of protooncogenes or in the functional inactivation of tumor-suppressor genes are associated with the progression pathway that leads to human tumors (1). The genetic approach led in several cases to the discovery of candidate tumor-suppressor genes (*DCC*, *MCC*, *APC*).

Another approach has been to identify candidate genes by their ability to restore growth arrest *in vitro* (2–4). In fact, the most distinctive feature of normal cells cultured *in vitro* is their ability to stop proliferation under growth-restricting conditions such as diminished concentrations of growth factors or high cell density. This feature is universally and overtly lost in transformed cells. It follows that the genetic response induced at growth arrest in normal cells, and lost in transformed cells, could reveal potential cellular targets for transformation.

This laboratory has previously cloned genes (*gas* genes) (5) whose expression is induced in NIH 3T3 mouse cells under growth-arrest conditions such as serum deprivation and high cell density. We have shown that *gas1*, when overexpressed, is able to exert a proliferation block in both normal and transformed cells, thus indicating its involvement in growth suppression (6). To establish a potential role for *gas1* in the origin of tumors, we here present the structural and functional characterization of the human homologue of *gas1* (h-*gas1*).¶ Together with its chromosomal localization, the results described will help to define whether the *gas1* gene could be a target for genetic alterations that might lead to defined forms of cancer.

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MATERIALS AND METHODS

Cell Lines and RNA Extraction. Human embryo fibroblasts (HEF) and A549, SAOS2, and 293 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). T24 cells were grown in Iscove's modified DMEM supplemented with 10% FCS. For serum starvation, subconfluent cells were left for 72 hr in DMEM containing 0.5% FCS. For density-dependent arrest, cells were left for 6 days in DMEM containing 10% FCS; fresh medium was provided every 2 days. For Northern analysis, total RNA was extracted from cultured cells and frozen tissue samples as previously described (7); 15 μg of total RNA was blotted by the downwards method (8).

Isolation of cDNA and Genomic Clones and PCR Amplification. To isolate the h-*gas1* cDNA approximately 4×10^4 clones of a λ human liver cDNA library were screened, using a 1.1-kb *Eco*RI fragment from the 3′ end of murine *gas1* cDNA (6). Hybridization was performed for 24 hr in 1 M NaCl/1% SDS at 52°C. Washes were done at room temperature in 2× SSC and at 50°C in 2× SSC/1% SDS for 30 min (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Both strands were sequenced, using 7-deazaguanosine mixes and an automated fluorescent DNA sequencer (European Molecular Biology Laboratory; EMBL). To isolate the λ-GEN17 genomic clone, approximately 1×10^6 clones from a λ-EMBL3 human genomic library were screened in parallel with two nonoverlapping segments of h-*gas1* cDNA. Hybridization and washes were done essentially as described (6). To clone the 5′ end of h-*gas1* cDNA, a rapid amplification of cDNA ends (RACE)–PCR amplification of a G-tailed primer extension product was performed essentially as previously described (6). The reverse transcription was carried out at 70°C on 10 μg of total RNA extracted from quiescent human embryo fibroblasts, using a thermostable reverse transcriptase (Retrotherm, Epicentre Technologies, Madison, WI) and a 24-nt synthetic primer (5′-TCGGCGTACTGGTTG-TAGGCGTAG-3′). Extension products were G-tailed with terminal deoxynucleotidyltransferase (Bethesda Research Laboratories) and successively PCR amplified using a poly(C) upstream primer with an *Eco*RI linker and a downstream primer complementary to bases 468–485, which is located 74 nucleotides after the first *Not* I site. PCRs were performed under the following conditions: 40 cycles of 1 min at 94°C, 30 sec at 60°C, and 1 min at 72°C, in a total volume of 50 μl, with 25 pmol of each primer, 1.5 mM $MgCl_2$, and 5% (vol/vol) dimethyl sulfoxide in a Perkin–Elmer/Cetus Gene-

Abbreviations: FCS, fetal calf serum; SV40, simian virus 40; FITC, fluorescein isothiocyanate; RACE, rapid amplification of cDNA ends; BrdUrd, 5-bromodeoxyuridine; UTR, untranslated region.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L13698).

Amp 9600 thermal cycler. For amplification of genomic h-*gas1*, the dimethyl sulfoxide concentration was raised to 10% and $MgCl_2$ to 2.5 mM.

Plasmid Constructions, Transfection, and Microinjection. For *in vitro* translation of h-*gas1*, a fragment containing the complete open reading frame was cloned in the *Nco* I/*Xba* I sites of the pCITE-1 vector (Novagen). Immunoprecipitation of translated products was performed as described (9). All translation products were analyzed on SDS/15% polyacrylamide gels. For expression in human cells a cDNA fragment of h-*gas1* (base pairs 288–2020) containing the complete open reading frame was inserted in the *Eco*RI site of pGDSV7 vector (6).

SAOS-2 cells (10^5) were plated on coverslips in 35-mm Petri dishes 24 hr prior to transfection. Cells were transfected with 4 μg of pGDSV7/h-*gas1* DNA, using the calcium phosphate transfection procedure (10). After 8 hr of incubation, cells were shocked with glycerol and then incubated in growth medium for an additional 24 hr before immunofluorescence analysis. For microinjection experiments, 10^5 cells were seeded on coverslips in 35-mm Petri dishes and used 24 hr later. Automated microinjections and immunofluorescence were performed as previously described (6). h-Gas1 was revealed by the affinity-purified anti-Gas1 antibody followed by fluorescein isothiocyanate (FITC)-conjugated goat antibody to rabbit IgG (Dako). For control, 1% FITC-dextran (M_r 150,000; Sigma) was added to pGDSV7-huTR (6) at 100 ng/μl. Given the high endogenous expression of human transferrin receptor, microinjected cells were scored for FITC-dextran after paraformaldehyde fixation, which allowed the concurrent incorporation of 5-bromodeoxyuridine (BrdUrd) to be assayed (6).

***In situ* Hybridization.** The genomic clone λ-GEN17 DNA was labeled by nick translation with biotin-16-dUTP (Boehringer) and hybridized at a concentration of 5 ng/μl to metaphase chromosome spreads obtained from phytohemagglutinin-stimulated lymphocyte cultures of normal male individuals. To suppress background from repeated sequences present in the probe, hybridization was carried out in the presence of unlabeled sonicated human genomic DNA at 2.5 μg/μl. Hybridization and detection with FITC-conjugated avidin were performed as previously described (11). Metaphase spreads were stained with propidium iodide in antifade medium and photographed. The same metaphase plates were subsequently G-banded with Wright's stain and rephotographed. Mapping was carried out by overlap image.

RESULTS

Sequence and Structure of h-*gas1* cDNA. A fragment of murine *gas1* cDNA containing the 3′ untranslated region (UTR) and having a lower C+G content (6) was used as a probe to screen a human liver cDNA library with low-stringency hybridization. A clone was isolated that, by sequence analysis, starts at nucleotide 526 relative to the mouse cDNA sequence, thus missing the 5′ UTR sequence. To define the 5′ end region of h-*gas1* cDNA, a RACE–PCR amplification was performed. The mRNA template was isolated from human embryo fibroblasts previously arrested by serum deprivation for 72 hr and primed with an oligonucleotide complementary to bases 596–620. The resulting amplified cDNA (Fig. 1*A*) extended the upstream sequence of the human liver cDNA clone by 388 nucleotides. Fig. 1*B* shows the composite cDNA sequence combining the liver cDNA clone and the RACE–PCR products of h-*gas1*. The resulting 5′ UTR is 410 nucleotides long and does not contain stop codons that are in frame with the first putative ATG. To confirm that no other ATG is present upstream to the sequenced RACE–PCR product, a genomic clone was obtained that contains the 5′ UTR (not shown). From the sequence analysis of the isolated genomic clone, neither

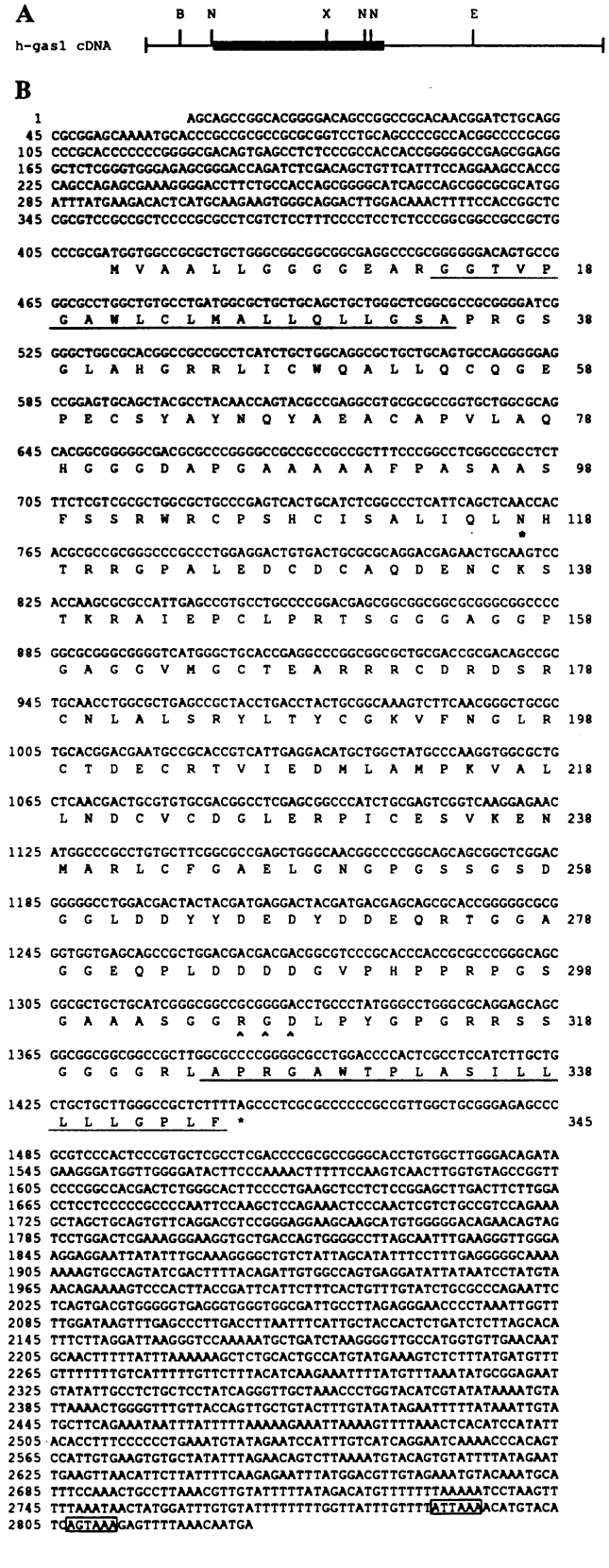

FIG. 1. Partial restriction map and DNA sequence with deduced amino acid sequence of h-*gas1* cDNA. (*A*) Restriction map of the h-*gas1* clone analyzed. B, *Bgl* II; N, *Not* I; X, *Xho* I; E, *Eco*RI. (*B*) DNA sequence with the encoded amino acid sequence. Potential transmembrane domains are indicated by underlining. Potential N-glycosylation sites are indicated by a star. The RGD consensus recognition structure for integrin type receptors is identified by carets. Polyadenylylation signals are boxed.

upstream ATG nor in-frame stop codons were observed. Furthermore, subsequent comparison of genomic and reverse transcription–PCR products containing the 5′ UTR region revealed the absence of intervening sequences (not shown). The colinearity between genomic and cDNA by both sequence and restriction analysis was observed over the entire length of the cDNA clones. Moreover, when Southern blotting was performed on human genomic DNA, only the expected restriction pattern was observed, thus confirming that the isolated h-*gas1* is not a member of a gene family (not shown).

We can thus conclude that the open reading frame starts at position 411 and encodes a polypeptide of 345 amino acid residues. Within the first 37 residues of the predicted protein sequence a stretch of predominantly hydrophobic residues (residues 14–34) is present that resembles a putative transmembrane domain (12). Twenty cysteine residues are distributed between residues 48 and 243. An RGD sequence is recognized at residues 306–308, while only one putative glycosylation site is present, at residue 117. The coding region ends with a second putative transmembrane domain between residues 325 and 345.

Comparative Analysis of h-Gas1. Alignment of human and mouse Gas1 amino acid sequences reveals that the two proteins bear 82% amino acid identity (Fig. 2). All 20 cysteines present in the putative extracellular region are conserved at the same position. Only one of the two putative glycosylation sites present in the mouse protein is conserved in the human sequence. This observation suggests that the N-conserved glycosylation site is the site used to add the only *N*-glycan chain that was previously observed in the analysis of the murine product (6). Both proteins contain the RGD motif, which is a potential recognition site for integrin-type receptors (13). Interestingly the starting methionine of h-Gas1 corresponds to Met-42 in mouse Gas1. This may indicate that Met-42 corresponds to the initiation codon also in mouse Gas1, especially since the consensus pattern at the first murine ATG is less conserved (not shown).

Analysis of h-*gas1* Product. To confirm that the h-*gas1* cDNA gives rise to the predicted product, an *in vitro* transcription/translation analysis was performed. Fig. 3*A* shows the products derived from *in vitro* transcription/translation of h-*gas1*. The *gas1* primary translation product has an apparent molecular mass of 45 kDa and is specifically immunoprecipitated by the anti-Gas1 antibody, a monospecific affinity-purified antibody raised and purified against murine Gas1 (6). The h-*gas1* cDNA obtained was then cloned in pGDSV7, transfected into SAOS-2 cells, and transiently expressed. Surface immunofluorescence staining (without cell permeabilization) was performed after 24 hr by using the described antibody (Fig. 3*B*). This analysis thus reveals that h-Gas1 is targeted to the plasma membrane.

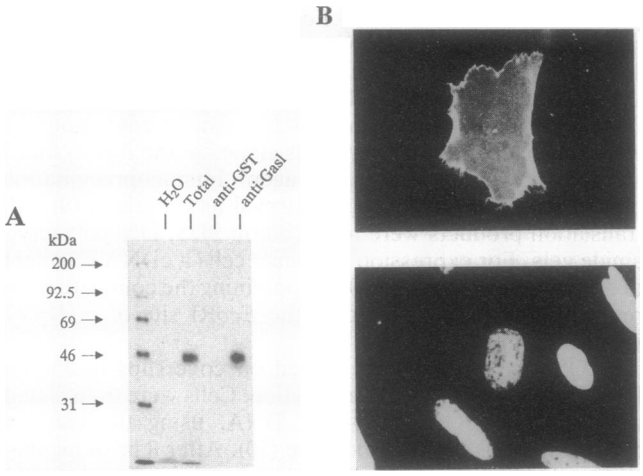

FIG. 3. Analysis of h-Gas1 protein by a specific affinity-purified antibody raised against mouse Gas1-glutathione *S*-transferase (GST) bacterial fusion protein. (*A*) SDS/polyacrylamide gel analysis of the primary *in vitro* translated product (Total), immunoprecipitation of the same product by the affinity-purified anti-Gas1 antibody or by a control affinity-purified anti-GST antibody. (*B*) The antibody to mouse Gas1 was used to reveal h-Gas1 expressed in transfected SAOS-2 cells by immunofluorescence. (*Upper*) Twenty-four hours after transfection cells were fixed without permeabilization and stained with rabbit anti-Gas1 antibody followed by goat FITC-conjugated anti-rabbit IgG. (*Lower*) Nuclei of the same field of cells stained with Hoechst 33342.

h-*gas1* Expression in Fibroblasts and in Human Tissues. We have shown that expression of *gas* genes in NIH 3T3 cell lines is induced under growth-arrest conditions. We therefore performed a Northern blot analysis to monitor h-*gas1* mRNA expression in human embryo fibroblasts cultured under different growing conditions. Fig. 4*A* shows that h-*gas1* expression is low in growing cells (lanes G), while it is significantly increased when cells are arrested either by serum deprivation (lane S) or by density-dependent inhibition (lane DDI). A similar pattern of expression was also obtained in Western analysis using the anti-Gas1 antibody (not shown). h-*gas1* expression was also monitored by Northern blot analysis of total RNA isolated from different human tissues. Fig. 4*B* shows that h-*gas1* is expressed in several tissues such as skeletal muscle, kidney, heart, and placenta. In brain, ex-

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h-Gas1     1 MVAALLGGGGEARGGTVPGAWLCLMALLQLLGSAPRGSGLAHGRRLICWQALLQCQGEPECSYAYNQYAEAC
Gas1      42 MLAALLGGAG-ARTGTLPGALLCLMALLQLLCSAPRGSGLAHGRRLICWQALLQCQGEPDCSYAYSQYAEAC

          73 APVLAQHGGGDAPGAAAAAFPASAASFSSRWRCPSHCISALIQLNHTRRGPALEDCDCAQDENCKSTKRAIE
         113 APVLAQRGGADAPG-PAGAFPASAAS-SPRWRCPSHCISALIQLNHTRRGPALEDCDCAQDEHCRSTKRAIE

         145 PCLPRTSGGGAGGPGAGGVMGCTEARRRCDRDSRCNLALSRYLTYCGKVFNGLRCTDECRTVIEDMLAMPKV
         183 PCLPRTSSVGPGA-GAGSVMGCTEARRRCDRDSRCNLALSRYLAYCGKLFNGLRCTDECRAVIEDMLAVPKA

         217 ALLNDCVCDGLERPICESVKENMARLCFGAELGNGPGSSGSDGGLDDYYDEDYDDEQRTGGAGGEQPLDDDD
         254 ALLNDCVCDGLERPICESVKENMARLCFGPDASNGPGSSGSDGGLDDYYDEEYDDEQRAGAAGGEQPLDDDD

         289 GVPHPPRPGSGAAASGGRGDLPYGPGRR--SSGGGGRLAPRGAWTPLAS---ILLLLLGPLF        345
         326 GL---ARPGGGAAAAGGRGDLPHGPGRRTSSSGSGGHWANRSAWTPFACLLLLLLLLLGSHL        384
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FIG. 2. Alignment of the h-Gas1 and mouse Gas1 amino acid sequences. The stars denote cysteine residues, the box indicates the potential N-glycosylation site; vertical lines mark identities, and colons indicate similar residues. The RGD sequences are denoted by heavy lines.

pression of h-*gas1* is observed only in fetal tissue (compare lane fB with lane B).

Antiproliferative Effect of Ectopic h-*gas1* Expression. We have recently demonstrated that overexpression of mouse *gas1* is able to block cell proliferation of nontransformed and single oncogene-transformed NIH 3T3 cells with the exception of the simian virus 40 (SV40)-transformed NIH 3T3 cell line. We therefore analyzed if h-*gas1* plays a similar role in human cells by overexpressing h-Gas1, through microinjection of the cloned cDNA, in various transformed human cell lines. pGDSV7/h-*gas1* containing the entire coding region was injected in asynchronously growing cells. After injection, cells were left in the same medium containing 10% FCS for an additional 18 hr before BrdUrd was added for 6 hr to monitor entry into S phase. Cells were then fixed and processed for double immunofluorescence microscopy by using anti-Gas1 and anti-BrdUrd antibodies. The results shown in Table 1 represent mean values from several microinjection experiments. The human transferrin receptor cloned in the same vector (pGDSV7/hu-TR) was separately injected as a negative control. h-*gas1* overexpression leads to a significant inhibition of S-phase entry in the T24 bladder carcinoma cell line and in the A549 lung carcinoma cell line (Table 1). On the contrary, when h-*gas1* was overexpressed in the sarcoma-derived cell line SAOS-2 and in the adenovirus type-5-transformed kidney cell line 293 no effect on cell proliferation was observed. Under the same conditions expression of human transferrin did not inhibit entry into the S-phase (see Table 1 legend).

Chromosomal Localization of h-*gas1*. Chromosomal localization of the h-*gas1* gene was determined by *in situ* hybridization of the genomic clone λ-GEN17 to human metaphase chromosomes. This clone contains most of the h-*gas1* coding sequences, up to nucleotide 520 of the cDNA, and the 3′ flanking region (Fig. 5). The biotinylated DNA was hybridized to human metaphase spreads, and hybridization was detected with avidin conjugated to FITC. Analysis of 29 metaphases revealed a total of 102 fluorescent signals: 84 (82%) were localized on chromosome 9, with peak signal density (79 spots, corresponding to 77% of the total) occurring in the q21.3–22.1 region (Fig. 5). No other significant signal cluster was detected on any other chromosome. These results indicate that the h-*gas1* gene resides on chromosome 9q21.3–22.1.

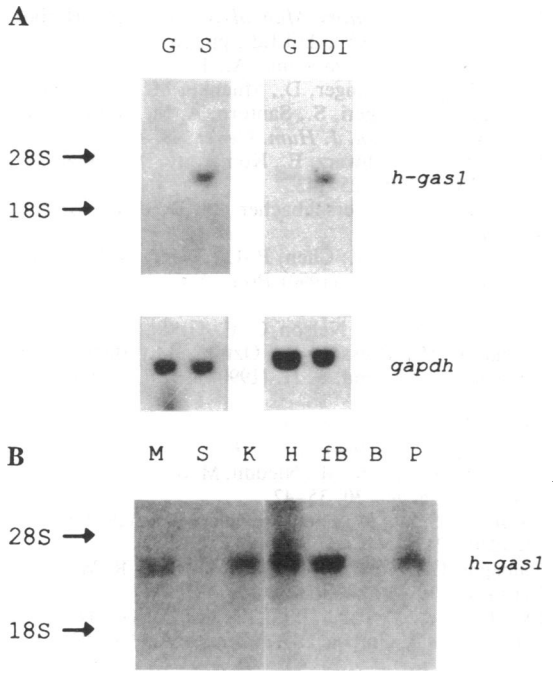

FIG. 4. Analysis of h-*gas1* steady-state expression in human embryo fibroblasts and in human tissues. (*A*) RNA was extracted from human embryo fibroblasts growing asynchronously (lane G), serum starved (lane S), or growth-arrested by density-dependent inhibition (lane DDI). The same Northern blots were also probed with glyceraldehyde-3-phosphate dehydrogenase cDNA (*gapdh*) to normalize for the RNA content. (*B*) Total RNA from various human tissues was extracted and equal amounts were separated on Northern blots. M, muscle; S, suprarenal gland; K, kidney; H, heart; fB, fetal brain; B, adult brain; and P, placenta.

Table 1. Inhibition of DNA synthesis by h-*gas1* in transformed human cells

Cell line	No. of injected cells analyzed	BrdUrd-positive cells, %		Relative inhibition, %
		Injected	Uninjected	
A549	221	19.5 ± 2.7	44.0 ± 2.3	55.2 ± 8.0
T24	287	23.3 ± 2.2	42.5 ± 4.3	44.5 ± 8.1
293	162	51.8 ± 5.8	50.8 ± 3.9	0.1 ± 7.2
SAOS-2	259	33.8 ± 4.8	34.2 ± 4.0	1.1 ± 5.7

Transformed human cells were cultured in medium containing 10% FCS and microinjected with pGDSV7/h-*gas1* (100 ng/μl). Eighteen hours after injection cells were labeled for 6 more hr with BrdUrd (50 μM), fixed, and processed for immunofluorescence. Injection of pGDSV7/hu-TR (100 ng/μl) in T24 carcinoma cell line gave a relative inhibition of 9.9% ± 1.3% in 288 scored cells. Injection of pGDSV7/hu-TR in the A549 carcinoma cell line gave a relative inhibition of 2.2% ± 7.6% in 155 scored cells. Relative inhibition of DNA synthesis in injected cells was calculated according to the formula % = 100 × [% of BrdUrd-positive cells (uninjected) − % of BrdUrd-positive cells (h-Gas1-positive)]/% of BrdUrd-positive cells (uninjected). Results are presented as mean ± SD.

DISCUSSION

The h-*gas1* cDNA clone was isolated from a normal human liver cDNA library, and it has a high degree of homology with the murine cDNA. The coding region of h-*gas1* results in a protein with 82% amino acid identity with the murine Gas1, maintaining the typical domains of an integral membrane protein. All the cysteines present are conserved and match with the positions in the murine form; in addition, the RGD motif is conserved at the same location. A noticeable difference from the murine Gas1 is at the starting methionine: the murine Gas1 in fact contains 41 amino acid residues before the common AUG, suggesting that the second AUG is the one used. Membrane localization of h-Gas1 was shown by transient expression of the cDNA in SAOS-2 cells, where a diffuse membrane staining was observed in the absence of detergent permeabilization. h-Gas1 is upregulated in human fibroblasts when arrested by either serum deprivation or high cell density, while its level is significantly lower during exponential growth.

We have previously shown that overexpression of murine *gas1* blocks cell proliferation in various transformed murine fibroblasts and does not allow the G_0 to S-phase transition in serum-stimulated quiescent fibroblasts (6). Here we extended the analysis on a panel of human tumor cell lines.

Ectopic expression of h-*gas1* in the T24 bladder carcinoma and in the A549 lung carcinoma cell lines is able to suppress cell growth, but this effect is not observed in the sarcoma-derived cell line SAOS-2 and in the adenovirus type-5-transformed cell line 293. Lack of response to h-*gas1* overexpression in the last two cell lines could be related to the behavior previously noticed in the SV40-transformed NIH 3T3 cell line when murine *gas1* was used (6). In fact, the target elements (pRb and p53) involved in transformation by SV40 large T antigen seem also to be implicated in the adenovirus type-5-transformed cell line. However, in con-

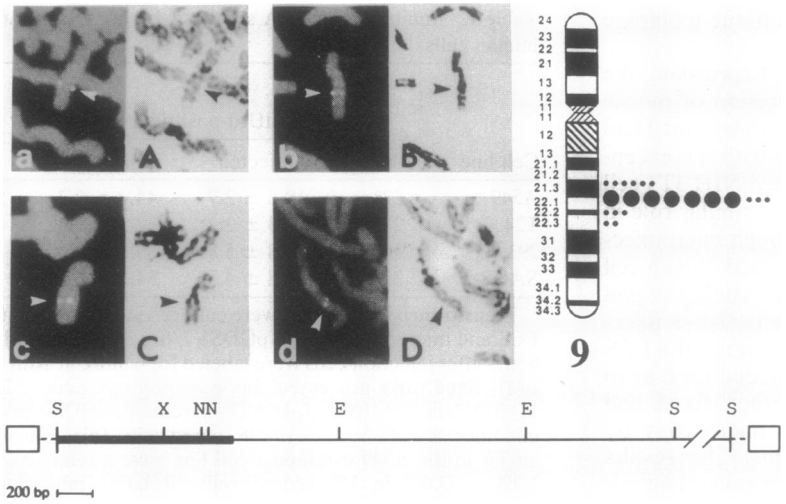

FIG. 5. *In situ* hybridization of h-*gas1* gene probe to chromosome 9q21.3–22.1 and schematic representation of the genomic clone (λ-GEN17) used as probe. Hybridization with biotin-labeled λ-GEN17 DNA on partial metaphases was detected by FITC-conjugated avidin on the background of propidium iodide-counterstained chromosomes (*a–d*). The same partial metaphases were G-banded and Wright's stained, and the corresponding regions are marked by arrowheads (*A–D*). On the right is an idiogram of human G-banded chromosome 9 illustrating the distribution of the hybridization signals. Each large dot represents ten hybridization signals.

trast with the concerted action of oncogenic DNA transforming virus products in the 293 line, the SAOS-2 cell line reflects a different mechanism of *gas1* lack of response, probably related to the absence of both functional p53 and *RB* products (14, 15).

The findings reported here establish that (*i*) h-*gas1* expression is induced under restricting growth conditions; (*ii*) its ectopic expression can block proliferation of normal and some transformed cells; and (*iii*) its growth-suppressive effect correlates to the presence of functionally active RB and/or p53. All these results imply that h-*gas1* is actively involved in controlling proliferation of human cells and could thus be considered a potential target for genetic alterations that might lead to its inactivation. We do not yet know if either RB or p53 is responsible for Gas1-mediated growth suppression. If one or the other is, it may define a negative regulatory circuit that originates from the plasma membrane. In this regard, the localization of the h-*gas1* gene on chromosome 9q21.3-22.1 will help to establish a link between lesions in this region, which are very frequent in certain tumors, particularly myeloid neoplasias, and potential genetic alterations of h-*gas1* leading to inactivation of its established growth-suppressing effect. In fact, genetic alterations in chromosome 9q have previously been implicated as early events during the progression of bladder carcinoma (16, 17). Moreover, preliminary mapping of the deleted region of chromosome 9 in bladder tumors suggests 9q22 as one of the target regions (18, 19).

Note. h-*gas1* has been independently mapped on the same region of chromosome 9 by Evdokiou *et al.* (20).

This work was supported by grants from the Progetto Speciale Oncosoppressori–Associazione Italiana per la Ricerca contro il Cancro to C.S., from the Associazione Italiana per la Ricerca contro il Cancro to G.D.V., and from the Progetto Finalizzato Ingegneria Genetica, Consiglio Nazionale delle Ricerche Rome, to C.S. and G.D.V.; L.C. was supported by the Scuola Internazionale Studi Superiori Avanzati Ph.D. program.

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