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Extension of the Replicative Life Span of Human Diploid Fibroblasts by Inhibition of the p33^{ING1} Candidate Tumor Suppressor

IGOR GARKAVTSEV* AND KARL RIABOWOL

Departments of Medical Biochemistry and Oncology and Southern Alberta Cancer Research Centre, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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Previous studies suggest that tumor suppressors may play significant roles in blocking the growth of cells during cellular senescence. We therefore studied the potential involvement of a novel growth inhibitor and candidate tumor suppressor gene called *ING1*, which we have cloned recently (I. Garkavtsev, A. Kazarov, A. Gudkov, and K. Riabowol, Nat. Genet. 14:415–420, 1996), in the process of cellular senescence. Our results show that the RNA and protein levels of *ING1* were 8- to 10-fold higher in senescent cells than in young, proliferation-competent human diploid fibroblasts. Expression of the nuclear p33^{ING1} protein was regulated during the cell cycle, reaching maximal levels during DNA synthesis. Chronic expression of antisense *ING1* RNA reproducibly resulted in extension of the proliferative life span of normal human fibroblasts by approximately seven population doublings.

Primary normal human diploid fibroblasts (HDFs) undergo a limited number of population doublings in vitro that is proportional to the age of the donor and to the life span of the species from which they were explanted (13, 21), suggesting a direct connection between senescence in vivo and HDF senescence in vitro. The same phenomenon of replicative senescence in culture has also been confirmed with several other human cell types, including smooth muscle cells (2), endothelial cells (28), and lymphocytes (5). A limited replication potential has often been compared to a state of antioncogenesis in which cellular senescence serves as a mechanism for limiting the growth of cancer cells (4, 10). This view is supported by the observations that, upon immortalization, cells become much more susceptible to transformation and that malignant tumor cells are often found to have unlimited growth potential (14, 32). Thus, the genetic events underlying senescence likely represent the same cell cycle control mechanisms that are abrogated in the spontaneous immortalization of cells, an event that is highly correlated with malignant transformation (25).

Treatment of normal cells with oncogenes or viral oncoproteins (such as simian virus 40 [SV40] large T antigen [Tag], adenovirus E1a/E1b, or papillomavirus E6/E7) or mutation or down regulation of tumor suppressors (Rb and p53) increases the frequency with which cells can escape senescence (15, 31). These observations indicate that this escape from senescence is important in cancer induction. To date, only two growth suppressor genes, those encoding the retinoblastoma and p53 tumor suppressors, have been reported to extend the life span of normal cells by mutation or down regulation (3, 11, 25), and both are more active in senescent cell populations than in young cell populations (1, 27).

We have used a novel positive selection procedure that combines subtractive hybridization with an in vivo selection assay to clone a new candidate tumor suppressor gene, *ING1*, whose suppression is associated with loss of cellular growth control and immortalization (7). Several methods were used to show that overexpression of this growth inhibitor efficiently arrests cells in the G_0/G_1 phase of the cell cycle. Because ectopic overexpression of the *Rb* and *p53* tumor suppressor genes also effectively blocks cell growth, we examined the possibility that p33^{ING1} is similarly involved in regulating the proliferative life span of normal HDFs. We provide evidence for the direct involvement of this new candidate tumor suppressor gene in the process of cellular senescence.

MATERIALS AND METHODS

Cell culture and retroviral gene transfer. The normal HDF cell strain Hs68 (ATCC CRL 1635) and a phenotypically normal mouse epithelial cell line from a mammary gland (NMuMG) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Hs68 cells were used at 30 ("young"), 70 ("preaged"), and 80 ("old") mean population doublings (MPDs) for expression and life span experiments. After retroviral infection, HDFs were repeatedly passaged in 10-cm-diameter plates and were split at a ratio of 1:2 when confluent.

For infection of fibroblasts, the retroviral vector pLNCX was used (17). Highly efficient ecotropic (BOSC23) and amphotropic (CAK8) packaging cell lines were also used (20). A fragment of the *ING1* gene (7) was cloned in the antisense orientation into the pLNCX vector, and the resulting construct (pLNCX-ING1-αS) or pLNCX alone was transfected into the BOSC23 virus-packaging cell line. The amphotropic cells were infected by viruses from the BOSC23 supernatant. Fibroblasts were plated at 10^5 cells per 10-cm plate and infected with undiluted viral supernatant from amphotropic producer cells. Infection efficiencies ranged from 85 to 95% in individual trials.

PCRs. *ING1* mRNA expression in young and old cells was monitored by reverse transcription-PCR (RT-PCR). RT with 1 μg of total RNA from young and old Hs68 cells was performed with 50 U of RNasin (Pharmacia) and 200 U of Moloney murine leukemia virus reverse transcriptase for 50 min at 42°C in 20-μl reaction volumes. Two microliters of each RT reaction product was amplified with 2 U of *Taq* polymerase. The two sets of primer pairs for the *ING1* gene and for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were 5′-GAAGCGGCGGATGCTGCACT-3′ and 5′-ACGCACGAGAAGTG GAACCA-3′ (*ING1*) and 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (*GAPDH*). Thirty-two PCR cycles for *ING1* and 22 PCR cycles for *GAPDH* were performed under standard conditions (22). Primers for *GAPDH* were added to PCR tubes at the end of the 10th cycle (33).

Western blotting. Hs68 and NMuMG cells were harvested, and 20 μg of total protein was used in each lane. Proteins were separated by electrophoresis in 12.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels and transferred to membranes for 1 h with an electroblotter. The membranes were blocked in TBS (100 mM Tris, 150 mM NaCl) containing 10% nonfat dried milk and 0.1% Tween 20 for 2 h. The membranes were incubated with p33^{ING1} antiserum in TBS containing 5% nonfat milk and 0.1% Tween 20 for 1 h and then washed with TBST solution (TBS containing 0.1% Tween 20) for 30 min. Horseradish peroxidase-conjugated goat anti-rabbit antibody was then applied to the filters for

* Corresponding author. Phone: (403) 220-8865. Fax: (403) 283-8727. E-mail: igarkavt@acs.ucalgary.ca.

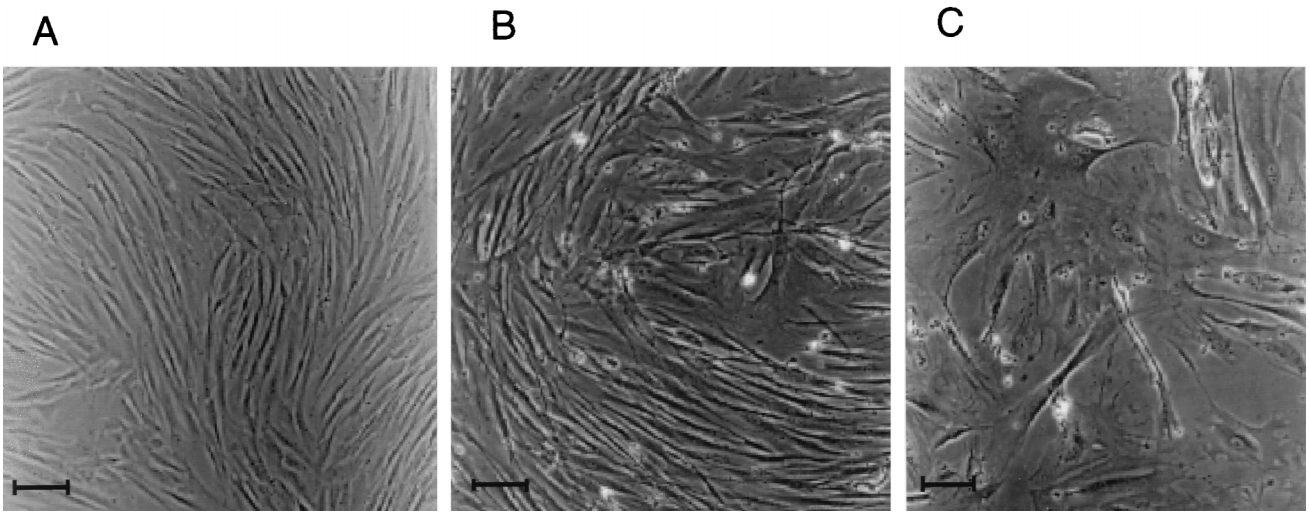


FIG. 1. Morphology of senescing HDFs. Young (30 MPDs) (A), preaged (70 MPDs) (B), and senescent (80 MPDs) (C) confluent Hs68 fibroblasts were grown in the same type of medium and photographed under identical conditions. Bars = 50 μm.

1 h in TBST. Peroxidase activity was detected with an ECL kit (Amersham), and relative band intensities were determined by scanning densitometry.

Indirect immunofluorescence. For indirect immunofluorescence, normal HDFs (Hs68 cells) were grown on glass coverslips for 48 h at 37°C to 60% confluence. The cells were fixed in 3.7% formaldehyde; they were then washed first in 0.5% Triton X-100 and then in 0.05% Tween 20 in phosphate-buffered saline for 10 min each time at room temperature. The cells were incubated with a 1:100 dilution of rabbit p33^{ING1} antiserum for 30 min, washed in phosphate-buffered saline with 0.05% Tween 20, and incubated with goat anti-rabbit immunoglobulin G–biotin antibody and then with streptavidin-conjugated Texas Red. Samples were examined with a Zeiss Axiophot fluorescence microscope, and images were photographed on Kodak TMAX 400 film.

RESULTS

Expression levels of *ING1* in young and senescent fibroblasts. Since the activity or expression levels of several tumor suppressors increase in senescent cells (1, 12, 27), we first checked the levels of *ING1* expression in low- and high-passage-number cells. All experiments were performed with the Hs68 strain of primary normal HDFs. Typical morphologies of young (30 MPDs), preaged (70 MPDs) and old (80 MPDs) fibroblasts are shown in Fig. 1. Senescent cells were obtained by passaging early-passage (young) fibroblasts continuously to a point at which one population doubling required from 2 to 3 weeks to complete, compared to 24 h on average for young HDFs. Hs68 cells at 80 MPDs exhibited characteristics typical of senescent cells, such as an inability to respond to growth factors and altered morphology, including increased size and decreased saturation density, as shown in Fig. 1.

To study the level of expression of *ING1* mRNA, RT-PCR with total RNA isolated from young and old cells was performed (Fig. 2A). The relative levels of the *ING1* transcript were compared to those of the internal control gene *GAPDH* by using PCR primers specific for the *ING1* and *GAPDH* genes. *ING1* and *GAPDH* were amplified in the same reaction tube by the primer dropping approach (33), which internally controls for efficiency of reverse transcription and amplification by PCR. The levels of *ING1* mRNA were estimated by scanning densitometry to be approximately 10-fold higher in senescent fibroblasts than in young fibroblasts. In order to see if increased mRNA levels resulted in increased protein levels, Western blotting experiments were performed with a rabbit polyclonal antibody that was raised against a bacterially expressed glutathione *S*-transferase–p33^{ING1} fusion protein and that reacted with a 33-kDa protein in human and mouse cell lysates (7). As shown in Fig. 2B, the level of p33^{ING1} protein increases approximately eightfold when cells approach the end of their in vitro replicative life span, consistent with the results obtained by RT-PCR.

***ING1* expression changes during the cell cycle.** Since *ING1* appears to arrest cells in G_1 when overexpressed (7) and senescent cells are arrested primarily in the G_1 phase of the cell cycle (23), we tested whether p33^{ING1} protein levels changed during the cell cycle. Quiescent, proliferation-competent NMuMG cells were serum stimulated, lysates were prepared at different times after serum addition, and samples were analyzed by Western blotting with anti-p33 antibodies. The level of p33^{ING1} was found to decrease as cells exited from G_0, to increase during late G_1, and to reach a maximum in S phase. This was followed by a decrease in G_2 (Fig. 3B). *CDK2* expression was used as a control for cell cycle progression (29) and changed as reported previously (Fig. 3A). Figure 3C shows the results of DNA content analysis by fluorescence-activated cell sorting (FACS) with parallel cultures, indicating that cells enter S phase at 16 h under these experimental conditions. These results indicate that *ING1* is regulated after addition of mito-

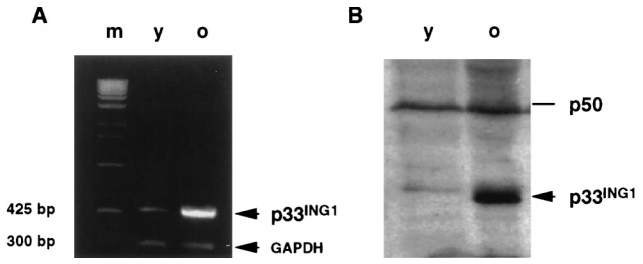
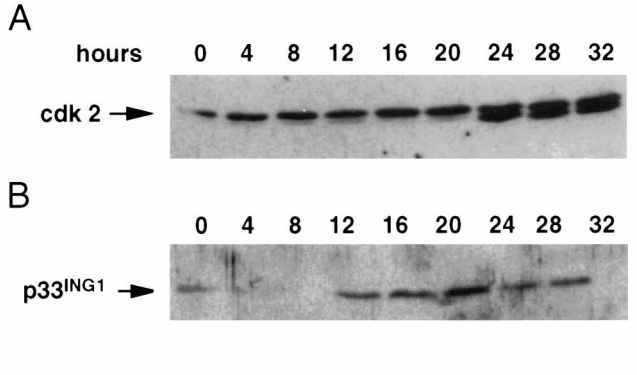


FIG. 2. Levels of *ING1* mRNA and protein in normal young and senescent fibroblast cells. (A) RNA expression. *ING1* and *GAPDH* mRNA expression in young (lane y) and senescent (lane o) Hs68 cells (30 and 82 population doublings, respectively). *GAPDH* served as an internal control for RT and PCR amplification. Lane m, molecular size markers. (B) Protein expression. The arrowhead indicates the signals obtained when cell lysates containing equal amounts of protein from low-passage (lane y) or high-passage (lane o) Hs68 cells were examined by immunoblot analysis with anti-p33^{ING1} antibody. The 48-kDa band represents a nonspecific signal observed in all lysates. p50 is protein from prestained SDS-polyacrylamide gel electrophoresis broad-range standards.



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	0	4	8	12	16	20	24	28	32
G0/G1	87.0	87.3	88.0	89.2	72.2	61.7	50.7	50.8	58.9
S	6.2	4.7	3.1	5.0	17.7	30.4	28.5	22.3	19.1
G2/M	6.8	8.0	8.9	5.8	10.1	8.0	20.8	21.9	22.0

FIG. 3. Level of p33^{ING1} protein throughout the cell cycle. Quiescent NMuMG cells, starved for 72 h, were stimulated to reenter the cell cycle by addition of fresh medium containing 10% fetal bovine serum. Cell lysates were harvested at the indicated times, and 20 μg of protein per lane was analyzed by blotting with an anti-CDK2 antibody as a positive control (A) or with anti-p33 antibodies (B). (C) The cell cycle profile at each point was determined by FACS by a standard propidium iodide staining method. The data are the percentages of cells in each cell cycle phase at each time point.

gen to quiescent cells, with expression reaching a peak during DNA synthesis.

Suppression of the growth-inhibitory effect of *ING1* by SV40. We have previously found that overexpression of *ING1* efficiently blocked cell growth (7), similar to observations made upon overexpression of the tumor suppressors p53 and Rb (15). Since the SV40 oncoprotein Tag contains a site that binds and inactivates both the p53 and Rb tumor suppressors (16), we asked whether Tag could also block the growth-inhibitory effect of *ING1*. The *ING1* gene was cloned in the sense orientation into the mammalian expression vector pBK, which contains a neomycin resistance gene and a cytomegalovirus promoter. The resulting construct, pBK-ING1-S, was transfected into normal HDFs (WI38) and a syngeneic line immortalized by SV40 (VA13). Following growth of the cells in antibiotic-containing medium for 3 weeks, a large number of stable transformants were recovered from VA13 cells, whereas very few colonies were seen in plates of WI38 cells transfected with pBK-ING1-S (Fig. 4). These preliminary results suggest that the SV40 Tag oncoprotein can inactivate the growth-inhibitory effect of *ING1* in a manner similar to that seen with p53 and Rb.

Suppression of *ING1* expression can extend the proliferative life span of normal fibroblasts. To determine the effects of reducing the levels of *ING1* mRNA on the replicative life span of HDFs, cells were infected with a retroviral construct carrying a 182-bp fragment in the antisense orientation representing nucleotides 942 to 1124 of the *ING1* cDNA (7). This antisense fragment effectively inhibits translation of *ING1* mRNA, as shown in a previous study where chronic expression of the antisense construct resulted in 90% inhibition of the expression of the endogenous p33^{ING1} protein in cells that had acquired the ability to grow in soft agar (7).

Young HDFs at 30 MPDs were preaged by continuous subculturing until they reached 70 MPDs. These cells were then used as recipients for retroviral infection with the vector pLNCX (as a control) or with pLNCX expressing antisense *ING1* (pLNCX-ING1-αS). The amphotropic and ecotropic packaging cells that were used for infection are capable of producing retroviruses with titers higher than 10^6 per ml upon transient transfection, which allows delivery of the retroviral construct to HDFs with efficiencies of approximately 90%, as monitored by expression of a retroviral β-galactosidase construct (data not shown).

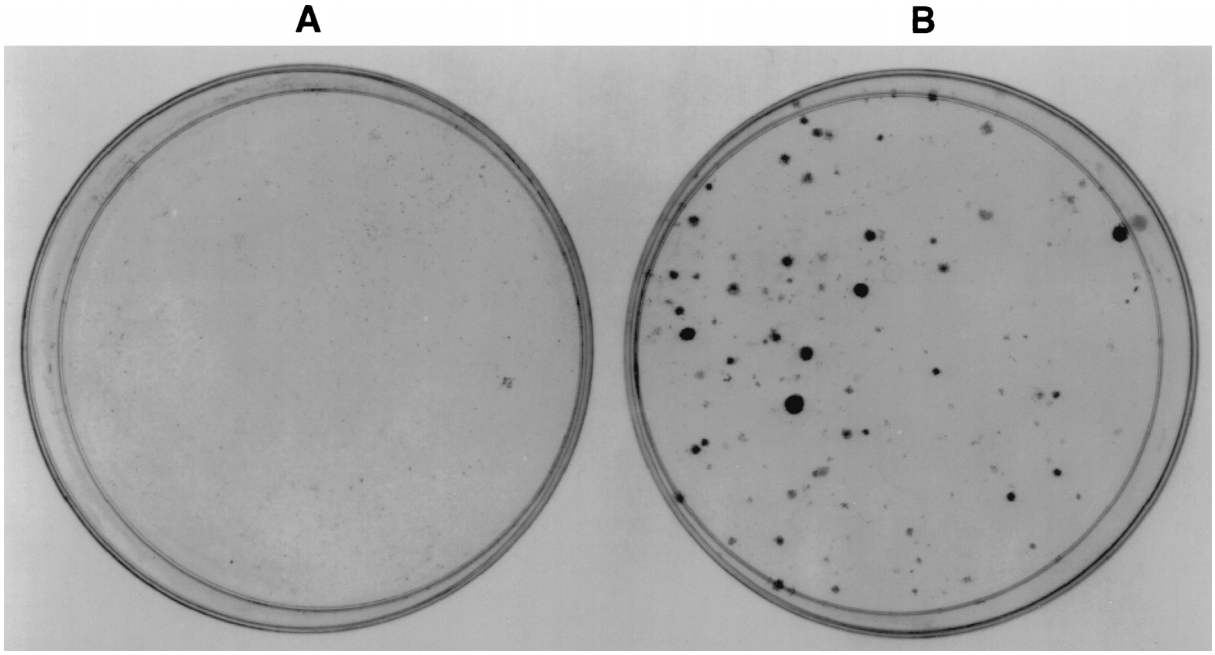

FIG. 4. Inactivation of the growth-inhibitory effect of p33^{ING1}. The normal human fibroblast strain WI38 (A) and WI38 cells immortalized with SV40 (VA13) (B) were transfected with plasmid pBK-ING1-S. Following growth for 3 weeks in medium containing G418, plates were fixed and stained with Coomassie brilliant blue to identify surviving colonies.

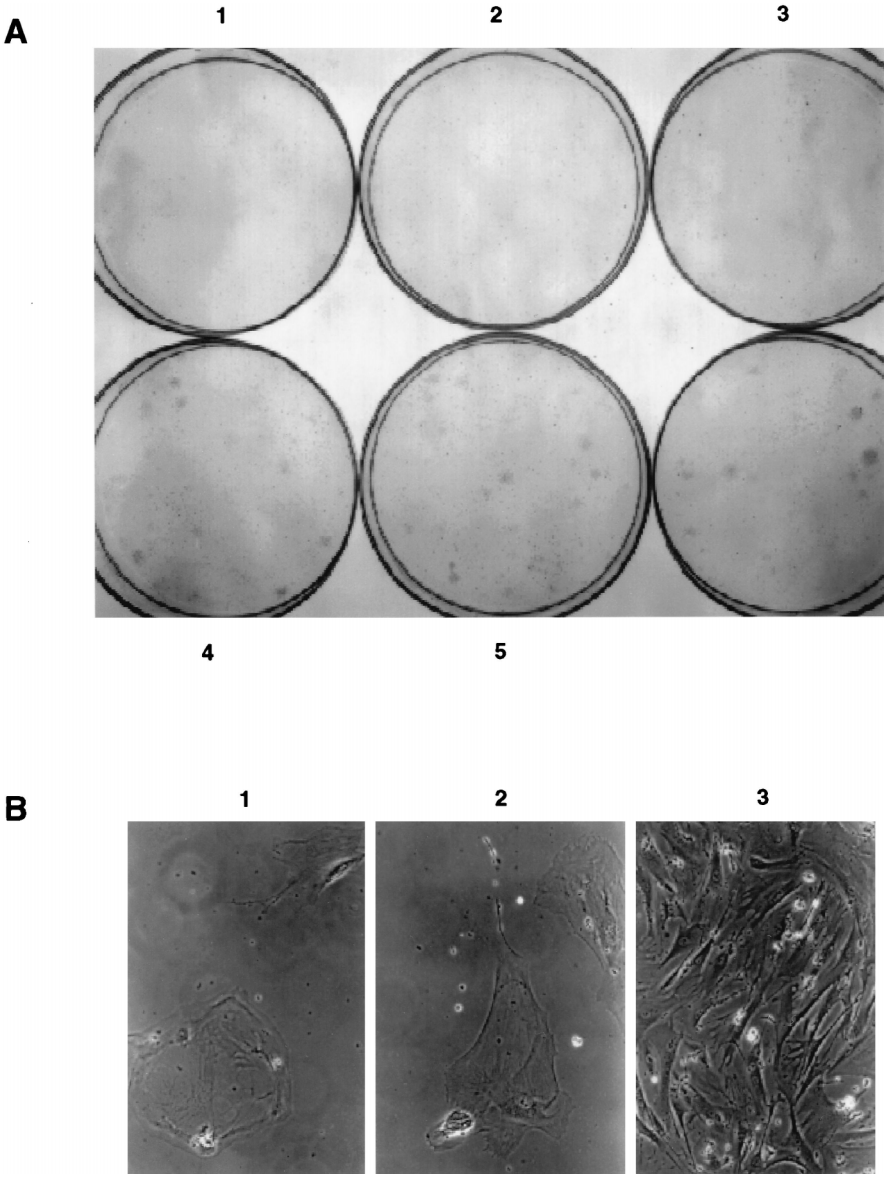

FIG. 5. Inhibition of *ING1* expression extends proliferative life span in normal diploid cells. (A) Colony formation assays with HDFs that were infected with control virus (plates 1 to 3) and with virus containing antisense *ING1* (plates 4 to 6). (B) Morphology of HDFs grown at low density and infected with pLNCX (panel 1) or with pLNCX-ING1-αS (panels 2 and 3).

Hs68 cells at 70 MPDs were infected with the retroviral vector pLNCX as a control or with pLNCX-ING1-αS (ING1-αS cells) and were subcultured in parallel with a subculturing ratio of 1:2. Infected cells were propagated for an additional 10 MPDs, after which 10^5 control and 10^5 ING1-αS cells at 80 MPDs were split among 12 10-cm plates and cultivated for 2 months, with weekly refeeding with complete medium. A small number of the cells infected with the retrovirus alone were observed to divide once or twice during this time and were rarely capable of forming colonies. In contrast, a larger number of cells containing pLNCX-ING1-αS continued to grow and created visible colonies (Fig. 5A). The morphology of fibroblasts infected with the viral vector alone is similar to the morphology of fully senescent cells (Fig. 5B).

To confirm the effect of the antisense fragment of *ING1* in cells, indirect immunofluorescence with a rabbit polyclonal antibody (7, 8) that was raised against $p33^{ING1}$ was performed. Senescent vector-infected fibroblasts and fibroblasts from colonies resulting from antisense *ING1* retrovirus infection were transferred to glass coverslips. Two days after transfer, the cells were fixed and incubated sequentially with rabbit $p33^{ING1}$ antiserum, goat anti-rabbit immunoglobulin G–biotin antibody, and streptavidin-conjugated Texas Red in order to visualize p33 protein. Staining with anti-p33 antibody was observed in the nuclear compartment of senescent cells containing the control virus (Fig. 6A) but not in cells obtained from colonies that had been infected with the antisense *ING1* retrovirus (Fig. 6B). These results corroborate our previous observation that $p33^{ING1}$ is a nuclear protein (8) and confirmed that the levels of $p33^{ING1}$ protein decrease in cells from colonies resulting from antisense *ING1* retrovirus infection (7). Similar results were seen in cells from three individual colonies and in 20

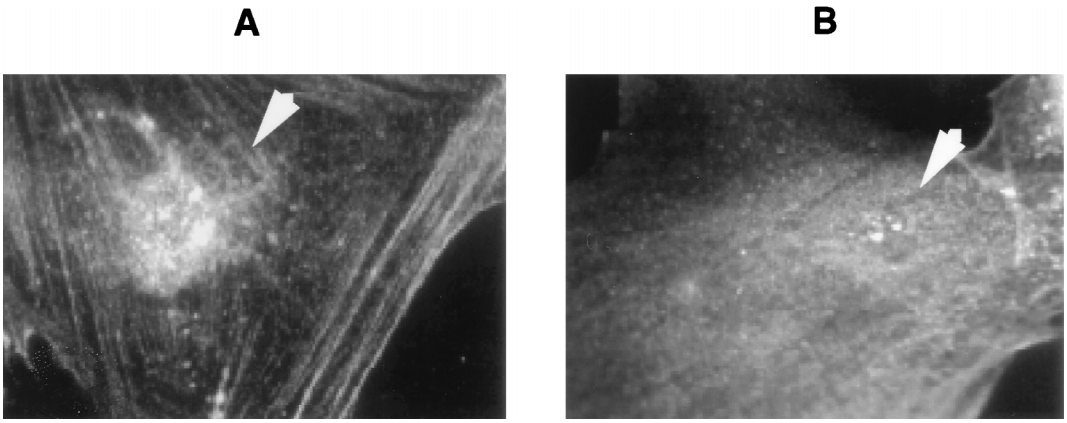

FIG. 6. Reduced levels of *ING1* gene expression in cells containing an antisense construct. Indirect immunostaining of senescent Hs68 fibroblasts infected with a control viral vector (A) or with a viral vector containing antisense *ING1* (B) is shown. The arrowheads show the staining intensity associated with infection with the vector (A) and the reduction of nuclear staining in cells containing the antisense *ING1* fragment (B).

independent senescent cells containing the control retrovirus. Levels of *ING1* in colonies expressing antisense *ING1* were also tested by RT-PCR. While the results varied considerably due to the large number of PCR cycles required to amplify *ING1* mRNA from small numbers of cells, levels were clearly reduced (data not shown), consistent with results obtained previously by this method (7).

To estimate the efficiency with which down regulation of the *ING1* gene was able to extend the proliferative life span of normal fibroblasts, the number of cells in each colony was counted. Results of these calculations are shown in Fig. 7, in which colonies are divided into four groups depending on the number of cells in the colony. Most colonies contained 100 to 159 cells; therefore, if cells divided in an arithmetic progression (2, 4, 8, . . ., n) this class corresponds to approximately seven additional MPDs ($2^7 = 128$). Colonies in the largest category (220 to 280 cells) correspond to eight cell doublings ($2^8 = 256$). Similar results were obtained in two separate trials (Fig. 7), strongly indicating that down regulation of p33^{ING1} protein is sufficient to extend the proliferative life span of normal fibroblasts by approximately 10%, as previously reported for the p53 tumor suppressor (3).

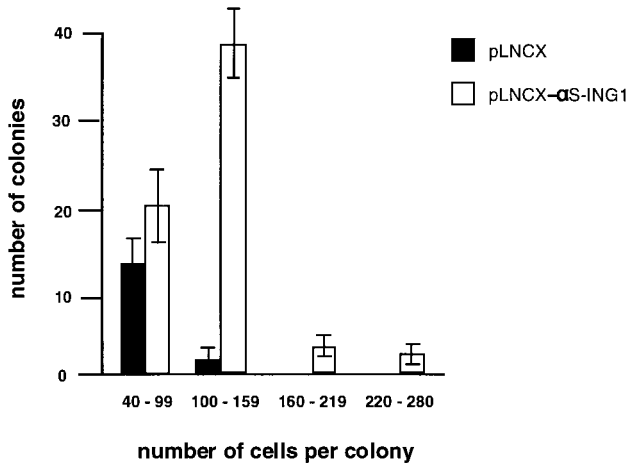

FIG. 7. Colony formation by cells in which *ING1* expression is blocked. Colonies formed after retroviral infection were divided into four groups depending on the number of cells per colony. Error bars indicate standard deviations for two separate trials.

DISCUSSION

In this study, several observations were made: (i) the levels of *ING1* mRNA and protein are severalfold higher in senescent normal fibroblasts than in young cells, (ii) p33^{ING1} protein expression is regulated through the cell cycle, (iii) the growth-inhibitory effect of *ING1* can be suppressed by the SV40 Tag oncoprotein, and (iv) repression of p33^{ING1} protein expression can extend the in vitro life span of normal fibroblasts. These results suggest a relationship between the p33^{ING1} negative growth regulator and in vitro cellular senescence.

Several hypotheses have been raised to explain the phenomenon of cellular senescence. In one model, senescence results from the accumulation of errors or genetic damage during cellular and organismal aging. In another model, which is supported by substantial molecular and cellular evidence (4, 10), senescence is a genetically controlled process in which tumor suppressors might play a major role. For example, Rb and particularly p53 are frequent targets of inactivation in a wide variety of cancer cell lines and tumor types, and inactivation of these tumor suppressors has been reported to be able to extend the proliferative life span of normal HDFs (3, 11). Conversely, the activity of these growth inhibitors has been reported to increase as primary HDFs approach the end of their in vitro life span and become senescent (1, 27).

Tumor suppressors have been shown to contribute to the regulation of the mammalian cell cycle, largely through interaction with multiple proteins including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (18). One of the major mechanisms of negative regulation of CDK activity is the binding of inhibitory subunits (26). It is interesting that the expression of certain CDK inhibitors, including p21 and p16, is 10- to 20-fold higher in senescent cells than in young cells (12, 19) and that p21 expression is induced by the p53 tumor suppressor (6). Experiments in which p16 was ectopically expressed suggest a mechanism by which members of the p16 family might inhibit cell growth by inhibiting the activity of CDK4 and CDK6 kinases through formation of p16-CDK complexes that are inactive as kinases and therefore unable to phosphorylate Rb (24). Although several CDK-regulatory mechanisms have been identified to date, our present under-

standing of the regulation of CDKs is clearly incomplete, and new components of this cellular machinery undoubtedly remain to be discovered. The limited sequence homology between $p33^{ING1}$ and retinoblastoma binding protein 2 (7) raises the possibility that $p33^{ING1}$, like p53 and Rb, interacts with cell cycle regulators. In agreement with this idea, we have found that the growth suppression associated with *ING1* overexpression is abrogated in cells infected with SV40, raising the possibility that, like p53 and Rb, $p33^{ING1}$ interacts with and is inactivated by viral oncoproteins. Although $p33^{ING1}$ can efficiently block cell growth when overexpressed and can promote growth under inappropriate conditions, the expression of *ING1* was seen to be induced upon serum stimulation of quiescent cells, peaking in the S phase of the cell cycle. Similar kinetics of serum induction are seen for the *BRCA1* and *BRCA2* (30) tumor suppressor genes.

Genes that are involved in regulating cellular aging are expected to have several characteristics. First, they should be overexpressed and/or have increased activity in senescent cells in comparison to young cells. Second, mutations in or down regulation of these genes should extend the life span of normal cells. Third, ectopic overexpression of proteins encoded by these genes should induce growth arrest in normal dividing cells. Fourth, mutations in these genes should be observed in cancer cells that have escaped the growth limitation of cellular senescence. In addition, senescence genes might be directly involved in or act through mediators of cell cycle control. To date only two tumor suppressor genes, *Rb* and *p53*, have shown features that fulfill all of the criteria mentioned above (9, 31). The data presented here also implicate *ING1* as a gene involved in the regulation of cellular senescence on the basis of these criteria. The participation of *ING1* in the program of cellular senescence may therefore represent a significant event in the complex processes that lead to cellular immortalization and the neoplastic changes that occur during tumorigenesis in vivo.

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