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Cloning of Senescent Cell-Derived Inhibitors of DNA Synthesis Using an Expression Screen

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We here describe a rapid and simple expression screen method that has allowed us to isolate cDNAs coding for inhibitors of DNA synthesis from senescent human diploid fibroblasts. The assay involved transient transcriptional overexpression of a gene product encoded by a cDNA in a proliferating cell, on the assumption that this would be sufficient to block DNA synthesis in a short-term assay using tritiated thymidine autoradiography. Three cDNAs, referred to as *s*enescent cell-*d*erived *i*nhibitors (*sdi*), that exhibit DNA synthesis-inhibitory activity when introduced into young cycling cells, were successfully identified. Expression of one of the cDNAs, *sdi1*, increased 10- to 20fold in senescent compared with young cells and the increase in RNA closely paralleled the onset of the senescent phenotype and loss of cell proliferation. *sdi1* expression was also increased in young cells made nondividing (quiescent) by deprivation of growth factors or contact inhibition. Following serum stimulation, RNA levels of *sdi1* in quiescent cells were initially increased, but then declined to low levels just prior to the entry of the cells into S phase. In contrast, RNA levels of *sdi1* in senescent cells failed to decline, suggesting a role for this gene in maintaining the senescent phenotype. The *sdi1* gene has been mapped to the p arm of chromosome 6.

INTRODUCTION

Hayflick and Moorhead [1] first described the fact that normal human fibroblasts exhibit limited proliferative potential in culture. Hayflick [2] proposed that this loss of division capability reflected what occurred in normal cells *in vivo* and that the phenomenon provided an *in vitro* model for cellular senescence. The model is now established and accepted [3]. A number of studies comparing young and senescent human cells have revealed differences in gene expression. Some of the genes that are not expressed at senescence are cell cycle-related genes, e.g., c-*fos*, cdc2, and cyclins A and B [4, 5]. However, reintroduction of these genes into senescent cells does not result in reentry into the cell cycle, indicating that some other regulatory genes are involved. A candidate for such a gene is one that codes for an inhibitor of DNA synthesis, which is expressed in senescent but not proliferating young cells. This inhibitory activity was initially detected in whole cell heterokaryons following fusion of young and senescent cells [6, 7] and also in hybrids of senescent cytoplasts and young cells [8, 9]. Other experiments indicated that the activity was mediated by a protein, which was most likely associated with surface membrane enriched preparations of cells [10, 11]; however, biochemical approaches proved unsuccessful in purifying this protein inhibitor of DNA synthesis. Since we had determined, from microinjection experiments, that senescent cells contained a large amount of DNA synthesis inhibitory mRNA [12], we used the expression screening strategy described here to identify the cDNAs involved. The use of a modified vector that was strongly expressed in young, normal human fibroblasts, coupled with high transfection efficiency and cotransfection of a marker plasmid, allowed us to clone three cDNAs from a senescent cell cDNA library. All three caused inhibition of DNA synthesis following transfection into young, proliferating cells. The mRNA level of one of the cDNAs increased 10- to 20fold in senescent compared with young cells, suggesting that it might code for the previously described DNA synthesis-inhibitory protein. The mRNA levels of the other two cDNAs were equivalent in young and senescent cells.

MATERIALS AND METHODS

Cells and culture conditions. Normal human neonatal foreskin fibroblasts HCA2, isolated in this laboratory, were grown in Eagle's minimum essential medium with either Earle's or Hanks' balanced salt solution, supplemented with 10% fetal bovine serum (FBS). Details of cell culture conditions and determination of *in vitro* lifespan of the cultures have been described previously [13]. Tritiated thymidine autoradiography was used to determine the percentage of cells capable of synthesizing DNA in cell cultures. Eighty to ninety percent of young, early passage cells synthesized DNA during a 24-h labeling with tritiated thymidine, whereas in senescent cultures <2% of the cells were labeled.

Plasmids and DNA fragments. The plasmid pcDSRα296, which includes the Okayama–Berg SV40 promoter and the LTR from

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HTLV-1 [14], was provided by Dr. M. Yoshida (University of Tokyo). After 336 bp of the *Pst*I–*Kpn*I fragment was removed, 28 bp of a *Pst*I–*Kpn*I fragment from pUC19 was inserted in its place. The resulting 3.4-kb plasmid, pcDSR$\alpha\Delta$, was used as the cloning and expression vector for these studies. For cDNA antisense expression, full-length cDNA fragments were excised by *Bam*HI digestion from pcDSR$\alpha\Delta$ and religated in the reverse direction.

pcDcat has the CAT gene inserted between the SV40 promoter and the SV40 poly(A) signal of the pcD vector [15] and was provided by Dr. H. Okayama (Osaka University). pSV2cat and pSV0cat [16] were provided by Dr. Gretchen Darlington (Texas Children's Hospital). pcDSR$\alpha\Delta$-cat was constructed by the insertion of 0.8 kb of a *Hin*dIII–*Sma*I-digested SRα promoter fragment from pcDSR$\alpha\Delta$ into *Hin*dIII-digested pSV0cat, via a two-step ligation.

pCMVβ, which carries the *Escherichia coli* β-galactosidase gene driven by the human cytomegalovirus immediate early gene promoter [17], was provided by Dr. Grant MacGregor (Baylor College of Medicine).

pβ440, which carries 443 bp of the human β-actin sequence [18], was provided by Dr. Kozo Makino (Osaka University).

pHcGAP [19], which carries a full-length human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, was obtained from the American Type Culture Collection.

cDNA library construction. Total cellular RNA was isolated by a guanidium thiocyanate/phenol method [20; RNAzol B, Biotecx Laboratory Inc.]. Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose column chromatography (Collaborative Research). Ten micrograms of poly(A)$^+$ RNA derived from senescent cells was converted to double-stranded cDNAs using RNase H$^-$ MMLV reverse transcriptase according to the instructions of the supplier (BRL) and blunt-ended by T4 polymerase treatment. The double-stranded cDNA preparations were size fractionated by agarose gel electrophoresis, and fractions of 0.5–2, 2–4.5, and 4.5–10 kb were isolated. cDNAs (2–4.5 kb) recovered from the agarose gel were directly inserted into a calf intestine alkaline phosphatase-treated *Sma*I site of pcDSR$\alpha\Delta$ and transformed into *E. coli* MC1061 or DH-1. Ampicillin-resistant colonies were picked randomly and plasmid sizes were determined to ensure that cDNA insertions of the correct size were present. Each *E. coli* colony was picked with a toothpick and combined into cDNA pools, each composed of five cDNAs. More than 400 cDNA pools were prepared, grown in 96-well microtiter plates, and stored in 14% glycerol at −70°C. *E. coli* from each cDNA pool was cultured in 200 ml LB broth and subjected to the standard method of ethidium bromide/CsCl ultracentrifugation [21] to isolate DNA. The DNA was dialyzed against TE (10 m*M* Tris, pH 8.0, 1 m*M* EDTA).

DEAE dextran-mediated transfection. Young, rapidly dividing HCA2 cells were seeded at 1×10^5 cells/35-mm tissue culture dish, 18 h prior to transfection. Transfection was performed according to Cullen [22] with minor modifications. For each transfection, 100 ng of pCMVβ and 400 ng of a cDNA pool were mixed, suspended in 190 μl of phosphate-buffered saline (PBS), and 10 μl of 10 mg/ml DEAE dextran (Pharmacia, MW ~500,000) added. pCMVβ was included as a marker to identify those cells capable of incorporating and expressing exogenous DNA. As controls, 400 ng of either the cloning vector pcDSR$\alpha\Delta$ or the plasmid pcDSR$\alpha\Delta$-cat, was transfected with pCMVβ. Young cells to be transfected were washed with PBS, DNA solutions were added, and the cells were incubated for up to 45 min at 37°C in a CO_2 incubator. Two milliliters of cell culture medium with FBS, containing 80 μM chloroquine (Sigma), was then added and incubation continued for another 2.5 h. The transfection mixture was removed and the cells were treated with 10% dimethyl sulfoxide in cell culture medium with serum for 2 min. Fresh cell culture medium with serum was then added and the cells incubated to allow for expression of the transfected DNA.

Screening for DNA synthesis-inhibitory activity. Eighteen hours after transfection, 0.5 μCi/ml of [^{3}H]thymidine was added to the cells and the incubation continued for 48 h. Cells were fixed by the addition of 25 μl of a 25% glutaraldehyde solution to the 2 ml of cell culture medium, followed by incubation for 5 min at room temperature and three washings with PBS. Immediately thereafter, the cells were treated with the X-gal reaction mixture (1 m*M* $MgCl_2$, 3 m*M* $K_4[Fe(CN)_6]$, 3 m*M* $K_3[Fe(CN)_6]$, 0.1% Triton X-100, and 1 m*M* X-gal dissolved in 0.1 *M* sodium phosphate buffer, pH 7.5, containing 10 m*M* KCl) for up to 20 min to allow light-blue staining of the transfected cells. The cells were then washed with water, dried, and processed for autoradiography using Kodak NTB nuclear track emulsion (Kodak). DNA synthesis activity in β-galactosidase-positive cells was determined. The percentage inhibition of DNA synthesis was calculated by the formula [(% labeled nuclei in cells transfected with control plasmid − % labeled nuclei in cells transfected with *s*enescent cell-derived *i*nhibitors (*sdi*) plasmid)/% labeled nuclei in cells transfected with control plasmid] × 100.

Individual cDNAs were isolated from pools that had caused inhibition of DNA synthesis and screened to identify the specific cDNA sequences having the inhibitory activity.

Microinjection. Nuclear microinjection of young cycling cells was performed as described previously [12]. Briefly, 5×10^4 cells were plated onto 22-mm-square, etched grid coverslips (Bellco) in 35-mm tissue culture dishes. Three or four days later, nuclear microinjections were performed on a minimum of 300 cells, using either pCMVβ + pcDSR$\alpha\Delta$-*sdi* or pCMVβ + pcDSR$\alpha\Delta$. The latter served as the control. Plasmids were comicroinjected at a concentration of 50 μg/ml of each DNA. Eighteen hours after microinjection, the cells were labeled with [^{3}H]thymidine for 24 h, fixed, stained with X-gal and processed for autoradiography. The percentage inhibition of DNA synthesis was calculated as above.

Northern analysis. Northern analysis was performed by standard methods using 1 μg poly(A)$^+$ RNA [23, 24]. Radioactive probes were prepared by the method of random priming and blots were hybridized according to standard procedures [23, 24]. Membranes were stripped of the labeled probe following manufacturer instructions and rehybridized with either β-actin or GAPDH. The membranes were scanned with an Ambis radioanalytic scanning system to quantitate the amount of radioactivity in the individual bands.

CAT assay. Young cycling cells were transfected with 500 ng of pcDSR$\alpha\Delta$-cat as described above. Twenty-four hours after transfection, the cells were scraped from the dish, and a CAT assay was performed as described by Gorman *et al.* [16].

DNA sequencing and sequence analysis. DNA fragments to be sequenced were subcloned into the pUC19 plasmid. Double-strand DNA sequencing was performed using the Exo-Mung deletion system (Stratagene) and Sequenase 2.0 (U.S. Biochemical) according to the manufacturer's directions. Comparison of the sequences of the *sdi* cDNAs with those in the Genbank and EMBL database was performed using the EuGene system of Baylor College of Medicine.

Chromosomal localization of the sdi1 gene. Southern analysis of normal human cells and monochromosomal hybrids was performed according to standard protocols [24]. Metaphase spreads of normal human fibroblasts were submitted to fluorescence *in situ* hybridization using *sdi1* probes and the hybridization and labeling kit (Oncor) following the manufacturer's directions.

RESULTS

Identification of a promoter expressed at high efficiency in young cells. To allow for efficient expression screening of the cDNA library, a promoter that was highly transcribed in normal human fibroblasts was required. We tested several mammalian expression vectors using CAT as a reporter gene and determined that the SRα promoter was expressed at high levels in young, cycling

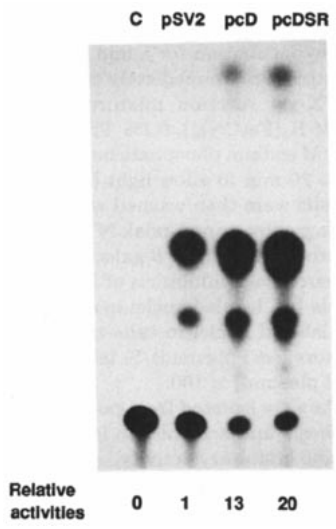

FIG. 1. Identification of a promoter efficiently transcribed in young, human cells. The expression vectors pSV2-cat, pcD-cat, and pcDSR$\alpha\Delta$-cat were transfected into young cells and relative CAT activities were calculated by normalizing to the amount of protein used for each reaction.

cells (Fig. 1). CAT transcription from the pcDSRα promoter was greater than that observed with other vectors utilizing the SV40 promoter: pSV2-cat and pcD-cat (Fig. 1). The difference in promoter activities was most likely due to the fact that the pcD plasmid was derived from pSV2 by deletion of extra ATG and leader sequences and the pcDSRα was derived by the insertion of the HTLV-1 LTR enhancer into the pcD vector. The plasmid pcDSR$\alpha\Delta$ was therefore utilized for expression of the cDNAs.

cDNA cloning of the senescent cell-derived inhibitors of DNA synthesis. Pools of cDNAs were cotransfected with a marker plasmid pCMVβ, to allow for the identification of transfected cells in which DNA synthesis could be determined. The marker plasmid was required because the frequency of transfection varied, with a range of 30–90%. After cotransfections with pCMVβ and either the plasmid pcDSR$\alpha\Delta$ or pools of cDNA negative for DNA synthesis inhibitory activity, most of the β-galactosidase-producing cells were able to synthesize DNA (Fig. 2A). However, cells cotransfected with pCMVβ and a cDNA pool positive for inhibition resulted in only a small number of blue cells with labeled nuclei (Fig. 2B). This difference in nuclear labeling provided a relatively quick screen for cDNA pools positive for DNA synthesis inhibitory activity. Screening of 200 cDNA pools led to the identification of three that were positive for DNA synthesis inhibition through five repeated transfections. The individual cDNAs from these candidate pools were isolated and further screened. Only one plasmid in each cDNA pool (A–C) was capable of inhibiting DNA synthesis (Figs. 3A–3C). These cDNA sequences were designated senescent cell-derived inhibitors, *sdi1, -2,* and *-3.* The size of each cDNA insert was determined by agarose gel electrophoresis. They were approximately 2.1, 1.2, and 2.7 kb, respectively.

To eliminate the possibility that nonspecific DNA synthesis inhibition would be caused by any full-length cDNA expressed at a high level, we constructed the plasmid pcDSR$\alpha\Delta$-cat. When this was transfected along with pCMVβ into young, proliferation-competent cells, no inhibition of DNA synthesis was observed (Fig. 3D). As an additional control, antisense expression vectors of *sdi1* and *sdi2* sequences were transfected into young cells and no inhibition or enhancement of DNA synthesis was observed (Fig. 3D). These results indicate that the effect of the *sdi*s are sequence orientation specific and not due to general perturbances in the cell.

Microinjection of sdi sequences into young cycling cells. To verify the functional activity of *sdi* sequences and to eliminate any possible artifact due to DEAE dex-

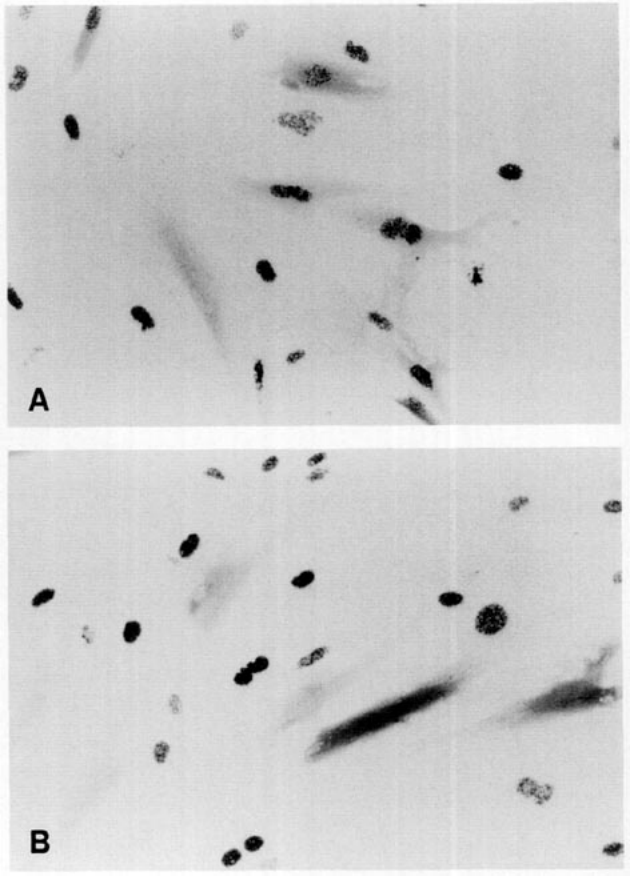

FIG. 2. Assay for DNA synthesis inhibitory activity. Young (PD28) HCA2 cells were transfected as described under Materials and Methods section. (A) Cells cotransfected with pCMVβ and pcDSR$\alpha\Delta$. DNA synthesis is indicated by dense silver deposits over nuclei. (B) Cells cotransfected with pCMVβ and a cDNA pool containing pcDSR$\alpha\Delta$-*sdi1.*

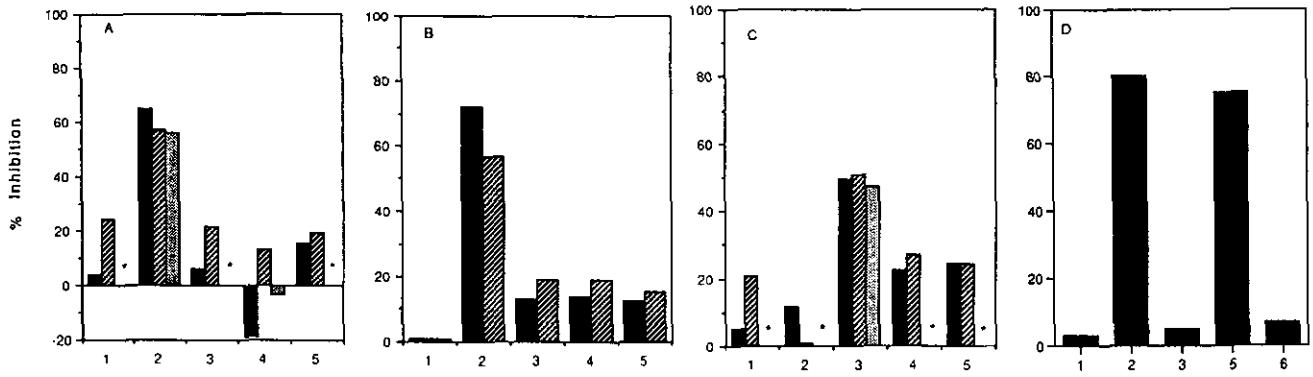

FIG. 3. Identification of cDNA clones with DNA synthesis-inhibitory activity. Individual plasmids from the cDNA pools (A, B, and C), which tested positive for inhibitory activity in the initial screenings, were transfected into young, cycling human cells. For each pool, the numbers 1 to 5 represent the individual cDNAs and the bars represent independent transfection experiments. A negative number reflects DNA synthesis greater than control. (D) pcDSR$\alpha\Delta$-cat or antisense cDNA expression plasmids were cotransfected with pCMVβ into young cells and inhibition of DNA synthesis was determined. Lane 1, control pcDSR$\alpha\Delta$-cat; lane 2, pcDSR$\alpha\Delta$-*sdi1;* lane 3, pcDSR$\alpha\Delta$-anti*sdi1;* lane 4, pcDSR$\alpha\Delta$-*sdi2;* lane 5, pcDSR$\alpha\Delta$-anti*sdi2.*

tran transfection, microinjections were also performed. pcDSR$\alpha\Delta$-*sdi1* or pcDSR$\alpha\Delta$-*sdi2* were comicroinjected with pCMVβ into the nuclei of young cycling cells and DNA synthesis inhibition in blue cells determined. The cDNAs caused strong inhibition of DNA synthesis in comparison with the vector pcDSR$\alpha\Delta$ (Table 1) although the latter did cause a minor inhibition of DNA synthesis compared to uninjected cells. Similar observations had been made in transfection experiments. Microinjection of pcDSR$\alpha\Delta$-*sdi3* was not performed because the inhibitory activity was lower and more variable than that of the *sdi1* and *sdi2* cDNAs in transfection experiments.

Expression of sdi mRNAs during cellular senescence. Changes in *sdi* mRNA expression during cellular senescence was examined by Northern analysis. One microgram of poly(A)$^+$ RNA from young or senescent cells was sequentially hybridized to ^{32}P-labeled *sdi* cDNAs, β-actin, and GAPDH. The *sdi1* probe hybridized to a 2.1-kb cellular transcript, *sdi2* to a 1.4-kb transcript, and *sdi3* to a 2.7-kb transcript (Fig. 4A). *sdi1* was found to increase significantly at senescence, whereas expression of *sdi2* and *sdi3* was the same in young and senescent cells. The expression of β-actin and GAPDH, respectively, was equivalent in young and senescent cells [5]. Since *sdi1* was the only gene that was overexpressed in senescent cells, we chose to concentrate on its characterization.

Northern analysis, using the full-length cDNA as a probe, determined that levels of the 2.1-kb *sdi1* transcript increased about 20-fold during the *in vitro* lifespan of the cells. While the increase was gradual, the major change in mRNA level occurred during the final few population doublings (Fig. 4B) of the culture, corre-

TABLE 1

Microinjection of Various Plasmids Into Young Cycling Cells

Plasmids injected	No. of cells injected	No. of labeled nuclei per total No. blue cells[a]	Labeling index (%)	% Inhibition
Exp+ 1				
pCMVβ +pcDSR$\alpha\Delta$[b]	335	58/97	59.8	0
pCMVβ +pcDSR$\alpha\Delta$-*sdi1*	380	20/89	22.5	62.4
pCMVβ +pcDSR$\alpha\Delta$-*sdi2*	380	6/82	7.3	87.8
Exp+ 2				
pCMVβ +pcDSR$\alpha\Delta$[b]	423	68/109	62.3	0
pCMVβ +pcDSR$\alpha\Delta$-*sdi1*	465	26/98	26.5	57.5
pCMVβ +pcDSR$\alpha\Delta$-*sdi2*	475	27/118	22.9	63.2

Note. The concentration of each DNA was 50 μg/ml. Nuclear microinjection of young, cycling cells was performed as described previously [12].

[a] This is the number of cells expressing detectable levels of β-galactosidase.

[b] Control plasmid.

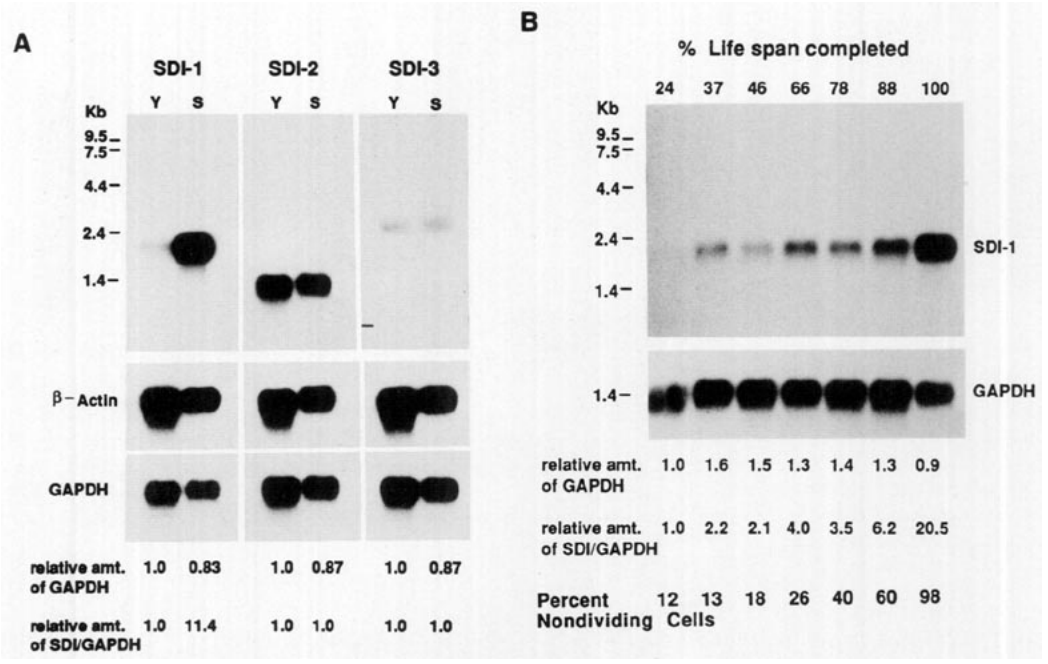

FIG. 4. Northern analysis of *sdi* gene expression in young and senescent cells. (A) One microgram of poly(A)$^+$ RNA from young (Y) and senescent (S) cells was used for Northern analysis. The relative amount of *sdi* mRNA in each sample was calculated as the ratio of *sdi*/GAPDH. (B) Northern blot of 1 μg poly(A)$^+$ RNA isolated from cells at different population doublings during serial subculture to senescence were probed sequentially with *sdi1* and GAPDH (as a loading control) cDNAs. The relative amount of *sdi1* mRNA in each sample was determined as the ratio of *sdi1*/GAPDH. The percentage of *in vitro* lifespan completed and the percentage of nondividing cells are indicated for each sample.

lating closely with expression of the senescent phenotype and loss of ability to synthesize DNA and divide.

sdi1 RNA increases as young cells become growth arrested. Since previous studies had demonstrated that young cells made nondividing by either serum deprivation or contact inhibition also produced an inhibitor of DNA synthesis, we examined *sdi1* expression in quiescent cells. Total RNA was harvested from cells maintained for up to 3 weeks in medium containing low serum, and the amount of *sdi1* message was determined by Northern analysis. *sdi1* expression increased significantly in quiescent cells compared with actively proliferating cells and the increase was similar to that observed in senescent cells (Fig. 5A). Increased expression of *sdi1* was also observed in nondividing, contact-inhibited cells that were grown to confluence in the presence of 10% FBS (Fig. 5B).

sdi1 expression is modulated during transition from G_0 to S phase. To determine whether *sdi1* was regulated as cells traversed the cell cycle, we examined expression of the gene in quiescent early passage cells synchronized in G_0 by deprivation of serum growth factors for 7 days and then stimulated to enter the cell cycle by the addition of serum. Quiescent cells expressed high levels of *sdi1* mRNA prior to serum stimulation. Following the addition of serum, mRNA levels rose, increasing about twofold at 4 h and then declining to the low levels observed in actively growing cells by 16 h, just prior to entry into S phase (Fig. 6).

sdi1 mRNA in senescent cells remains high following serum stimulation. mRNA was isolated from senescent cells deprived of growth factors by maintenance on 0.5% FBS for a week and following stimulation of the cells with 10% FBS. *sdi1* mRNA levels increased about 4-fold in senescent cells after a week in 0.5% FBS (Fig. 6). Addition of medium containing 10% FBS to the cells resulted in a transient increase of *sdi1* RNA level followed by a decrease (Fig. 6). Although the pattern of *sdi1* RNA response to serum stimulation was similar in young and senescent cells, the level of the RNA in senescent cells never fell below that observed in these cells constantly maintained in 10% serum (Fig. 6), which was 10- to 20-fold higher than that observed in young rapidly dividing cells (Fig. 6). Therefore, the inability of senescent cells to downregulate *sdi1* message may be a major contributing factor in their failure to initiate DNA synthesis.

sdi1 codes for a novel gene product. The full-length cDNA sequence of *sdi1* was determined and the protein sequence was deduced from it (Fig. 7). An examination of DNA sequence databases revealed no significant sequence similarity to any other known genes except for two "Expressed Sequence Tag" cDNAs, from more than 2000 random sequences obtained from a human

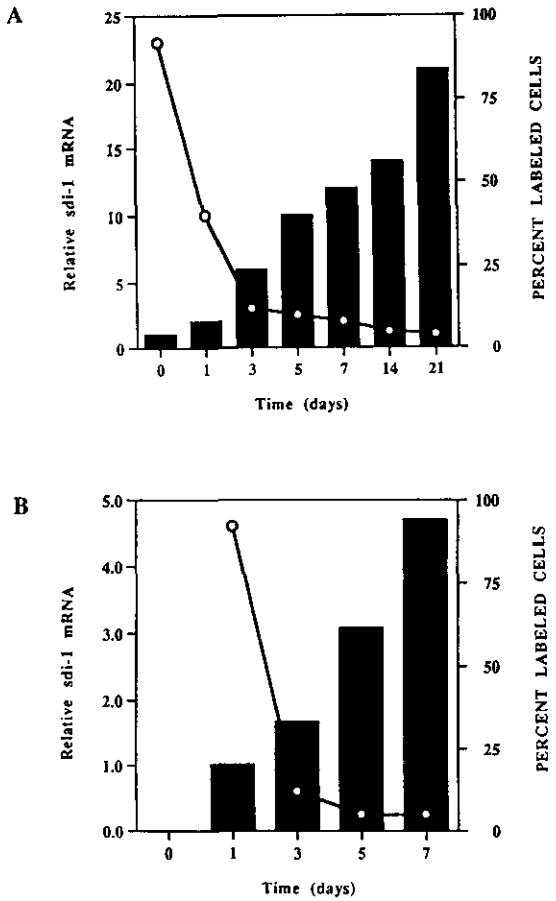

FIG. 5. *sdi1* RNA levels in proliferating and growth arrested young cells. (A) Young, proliferating cells were subcultured into medium with 10% FBS. Six hours later they were changed to and maintained in medium with 0.5% FBS. At the indicated times RNA was harvested and subjected to Northern analysis. The bands were quantitated as described under Materials and Methods and the *sdi1*/GAPDH ratio at each time point was normalized to the *sdi1*/GAPDH ratio of rapidly proliferating cells (bars). The percentage of cells synthesizing DNA at each time point (circles) was determined as described under Materials and Methods. (B) The same as (A) except cells were maintained in medium with 10% FBS.

brain cDNA library ([25] GenBank accession No. M79002, [26] GenBank accession No. T08389). One of the EST sequences corresponds to a portion of the 3′ noncoding region of the *sdi1* gene, while the other is 171 bp of the 5′ coding region. The longest open reading frame (ORF) of *sdi1*, beginning with a methionine codon at nucleotide 79 within a Kozak consensus sequence [27], codes for a protein of 164 amino acids, with a calculated molecular weight of 18 kDa. When transcribed and translated *in vitro* a product of about 21 kDa was observed by SDS–polyacrylamide gel electrophroetic analysis (data not shown). Although there is no in-frame termination codon preceding the putative initiation codon, *sdi1* mRNA detected by Northern analysis (Fig. 4A) is approximately 2.1 kb, indicating that the *sdi1* cDNA (2109 bp) is likely full-length. Furthermore, polyclonal antisera were obtained against a bacterially produced histidine-tagged fusion protein containing 12 amino acids (Met–Arg–Gly–Ser–6XHis–Gly–Ala) in front of the putative ORF *sdi1* sequence. Western analysis of cell extracts from human cells compared with the histidine-tagged protein demonstrated bands at the appropriate MW, indicating that the cDNA contained the entire coding sequence for the Sdi1 protein (data not shown). The predicted amino acid sequence does not display significant similarity to any other known protein sequences in the database, suggesting that Sdi1 may be a novel inhibitor of DNA synthesis. The sequence of *sdi1* has been deposited in GenBank under accession No. L26165.

Chromosomal localization of sdi1. Genomic DNA isolated from normal human fibroblasts and a battery of monochromosomal hybrids composed of a single human chromosome in the mouse A9 or hamster CHO background was submitted to Southern analysis, using *sdi1* cDNA as a probe. Following *Eco*RI digestion, two bands at 5 and 10 kb were detected in the normal human cells (Fig. 8A). Similar bands were observed only in the hybrid cell line that contained a human chromosome 6 (Fig. 8A). No bands were detected in any of the other monochromosomal hybrid cell lines or in the hamster and A9 cells that were the background for the human chromosomes (Fig. 8A). The 5-kb band was excised from a gel and cloned into pBluescript and the resulting colonies were screened with the *sdi1* cDNA. A positive genomic clone and the *sdi1* cDNA were biotin labeled

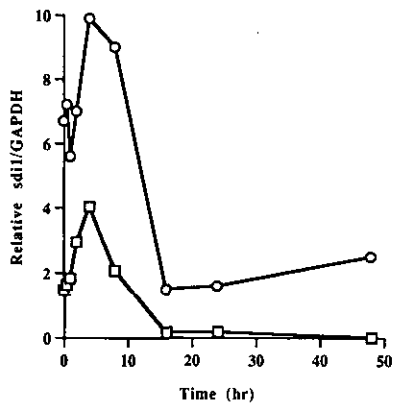

FIG. 6. Northern analysis of *sdi1* gene expression in young and senescent human cells following mitogen stimulation. Young (PD28) (□) and senescent (PD86) (○) HCA2 cells were seeded into 150-cm^2 flasks in medium containing 10% FBS. Six hours later, the medium was replaced with medium containing 0.5% serum. Seven days later, the cultures were stimulated with fresh medium containing 10% FBS. RNA was extracted at the indicated times and subjected to Northern analysis. The membrane was probed sequentially with *sdi1* and GAPDH ^{32}P-labeled cDNA. Quantitation was performed as described and the ratio of *sdi1*/GAPDH intensity plotted for the given times after serum stimulation.

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   1                                                        CCTGCCGAAGTCAGTTCCT
  20 TGTGGAGCCGGAGCTGGGCGCGGATTCGCCGAGGCACCGAGGCACTCAGAGGAGGCGCC
  79 ATG TCA GAA CCG GCT GGG GAT GTC CGT CAG AAC CCA TGC GGC AGC
   1 Met Ser Glu Pro Ala Gly Asp Val Arg Gln Asn Pro Cys Gly Ser

 124 AAG GCC TGC CGC CGC CTC TTC GGC CCA GTG GAC AGC GAG CAG CTG
  16 Lys Ala Cys Arg Arg Leu Phe Gly Pro Val Asp Ser Glu Gln Leu

 169 AGC CGC GAC TGT GAT GCG CTA ATG GCG GGC TGC ATC CAG GAG GCC
  31 Ser Arg Asp Cys Asp Ala Leu Met Ala Gly Cys Ile Gln Glu Ala

 214 CGT GAG CGA TGG AAC TTC GAC TTT GTC ACC GAG ACA CCA CTG GAG
  46 Arg Glu Arg Trp Asn Phe Asp Phe Val Thr Glu Thr Pro Leu Glu

 259 GGT GAC TTC GCC TGG GAG CGT GTG CGG GGC CTT GGC CTG CCC AAG
  61 Gly Asp Phe Ala Trp Glu Arg Val Arg Gly Leu Gly Leu Pro Lys

 304 CTC TAC CTT CCC ACG GGG CCC CGG CGA GGC CGG GAT GAG TTG GGA
  76 Leu Tyr Leu Pro Thr Gly Pro Arg Arg Gly Arg Asp Glu Leu Gly

 349 GGA GGC AGG CGG CCT GGC ACC TCA CCT GCT CTG CTG CAG GGG ACA
  91 Gly Gly Arg Arg Pro Gly Thr Ser Pro Ala Leu Leu Gln Gly Thr

 394 GCA GAG GAA GAC CAT GTG GAC CTG TCA CTG TCT TGT ACC CTT GTG
 106 Ala Glu Glu Asp His Val Asp Leu Ser Leu Ser Cys Thr Leu Val

 439 CCT CGC TCA GGG GAG CAG GCT GAA GGG TCC CCA GGT GGA CCT GGA
 121 Pro Arg Ser Gly Glu Gln Ala Glu Gly Ser Pro Gly Gly Pro Gly

 484 GAC TCT CAG GGT CGA AAA CGG CGG CAG ACC AGC ATG ACA GAT TTC
 136 Asp Ser Gln Gly Arg Lys Arg Arg Gln Thr Ser Met Thr Asp Phe

 529 TAC CAC TCC AAA CGC CGG CTG ATC TTC TCC AAG AGG AAG CCC TAA
 151 Tyr His Ser Lys Arg Arg Leu Ile Phe Ser Lys Arg Lys Pro ...

 574 TCCGCCCACAGGAAGCCTGCAGTCCTGGAAGCGCGAGGGCCTCAAAGGCCCGCTCTACA
 633 TCTTCTGCCTTAGTCTCAGTTTGTGTGTCTTAATTATTATTTGTGTTTTAATTTAAACA
 692 CCTCCTCATGTACATACCCTGGCCGCCCCCTGCCCCCCAGCCTCTGGCATTAGAATTAT
 751 TTAAACAAAAACTAGGCGGTTGAATGAGAGGTTCCTAAGAGTGCTGGGCATTTTTATTT
 810 TATGAAATACTATTTAAAGCCTCCTCATCCCGTGTTCTCCTTTTCCTCTCTCCCGGAGG
 869 TTGGGTGGGCCGGCTTCATGCCAGCTACTTCCTCCTCCCCACTTGTCCGCTGGGTGGTA
 928 CCCTCTGGAGGGGTGTGGCTCCTTCCCATCGCTGTCACAGGCGGTTATGAAATTCACCC
 987 CCTTTCCTGGACACTCAGACCTGAATTCTTTTTCATTTGAGAAGTAAACAGATGGCACT
1046 TTGAAGGGGCCTCACCGAGTGGGGGCATCATCAAAAACTTTGGAGTCCCCTCACCTCCT
1105 CTAAGGTTGGGCAGGGTGACCCTGAAGTGAGCACAGCCTAGGGCTGAGCTGGGGACCTG
1164 GTACCCTCCTGGCTCTTGATACCCCCCTCTGTCTTGTGAAGGCAGGGGGAAGGTGGGGT
1223 CCTGGAGCAGACCACCCCGCCTGCCCTCATGGCCCCTCTGACCTGCACTGGGGAGCCCG
1282 TCTCAGTGTTGAGCCTTTTCCCTCTTTGGCTCCCCTGTACCTTTTGAGGAGCCCCAGCT
1341 ACCCTTCTTCTCCAGCTGGGCTCTGCAATTCCCCTCTGCTGCTGTCCCTCCCCCTTGTC
1400 CTTTCCCTTCAGTACCCTCTCAGCTCCAGGTGGCTCTGAGGTGCCTGTCCCACCCCCAC
1459 CCCCAGCTCAATGGACTGGAAGGGGAAGGGACACACAAGAAGAAGGGCACCCTAGTTCT
1518 ACCTCAGGCAGCTCAAGCAGCGACCGCCCCCTCCTCTAGCTGTGGGGGTGAGGGTCCCA
1577 TGTGGTGGCACAGGCCCCCTTGAGTGGGGTTATCTCTGTGTTAGGGGTATATGATGGGG
1636 GAGTAGATCTTTCTAGGAGGGAGACACTGGCCCCTCAAATCGTCCAGCGACCTTCCTCA
1695 TCCACCCCATCCCTCCCCAGTTCATTGCACTTTGATTAGCAGCGGAACAAGGAGTCAGA
1754 CATTTTAAGATGGTGGCAGTAGAGGCTATGGACAGGGCATGCCACGTGGGCTCATATGG
1813 GGCTGGGAGTAGTTGTCTTTCCTGGCACTAACGTTGAGCCCCTGGAGGCACTGAAGTGC
1872 TTAGTGTACTTGGAGTATTGGGGTCTGACCCCAAACACCTTCCAGCTCCTGTAACATAC
1931 TGGCCTGGACTGTTTTCTCTCGGCTCCCCATGTGTCCTGGTTCCCGTTTCTCCACCTAG
1990 ACTGTAAACCTCTCGAGGGCAGGGACCACACCCTGTACTGTTCTGTGTCTTTCACAGCT
2049 CCTCCCACAATGCTGATATACAGCAGGTGCTCAATAAACGATTCTTAGTGAAAAAAAA
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FIG. 7. Primary nucleotide sequence and predicted translation product of *sdi1* cDNA. The putative translation initiation condon within a Kozak consensus sequence [27] and polyadenylation site are underlined.

and hybridized to metaphase spreads of normal human fibroblasts. Fluorescein-labeled avidin detected the hybridized probe on the p arm of chromosome 6 (Fig. 8B). Chromosome X is cytogenetically very similar to chromosome 6. However, it was eliminated as a candidate for the location of the *sdi1* gene because Southern analysis of DNA from a cell line containing chromosome X in the A9 background, probed with *sdi1* cDNA, revealed no hybridizing bands (Fig. 8A).

DISCUSSION

We here describe the molecular cloning of DNA synthesis-inhibitory sequences present in senescent human diploid fibroblasts using expression screening, the most direct way to identify desired inhibitor genes. However, there are some potential problems with this approach. For example, introduction of large amounts of plasmid DNA or continued overexpression of genes not involved in the cell cycle, such as housekeeping genes, could cause depletion of the transcriptional and translational machinery, resulting in inhibition of DNA synthesis in the transfected cells. In fact, in the case of cells transfected with the plasmid, pcDSR$\alpha\Delta$, the labeling index was sometimes 10–20% lower than that of mock-transfected cells. We attributed this minor, nonspecific inhibition to the fact that a large amount of DNA was being introduced. However, to be certain that transfection of any gene in the expression vector pcDSR$\alpha\Delta$ would not have a major DNA synthesis inhibitory effect, we examined DNA synthesis in cells transfected with the plasmid pcDSR$\alpha\Delta$-cat and found that it was comparable to that in cells transfected with the plasmid pcDSR$\alpha\Delta$. This indicates that nonspecific inhibition of DNA synthesis did not occur in our assay and that the transcriptional and translational machinery of the cell was sufficient to maintain normal metabolism, even when an exogenous gene expressed from a strong promoter was introduced into these cells. In addition, the *sdi* sequences were functional only in a positive orientation, as antisense constructs were not inhibitory.

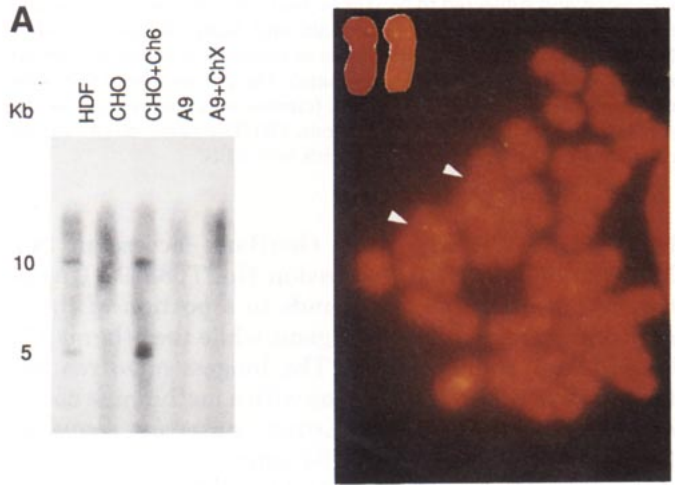

FIG. 8. Chromosomal localization of the *sdi1* gene. (A) Genomic DNA from the cells was digested with *Eco*RI and run on 0.7% agarose gels. Following transfer to membrane, the DNA was hybridized with *sdi1* cDNA. (B) Metaphase spread of HCA2 cells hybridized to biotin-labeled 5-kb *sdi1* genomic clone plus the biotin-labeled 2.1-kb *sdi1* cDNA and detected by fluorescein-labeled avidin. Arrowheads indicate the *sdi1* signal on the short arm of chromosome 6.

In order for expression screening for inhibitors of DNA synthesis to be practical, efficient transfection and a rapid assay for DNA synthesis are important requisites. We here describe conditions for DEAE dextran-mediated transfection that resulted in transient expression in a high percentage of young, cycling human cells. The problem of variation in transfection frequencies was overcome by cotransfection of a marker plasmid that allowed us to determine DNA synthesis in only transfected cells. We had found that coexpression of two introduced plasmids occurred in 90% of cells transfected with pCMVβ and an SV40 T-antigen-expressing plasmid, pBSV-1 (data not shown). Expression of these gene products was monitored by double-label immunofluorescence staining for SV40 T antigen and β-galactosidase. Another factor that aided in the success of this screening approach was the division of the cDNA library into pools, composed of five independent cDNA clones, to increase the relative amount of any cDNA coding for a DNA synthesis inhibitor.

Northern blot analyses revealed that the size of the cellular transcripts of the *sdis* were compatible with the size of the cDNAs and that expression levels of *sdi2* and *-3* remained unchanged throughout the *in vitro* life span. In contrast, *sdi1* message increased up to 20-fold as cells became senescent. Based on the results of previous microinjection studies we estimated that the abundance of DNA synthesis-inhibitory mRNA in senescent cells was 0.1–1% of the total mRNA present [12]. When the original library was probed with *sdi1*, this sequence was found to be present at a frequency of 5×10^{-2} to 10^{-3} in the represented cDNAs, which is in line with what was predicted from the microinjection studies. These results suggest that *sdi1* may code for the previously described senescent cell inhibitor of DNA synthesis [10, 11], whereas *sdi2* and *sdi3* are more general inhibitors, perhaps involved in metabolic functions of the cell.

When young or senescent cells are placed under conditions unfavorable for proliferation, such as decreased serum growth factors or growth to high density, *sdi1* RNA increases. However, when 10% fetal bovine serum is added to serum-deprived young cell cultures, *sdi1* RNA decreases to low levels before the onset of DNA synthesis. While the *sdi1* RNA level in senescent cells decreases following serum stimulation, it never falls below the level found in low serum-arrested young cells. It is tempting to speculate that the normal cellular function of *sdi1* is to act as a negative regulator of cell proliferation. In young cells, the expression is modulated according to availability of growth factors and other external signals. It is possible that the inability of senescent cells to sufficiently downregulate *sdi1* is responsible for their inability to synthesize DNA.

The localization of the *sdi1* gene to the p arm of chromosome 6 is of interest because this chromosome has been implicated in immortalization of SV40-transformed cells (Ozer, personal communication). However, the studies of Ozer's group indicate that the q arm of the chromosome carries the gene related to immortalization. As various growth-regulatory genes are cloned, their role in cellular senescence versus cell cycle control will become evident as well as the manner in which they interact with each other.

In summary, we have successfully cloned three inhibitors of DNA synthesis from senescent cells using a functional assay. Their role in senescence has yet to be determined. However, it should be possible to apply this method to clone other negative regulatory genes, such as those involved in tissue-specific differentiation and tumor suppression.

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