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Diverse Gene Sequences Are Overexpressed in Werner Syndrome Fibroblasts Undergoing Premature Replicative Senescence

SHUNICHI MURANO,† RAY THWEATT, ROBERT J. SHMOOKLER REIS, RICHARD A. JONES, ELENA J. MOERMAN, AND SAMUEL GOLDSTEIN*

Departments of Medicine and Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, and Geriatric Research Education and Clinical Center, John L. McClellan Memorial Veterans Hospital, Little Rock, Arkansas 72205

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Genes that play a role in the senescent arrest of cellular replication are likely to be overexpressed in human diploid fibroblasts (HDF) derived from subjects with Werner syndrome (WS) because these cells have a severely curtailed replicative life span. To identify some of these genes, a cDNA library was constructed from WS HDF after they had been serum depleted and repleted (5 days in medium containing 1% serum followed by 24 h in medium containing 20% serum). Differential screening of 7,500 colonies revealed 102 clones that hybridized preferentially with [^{32}P]cDNA derived from RNA of WS cells compared with [^{32}P]cDNA derived from normal HDF. Cross-hybridization and partial DNA sequence determination identified 18 independent gene sequences, 9 of them known and 9 unknown. The known genes included α1(I) procollagen, α2(I) procollagen, fibronectin, ferritin heavy chain, insulinlike growth factor-binding protein-3 (IGFBP-3), osteonectin, human tissue plasminogen activator inhibitor type 1, thrombospondin, and αB-crystallin. The nine unknown clones included two novel gene sequences and seven additional sequences that contained both novel segments and the *Alu* class of repetitive short interspersed nuclear elements; five of these seven *Alu*$^{+}$ clones also contained the long interspersed nuclear element 1 (*Kpn*I) family of repetitive elements. Northern (RNA) analysis, using the 18 sequences as probes, showed higher levels of these mRNAs in WS HDF than in normal HDF. Five selected mRNAs studied in greater detail [α1(I) procollagen, fibronectin, insulinlike growth factor-binding protein-3, WS3-10, and WS9-14] showed higher mRNA levels in both WS and late-passage normal HDF than in early-passage normal HDF at various intervals following serum depletion/repletion and after subculture and growth from sparse to high-density confluent arrest. These results indicate that senescence of both WS and normal HDF is accompanied by overexpression of similar sets of diverse genes which may play a role in the senescent arrest of cellular replication and in the genesis of WS, normal biological aging, and attendant diseases.

Biological aging, an inevitable process in multicellular organisms, features two sets of interdependent events: functional decline and an exponential rise in the incidence of degenerative and neoplastic diseases (27). The mechanism of aging remains unknown, but it seems clear that the progressive loss of cellular replicative capacity in many tissues is inextricably involved (26, 27, 51, 55). Human diploid fibroblasts (HDF) have a limited replicative life span and provide a valuable model for studies of replicative senescence (14, 36). Earlier studies have pursued the error catastrophe and genetic damage hypotheses as possible mechanisms, but little evidence has been marshalled to support these ideas. On the other hand, the concept of a genetically programmed, quasidifferentiative process has increasingly been invoked for several reasons (28). First, senescence of HDF is linked to the number of times the cell population doubles up to a critical limit rather than to the metabolic or calendar time (see reference 28 and references therein). Second, cell fusion experiments involving heterokaryons, hybrid cells containing a late-passage senescent (old) nucleus and an early-passage proliferation competent (young) nucleus in a single cytoplasm, clearly demonstrate that the old cell has a dominant inhibitory effect on nuclear DNA synthesis in the young cell. Additionally, this inhibition can be abolished by drugs which block protein and RNA synthesis (55). Third, microinjection of mRNA from senescent cells into young HDF can inhibit DNA synthesis (47). Fourth, the life span of HDF has been divided into seven stages, three mitotic and four postmitotic, on the basis of morphology and protein profiles identified by two-dimensional gel chromatography (4). In total, the data support the concept that senescence of HDF resembles cytodifferentiation and is effected via elaboration of one or more proteins which inhibit DNA synthesis.

To pursue the concept that dominant, negative growth-regulatory genes are expressed during HDF senescence, we elected to study HDF from a subject with Werner syndrome (WS), a rare inherited disorder. WS features impaired somatic growth, a readily discernible phenotype of premature aging and functional decline, coupled with the early appearance of a variety of severe age-related pathologies such as atherosclerosis, malignancy, osteoporosis, soft tissue calcification, insulin-resistant diabetes mellitus, lenticular cataracts, and skin atrophy and ulceration (22). WS HDF grow more slowly than normal cells, develop the senescent morphology earlier, and display a severe reduction in their replicative life span compared with age-matched controls (26, 51, 64). Because of this premature replicative senescence and the dominance of WS cells in cell fusion hybrids with young normal HDF (75; see also reference 30), we used WS fibroblasts to construct a cDNA library with the rationale that these cells might overexpress one or more senescence-specific, potentially growth-inhibitory genes which we

* Corresponding author.
† Present address: Second Department of Internal Medicine, Chiba University School of Medicine 1-8-1, Inohana, Chiba City, 280 Japan.

could then identify more readily. Moreover, since the genetic, biochemical, and metabolic consequences of many inherited mutant genes are expressed in cultured HDF (4a), we also reasoned that identification of any overexpressed genes might provide valuable insights into the pathogenesis of the WS phenotype and the diseases that accompany normal aging. We describe here the construction of a cDNA library from WS cells followed by the isolation and characterization of a diverse array of overexpressed cDNA clones identified by differential hybridization of this library with total cellular cDNAs prepared from either WS or normal HDF.

MATERIALS AND METHODS

Cell culture. HDF strain WS8, derived from skin of a 47-year-old male subject with classical WS, was used in all experiments. These cells have a severely abbreviated replicative life span of 18 mean population doublings (MPD) (29). HDF derived from skin of a normal 56-year-old male, used as control cells (strain J065), have a life span of 46 MPD (29). Normal cells designated as young HDF were at ≤20 MPD ([^{3}H]thymidine labeling index [TLI], >90%), whereas old HDF were at 40 to 42 MPD (TLI, ≤13%). Cells were routinely propagated in Eagle's medium supplemented with 15% fetal bovine serum, without antibiotics, and used at different MPD levels as described below.

Construction of cDNA library. WS cells at 9 MPD, i.e., halfway through their replicative life span (TLI, ~10%), were grown to half confluence in Eagle's medium supplemented with 15% fetal bovine serum. Cells were rinsed with phosphate-buffered saline and incubated at 37°C for 5 days in serum-depleted medium containing 1% serum, the minimum concentration required to maintain these cells as intact monolayers. Cells were then refed with serum-repleted medium containing 20% fetal bovine serum and harvested 24 h later. RNA was prepared from the cells by using guanidinium thiocyanate as described previously (71), passed through two cycles of oligo(dT) chromatography to isolate poly(A)$^+$ mRNA, and then inserted as cDNA into a eukaryotic expression vector (56). The quality of the cDNA library was assessed by screening 7,200 colonies on nitrocellulose filters with a probe derived from the 3′ untranslated region of human β-actin cDNA, a G+C-rich sequence (60). Forty-four colonies (0.62%) were found to hybridize to the β-actin probe, within the range (0.5 to 1.2%) reported for β-actin mRNA abundance in various animal tissues. The length of cDNA inserts was estimated on 1% agarose gels following excision with *Bam*HI endonuclease, and nine clones (~20%) appeared to be full length (~1.9 kb [60]).

Differential (plus/minus) screening by colony hybridization. The same WS poly(A)$^+$ mRNA used in construction of the cDNA library was used to prepare the plus probe, whereas young normal HDF, serum depleted and repleted identically to WS cells (see above), were used to isolate poly(A)$^+$ mRNA for the minus probe. Radiolabeled ^{32}P-probes were prepared as single-stranded cDNAs by random priming of RNA (23) or by oligo(dT) priming of RNA (34) from these poly(A)$^+$ mRNAs.

Bacterial strain DH5.1 was transformed with the cDNA library and then plated and grown overnight at 37°C on nitrocellulose filters (82-mm diameter) on LB agar plates containing 35 μg of ampicillin per ml. A duplicate set of nitrocellulose filters was prepared as lifts of each original filter. The filters were incubated at 37°C until the size of each clone was ~1 to 1.5 mm in diameter. Colonies were lysed on the filter, and the liberated DNA was bound (33). One replicate filter was hybridized with the plus probe (WS [^{32}P]cDNA), and the other was hybridized with the minus probe (young normal [^{32}P]cDNA), followed by washing (11) and exposure to Kodak GB XAR-5 film.

Isolation of overexpressed cDNA clones. Duplicate filters were compared for intensity of colony signals. Clones exhibiting clearly increased intensities of signal on the filters hybridized with the WS probe were selected with sterile toothpicks and dotted in arrays on fresh nitrocellulose filters. Several clones of equal intensity were also selected and spotted on the same filter as equally expressed standards. Replicas of these filters were prepared for a secondary round of plus/minus screening. Clones again exhibiting substantially increased signal on WS [^{32}P]cDNA-probed filters were judged to be overexpressed in WS HDF.

Identification and characterization of clones. Overexpressed clones isolated as described above were grown in 250 ml of LB medium overnight and lysed to liberate the DNA, which was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (65). Approximate sizes of cDNA inserts were measured by excision with *Bam*HI and resolution on 1% agarose gels. Following Southern transfer, blots were cross-hybridized with the various cDNAs to determine homologies. Selected cDNAs were partially sequenced from 5′ and 3′ ends, using Sequenase kit version 2.0 (United States Biochemical Co., Cleveland, Ohio), by the dideoxy-mediated chain termination method (66). A synthetic sense strand 18-mer (TCTAGGCCTGTACGGAAG) which corresponds to the pcD-X vector, just upstream from the cDNA insert, was used as primer for the 5′ end. A mixture of synthetic 21-mer DNAs, $T_{20}A$, $T_{20}C$, and $T_{20}G$, was used as a universal primer for the 3′ end of cDNAs (76). Sequence data thus derived were compared with data in the GenBank (release 61.1) and EMBL (release 21) data bases to seek identities or homologies to known DNA sequences (59).

Northern (RNA) blot analysis. Total RNA was prepared (8) from multiple petri dishes of WS and control HDF at various intervals after serum repletion or following subculture; 10-μg samples were run on 1.2% agarose gels containing 1.9% formaldehyde and then blotted onto Zeta probe membranes (Bio-Rad Laboratories, Richmond, Calif.). Prehybridization and hybridization of Northern blots were performed as described previously (71). Membranes were probed individually with ^{32}P-labeled cDNA inserts prepared from the purified plasmids. Four additional DNA probes included a synthetic consensus sequence of the *Alu* class of repetitive short interspersed nuclear elements (SINE) (38), BLUR 8, a specific *Alu* sequence (16), a *Kpn*I consensus sequence of the long interspersed nuclear element 1 (LINE-1) class of repetitive elements (46), and a specific *Kpn*I sequence (69). Autoradiographic bands were quantified within the near-linear range of film and a model 300A laser densitometer using ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.) and corrected as necessary for variations in loading, using 28S rRNA visualized on negatives of ethidium bromide-stained agarose gels photographed prior to membrane transfer.

RESULTS

Preparation and screening of the WS cDNA library. We constructed the cDNA library in a eukaryotic expression vector (56), thus enabling direct functional assays of one or more cDNA clones, as desired, for negative growth-regulatory activity (29). WS cells, the source of mRNA for library

construction and for the plus probe, were primed by serum depletion/repletion in a manner analogous to recent approaches used to isolate positive growth-regulatory genes from mouse 3T3 cells (2, 45) and by extending the logic applied in the isolation of growth arrest-specific genes (68). We reasoned that genes playing a role in the senescent inhibition of DNA synthesis or cell division would be expressed at constitutively high levels in WS HDF and that these elevated levels might be further augmented following a serum stimulus in order to override growth-stimulatory signals. Moreover, such a serum depletion/repletion protocol, when applied to young normal HDF (used to prepare the minus probe), would further reduce their levels, if any, of such negative growth transcripts, allowing potential differences between WS and control cDNAs to stand out in bolder relief.

The WS cDNA library, found to contain $\sim 7 \times 10^5$ recombinant clones, was screened for WS-overexpressed gene sequences by differential hybridization. Duplicate filters were probed with either a single-stranded ^{32}P-labeled cDNA probe from poly(A)$^+$ RNA of normal HDF or from WS cells harvested 24 h after serum repletion. Hybridization signals of the two probes were compared for each colony, and plaques hybridizing preferentially with the WS cDNA probe were picked as presumptive WS-overexpressed gene sequences. A total of 7,500 colonies were screened in two stages: an initial screen of 2,300 clones to determine the magnitude and nature of overexpressed clones, followed by a second stage of screening 5,200 clones. Thus, cDNA clones isolated and characterized in the first stage were used as hybridization probes at high stringency to ascertain homologous clones identified in the second stage. After both stages of screening, each involving two rounds of hybridization, 102 clones were deemed to be overexpressed.

Identification of cDNA clones containing distinct overexpressed gene sequences by cross-hybridization and partial DNA sequencing. To ascertain the number of distinctive cDNA species represented among the 102 clones, cDNA inserts were tested for sequence homology to one another by cross-hybridization. Recombinant clones showing cross-hybridization after high-stringency washes, i.e., in buffer equivalent to 0.6× SSC (11) at 65°C, were judged to be derived from identical mRNAs. At least one representative of each cross-hybridizing group plus the other single isolates of cDNA were analyzed by partial DNA sequence determinations totaling 300 to 400 bp at the 5′ and 3′ ends, and the results were compared with data in the GenBank and EMBL data bases. Table 1 summarizes the identities and abundances of all cDNAs within the total of 102 clones, along with the sizes of the corresponding RNA transcripts. The clones are classified into two major groups according to whether their identities were known (i.e., ≥95% homology to DNA sequences entered in the GenBank and EMBL data bases) or unknown (≤50% homology to known sequences). On this basis, the 92 known clones could be reduced to nine distinct gene sequences, whereas an additional nine remained unknown. The latter clones could be further resolved into two groups: three clones that appeared to represent two distinct and novel gene sequences and a group of seven clones, each with unique and novel 5′ and 3′ ends, that also contained *Alu* repeat sequences of the SINE family. The latter group of seven all hybridized to a ^{32}P-labeled *Alu* consensus DNA probe (38), to the specific BLUR 8 probe (16), and to each other. Additionally, when these *Alu*-containing sequences were probed against Southern blots of human genomic DNA, a heterodisperse smear was seen (not shown). Five of the seven *Alu*-containing clones also hybridized to a consensus LINE-1 repeat sequence probe (46) and a specific LINE-1 sequence probe (*Kpn*I) (69).

TABLE 1. Identity, abundance, and corresponding RNA transcripts of individual cDNAs among 102 WS cDNA clones[a]

cDNA	Abundance	Approx size of corresponding RNA (kb)
Known		
α1(I) procollagen	21	7.5, 5.8
α2(I) procollagen	21	5.6, 4.5
Fibronectin	19	8.0
Ferritin heavy chain	12	1.3
IGFBP-3	9	2.5
Osteonectin	7	4.0, 2.8
PAI 1	1	4.4, 2.9
Thrombospondin	1	7.2, 5.0
αB-crystallin	1	0.9–1.4
Unknown		
WS3-10	2	1.5
WS9-14	1	4.2
WS3-13[b]	1	>23, 0.3
WS9-9[b]	1	>23, 0.3
WS19-7[c]	1	>23, 0.3
WS19-9[c]	1	>23, 0.3
WS19-15[c]	1	>23, 0.3
WS6-7[c]	1	>23, 4.7
WS11-12[c]	1	2.4

[a] Each cDNA insert was labeled with ^{32}P and hybridized to the other cDNAs and/or partially sequenced at the 5′ and 3′ ends. Cross-hybridization or sequence identity was taken as evidence that the cDNAs were derived from the same mRNA species. Underlined RNA species represent novel transcripts not previously reported.
[b] Contains *Alu* sequences.
[c] Contains both *Alu* and LINE-1 sequences.

Northern analysis of overexpressed cDNAs. To confirm that overexpression in fact occurs at the RNA level and to acertain the sizes and numbers of RNA transcripts for each cDNA, we carried out Northern analysis of young normal and WS RNAs, with normalization of RNA loads and gel transfers for 28S rRNA (Fig. 1). Among the various clones, a wide range of WS RNA overexpression was seen, including ratios of 1.5-fold [α2(I) procollagen, lower band], almost 3-fold (ferritin heavy chain and thrombospondin, upper band), 85-fold [α1(I) procollagen, upper band], 132-fold (WS3-10), 723-fold (tissue plasminogen inhibitor type 1 [PAI 1], upper band), and >1,000-fold [PAI 1, lower band, and α2(I) procollagen, upper band]. In general, mRNA bands agreed with previously published sizes and numbers, but in certain cases, additional higher-molecular-weight bands were evident in our analysis, likely due to tissue- and cell-specific variants or population polymorphisms not previously reported. Specifically, probing with osteonectin cDNA revealed a 4.0-kb band, probing with PAI 1 cDNA revealed a 4.4-kb RNA band, and probing with thrombospondin cDNA revealed a 7.2-kb band. αB-crystallin cDNA gave an apparent smear from ~0.9 to 1.4 kb, most of which appeared overexpressed in WS cells.

Among the seven unknown clones containing *Alu* repeats, three distinctive RNA patterns were observed. Six *Alu*$^+$ clones, including four that also hybridized to the LINE-1 sequence probe, showed a >23-kb band that was substantially overexpressed in WS cells (e.g., ~18-fold for WS3-13 and ~3-fold for WS19-7); all but one of these also hybridized to a 0.3-kb band that was moderately overexpressed (~1.5- to 2-fold) in WS versus normal cells (Table 1 and Fig. 1). The

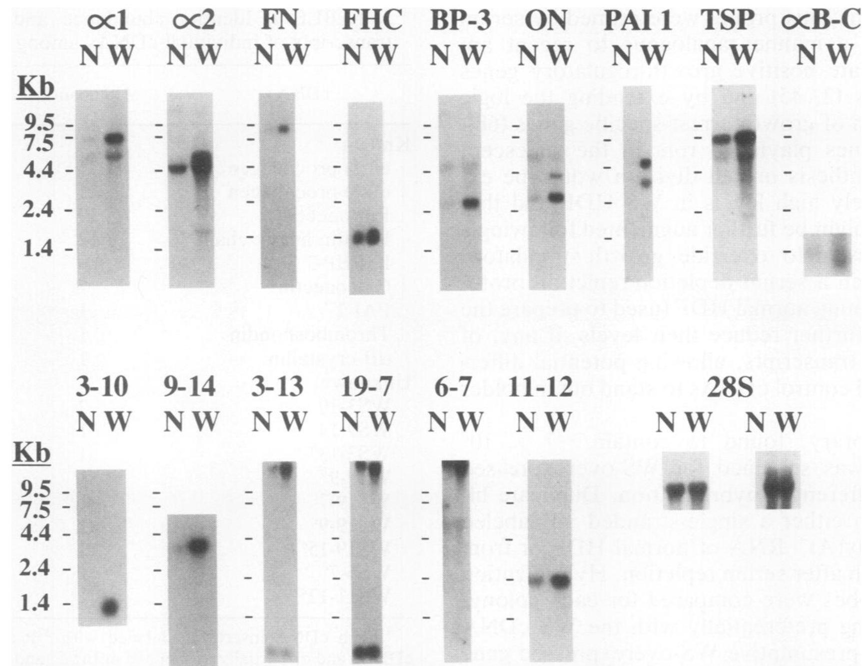

FIG. 1. Northern analysis of RNA transcripts in WS fibroblasts and normal young fibroblasts. Replicate lanes of WS (W) and normal (N) cellular total RNA (10 μg per lane) were prepared from cells first exposed to serum-depleted medium (1% serum) for 5 days and then repleted for 24 h with regular growth medium (15% serum). RNAs were run along with an RNA size ladder on agarose-formaldehyde gels and transferred to nylon filters, which were then cut into replicate strips each containing three lanes, corresponding to normal and WS RNAs and the size ladder. Individual strips were probed with each of the cDNAs as indicated. α1, α1(I) procollagen; α2, α2(I) procollagen; FN, fibronectin; FHC, ferritin heavy chain; BP-3, insulinlike growth factor-binding protein-3; ON, osteonectin; PAI 1, human tissue plasminogen activator inhibitor type 1; TSP, thrombospondin; αB-C, αB-crystallin. Other abbreviations have WS prefix as in Table 1. High-molecular-weight bands in WS3-13, WS19-7, and WS6-7 are >23 kb, whereas low-molecular-weight bands in WS3-13 and WS19-7 are 300 bases. Even loading, quantitative transfer, and integrity of RNA for two strips representative of all replicate strips are shown as bands hybridizing to a DNA probe specific for 28S rRNA (22a).

high-molecular-weight band was unlikely to be DNA because it persisted after RNA samples were digested with RNase-free DNase. Moreover, when electrophoresis was carried out for longer periods, Northern analysis revealed a heterodisperse smear of high-molecular-weight RNA species which were cumulatively overexpressed in WS cells (not shown). The 0.3-kb bands, but not the >23-kb bands, disappeared after RNA samples were subjected to oligo(dT) chromatography. One of the *Alu*- and LINE-1-containing cDNAs (WS6-7) gave a Northern pattern with the >23-kb band and a second band of 4.7 kb, whereas clone WS11-12 gave a single band of 2.4 kb.

Kinetic analysis of WS-overexpressed mRNAs following serum depletion/repletion and following subculture in WS and normal cells at early and late passage. To extend the observations in Fig. 1, we determined the kinetic profiles for five differentially expressed RNA transcripts in WS cells, in the early-passage (young) normal HDF, and also in the same normal cells at late passage (old). These five sequences were chosen because α1(I) procollagen, fibronectin, and insulinlike growth factor-binding protein 3 (IGFBP-3) were representative of highly overexpressed known sequences identified early in our screening as multiple, independent cDNA isolates, whereas WS3-10 and WS9-14 were unknown sequences which gave simple banding patterns on Northern analysis (Table 1 and Fig. 1). We isolated total RNA from HDF following 5 days of serum depletion and at intervals up to 48 h following serum repletion (Fig. 2A) and at several intervals following subculture and proliferation from sparse to confluent and superconfluent cultures up to 15 days (Fig. 2B). Following Northern analysis, we carried out densitometric scanning of autoradiograms (Fig. 3). In the serum depletion/repletion experiments (Fig. 2A and 3A), with the exception of fibronectin in WS cells at 0.5 h, mRNA levels were consistently higher in old and WS cells than in young cells, at ratios of overexpression ranging from 2- to ≥100-fold. Patterns differed for the five mRNAs in old and WS cells; both cell types tended to exhibit peak mRNA levels after 5 days of serum depletion (0 time) or at early times after serum repletion, but the levels remained relatively high throughout the 48 h. The mRNAs for fibronectin, IGFBP-3, and WS9-14 (WS cells only) appeared to rise at 48 h. Young cells showed relatively flat profiles for each mRNA except for α1(I) procollagen and fibronectin, which rose to peak levels at 48 h.

Analysis of mRNA profiles following subculture (Fig. 2B and 3B) revealed that α1(I) procollagen and fibronectin mRNAs were at clearly higher levels in WS cells only at days 6 to 15, whereas IGFBP-3 mRNA was at higher levels in WS cells at days 6 to 15 and in old cells throughout the 15 days relative to young cells. The two other gene sequences, WS3-10 and WS9-14, were expressed at higher levels in WS and old cells than in young cells across the growth cycle from sparse to superconfluent cultures. Temporal profiles for each mRNA in all three cell types following subculture generally started low, tending to rise as cells approached confluence (days 4 to 6); mRNA levels then either fell or leveled off toward superconfluence (IGFBP-3, WS3-10, and

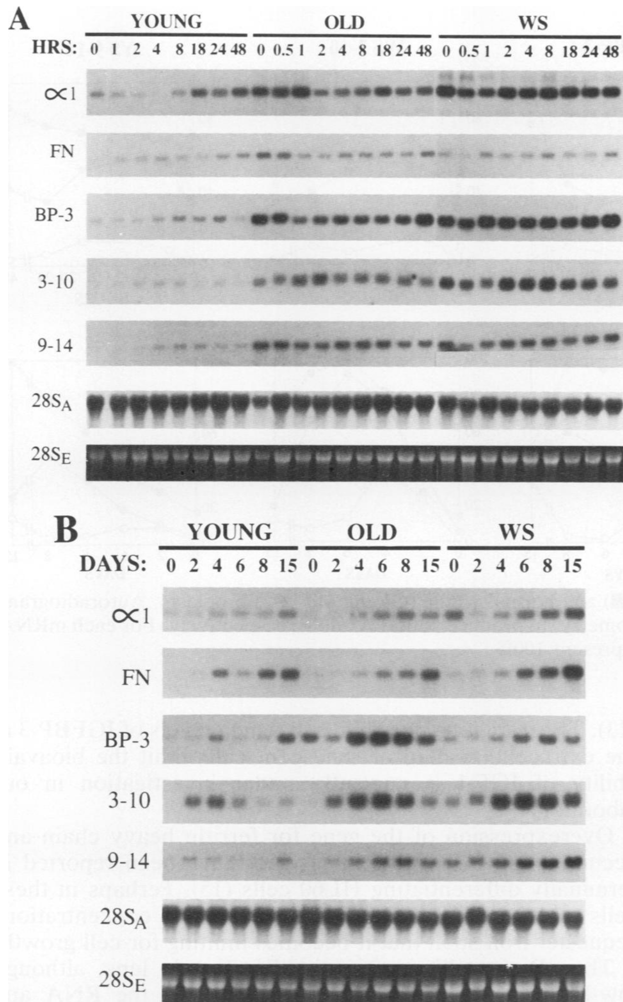

FIG. 2. Northern analysis of five mRNAs in WS and normal young and old fibroblasts. Cells exposed to serum-depleted medium for 5 days were repleted with regular growth medium (A) or subcultured into regular growth medium (B). Total cellular RNA was isolated at the intervals shown, fractionated on agarose-formaldehyde gels, transferred to nylon membranes, and hybridized to each of the five ^{32}P-labeled cDNA inserts. The cDNA used to detect α1(I) procollagen was homologous to a region at the 3′ end of the mRNA specific for the larger (7.5-kb) transcript. Gels stained with ethidium bromide were photographed before transfer to demonstrate equivalent sample loading and RNA integrity as judged by 28S rRNA bands ($28S_E$). Autoradiographs of RNA bands on blots probed with 28S rDNA ($28S_A$) demonstrate even transfer and relatively constant expression. RNA was not available for young cells at 30 min in panel A. For abbreviations, see legend to Fig. 1.

WS9-14) or continued to rise in WS cells to day 15 [α1(I) procollagen, fibronectin, and WS9-14].

DISCUSSION

In an attempt to define the molecular genetic basis of replicative senescence of HDF, we have isolated and begun to characterize a diverse constellation of overexpressed clones from a WS cDNA library. Many of the known cDNA clones identified here encode proteins that are secreted by cells and either represent important components of the extracellular matrix (ECM) or are closely associated with it. These include α1(I) procollagen, α2(I) procollagen, fibronectin, osteonectin, PAI 1, and thrombospondin. IGFBP-3 is also secreted into the extracellular fluid and plasma, but it is still unclear whether it associates with the ECM (12, 52). In contrast, the proteins for ferritin heavy chain (5) and αB-crystallin (19, 40) are believed to reside primarily if not exclusively inside the cell.

That several genes encoding ECM proteins are overexpressed during replicative senescence is of great interest. Strong inverse relationships between expression of one or more of these genes and cellular proliferation have been reported in a variety of systems undergoing the growth arrest that accompanies differentiation or high-density contact inhibition, including osteoblasts (72), endothelial cells (48), myoblasts (61), chondroblasts (78), fibroblasts (57), and smooth muscle cells (73). Indeed, production of fibronectin and collagen, essential ECM elements involved in development, cell adhesion, blood clotting, and maintenance and repair of virtually all tissues, is often coordinately linked, as appeared to be the case here (Fig. 3). Moreover, each of these molecules has a specific binding site for the other, as well as for fibrin, heparin, glycosaminoglycans, proteoglycans, and thrombospondin (54, 62). Our visualization of two major mRNA species on Northern analysis for both α1(I) and α2(I) procollagen is consistent with earlier reports and is explained by the presence of two different transcriptional stop sites at the 3′ end of these genes (10, 18). Although as many as 20 different species of fibronectin mRNA may exist as a result of combinatorial splicing of the initial RNA transcript, these cannot be readily distinguished on the basis of molecular weight alone (35). In any case, our data agree with a recent report in which fibronectin cDNA was isolated from a library derived from senescent neonatal foreskin HDF and confirmed to be overexpressed by Northern analysis of RNA from these cells compared with their young counterparts (42). However, we did not identify any clones in this study related to pSEN (25a), a senescence-specific cDNA whose cognate RNA is overexpressed in old WI-38 cells, HDF derived from fetal lung; sequencing of the ~1.3-kb pSEN reveals a large segment homologous to elongation factor Iα plus a small novel segment (25a).

Thrombospondin, a large multifunctional protein released from activated platelets and other cells, binds to components of the cell surface and ECM. Its various actions include modulation of the formation and lysis of fibrin and growth regulation. Indeed, thrombospondin is inversely correlated with growth not only via a direct action on the proliferating cell (54) but also via inhibition of angiogenesis (32). The reported size of thrombospondin mRNA in endothelial cells is 5.8 kb, although a smaller species, perhaps corresponding to our 5.0-kb transcript, has also been reported (54). However, the presence of a 7.2-kb RNA transcript appears to be unique to our study.

The gene for osteonectin, also known as SPARC, is highly expressed at both the mRNA and protein levels in bone and a wide variety of adult and embryonic tissues, in particular those involved in ECM assembly and remodeling (37). High expression also occurs in mouse embryonic parietal endoderm cells and mouse F9 teratocarcinoma cells in which differentiation to parietal endoderm has been induced by retinoic acid and cyclic AMP (53). In view of the osteonectin overexpression described here in WS cells, it warrants emphasis that osteonectin can inhibit entry of endothelial cells into S phase (24a). Furthermore, as the major noncollagenous protein in bone, osteonectin binds to hydroxyapatite and collagen and may thus play a role in regulating bone

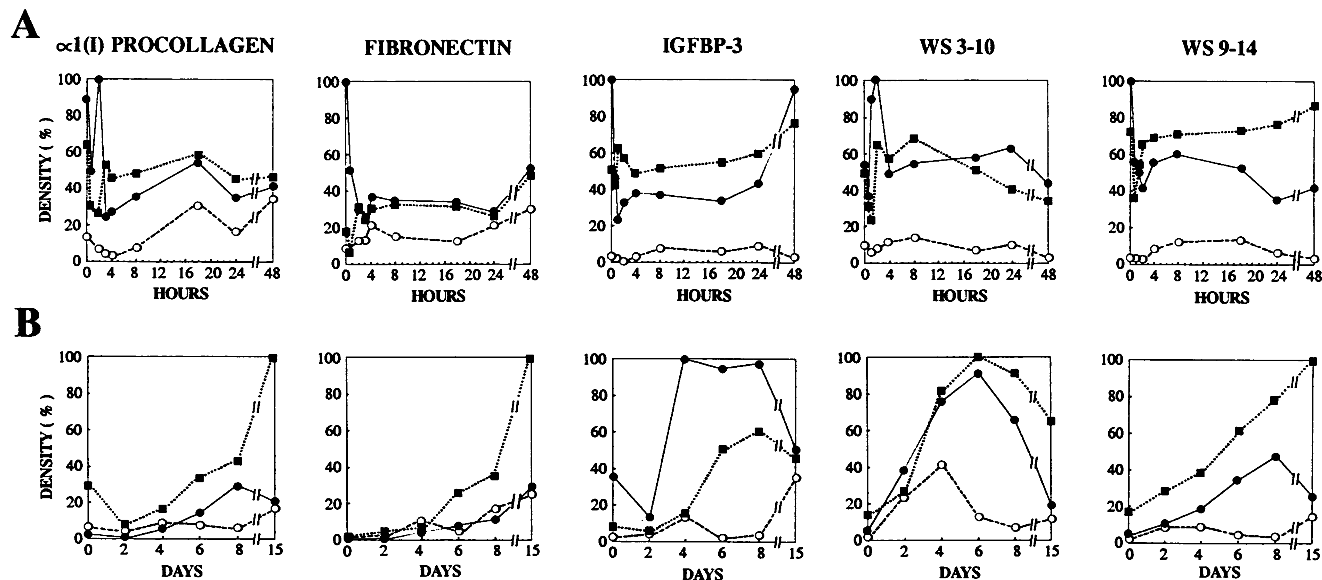

FIG. 3. Densitometric analysis of five mRNAs overexpressed in WS (■) and normal young (○) and old (●) fibroblasts. Autoradiograms of the Northern blots shown in Fig. 2A and B were scanned by laser densitometry, to produce panels A and B, respectively. For each mRNA, the highest-density band among the three cell types was normalized to represent 100%.

mineralization, perhaps in part through the presence of two calcium-binding sites (21). Thus, overexpression of osteonectin and thrombospondin, an ECM protein with multiple calcium-binding sites, may not only be involved in senescent inihibition of cell growth but may also relate to two other prominent clinical features of WS: severe osteoporosis and widespread soft tissue and vascular calcification (22, 26).

PAI 1, a serine protease inhibitor, is involved in regulating fibrinolysis, by maintaining the balance between plasminogen activation and inhibition (25). The protein is encoded in a 12.2-kb gene which can produce two mRNAs of 3.0 and 2.2 kb in endothelial cells (63). Our finding, therefore, of a 4.4-kb transcript in addition to the 3.0-kb species suggests that the former is an incompletely spliced pre-mRNA or an aberrant transcript. Overexpression of thrombospondin and PAI 1, combined with extremely high levels of the procoagulant tissue factor, found in WS HDF (31), can readily be imagined to lead to hypercoagulability and thrombosis and, in concert with other ECM components such as fibronectin and collagen, to play a role in atherogenesis (44). Conversely, the genes for fibronectin, α1(I) and α2(I) collagen, and human PAI 1 appear to be repressed in several transformed and malignant cells (1, 67), particularly those actively undergoing invasion and metastasis (81). Since WS cells feature chromosomal instability (64) and high rates of genetic mutability (24) and recombination (7), it is possible that loss of function or loss of expression of one or more of these ECM genes and other genes which normally regulate cell proliferation could lead to the high tumor incidence in this disorder (22, 26).

IGFBP-3 binds insulinlike growth factor I (IGF-I) and IGF-II with high affinity in human plasma (52) and displays a variable effect on growth of HDF. When coincubated with IGF-I, IGFBP-3 inhibits IGF-I-stimulated DNA synthesis of HDF, whereas when preincubated with these cells prior to addition of IGF-I, DNA synthesis is augmented (12, 17). IGF-I receptor number and affinity remain essentially unchanged in old compared with young HDF, although old cells became unresponsive to IGF-I and other growth factors (13). The intriguing possibility that high levels of IGFBP-3 in the extracellular fluid of senescent cells limit the bioavailability of IGF-I is currently under investigation in our laboratory.

Overexpression of the gene for ferritin heavy chain and accumulation of its protein product have been reported in terminally differentiating HL60 cells (15). Perhaps in these cells and in senescent HDF, high ferritin concentrations sequester iron such that it becomes limiting for cell growth.

The αB-crystallin protein is abundant in lens, although low-level expression of this gene at both the RNA and protein levels has been demonstrated in a variety of extralenticular tissues and cultured glial cells, in which it may serve in a structural or enzymatic role (19, 40). Elevated expression has also been reported in scrapie-infected brain (20) and in the brains of subjects with Alexander's disease (40). We have no knowledge of αB-crystallin expression in WS brain or other WS tissues, but our detection of a smear of RNA between 0.9 and 1.4 kb, most of which is overexpressed in WS HDF, is consistent with previous findings (19, 40). Indeed, a recent report indicates that a variety of transcriptional initiation sites can account for this mRNA microheterogeneity (39). In any case, it is tempting to speculate that the αB-crystallin overexpression in HDF derived from WS skin is somehow related to the precocious appearance of cataracts in the WS lens (22, 26).

We emphasize that further analysis is needed for each overexpressed gene sequence to verify that commensurate overexpression occurs at the protein level. Several reports indicate that a dichotomy may exist in senescent HDF between the steady-state level of a given mRNA and synthesis of its cognate protein (see references 13 and 28 and references therein), thus implying disregulation at the translational level. Indeed, reduced collagen synthesis has been reported in old compared with young WI-38 cells (58) and in early-passage gingival HDF from old donors compared with young donors (41), whereas no difference was found between young and old foreskin HDF in the level of endogenously produced fibronectin accumulating in the medium

(49). Nonetheless, consonant with our results of high collagen mRNA levels in skin-derived WS HDF, increased collagen synthesis has been reported for other strains of WS skin fibroblasts (3, 74). Moreover, we have found overproduction of IGFBP-3 by radioimmunoassay of conditioned medium of old normal and WS HDF compared with young HDF (28a).

It is noteworthy that levels of hyaluronic acid, an abundant glycosoaminoglycan often associated with collagen in the ECM, are also increased in WS fibroblasts, and this increase is associated with excessive urinary excretion of hyaluronic acid in WS subjects (6). Similar derangements of hyaluronic acid metabolism occur in HDF and urine of subjects with the Hutchinson-Gilford (progeria) syndrome of premature aging (a disorder genetically and phenotypically distinct from WS [26]) and as a function of chronological age in normal persons (6). It is also of interest that increased production of fibronectin and collagen has been reported in HDF from a child with progeria (50). These reports, taken together with the present findings, indicate that aging is accompanied by alterations in several components of the ECM, which are accelerated or exaggerated during premature aging.

Little information is available with respect to the functional significance of overexpressed RNA sequences containing the *Alu* and LINE-1 repeat sequences. The differing band patterns obtained on Northern analysis of these cDNAs (Table 1 and Fig. 1) can be explained by the relatedness but nonidentity of *Alu* and LINE-1 family members plus the unique sequences present at the 5′ and 3′ ends of each of these seven clones. It is emphasized that construction of the cDNA library and Northern analyses were carried out with total (i.e., nuclear and cytoplasmic) RNA preparations, which include heterogeneous nuclear RNA (15, 38, 43). The large heterodisperse RNAs that we identified, therefore, would appear to contain multiple heterogeneous nuclear RNAs, each containing *Alu* or LINE-1 elements homologous to our cDNA probes. The *Alu*-related 7SL RNA is a ~300-nucleotide component of the signal recognition particle (77), a ribonucleoprotein complex involved in cotranslational insertion of signal sequence-containing nascent polypeptides into the endoplasmic reticulum (79). The small RNA species (0.3 kb) that we detect as moderately overexpressed in WS compared with normal cells on probing with our *Alu* sequence-containing clones would thus appear to be the 7SL RNA. Induction of other *Alu*-containing mRNA transcripts, however, has been reported in the cytoplasm of terminally differentiating HL60 cells (15) and in human thymocytes following human immunodeficiency virus infection (43). *Alu* transcripts have also been invoked as a mechanism for repression of the embryonic ε-globin gene (80). In short, the significance of *Alu*-containing RNA sequences in human biology remains unknown, but a speculative role has been fashioned for such repetitive elements in regulation, via both RNA-RNA and RNA-protein interactions, of cellular proliferation and terminal differentiation (38).

LINE-1 elements comprise a family of long repetitive, interspersed, often truncated sequences that originated as retrotransposons and are found in all mammalian genomes (70). Although their role is still unclear, some of these elements appear to represent functional genes and pseudogenes, whereas others may modulate expression of neighboring genes (46).

This study was undertaken as an initial survey to find overexpressed cDNAs corresponding to RNA transcripts present at relatively high abundance in WS HDF. That both WS and old normal HDF overexpress the five genes studied here in detail indicates that this phenomenon is not unique to WS but rather is a more general feature of HDF senescence. Because the low-level expression of novel genes WS3-10 and WS9-14 is evident in young cells, it cannot be construed as absolutely senescence specific, although the small admixture of senescent cells present in early-passage HDF cultures could account for this picture (28, 55). Clearly, however, collagen, fibronectin, and IGFBP-3 genes are expressed by young HDF in these and many other studies in vitro, just as they are expressed in vivo by fibroblasts and several additional cell types through life. In any case, the senescence-dependent overexpression of these five genes differs from expression of the six growth arrest-specific genes isolated from a cDNA library of serum-depleted NIH 3T3 cells (68). Whereas mRNA levels of our genes tend to fall in the first few hours following serum repletion, they generally rebound and are maintained at relatively high levels in WS and old normal cells up to 48 h, in contrast to all six growth arrest-specific gene transcripts, which reach a nadir between 3 and 6 h after serum repletion (68). As yet, we have no data bearing directly on the antiproliferative potential of these five gene sequences, but persistent overexpression would be expected for genes causally involved in HDF senescence.

In summary, we have identified a diverse constellation of gene sequences that are overexpressed during the premature replicative senescence of WS fibroblasts. That five of these genes are also generally overexpressed in senescent normal HDF bolsters the concept that HDF senescence in vitro is related to cellular senescence in vivo, including its concomitants of functional decline and the emergence of degenerative and neoplastic diseases (26, 28). Among the 18 overexpressed genes identified here, two (WS3-10 and WS9-14) appear to be entirely novel and seven appear to be partially novel. Overexpression in senescent HDF of several gene sequences, whose proteins form the ECM [α1(I) and α2(I) procollagens, fibronectin, osteonectin, PAI 1, and thrombospondin], along with overexpression of IGFBP-3, ferritin heavy-chain, and *Alu*-containing genes, all of which have the potential to inhibit DNA synthesis and cell growth, is consistent with the notion that HDF senescence is a form of terminal differentiation. The simultaneous overexpression of such genes also offers an explanation for the stunted somatic growth seen in WS and the defective tissue repair observed in WS and in many aging normal persons. Further studies should determine the precise role of these varied sequences, acting singly and in concert, in the mechanism of HDF senescence, of biological aging, and of age-dependent pathology.

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ADDENDUM IN PROOF

A recent report indicates that αB-crystallin is a member of the class of small, heat shock proteins that accumulate under stress conditions (R. Klemenz, E. Fröhli, R. H. Steinger, R. Schäfer, and A. Aoyama, Proc. Natl. Acad. Sci. USA **88**:3652–3656, 1991).

REFERENCES

1. **Adams, S. I., J. C. Alwine, B. de Crombrugghe, and I. Pastan.** 1979. Use of recombinant plasmids to characterize collagen RNAs in normal and transformed chick embryo fibroblasts. J. Biol. Chem. **254**:4935–4938.
2. **Almendral, J. M., D. Sommer, H. MacDonald-Bravo, J. Burchkardt, J. Perera, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140–2148.
3. **Arakawa, M., A. Hatamochi, K. Takeda, and H. Ueki.** 1990. Increased collagen synthesis accompanying elevated m-RNA levels in cultured Werner's syndrome fibroblasts. J. Invest. Dermatol. **94**:187–190.
4. **Bayreuther, K., H. P. Rodemann, R. Hommel, K. Dittman, M. Albiez, and P. I. Francz.** 1988. Human skin fibroblasts in vitro differentiate along a terminal cell lineage. Proc. Natl. Acad. Sci. USA **85**:5112–5116.

4a. **Beaudet, AL, C. R. Scriver, W. S. Sly, D. Valle, D. N. Cooper, V. A. McKusick, and J. Schmidke.** 1989. Genetics and biochemistry of variant human phenotypes, p. 3–53. *In* C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (ed.), The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York.

5. **Boyd, D., C. Vecoli, D. M. Belcher, S. K. Jain, and J. W. Drysdale.** 1985. Structural and functional relationships of human ferritin H and L chains deduced from cDNA clones. J. Biol. Chem. **260**:11755–11761.
6. **Brown, W. T.** 1990. Genetic diseases of premature aging as models of senescence. Annu. Rev. Gerontol. Geriatr. **10**:23–42.
7. **Cheng, R. Z., S. Murano, B. Kurz, and R. J. Shmookler Reis.** 1990. Homologous recombination is elevated in some Werner-like syndromes but not during normal in vitro or in vivo senescence of mammalian cells. Mutat. Res. **237**:259–269.
8. **Chomczynsky, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid-guanidinium-thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
9. **Chou, C.-C., R. A. Gatti, M. L. Fuller, P. Concannon, A. Wong, S. Chada, R. C. Davis, and W. A. Salser.** 1986. Structure and expression of ferritin genes in a human promyelocytic cell line that differentiates in vitro. Mol. Cell. Biol. **6**:566–573.
10. **Chu, M. L., W. deWet, M. Bernard, and F. Ramirez.** 1985. Fine structural analysis of the human pro-α-1(I)collagen gene. Promoter structure, Alu I repeats, and polymorphic transcripts. J. Biol. Chem. **260**:2315–2320.
11. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
12. **Conover, C. A., M. Ronk, F. Lombana, and D. R. Powell.** 1990. Structural and biological characterization of bovine insulin-like growth factor binding protein-3. Endocrinology **127**:2795–2803.
13. **Cristofalo, V. J., D. L. Doggett, K. M. Brooks-Frederich, and P. D. Phillips.** 1989. Growth factors as probes of cell aging. Exp. Gerontol. **24**:367–374.
14. **Cristofalo, V. J., and B. M. Stanulis-Praeger.** 1982. Cellular senescence in vitro, p. 1–68. *In* K. Maramorosch (ed.), Advances in tissue culture, vol. 2. Academic Press, New York.
15. **Davis, R., A. R. Thomason, M. L. Fuller, J. P. Slovin, C.-C. Chou, S. Chada, R. A. Gatti, and W. A. Salser.** 1987. mRNA species regulated during the differentiation of HL-60 cells to macrophages and neutrophils. Dev. Biol. **119**:164–174.
16. **Deininger, P. L., D. J. Jolly, C. W. Rubin, T. Friedmann, and C. W. Schmid.** 1981. Base sequence studies of 300 nucleotide renatured repeated human DNA clones. J. Mol. Biol. **151**:17–33.
17. **DeMellow, J. S. M., and R. C. Baxter.** 1988. Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-1-stimulated DNA synthesis in human skin fibroblasts. Biochem. Biophys. Res. Commun. **156**:199–204.
18. **deWet, W., M. Bernard, V. Benson-Chanda, M.-L. Chu, L. Dickson, D. Weil, and R. Ramirez.** 1987. Organization of the human pro-α2(I) collagen gene. J. Biol. Chem. **262**:16032–16036.
19. **Dubin, R. A., A. H. Ally, S. Chung, and J. Piatigorsky.** 1990. Human αB-crystallin gene and preferential promoter function in lens. Genomics **7**:594–601.
20. **Duguid, J. R., R. G. Rohwer, and B. Seed.** 1988. Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. Proc. Natl. Acad. Sci. USA **85**:5738–5742.
21. **Engel, J., W. Taylor, M. Paulsson, H. Sage, and B. Hogan.** 1987. Calcium binding domains and calcium induced conformational transition of SPARC/BM-40/osteonectin, an extracellular glycoprotein expressed in mineralized and non-mineralized tissues. Biochemistry **26**:6958–6965.
22. **Epstein, C. J., G. M. Martin, A. L. Schultz, and A. G. Motulsky.** 1966. Werner's syndrome. A review of its symptomatology, natural history, pathologic features, genetics, and relationship to the natural aging process. Medicine **45**:177–221.

22a. **Erickson, J. M., J. M. Rushford, D. J. Dorney, G. N. Wilson, and R. D. Schmickel.** 1981. Structure and variation of human ribosomal DNA: molecular analysis of cloned fragments. Gene **16**:1–9.

23. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
24. **Fukuchi, K.-I., G. M. Martin, and R. J. Monnat, Jr.** 1989. Mutator phenotype of Werner syndrome is characterized by extensive deletions. Proc. Natl. Acad. Sci. USA **86**:5893–5897.

24a. **Funk, S. E., and E. H. Sage.** 1991. The Ca^{2+}-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. Proc. Natl. Acad. Sci. USA **88**:2648–2652.

25. **Ginsburg, D., R. Zeheb, A. Y. Yang, U. M. Rafferty, P. A. Andreasen, L. Nielsen, K. Dano, R. V. Lebo, and T. D. Gelehrter.** 1986. cDNA cloning of human plasminogen activator-inhibitor from endothelial cells. J. Clin. Invest. **78**:1673–1680.

25a. **Giordano, T., and D. N. Foster.** 1989. Identification of a highly abundant cDNA isolated from senescent WI-38 cells. Exp. Cell Res. **185**:399–406.

26. **Goldstein, S.** 1978. Human genetic disorders which feature accelerated aging, p. 171–224. *In* E. L. Schneider (ed.), The genetics of aging. Plenum Press, New York.
27. **Goldstein, S.** 1989. Cellular senescence, p. 2525–2549. *In* L. J. Degroot, L. Martini, J. Potts, D. Nelson, A. Winegrad, W. Odell, E. Steinberger, and G. Cahill (ed.), Endocrinology, vol. 3. Grune & Stratton, New York.
28. **Goldstein, S.** 1990. Replicative senescence: the human fibroblast comes of age. Science **249**:1129–1133.

28a. **Goldstein, S., et al.** Submitted for publication.

29. **Goldstein, S., S. Murano, H. Benes, E. J. Moerman, R. A. Jones, R. Thweatt, R. J. Shmookler Reis, and B. H. Howard.** 1989. Studies on the molecular genetic basis of replicative senescence in Werner syndrome and normal fibroblasts. Exp. Gerontol. **24**:461–468.
30. **Goldstein, S., S. Murano, and R. J. Shmookler Reis.** 1990. Werner syndrome: a molecular genetic hypothesis. J. Gerontol. **45**:B3–B8.
31. **Goldstein, S., and S. Niewiarowski.** 1976. Increased procoagulant activity in cultured fibroblasts from progeria and Werner's syndromes of premature ageing. Nature (London) **260**:711–713.
32. **Good, D. J., P. J. Polverini, F. Rastinejad, M. M. LeBeau, R. S. Lemons, W. A. Frazier, and N. P. Bouck.** 1990. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc. Natl. Acad. Sci. USA **87**:6624–6628.
33. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
34. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient

method for generating cDNA libraries. Gene **25**:263–269.

35. **Gutman, A., and A. R. Kornblihtt.** 1987. Identification of a third region of cell-specific alternative splicing in human fibronectin mRNA. Proc. Natl. Acad. Sci. USA **84**:7179–7182.
36. **Hayflick, L.** 1965. The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. **37**:614–636.
37. **Holland, P. W. H., S. J. Harper, J. H. McVey, and B. L. M. Hogan.** 1987. In vivo expression of mRNA for the Ca^{++}-binding protein SPARC (osteonectin) revealed by in situ hybridization. J. Cell Biol. **105**:473–482.
38. **Howard, B. H., and K. Sakamoto.** 1990. Alu interspersed repeats: selfish DNA or a functional gene family? New Biol. **2**:759–770.
39. **Iwaki, A., T. Iwaki, J. E. Goldman, and R. K. H. Liem.** 1990. Multiple mRNAs of rat brain α-crystallin B chain result from alternative transcriptional initiation. J. Biol. Chem. **265**:22197–22203.
40. **Iwaki, T., A. Kume-Iwaki, R. K. H. Liem, and J. E. Goldman.** 1989. αB-crystallin is expressed in nonlenticular tissues and accumulates in Alexander's disease brain. Cell **57**:71–78.
41. **Johnson, B. D., R. C. Page, A. S. Narayanan, and H. P. Pieters.** 1986. Effects of donor age on protein and collagen synthesis in vitro by human diploid fibroblasts. Lab. Invest. **55**:490–496.
42. **Kleinsek, D. A.** 1989. Selection of mRNAs expressed during cellular senescence in vitro. Age **12**:55–60.
43. **Koga, Y., E. Lindstrom, E. M. Fenyo, H. Wigzell, and T. W. Mak.** 1988. High levels of heterodisperse RNAs accumulate in T cells infected with human immunodeficiency virus and in normal thymocytes. Proc. Natl. Acad. Sci. USA **85**:4521–4525.
44. **Labat-Robert, J., and L. Robert.** 1988. Aging of the extracellular matrix and its pathology. Exp. Gerontol. **23**:5–18.
45. **Lau, L., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc. Natl. Acad. Sci. USA **84**:1182–1186.
46. **Leibold, D. M., G. D. Swergold, M. F. Singer, R. E. Thayer, B. A. Dombroski, and T. G. Fanning.** 1990. Translation of LINE-1 DNA elements in vitro and in human cells. Proc. Natl. Acad. Sci. USA **87**:6990–6994.
47. **Lumpkin, C. K., Jr., J. K. McClung, O. M. Pereira-Smith, and J. R. Smith.** 1986. Existence of high abundance and antiproliferative mRNA's in senescent human diploid fibroblasts. Science **232**:393–395.
48. **Maciag, T.** 1990. Molecular and cellular mechanisms of angiogenesis, p. 85–100. *In* V. T. DeVita, S. Hellman, and S. A. Rosenberg (ed.), Important advances in oncology. J. B. Lippincott, Philadelphia.
49. **Mann, D. M., P. J. McKeown-Longo, and A. J. T. Millis.** 1988. Binding of soluble fibronectin and its subsequent incorporation into the extracellular matrix by early and late passage human skin fibroblasts. J. Biol. Chem. **263**:2756–2760.
50. **Maquart, F. X., G. Bellon, P. Gillery, J. P. Borel, B. Labeille, B. Risbourg, and J. P. Denoeux.** 1988. Increased secretion of fibronectin and collagen by progeria (Hutchinson-Gilford) fibroblasts. Eur. J. Pediatr. **147**:442–448.
51. **Martin, G. M., C. A. Sprague, and C. J. Epstein.** 1970. Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype. Lab. Invest. **23**:86–92.
52. **Martin, J. L., and R. C. Baxter.** 1986. Insulin-like growth factor-binding protein from human plasma. Purification and characterization. J. Biol Chem. **261**:8754–8760.
53. **Mason, I. J., A. Taylor, J. G. Williams, H. Sage, and B. L. M. Hogan.** 1986. Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell "culture shock" glycoprotein of M_r 43,000. EMBO J. **5**:1465–1472.
54. **Mosher, D. F.** 1990. Physiology of thrombospondin. Annu. Rev. Med. **41**:85–97.
55. **Norwood, T. H., J. R. Smith, and G. H. Stein.** 1990. Aging at the cellular level: the human fibroblastlike cell model, p. 131–154. *In* E. L. Schneider and J. W. Rowe (ed.), Handbook of the biology of aging. Academic Press, San Diego, Calif.
56. **Okayama, H., M. Kawaichi, M. Brownstein, F. Lee, T. Yokota, and K. Arai.** 1987. High-efficiency cloning of full-length cDNA; construction and screening of cDNA expression libraries for mammalian cells. Methods Enzymol. **154**:3–28.
57. **Owen, T. A., R. Carter, M. M. Whitman, D. R. Soprano, and K. J. Soprano.** 1990. Evidence that density-dependent growth arrest is a two-stage process in WI-38 cells. J. Cell. Physiol. **142**:137–148.
58. **Paz, M. A., and P. M. Gallop.** 1975. Collagen synthesized and modified by aging fibroblasts in culture. In Vitro **11**:302–312.
59. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
60. **Ponte, P., S. Y. Ng, J. Engel, P. Gunning, and L. Kedes.** 1984. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. Nucleic Acids Res. **12**:1687–1696.
61. **Rao, J. S., R. L. Beach, and B. W. Festoff.** 1985. Extracellular matrix (ECM) synthesis in muscle cell cultures: quantitative and qualitative studies during myogenesis. Biochem. Biophys. Res. Commun. **130**:440–446.
62. **Ruoslahti, E.** 1988. Fibronectin and its receptors. Annu. Rev. Biochem. **57**:375–413.
63. **Saksela, O., and D. B. Rifkin.** 1988. Cell-associated plasminogen activation: regulation and physiological functions. Annu. Rev. Cell Biol. **4**:93–126.
64. **Salk, D., K. Au, H. Hoelm, M. R. Stenchever, and G. M. Martin.** 1981. Evidence of clonal attentuation, clonal succession, and clonal expansion in mass cultures of aging Werner's syndrome skin fibroblasts. Cytogenet. Cell Genet. **30**:108–117.
65. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 1.38–1.39, 1.42–1.46. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
66. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
67. **Schmidt, A., C. Setoyama, and B. de Crombrugghe.** 1985. Regulation of a collagen gene promoter by the product of the viral mos oncogene. Nature (London) **314**:286–289.
68. **Schneider, C., R. M. King, and L. Philipson.** 1988. Genes specifically expressed at growth arrest of mammalian cells. Cell **54**:787–793.
69. **Shafit-Zagardo, B., J. J. Maio, and F. L. Brown.** 1982. KpnI families of long, interspersed repetitive DNAs in human and other primate genomes. Nucleic Acids Res. **10**:3175–3193.
70. **Singer, M. F.** 1989. LINE-1 sequences: human transposable elements, p. 155–162. *In* M. Grunberg-Manago, B. F. C. Clark, and H. G. Zachau (ed.), Evolutionary tinkering in gene expression. Plenum Publishing Corp., New York.
71. **Srivastava, A., J. S. Norris, R. J. Shmookler Reis, and S. Goldstein.** 1985. c-Ha-ras-1 proto-oncogene amplification and overexpression during the limited replicative life span of normal human fibroblasts. J. Biol. Chem. **260**:6404–6409.
72. **Stein, G. S., J. B. Lian, and T. A. Owen.** 1990. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J. **4**:3111–3123.
73. **Stepp, M. A., M. S. Kindy, C. Franzblau, and G. E. Sonenshein.** 1986. Complex regulation of collagen gene expression in cultured bovine aortic smooth muscle cells. J. Biol. Chem. **261**:6542–6547.
74. **Tajima, T., K. Iijima, and T. Watanabe.** 1978. Collagen synthesis of cultured fibroblasts from Werner's syndromes of premature aging. Experientia **34**:1459–1460.
75. **Tanaka, K., T. Nakazawa, Y. Okada, and Y. Kumahara.** 1980. Roles of nuclear and cytoplasmic environments in the retarded DNA synthesis in Werner syndrome cells. Exp. Cell Res. **127**:185–190.
76. **Thweatt, R., S. Goldstein, and R. J. Shmookler Reis.** 1990. A universal primer mixture for sequence determination at the 3′ ends of cDNAs. Anal. Biochem. **190**:314–316.
77. **Ullu, E., and C. Tschudi.** 1984. Alu sequences are processed 7SL RNA genes. Nature (London) **312**:171–172.
78. **Urist, M. R.** 1983. The origin of cartilage: investigations in quest of chondrogenic DNA, p. 2–86. *In* B. K. Hall (ed.), Cartilage,

vol. 2. Development, differentiation, and growth. Academic Press, New York.
79. **Walter, P., and G. Blobel.** 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature (London) **299:**691–698.
80. **Wu, J., G. J. Grindlay, P. Bushel, L. Mendelsohn, and M. Allan.** 1990. Negative regulation of the human ε-globin gene by transcriptional interference: role of an Alu repetitive element. Mol. Cell. Biol. **10:**1209–1216.
81. **Zajchowski, D. A., V. Band, D. K. Trask, D. Kling, J. L. Connolly, and R. Sager.** 1990. Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells. Proc. Natl. Acad. Sci. USA **87:**2314–2318.