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Global Change of Gene Expression at Late G1/S Boundary May Occur in Human IMR-90 Diploid Fibroblasts During Senescence

JONG HWEI PANG AND KUANG YU CHEN*

Department of Chemistry and Graduate Program of Biochemistry, Rutgers-The State University of New Jersey, Piscataway, New Jersey 08855-0939

The hallmark of cellular aging is the failure of senescent diploid cells to enter or to complete the S phase of the cell cycle. The cause for such failure may hold the key for our understanding of the molecular basis of cellular aging. We have previously shown that aging of IMR-90 human diploid fibroblasts in culture is accompanied by a five to sevenfold decrease in both thymidine kinase activity and thymidine kinase mRNA level (Chang and Chen, 1988, J. Biol. Chem., *263*:11431–11435). To examine whether attenuation of gene expression at G1/S boundary is unique for thymidine kinase or it may involve most, if not all, of other G1/S genes, we compared the expressions of two classes of G1/S genes in young and in old IMR-90 cells following serum stimulation. We found that the expression of all these genes, including thymidylate synthase (TS), dihydrofolate reductase (DHFR), ribonucleotide reductase (PNR), proliferating cell nuclear antigen (PCNA), histone H1, histone H2A + 2B, histone H3, and histone H4, was induced to high levels in young IMR-90 cells but not in old IMR-90 cells. The mRNA levels of all G1/S genes in young cells were more than tenfold higher than that in old cells 12 hr after serum stimulation. The enzymes encoded by TS and DHFR genes and dUTPase also exhibited similar age-dependent attenuation in activities. In contrast, expression of growth-related genes such as eIF-5A, c-Ha-*ras*, and β-actin did not show significant differences between young and old cells after serum stimulation. Computer analysis of the promoter region of these G1/S genes revealed an Sp-1 binding site as the most common cis-element. Taken together, our results suggest that the suppression of G1/S gene expressions during senescence may be a global phenomenon and that G1/S genes may be coordinately controlled.

Normal diploid fibroblasts have a limited doubling potential in culture (Hayflick and Moorhead, 1961; Hayflick, 1979). The remarkable consistency of the life span of these cells in culture, which is inversely related to the age of the donor, and the species specificity of the life span (Martin, 1977) have made them a useful model to study the biochemistry of cellular aging. The hallmark of cellular aging is the failure of old cells to initiate DNA synthesis after growth stimulation (Cristofalo and Sharf, 1973). Earlier studies have shown that replication points of DNA in mammalian cells are situated on the nuclear membrane (e.g., Shearman and Kalf, 1977). Only membrane aggregates isolated from nuclei in cells undergoing DNA replication can synthesize DNA (Infante et al., 1976). Some studies have shown that these aggregates contain enzymes such as thymidine kinase (TK), thymidylate synthase (TS), dihydrofolate reductase (DHFR), and ribonucleotide reductase (RNR) that are essential for making DNA precursors (Reddy and Pardee, 1980). Kinetic studies support the notion that a coordinate expression of these genes at G1/S boundary may be a prerequisite essential for DNA replication (Das, 1981). Concomitant with the onset of DNA synthesis, these enzymes show dramatic increases of activities (Baserga, 1985; Pardee, 1989). The genes encoding for these enzymes are all serum inducible and highly expressed at late G1/S boundary. One possible cause for the loss of dividing potential in senescent cells could be due to an inactivation of enzymes such as TK, TS, DHFR, and RNR.

Alterations of gene regulation at late G1/S boundary during senescence have been proposed to play a key role in the aging of normal human cells (Chen et al., 1989; Goldstein, 1990). We have previously reported that the steady state level of TK mRNA in old IMR-90 cells was sevenfold less than that in young cells 24 hr after serum stimulation (Chang and Chen, 1988). Recently, expressions of H3 (Seshadri and Campisi, 1990), cdc2, cyclin A and B (Stein et al., 1991), and proliferating cell nuclear antigen (PCNA; Chang et al., 1991), all G1/S genes, have been shown to be suppressed in senescent cells. These studies raise an interesting question on whether the attenuation of gene expression at G1/S

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*To whom reprint requests correspondence should be addressed.

boundary is limited to certain genes or it may represent a more general phenomenon. To address this question, we carried out a systematic investigation on the expression of two classes of G1/S genes in human IMR-90 cells during senescence: (1) genes that encode for enzymes needed for DNA biosynthesis including TS, DHFR, RNR, and PCNA, and (2) genes that encode for the replication-dependent histones including H1, H2A, H2B, H3, and H4. In addition to the steady state mRNA levels, the activities of enzymes encoded by TS and DHFR genes and deoxyuridine triphosphate nucleotidohydrolase (dUTPase), another enzyme involved in DNA synthesis, were also compared in young and old IMR-90 cells following serum stimulation. The expression of all eight G1/S genes were significantly suppressed in senescent cells. The enzyme activities associated with TS and DHFR genes were also reduced in senescent cells. These findings suggest that coordinate regulation of gene expression at G1/S boundary may represent an important component in the aging program for normal human diploid cells.

MATERIALS AND METHODS

Cell culture

IMR-90 human embryonic lung fibroblasts at both low and high passage numbers (passage number = 5 and 16; population doubling level [PDL] = 10 and 38.5, respectively) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Confluent cultures of IMR-90 cells were expanded through subculturing at 1:4 or 1:8 split ratio to obtain cultures at a desired PDL as previously described (Chang and Chen, 1988).

Growth stimulation

Confluent cultures of young and old IMR-90 cells were serum deprived for 48 hr and then stimulated by replenishing the cultures with fresh Dulbecco's medium containing 10% fetal bovine serum. Cells were harvested at designated times after serum stimulation for RNA analysis and enzymatic assay.

Probe preparation

Escherichia coli cultures containing the desired plasmids were grown in LB media in the presence of an appropriate antibiotic with vigorous shaking at 37°C overnight. The plasmid DNA was purified according to the procedure as described (Sambrook et al., 1989). Nick translation of the plasmid DNA was carried as previously described (Chang and Chen, 1988; Chen and Chen, 1992).

Abbreviations

TK	thymidine kinase
TS	thymidylate synthase
DHFR	dihydrofolate reductase
PCNA	proliferating cell nuclear antigen
RNR	ribonucleotide reductase
dUTPase	deoxyuridine triphosphate nucleotidohydrolase
ODC	ornithine decarboxylase
PDL	population doubling level

Northern blot analysis

Total cellular RNA was isolated by lysis in a guanidinium thiocyanate buffer followed by a one-step phenol-chloroform-isoamyl alcohol extraction to remove DNA and protein as described by Chomczynski and Sacchi (1987). The 28S and 18S ribosomal RNA were used as internal size markers of 5.0 kb and 2.0 kb, respectively, and to ensure that the amount of loaded RNA in each lane was identical. Northern blot hybridization was carried out as previously described (Chen and Chen, 1992).

Enzyme activity assay

TS activity was determined essentially as described by Navalgund et al. (1980). The method measures the release of tritium, as tritiated water, from [5-^{3}H]dUMP during the formation of thymidylate. DHFR activity was determined with [^{3}H]methotrexate binding assay by scintillation counting and expressed as cpm/mg of proteins (Johnson et al., 1978). The activity of dUTPase was determined as described (Caradonna and Adamkiewicz, 1984). The activity of alkaline phosphatase was assayed following the protocols of Bessey et al. (1946). Protein concentration was determined by bicinchoninic acid (BCA) protein assay following the protocols given by the manufacturer (Pierce, IL).

Materials

All tissue culture media and sera were obtained from GIBCO (Grand Island, NY). [α-^{32}P]dCTP (3,000 Ci/mmol), [α-^{32}P]dGTP (3,000 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol) were from ICN Chemical Radioisotope Division (Irvine, CA). Restriction enzymes and other molecular biological supplies were from Pharmacia (Piscataway, NJ). All other chemicals were of reagent grade. Plasmids pMTS-3 was given by Dr. Lee F. Johnson (Ohio State University); pDHFR11 was given by Dr. Robert Schimke (Stanford University); pFO535 and PCNA-G4 were given by Dr. R. Baserga (Temple University Medical School); R1 and R2 cDNA in pUC18 were given by Dr. L. Thelander (University of Umea); and pFNC16A, pFF435B, pFF435C, and pFO108A were given by Dr. Janet Stein, (University of Massachusetts).

RESULTS

Induction of G1/S genes encoding for enzymes involved in DNA synthesis

The enzymes of the DNA synthesizing machinery constitute a group of gene products that are generally expressed at the G1/S boundary of the cell cycle (e.g., Baserga, 1985). Expression of these genes such as TK, TS, DHFR, and RNR has been shown to be serum inducible and cell cycle dependent (Baserga, 1985; Pardee, 1989). To determine whether the expressions of these genes also exhibit age-dependent characteristics, we have examined the time course of TS, DHFR, RNR, and PCNA gene expression in young and old IMR-90 cells following serum stimulation. Figure 1 shows that the mRNA levels all of these genes at quiescent state (time zero) were quite low in both young and old cells. All four genes were induced to high level in young cells

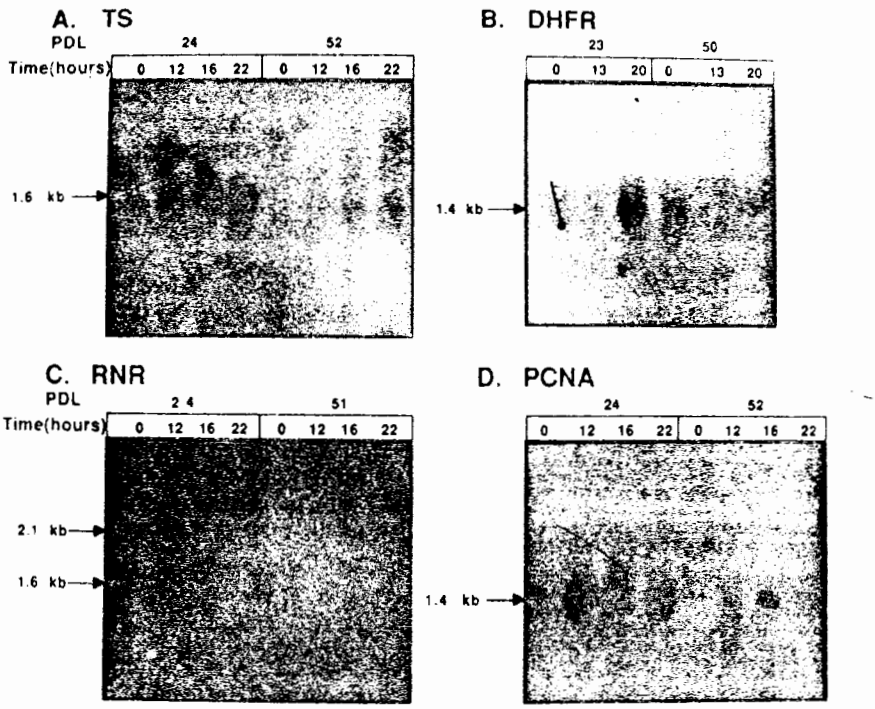

Fig. 1. The induction of G1/S gene expression in IMR-90 cells. Confluent cultures of young and old IMR-90 cells were serum deprived for 48 hr and then stimulated by fresh growth medium containing 10% fetal bovine serum. At designated time points after serum stimulation, cells were harvested for the determination of the steady state level of TS, DHFR, RNR, and PCNR mRNA. **A:** TS, the plasmid pMTS-3 containing 1 kb cDNA insert corresponding to part of the mouse TS mRNA, was used by hybridize the TS mRNA. Each lane contained 10 μg of total RNA. **B:** DHFR, the level of DHFR mRNA was determined by Northern blot analysis using plasmid pDHFR11 which contains all of the murine DHFR coding region, 50 bp of the 5′ noncoding region, and 950 bp of the 3′ noncoding sequence. Each lane contained 15 μg of total RNA. **C:** RNR, the nick-translated plasmid R2 was used to probe the ribonucleotide reductase mRNA. Each lane contained 20 μg of total RNA. **D:** PCNA, the nick-translated pPCNA-G4 was used to probe PCNA mRNA. Each lane contained 10 μg of total RNA.

12 hr after serum stimulation. Such an induction, however, was either strikingly dimished or absent in the old cells.

The induction kinetics and the level of induction that we observed for TS mRNA in young IMR-90 cells were similar to those reported for mouse cells (Geyer and Johnson, 1984) and for human TIG-1 cells (Ayusawa et al., 1986). The induction of TS mRNA in old cells, however, was barely detectable throughout the course of serum stimulation. Based on densitometric tracing, we estimated that the TS mRNA level in young cells was more than eightfold higher than that in old cells 22 hr after serum stimulation (Fig. 1A).

The DHFR gene encodes an enzyme involved in the production of purines, glycine, and thymidylate. The DHFR gene in mouse fibroblasts has been shown to be cell cycle dependent and regulated at transcriptional level (Liu et al., 1985; Farnham and Schimke, 1985). The induction of DHFR mRNA in young IMR-90 cells was evident 20 hr after serum stimulation. However, such induction was almost absent in old cells (Fig. 1B). The promoter sequence of human DHFR, similar to that in mouse gene, contains E2F and Sp1 binding sites. We have recently found that E2F binding activity was significantly reduced in IMR-90 cells during senescence (Good, L. and Chen, K.Y., unpublished data).

RNR catalyzes the first unique step on the biochemical pathway leading to DNA synthesis (Thelander and Reichard, 1979). In mammalian cells, RNR consists of two nonidentical subunits, M1 and M2. The level of protein M1 is constant throughout the cell cycle, whereas the level of M2 appears to be cell cycle dependent (Engstrom et al., 1985). Therefore, the plasmid R2 which contains most of the translated sequence of the protein M2 cDNA (Thelander and Berg, 1986) was used to determine the levels of RNR mRNA in IMR-90 cells. Two RNR M2 transcripts with sizes of 1.6 and 2.1 kb were detected in young IMR-90 cells, similar to those reported for mouse TA3 fibroblasts (Thelander and Berg, 1986). Both M2 transcripts in young cells started to increase 12 hr after serum stimulation and increased by more than eightfold 22 hr after serum stimulation. In contrast, the RNR mRNA level in old cells was barely detectable throughout the time course examined (Fig. 1C).

PCNA functions as an auxiliary subunit of the eukaryotic DNA polymerase δ and it is necessary for DNA replication and cell cycle progression (Celis et al., 1984; Bravo et al., 1987; Prelich et al., 1987). The pPCNA-G4 contains the full length cDNA for human PCNA (Almendral et al., 1987). Figure 1D shows that serum induced a significant increase in PCNA mRNA level in

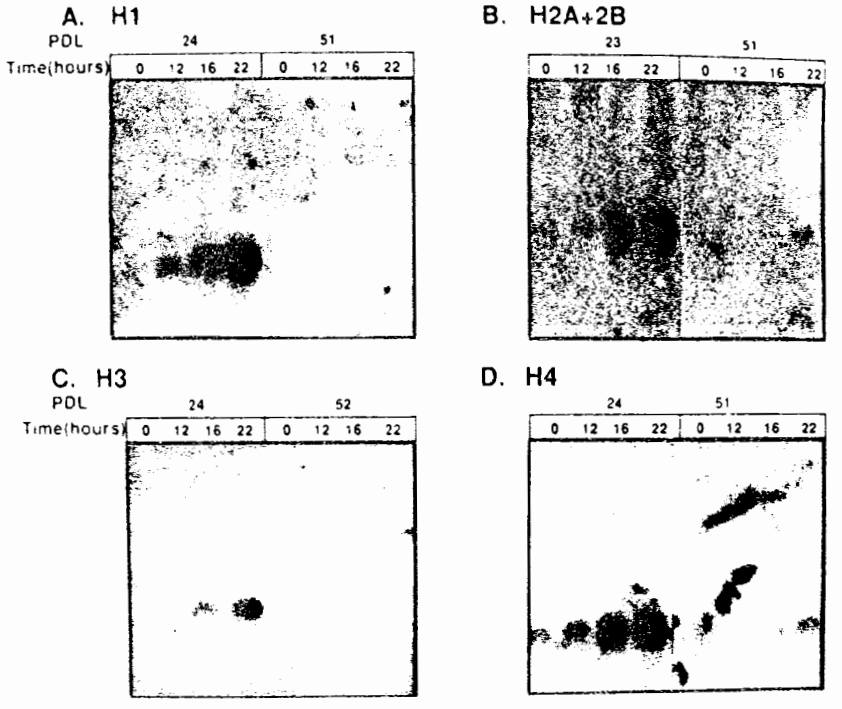

Fig. 2. Time course of the serum-induced histone mRNAs in young and old IMR-90 cells. Confluent cultures of young and old IMR-90 cells were serum deprived for 48 hr and then stimulated with 10% fetal bovine serum. At designated time points, cells were harvested and total RNA was prepared for Northern blot hybridization. The nick-translated plasmids, pFNC16A, pFF435B, pFF435C, and pFO108A were used to detect mRNAs of histone H1, H2A + 2B, H3, and H4, respectively. **A:** H1, each lane contained 20 μg of total RNA. **B:** H2A + 2B, each lane contained 20 μg of total RNA. C: H3, each lane contained 7 μg of total RNA. D: H4, each lane contained 20 μg of total RNA.

young IMR-90 cells, similar to that reported for other cell types (Celis et al., 1984; Morris and Mathews, 1989; Chang et al., 1991). Very little or almost no induction of PCNA mRNA was observed in old IMR-90 cells. The PCNA mRNA level in young cells was about 15-fold greater than that in old cells 16–22 hr after serum stimulation.

Expression of the histone genes in young and old IMR-90 cells

The histone genes encode a set of proteins essential for maintaining the integrity of eukaryotic chromosomal structure. The expression of the proliferation-specific histone genes is tightly coupled to DNA replication and appears to be cell cycle regulated (reviewed in Stein et al., 1984). If G1/S boundary represents a "hot" point for age-dependent gene regulation, one may expect that the expression of histone genes also exhibits age-dependent attenuation in human diploid fibroblasts. We therefore compared the expression of histone H1 gene and core histone genes, H2A and H2B, H3, and H4, in both young and old IMR-90 cells during the course of serum stimulation (Fig. 2). All histone mRNAs were at extremely low level when both young and old cells were at quiescent state. Serum stimulation caused a continuous increase in levels of all histone mRNAs in young cells from 12 to 22 hr following the addition of serum. The kinetics and magnitude of induction for these four histone genes in young IMR-90 cells are similar to those reported for other cultured cells (Plumb et al., 1984). In contrast to those in young cells, levels of the all four histone mRNAs in old IMR-90 cells were only barely detectable throughout the time course of serum stimulation.

G1/S enzyme activities in young and old IMR-90 cells after serum stimulation

Attenuation of gene expression at the mRNA level may not lead to the suppression of enzyme activity if the activity is controlled primarily at the posttranslational level. We therefore examined whether the attenuation of TS and DHFR gene expression in old IMR-90 cells correlated with decreases in the respective enzyme activities. Figure 3 shows that serum stimulation caused increases in TS and DHFR activities in both young and old IMR-90 cells. However, the increase was more pronounced in young cells than in old cells. Thus, TS and DHFR activities in young cells were, respectively, three to fourfold and five to tenfold higher than those in old cells 20–30 hr after serum stimulation. Since there was a slight increase in TS and DHFR activity in senescent cells in spite of low mRNA level, it is possible that there may exist a small component of translational and/or posttranslational control mechanism for both TS and DHFR gene expression. However, since the differences in activities for TS and DHFR between young and old cells could be accounted for by the differences in mRNA levels (Fig. 3 vs. Fig. 1A,B), we concluded that TS and DHFR gene expression in

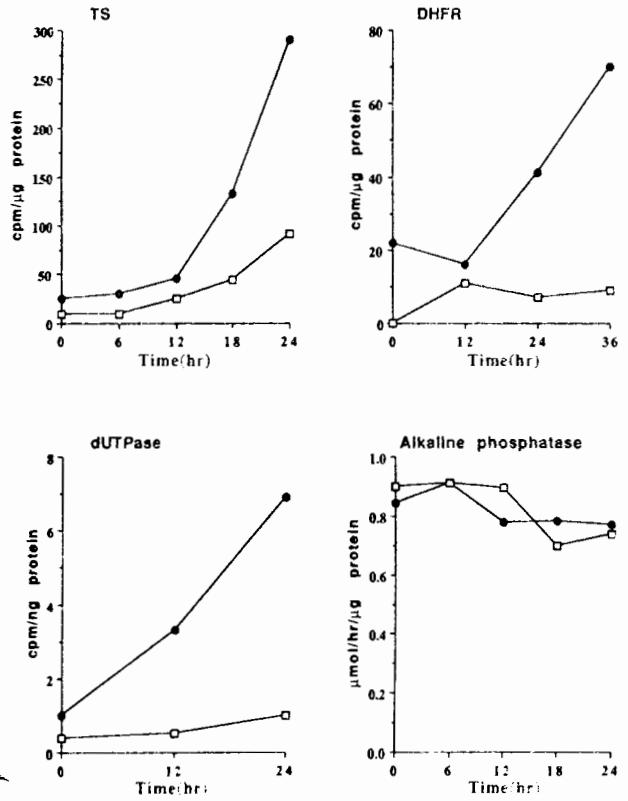

Fig. 3. Time course of the serum-induced activities of TS, DHFR, dUTPase, and alkaline phosphatase in young and old IMR-90 cells. Confluent cultures of young (PDL = 21) and old (PDL = 48) IMR-90 cells were serum deprived for 48 hr and then stimulated by 10% fetal bovine serum. At designated time points after serum stimulation, cells were harvested for the determination of enzyme activities as described in Materials and Methods.

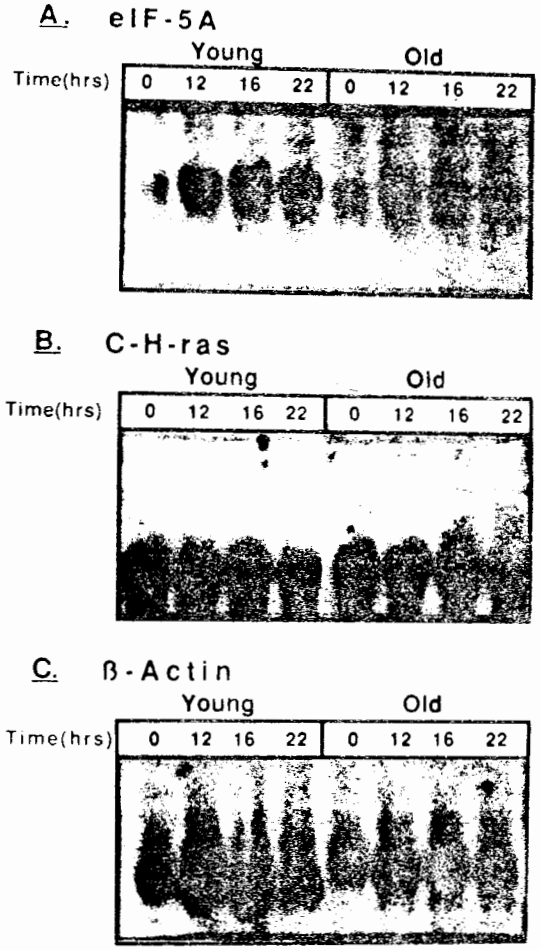

Fig. 4. The expression of eIF-5A, c-Ha-*ras*, and β-actin genes in young and old IMR-90 cells. Total RNA was isolated from both young (PDL = 24) and old (PDL = 51) IMR-90 cells at different time points after serum stimulation and processed for the Northern blot hybridization with eIF-5A (**A**), c-Ha-*ras* (**B**), and β-actin (**C**) cDNA probes. Each lane contained 20 μg of total cellular RNA.

IMR-90 cells during senescence is controlled primarily at the transcriptional or posttranscriptional level.

In addition to these two enzymes, we also measured the activity of dUTPase and alkaline phosphatase activity in young and old cells during serum stimulation. The enzyme dUTPase, which catalyzes the hydrolysis of dUTP to dUMP and inorganic pyrophosphate, plays a key role in keeping a significant amount of dUTP from participating DNA synthesis and in producing substrate for de novo dTMP synthesis. Figure 3 shows that dUTPase was serum inducible and its induction was significantly attenuated in old cells. In contrast, there was no difference in alkaline phosphatase activity between young and old cells throughout the time course during serum stimulation.

Expression of eIF-5A, c-Ha-*ras*, and β-actin in IMR-90 cells

Figure 4 shows the time course of the expression of three other growth-related genes, eIF-5A, c-Ha-*ras*, and β-actin, in young and old IMR-90 cells after serum stimulation. The expression of eIF-5A gene in both young cells and old cells appeared to be serum inducible, low at quiescent state, and started to increase after serum stimulation. The maximal expression of eIF-5A gene in both young and old cells occurred no later than 12 hr after serum stimulation, indicating that eIF-5A is a mid-G1 gene (Fig. 4A). The steady state of eIF-5A mRNA levels in old cells throughout the time course were comparable to, albeit 10–20% less than that in young cells. Both c-Ha-*ras* and β-actin genes in young and old IMR-90 cells did neither show cell cycle dependency nor age-dependent alteration in gene expression (Fig. 4B,C). These results indicate that old cells can enter the cell cycle and that not all growth-related genes are suppressed during senescence.

Expressions of various cell cycle-dependent genes in senescent human diploid fibroblasts

Figure 5 shows in a schematic diagram which represents a summary of all studies, including the present one, the expressions of cell cycle-dependent genes in human diploid fibroblasts during senescence. Altogether, 12 G1/S genes have been examined and all appear to be suppressed in serum-stimulated senescent IMR-90 cells. In contrast, among the 13 early G1 and

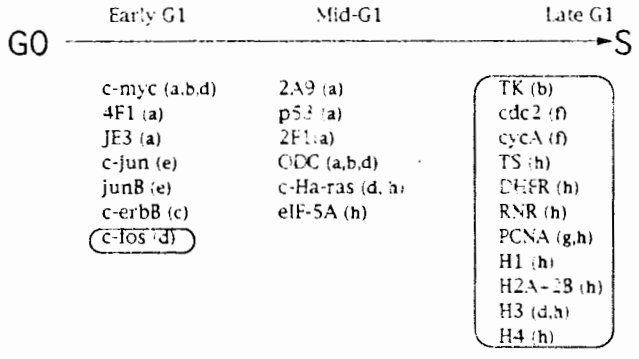

Fig. 5. Schematic diagram of the expression of various cell cycle-dependent genes in serum-stimulated senescent human diploid fibroblasts. Senescent human diplod fibroblasts at quiescent state can respond to serum stimulation by sequentially expressing most of cell cycle-dependent genes at mRNA levels during early G1 and mid-G1 phases to similar levels as those in young cells. However, the senescent cells appear to be blocked at the G1/S boundary. Among the early G1 and mid-G1 genes examined, only c-*fos* appears to be suppressed in senescent cells. In contrast, all the late G1 S genes examined appear to be suppressed in serum-stimulated senescent IMR-90 cells. Genes are identified by the gene product or the name of the cDNA clone. Genes whose expressions show age-dependent attenuation are circled. References indicated in the parathesis are: (a) Rittling et al. (1986); (b) Chang and Chen (1988); (c) Chen et al., (1989); (d) Seshadri and Campisi (1990); (e) Pignolo et al. (1990); (f) Stein et al. (1991); (g) Chang et al. (1991); (h) Pang and Chen, this study.

mid-G1 genes studies, only c-*fos* has been shown to exhibit an age-dependent attenuation in cells during senescence. Taken together, these data suggest that the attenuation of gene expression at the G1/S boundary in normal diploid fibroblasts during aging may represent a global and coordinate change and that G1/S boundary could be a hot spot for controlling expression of age-dependent genes.

Trans-acting factor binding sites in the promoter regions of G1/S genes

In light of possible coordinate suppression of G1/S genes in senescent cells, the controlling points upstream of the regulatory pathways of these genes are of interest. As an initial effort, we have examined whether these G1/S genes share any common cis-elements in their promoter region. Using GCG FIND program we have scanned the entire promoter region for trans-acting factor binding sites in nine G1/S genes. The results, summarized in Table 1, show that the most common cis-element in these genes is Sp-1 binding site. Other cis-elements shared by at least four G1/S genes include CCAAT, AP-1, and E2F. We have previously reported that Sp-1 binding in IMR-90 cells is neither serum responsive nor age dependent (Pang and Chen, 1993). Thus, at the transcriptional level of the regulatory hierarchy, it is unlikely that any particular trans-acting factor shown in Table 1 may serve as the master switch candidate responsible for the suppression of G1/S genes during cell senescence.

DISCUSSION

Since the hallmark of cellular aging is the loss of dividing potential, the suppression of some G1/S genes may play an important role in controlling the aging process. Whether only few or most, if not all, of G1/S genes are suppressed in normal cells during senescence is not clear. To address this question, we have focused on two classes of G1/S genes: one encodes enzymes such as TK (Chang and Chen, 1988), TS, DHFR, RNR, and PCNA, which are necessary for the biosynthesis of DNA; the other class encodes histones which are needed for maintaining the integrity of chromosomal structure during division. We found that all of these genes exhibit a significant age-dependent attenuation of expression during the course of serum stimulation (Figs. 1, 2). The degree of attenuation in both cases is striking; the steady state mRNA levels of all these genes in old cells were less than 15% of that in young cells 16–22 hr after serum stimulation. The suppression of G1/S genes occurs not only at mRNA levels, enzymes encoded by genes such as TS and DHFR also showed corresponding reduction in activities in old cells (Fig. 3).

In contrast to G1/S genes, all mid-G1 genes that we and others have examined, including eIF-5A appear to be induced by serum in senescent cells as well as in young cells (Fig. 4). The results on the study of cell cycle-dependent gene expression in human diploid fibroblasts (Fig. 5) indicate that (1) senescent cells can enter the cell cycle after mitogenic stimulation, (2) the expressions of many mid-G1 genes in senescent cells are not affected by the defect of c-*fos*, and (3) biochemical events that occur between mid-G1 and late-G1 phase may be crucial in causing the global suppression of G1/S genes in serum-stimulated senescent cells. In this regard, it is of interest to note that although expressions of mid-G1 genes such as eIF-5A and ornithine decarboxylase (ODC) do not show age-dependent attenuation at mRNA levels, there exists a significant difference in the translation and/or posttranslational modification of their gene products (Chang and Chen, 1988; Chen et al., 1992). Whether the attenuation of both ODC enzyme activity and posttranslational hypusine formation on eIF-5A in old cells can be related to suppression of late G1/S genes remains to be investigated.

The fact that all G1/S genes that have been examined exhibit an age-dependent attenuation of expression in senescent human diploid fibroblasts raises some interesting questions. For example, do these genes share a common or similar regulatory mechanism? Is there a master switch to turn off these genes? What are the upstream regulatory elements responsible for the attenuation of these genes in senescent cells? Since we cannot identify any common cis-element in the promoter region for all G1/S genes examined in the present study (Table 1), it seems necessary to examine the regulatory mechanism of each of these G1/S genes in human cells during senescence in detail in order to answer the above questions. In this regard, we have recently shown that a G1/S-specific trans-acting factor, CBP/tk, binds specifically to human TK promoter, and the binding appears to be not only serum responsive but also age dependent (Pang and Chen, 1993). It is possible that CBP/tk and other similar proteins may represent a class of trans-acting factors which not only control cell cycle-dependent, but also age-dependent, gene expression during cell senescence.

TABLE 1. Computer search for the trans-acting factor binding sites in the promoter regions of the human G1/S genes[1]

TF[2] Gene[3]	CCAAT	OCT	AP-1	NFkB	CRE	SP1	Yi	E2F	TATA
TK	−136 −106	—	—	—	—	−481 −296 −180 −117	−183	—	−83 −340
DHFR	—	—	—	—	—	−266 −119 −85	−94	−67	—
TS	—	−510	−634	—	—	−165 −45 −14	−185	−129	−489
PCNA	−95 −145	−349	−891	—	−50 −260	−129 −159 −193 −523	—	−42	—
H1	−112 −126	—	−197	—	—	−134 −359	—	—	−89
H2A	—	—	—	—	—	—	—	—	−84
H2B	−168 −125	−94	−136	—	−146	—	—	−250	−268 −75
H3	−94 −137	−379	—	—	−203	−153 −378	—	—	−108
H4	—	—	—	—	−142	−102 −125	—	—	−214

[1]The numbers indicate the positions of cis-elements relative to the translational initiation site identified in GenBank.
[2]TF indicates trans-acting factor recognition sites as searched by the program FIND using GCG Sequence Analysis Software Package.
[3]The sequence for human ribonucleotide reductase is not available. Instead, we included human TK gene.

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