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

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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 if 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 (RR), proliferating cell nuclear antigen (PCNA), histone H1, histone H2A + H2B, histone H3, and histone H4, was induced to high levels in young IMR-90 cells but not in old IMR-90 cells. The RNA levels of all G1/S genes in young cells were more than twofold higher than those in old cells 12 hr after serum stimulation. The enzymes encoded by TS and DHFR genes and PCNA also exhibited similar age-dependent attenuation in activities. In contrast, expression of growth-related genes such as Elf-3A, Elf-4, and β-fodrin did not show significant differences between young and old cells after serum stimulation. Considerable analysis of the promotore region of these G1/S genes revealed an Sp1-binding site as the most common cis-element. Taken together, our results suggest that suppression of G1/S gene expression during senescence may be a global phenomenon and that G1/S genes may be coordinately controlled.

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Normal diploid fibroblasts have a limited doubling potential in culture (Hayflick and Moorhead, 1961; Hayflick, 1975). 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 (Hodgkin and Sharp, 1973). Earlier studies have shown that replication points of DNA in mammalian cells are situated on the nuclear membrane (e.g., Searman and Kahl, 1977). Only membrane aggregates isolated from nuclei in cells undergoing DNA replication can synthesize DNA (Infante et al., 1970). Some studies have shown that these aggregates contain enzymes such as thymidylate kinase (TK), thymidylate synthase (TS), dihydrofolate reductase (DHFR), and ribonucleotide reductase (RR), and these enzymes are essential for making DNA precursors (Reddy and Pardee, 1985). Kinetic studies support the notion that coordinate expression of these enzymes at G1/S boundary may be a prerequisite essential for DNA replication (Das, 1988). Concomitant with the change in expression of DNA synthesis, these enzymes show dramatic increases in activities (Bazerga, 1985; Pardee, 1985). The gene-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 RR.

Alterations of gene regulation in 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; Golderstcin, 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, expression of Elf-3A (Bouck and Campisi, 1990; Oak, 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 boundary during senescence may be a global phenomenon and that G1/S genes may be coordinately controlled.

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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: (i) genes that encode for enzymes needed for DNA biosynthesis including TS, DHPR, RNR, and PCNA, and (ii) 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 DHPR 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 DHPR genes were also reduced in serum-supplemented culture medium containing 10% fetal bovine serum. Cells were harvested at designated times after serum stimulation for RNA analysis and enzymatic assay.

Northern blot analysis
Total cellular RNA was isolated by lids (in a g2/90 ncentration of 0.2 g/ml) followed by one-step phenol/chloroform-isooctyl alcohol extraction to remove DNA and protein as described by Chomczynski and Sacchi (1987). The 22S and 18S ribosomal RNAs were visualized as internal size markers of 5.9 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, 1999).

Enzyme activity assay
TS activity was determined essentially as described by Nasalud et al. (1980). The method measures the release of tritium, as tritiated water, from 3H-5HTP during the formation of thymidylate. DHPR activity was determined with [3H]thymidylate binding assay by scintillation counting and expressing as cpm mg of protein. The activity of dUTPase was determined as described by Caradonna and Adamiec (1984). The activity of alkaline phosphatase was assayed following the protocols of Bessey et al. (1982). Protein concentration was determined by bichromonic 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). Trizol (3,000 Cu mmol), [3H]-2HGTP (3,000 Ci mmol), and [3H]-2GATP (5,000 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. Plasmid pMFS-3 plasmid was given by Dr. Lee F. Johnson (Ohio State University). pDHFR-11 was given by Dr. Robert Schimke (Stanford University). pDHFR was given by Dr. R. Baserga (Temple University Medical School). R1 and R2 DNA in pHCC12 were given by Dr. L. Thielander (University of Umea) and pFNC16A, pFF435B, pFF435C, and pFO168A were given by Dr. Janet Stein, (University of Massachusetts).

RESULTS
Induction of G1/S gene expression for enzymes involved in DNA synthesis
The enzymes of the DNA synthesis 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, DHPR, and RNR has been shown to be serum-inducible and cell cycle dependent (Bosherg, 1985; Parsons, 1989). To determine whether the expression of these genes also exhibit age-dependent characteristics, we have examined the time course of TS, DHPR, RNR, and PCNA gene expression in young and old IMR-90 cells following serum stimulation. Figure 1 shows that the mRNA levels of all 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 GlS 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 media containing 10% fetal bovine serum. At designated times post-serum stimulation, cells were harvested for the determination of the steady state level of TS, DHFR, RNR, and PCNA mRNAs. A: The, the plasmid pMRS-3 containing 1 kb of DNA insert corresponding to a part of the mouse TS mRNA, was used to 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 pDHFR1 which contains all of the mouse DHFR coding region. 50 μg of the 3-microgram sample. Each lane contained 10 μg of total RNA. C: RNR, the nick-translated plasmid R1 was used to probe the ribonucleotide reductase mRNA. Each lane contained 20 μg of total RNA. D: PCNA, the nick-translated plasmid PCNA-G4 was used to probe PCNA mRNA. Each lane contained 20 μg of total RNA.

12 hr after serum stimulation. Such an induction, however, was either strikingly dimmed 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 scanning, 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 thymidine. The DHFR gene in mouse fibroblasts has been shown to be cell cycle dependent and regulated at transcriptional level (Liu et al., 1985; Pabst 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 in 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 FnR M2 transcripts with sizes of 1.6 and 2.1 kb were detected in young IMR-90 cells, similar to those reported for mouse TAg 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 (Celsi et al., 1984; Bravo et al., 1986; Pichler et al., 1987). The pCNA-G4 contains the full length cDNA for human PCNA (Ait- mulikof 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, pNC168, pFF43SS, pFF45SC, and pGG09A were used to detect mRNAs of histones H1, H2A + B, H3, and H4, respectively. A: H1, each lane contained 20 µg of total RNA. B: H2A + B, 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 (Celliat 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 Zain 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 IMR-90 cells, levels of 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 the presence 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
DMR-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 in 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 elf-5A, c-Ha-ras, and β-actin in DMR-90 cells

Figure 4 shows the time course of the expression of three other growth-related genes, elf-5A, c-Ha-ras, and β-actin, in young and old DMR-90 cells after serum stimulation. The expression of elf-5A gene in both young and old cells appeared to be serum inducible, low at quiescent state, and started to increase after serum stimulation. The maximal expression of elf-5A gene in both young and old cells occurred no later than 12 hr after serum stimulation, indicating that elf-5A is a mid-G1 gene (Fig. 4A). The steady state of elf-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 DMR-90 cells did not 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 DMR-90 cells. In contrast, among the 13 early G1 and
mid-G1 gene studies, only e5a 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 that both G1/S boundary and G1/S boundary could be used to test the hypothesis that G1/S gene expression 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 aging 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 in old cells were less than 15% of that in young cells 12-22 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 cells (Fig. 3). In contrast to G1/S genes, all mid-G1 genes that we and others have examined, including e1f-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. 3) 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 effect of e5a, 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 levels of normal cell cycle-acting enzymes (decarboxylase, ODC) do not show age-dependent attenuation at mRNA levels, there exist a significant difference in the transition and/or posttranslational modification of their gene products (Chang and Chen, 1988, 1993). Whether the attenuation of both ODC enzyme activity and posttranslational hypusine formation on e1f-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 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 this regard. Further studies will be required to answer the above questions. In this regard, we have recently shown that 1G1/S-specific trans-acting factor CBF1, 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 CBF1 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: G1/S Gene Expression in Normal and Old Cells

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### Figure 3: Schematic Diagram of the Expression of Various Cell Cycle-Dependent Genes in Serum-Stimulated Senescent Human Diploid Fibroblasts

The figure shows the expression of various cell cycle-dependent genes in senescent human diploid fibroblasts at different stages of the cell cycle. The genes are highlighted in different colors to indicate their expression levels in normal and old cells.

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*The numbers indicate the positions of the elements relative to the transcriptional initiation site identified in C6/F9.

**Table data taken from a larger compilation, as recorded by the program FONDO using GC/Sequence Analysis Software (Feng).**

**The sequence for human thymidine kinase is as follows:**

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**LITERATURE CITED**


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