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Expression of cell cycle-dependent genes in young and senescent WI-38 fibroblasts

(oncogenes/histone H3/G_1/S phase)

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ABSTRACT **We studied the expression of 11 cell cycle-dependent genes in senescent WI-38 fibroblasts and compared the results to those obtained in WI-38 cells from early passages (young cells). Every gene we examined is expressed in the senescent cells at levels similar to those in the young cells, including two genes maximally expressed at the G_1/S phase boundary—genes for thymidine kinase and histone H3. The results clearly show that senescent, noncycling WI-38 cells are not similar to quiescent cells. Rather, such senescent WI-38 cells may be blocked just prior to the onset of DNA synthesis.**

The human diploid fibroblast line WI-38 has a limited lifespan in culture (1, 2) and has been used frequently as a model of cellular senescence. Cultures are capable of a prolonged period of high proliferative activity followed by a gradual decrease in growth rate (1) and an increase in the fraction of cells arrested in the nonreplicative phase (3). The *in vitro* aging of these cells is characterized by an exponential decline in the percentage of the population capable of synthesizing DNA and an increase in cell cycle time (4). The primary increase in cell cycle time occurs in the G_1 phase, which progressively lengthens until the cells eventually become arrested (4). Several lines of evidence suggest that senescent cells are blocked in late G_1 rather than in G_0 phase, as are quiescent cells. First, the level of thymidine kinase (TK) activity in senescent, slowly proliferating cultures is similar to that of young, rapidly dividing populations (5). Second, the expansion of the thymidine triphosphate pool prior to DNA synthesis is similar in young and senescent cells (6). Finally, the nuclear fluorescence pattern of senescent cells after staining with quinacrine dihydrochloride is typical of cells that are blocked in late G_1 or at the G_1/S boundary (7, 8).

There are several genes whose expression is known to be cell cycle-dependent, the term expression being used here in one of its accepted usages—i.e., as levels of cytoplasmic mRNAs. Cell cycle-dependent genes include the oncogenes c-*myc* (9), c-*fos* (10–13), and c-*ras* (14–16); the gene encoding the cellular tumor antigen p53 (17); genes isolated as cDNA clones in our (18) and in other (19–21) laboratories on the basis of their cell cycle dependency of expression; and genes coding for well-characterized cellular proteins, such as ornithine decarboxylase (ODCase) (22, 23), β-actin (24, 14), thymidine kinase (TK) (25), and histones (26–28). Most of these genes are induced in different cell types stimulated by different mitogens (29), and their mRNA levels can be used as markers of a cell's progression through G_1 and S phases (29).

In this paper, we report our investigation of the expression of several of these cell cycle genes in senescent WI-38 cells and comparison of the results to those obtained in young cells. These experiments are similar to those performed with temperature-sensitive (ts) mutants of the cell cycle (25, 28, 30) in which the expression or lack of expression of cell cycle genes is used to map temporally the lesion in such cells. But in contrast to experiments with ts mutants, we find that every gene we have studied is fully expressed in senescent cells, including those that are preferentially expressed late in G_1 and in S phase. The results clearly demonstrate that the expression of several cell cycle genes is not diminished in cells that fail to enter S phase. The implication is that cell cycle progression is not arrested in senescent cells at least until they reach the G_1/S boundary.

METHODS

Cell Culture. Stock cultures of WI-38 cells were maintained in Earle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (vol/vol) as described (3, 31). *In vitro* age was determined by [^{3}H]thymidine incorporation into DNA (3). Young cultures were defined as those whose lifespan was <50% completed, and senescent cultures were defined as those whose lifespan was >90% completed.

Young and senescent cells were seeded at 1×10^4 cells per cm^2 into MEM containing 10% fetal bovine serum and allowed to grow to confluency (9 days for young cells and 14 days for senescent cells). Confluent monolayers were then rinsed and refed with MEM containing 0.1% fetal bovine serum and allowed to incubate for 40 hr to attain a uniform quiescent state. In some experiments, cells that were density-arrested but not serum-depleted were used. Similar results were obtained whether the cells were prepared by one method or the other. After synchronization, cells were refed with MEM containing 10% serum and harvested for RNA extraction at 0, 4, 17, or 24 hr after stimulation.

RNA Extraction. WI-38 cells were harvested as follows. The monolayers were washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (GIBCO), and the second wash was removed completely with a pipette. The cells were then rinsed in 3 ml of 0.25% trypsin in Ca^{2+}/Mg^{2+}-free Hanks' salts, which was quickly removed (<5 min). Phosphate-buffered saline (5 ml) was then added to each flask, and the cells were scraped into this solution and pelleted. Cells were lysed with 1% Nonidet P-40 in 0.15 M NaCl/0.01 M Tris, pH 7.9/1.5 mM $MgCl_2$ containing 10 mM vanadyl–ribonucleoside complex (Bethesda Research Laboratories). Cytoplasmic RNA was prepared from the postnuclear supernatant by repeated phenol extractions (18). Poly(A)$^+$ RNA was prepared by a single passage over oligo(dT)-cellulose (Collaborative Research, Waltham, MA) (32). Equal amounts, usually 1 or 3 μg, of poly(A)$^+$ RNA prepared from young and

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Abbreviations: TK, thymidine kinase; ts, temperature sensitive; ODCase, ornithine decarboxylase.

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senescent cells at different times after serum stimulation were fractionated on 1% agarose gels in the presence of 6.7% formaldehyde and blotted onto nitrocellulose (33). The blots were hybridized to nick-translated probes (34) in the presence of 50% formamide/10% dextran sulfate at 42°C. After the filters were washed in 0.015 M NaCl/0.0015 M sodium citrate/0.1% $NaDodSO_4$ at 52°C, they were exposed to Kodak X-Omat x-ray film for 4–24 hr. The p53 and TK cDNA probes were cloned into pSP64 (Promega Biotec, Madison, WI), and labeled complementary RNA (noncoding strand) was synthesized (35) and used as a probe. β-Actin cDNA was labeled by the method of Feinberg and Vogelstein (36). Several preparations of RNA were made from cells at each time point; representative data are shown. In each case, the amount of RNA actually blotted onto the nitrocellulose was monitored by hybridization to the control gene (plasmid pHE-7).

Plasmids. Plasmid DNA was isolated by phenol extraction followed by Sepharose chromatography. The following probes were used.

pJE-3 is a cDNA clone isolated from a mouse 3T3 cDNA library and provided by C. Stiles (19). p13-2A9, p13-2F1, and p13-4F1 were clones isolated in this laboratory from a Syrian hamster ts 13 cDNA library (18) and will be referred to here as clones 2A9, 2F1, and 4F1. The c-myc probe was a 3′ human cDNA obtained from Carlo Croce (37). The TK probe was pTK11, a nearly full-length cDNA clone isolated by Harvey Bradshaw (38). Histone H3 probe was pF0422, provided by Gary Stein. The β-actin probe was pHFβA-1, provided by Peter Gunning (39). The c-Ha-ras probe was pH-1 (40) provided by Edward Scolnick. The ODCase probe is a cDNA clone isolated from a human cDNA library (unpublished data). Two p53 probes, one human-specific (41) and one mouse-specific (42), were provided by Moshe Oren and gave essentially the same results. pHE-7 is a cDNA clone isolated from HeLa cells by Joseph Nevins (43), whose corresponding mRNA is not expressed in a cell cycle-dependent manner.

Densitometry Measurement. The intensity of the autoradiographic signal was quantitated by densitometry by using a Zeineh soft laser scanning densitometer. Underexposed films, or multiple exposures were scanned to ensure linearity in the response of the film. Several densitometer settings were used, so that the measurements were always made in the linear range of the densitometer. In every case, the densitometer values are plotted as a percentage of maximum, 100% being the highest value obtained in each particular experiment, and the results were normalized to the values for the control gene, clone pHE-7.

DNA Synthesis in Stimulated Cells. Cells made quiescent as described were stimulated and simultaneously labeled with [^{3}H]thymidine. The cells were plated in 60-mm dishes with coverslips at the same density and in the same volume of medium per cm^2 as for RNA extraction. Cells were stimulated at the same time as the cells for RNA extraction, only in the presence of [^{3}H]thymidine (1 μCi/ml; 2 Ci/mmol; 1 Ci = 37 GBq). Cells were fixed at various times after stimulation. Autoradiography was performed as described by Cristofalo and Sharf (3).

RESULTS

The genes or DNA segments whose expression was studied here are listed in Table 1, where they are divided into groups based on the time of maximal expression. The clones bearing these genes have been described in detail elsewhere (see *Methods*); the references listed are those describing the cell cycle modulation of the corresponding mRNA. c-*myc* has been shown to be cell cycle-dependent in mouse (9) and hamster cells (29), and in human lymphocytes (44). JE-3 is a cDNA clone selected by Cochran *et al.* (19) on the basis of increased expression 1 hr after stimulation of resting BALB/c 3T3 cells with platelet-derived growth factor. This clone is also cell cycle-dependent in different species and responds to different mitogens (45). 2F1, 4F1, and 2A9 are cDNA clones selected in this laboratory (18) on the basis of increased expression upon serum stimulation of hamster cells. These clones are maximally expressed in hamster and mouse fibroblasts and in human lymphocytes (44) in early (4F1 and 2F1) or in mid G_1 phase (2A9). c-*ras* has been shown to be cell cycle-dependent in regenerating liver (15, 16) and in 3T3 cells (12, 14). The time of maximal expression is mid–late G_1 phase. β-Actin mRNA levels have been measured in 3T3 (24) and BPA31 cells (14) and are usually low in G_0 cells, increase in G_1, and stay constant through S phase. ODCase mRNA levels are increased after serum stimulation of BALB/c (22) and Swiss (23) 3T3 cells. The level of this RNA increases 4–8 hr after serum stimulation and remains high throughout the G_1 phase (23). p53 mRNA level is low in G_0 3T3 cells and increases steadily after serum stimulation until S phase (17). Finally, the TK and histone genes are expressed at the G_1/S boundary in numerous species (25–28). The genes selected for this study then are cell cycle-dependent genes, not mitogen specific, and their modulation is a general characteristic of cells traversing the cell cycle. Even the cDNA clones containing genes whose products are unknown (JE-3, 2F1, 4F1, and 2A9) have been well characterized in this respect.

Table 1. Cell cycle-dependent genes studied

Gene*	Time of increased expression	Size of mRNA, kb	Ref(s).
4F1	Early G_1	2.4	18, 29, 44
c-*myc*	Early G_1	2.4	9, 29, 44
JE-3	Early G_1	1.2	19, 29, 44
2F1	Early–mid G_1	1.5	18, 29, 44
ODCase	Mid G_1	1.4	22, 23
2A9	Mid G_1	0.7	18, 29, 44
p53	Mid G_1	3.0	17
c-Ha-*ras*	Mid–late G_1	1.4	14, 16
β-actin	Variable	2.0	14, 24
TK	Late G_1–S	1.4	25
Histone H3	Late G_1–S	0.5	26–28

*Except for the cellular oncogenes, which are *MYC* and *HRAS* in human gene nomenclature, genes are identified by the name of the cDNA clone or by the gene product.

The number of cells entering S phase after quiescence and stimulation was constantly monitored throughout these experiments by the addition of [^{3}H]thymidine. In a stimulated culture, the cells begin to enter S phase after about 12–15 hr, and the maximal number of DNA-synthesizing cells plateaus at 24–30 hr. The maximal percentage of cells are labeled at this plateau stage. In a representative experiment, the maximal percentage of labeled cells for the young cells was 62%, while only 12% of the senescent cells eventually entered DNA synthesis. The time at which half the maximal percentage of cells was labeled ($t_{1/2}$) was slightly later in the senescent cells (21 hr) than in the young cells (17 hr), confirming the longer average G_1 period of still-cycling senescent cells.

Expression of Early G_1 Genes. The early G_1 genes that we have studied are c-*myc* and the genes of cDNA clones 4F1, JE-3, and 2F1. Young and senescent cells were made quiescent as described, stimulated with 10% serum, and harvested at 0, 4, 17, and 24 hr after stimulation. Fig. 1 shows the expression of 4F1 mRNA in WI-38 cells at 0, 4, and 17 hr after stimulation. The expression of clone 4F1 is cell cycle-dependent in young cells (Fig. 1*A*), with maximal expression

at 4 hr after stimulation. In senescent cells, on the other hand, the expression is increased at 4 hr but increases still further by 17 hr. Other experiments (data not shown) indicate that, even at 24 hr, the level of 4F1 mRNA in senescent cells remains high. Fig. 2 shows the results of a similar experiment in which c-myc and pJE-3 were used as probes. In this experiment, the autogradiograms of the blots were scanned densitometrically, and the areas under the peaks were integrated. These values are plotted as the percentage of maximum expression, 100% expression being the highest value of either young or senescent cell RNA. Both c-*myc* and pJE-3 are expressed in a cell cycle-dependent manner with peaks, respectively, at 4 and 16 hr. There are quantitative but no qualitative differences between young and senescent cells. Notice that pJE-3, whose expression increases within 1 hr in BALB/c 3T3 cells (19) and in human lymphocytes (44), increases after 4 hr in WI-38 cells. Although the peak of expression of this clone could be at any time between 4 and 17 hr, it appears that this clone is expressed somewhat later in WI-38 cells than in the other cell types examined. We have already noted, both in our laboratories and from the literature, that the time of increased expression for cell cycle-dependent genes may vary from cell type to cell type.

Fig. 3 shows the expression of p2F1 in young (Fig. 3*A*) and senescent (Fig. 3*B*) cells stimulated with serum. Whereas in young cells the expression is clearly maximal at 17 hr, falling to control levels by 24 hr, the expression in senescent cells remains elevated throughout the time examined, in a manner similar to that of clone 4F1.

Expression of Mid-G_1 Genes. We have examined the expression of several mid-G_1 genes. These include p2A9, c-Ha-*ras*, and the gene encoding ODCase. We include the genes encoding β-actin and p53 antigen in this group as well, as their mRNA levels increase in mid-G_1, although they do not decrease by the S phase. Fig. 4 shows results obtained with four mid-G_1 genes from densitometric scanning of autoradiograms. All four genes are expressed at least as well, if not slightly more, in senescent cells as in young cells. The patterns of expression are similar for the genes encoding β-actin, ODCase, and p53. c-Ha-*ras*, on the other hand, does not follow the same pattern of cell cycle-dependent expression in senescent cells as in the young cells. It is interesting that Srivastava *et al.* (45) have described an increased expression of c-Ha-*ras* in senescent cells, and this observation may be related to the results presented here. However, the growth state of the cells used in that paper (45) is unknown, so direct comparisons between these two sets of data cannot be made. Most important here though is that the c-Ha-*ras* gene is expressed in senescent cells at levels similar to those in young cells. The expression of p2A9 increases in young cells at 4 hr and falls again to control levels by 24 hr (data not shown). In senescent cells, the same pattern is observed as for p2F1 (data not shown). The level of expression of this RNA is similar in young and senescent cells.

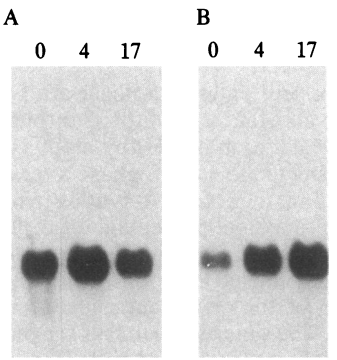

FIG. 1. Expression of p4F1 in young and senescent cells. WI-38 cells were plated as described and grown for 9–14 days. Fresh 10% serum was added, and the cells were harvested. Cytoplasmic poly(A)$^+$ RNA was prepared, and equal amounts were fractionated on 1% agarose-formaldehyde gels and transferred to nitrocellulose. The blot was hybridized to nick-translated 4F1 cDNA. The size of the mRNA band was determined by comparison to mouse and *Escherichia coli* ribosomal RNA run on the same gel. (*A*) Young cells. (*B*) Senescent cells. RNA was prepared from cells at 0, 4, and 17 hr after stimulation as indicated. The size of the 4F1 mRNA band shown is 2.4 kb.

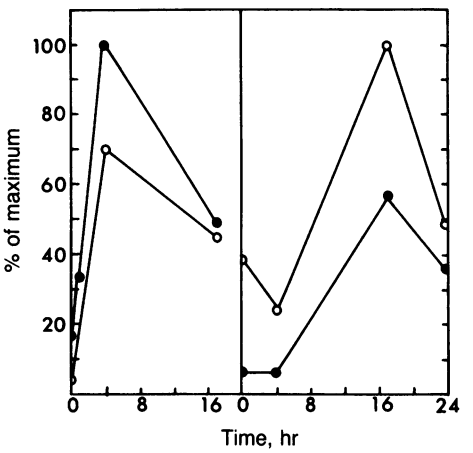

FIG. 2. Expression of c-*myc* (*Left*) and pJE-3 (*Right*) in WI-38 cells. Cells were grown, and RNA was extracted and hybridized as described in the legend to Fig. 1. Autoradiograms were scanned densitometrically, and the resulting peaks were integrated. The data are expressed as the percentage of maximal expression, maximal expression being the highest RNA level in young or senescent cells. ●, Young cells; ○, senescent cells.

Expression of G_1/S Genes. Lastly, we have examined the expression of two genes known to be preferentially expressed at the G_1/S boundary. These are the genes encoding thymidine kinase and histone H_3. Fig. 5*A* shows the expression of the TK gene in young and senescent cells. In young cells TK gene expression is low in G_0 and in cells 4 hr after stimulation but increases dramatically to a maximum at 17 hr. By 24 hr the level of this RNA has begun to fall. In senescent cells, the time of maximal expression is delayed until 24 hr, but the increase in expression is as dramatic as that seen in young cells. Fig. 6 shows data obtained from densitometry scanning of a blot of total RNA hybridized with a histone H3 probe. This figure shows that the expression of the histone gene in young cells is very low at 0 and 4 hr and increases at 17 and 24 hr. In senescent cells, the expression also increases at 17

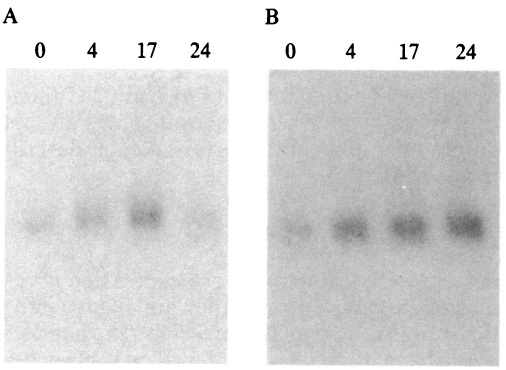

FIG. 3. Expression of p2F1. Conditions as described for Fig. 1. (*A*) Young cells. (*B*) Senescent cells. RNA was isolated at 0, 4, 17, and 24 hr after stimulation as indicated. The size of the band shown is 1.5 kb.

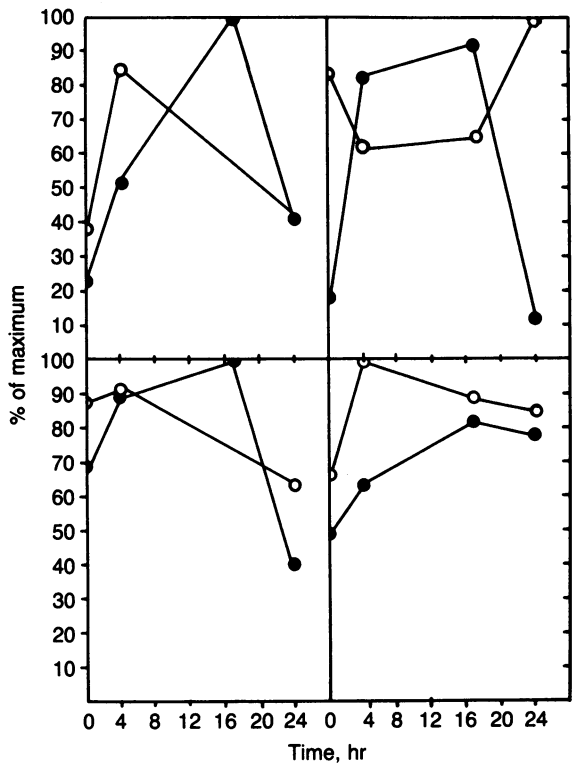

FIG. 4. Expression of c-Ha-*ras* (*Upper Right*) and genes for ODCase (*Upper Left*), β-actin (*Lower Left*), and antigen p53 (*Lower Right*). Conditions were as in the legend for Fig. 1; densitometry was as described for Fig. 2. ●, Young cells; ○, senescent cells.

and 24 hr. This experiment has been repeated several times, and there is no doubt that histone genes, at least *H3*, are fully expressed in senescent cells.

In this and in other experiments, the amount of RNA actually blotted on the nitrocellulose filter was monitored by hybridization to a control cDNA whose expression does not vary across the cell cycle. We have used pHE-7, isolated by Kao and Nevins (43) from a HeLa cell cDNA library, which has been shown to be expressed equally in cells in all phases of the cell cycle (43). When this probe was hybridized to blots containing total RNA or mRNA from WI-38 cells, a pattern was obtained such as that seen in Fig. 5*B*. Clearly, the gene contained in this clone is not cell cycle-dependent in WI-38

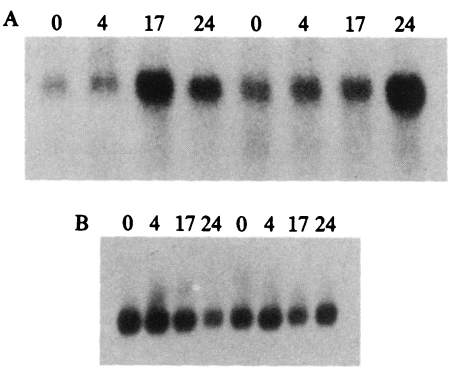

FIG. 5. (*A*) Expression of TK gene. (*B*) Expression of gene contained in clone pHE-7. Conditions were as in the legend to Fig. 1. In each case, the lanes on the left are young cells and those on the right are senescent cells. Times of harvest after stimulation are indicated above the lanes. The TK mRNA is 1.4 kb, while the pHE-7 mRNA is 1.0 kb.

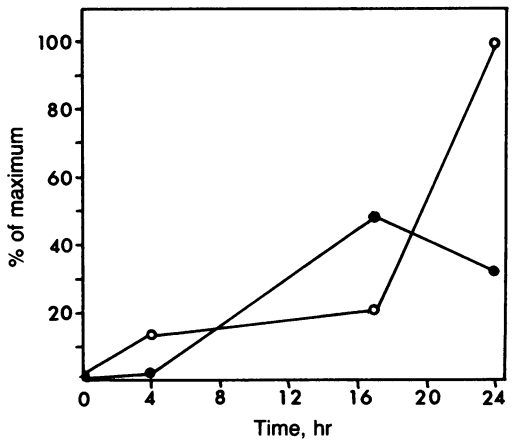

FIG. 6. Expression of the gene for histone H3. Conditions were as in the legend to Fig. 2. ●, Young cells; ○, senescent cells.

cells and is expressed equally in young and senescent cells. The maximum variability seen in this figure as determined by densitometry is less than 2-fold which is at the level of experimental variability in this system. The densitometry values shown in previous figures were normalized to those of the control gene hybridized on the same filter.

DISCUSSION

The modulation of the expression of the cell cycle genes studied in these experiments represents a series of events occurring throughout the G_1 period of the cell cycle and extending into the S phase. The occurrence, or lack thereof, of these events provides detailed information on the progression of cells through these periods. Monitoring these events, by means of measuring cytoplasmic mRNA levels, has provided detailed information on the temporal location of the lesions occurring in various ts mutants of the cell cycle. For example, it has been found that in various ts mutants that fail to enter S phase at the nonpermissive temperature, early- and mid-G_1 genes are expressed, while G_1/S genes are not expressed (refs. 18, 25, 28, and 30; S. Rittling, unpublished data). In other words, genes normally expressed prior to the ts block continue to be expressed at the nonpermissive temperature, while those expressed after the block fail to do so. In contrast to these results, senescent WI-38 cells fully express not only the early- and mid-G_1 genes examined but also the G_1/S genes. In particular, the cells fully express the genes encoding both TK and histone H3, which are usually tightly coupled to DNA synthesis (28). A detailed comparison of the expression in young and senescent cells is complicated by the slightly different lengths of the G_1 period in the two cell types. However, the level of expression is very similar in the two cell types, which indicates that it must be due to the noncycling cells in the senescent population and not just to the 12% of senescent cells that do enter S phase. If only the cycling cells expressed these genes, the level of expression in the senescent cells would be one-fifth of that in the young cells. Thus, these results clearly show that senescent WI-38 cells are not analogous to ts mutants blocked in G_1. Rather the block in the senescent cells must occur just at the time of entrance into S phase, as is discussed further below.

It has been hypothesized that a series of events occurs as cells traverse G_1 and that this series is analogous to a metabolic pathway (46). We have considered three possibilities, utilizing this model, that are consistent with our data and that could explain the failure of senescent cells to synthesize DNA. First, the lesion could exist in a pathway other than that terminating in the expression of TK and histone genes. It may be that there are several parallel

pathways that must be traversed simultaneously (46) and that the final genes of the various pathways must be activated concomitantly for the cells to reach S phase. Secondly, the lesion may occur in a gene whose expression is not cell cycle dependent. There may be genes necessary for cell cycle traverse that are expressed constitutively. However, if either of these first two possibilities were operative, one would expect reports of G_1 ts mutants that express the G_1/S genes at the restrictive temperature. Given the lack of such reports, it is unlikely that these mechanisms are responsible for the results presented here. Alternatively, the lesion may occur at the G_1/S boundary so that genes normally expressed late in G_1 or S are expressed in senescent cells, but additional events are required for the cells to actually synthesize DNA. It may be that very few events occur between the induction of TK and histone and the start of DNA synthesis, so that there is a low probability of ts mutants arising that are blocked between these points. This last possibility is supported by other data (5–8), which suggest that senescent cells are blocked very late in G_1.

There have been a number of reports in the literature detailing the results of fusion experiments between senescent cells and cycling cells. A general conclusion of such experiments (47–49) is that senescent cells contain an inhibitor of DNA synthesis, which is able to prevent DNA synthesis in the normally cycling nucleus in heterokaryons. Our results are not inconsistent with such data but indicate that such an inhibitor does not block the progression of senescent cells to the S phase. Furthermore, Pendergrass *et al.* (50) show that the levels of DNA polymerase activity in senescent cells is similar to that in young cells. This is another example of an S-phase gene product whose levels are undiminished in noncycling, senescent cells.

The results presented here indicate that senescent, noncycling WI-38 cells are blocked at the G_1/S boundary, in agreement with other data (5–8). Thus, study of this portion of the cell cycle should be fruitful in providing definitive data on cell cycle regulation in cellular senescence.

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