Evidence for Endogenous Polypeptide-mediated Inhibition of Cell-cycle Transit in Human Diploid Cells

GLENNA C. BURMER, CAROL J. ZEIGLER, and THOMAS H. NORWOOD
Department of Pathology, University of Washington, Seattle, Washington 98195

ABSTRACT Previous studies have shown that the senescent phenotype is dominant with respect to DNA synthesis in fusions between late passage and actively replicating human diploid fibroblasts. Brief postfusion treatments with the protein synthesis inhibitor cycloheximide (CHX) or puromycin have been found to significantly delay (by 24–48 h) the inhibition of entry into DNA synthesis of young nuclei in heterokaryons after fusion with senescent cells. A significant fraction of the senescent nuclei incorporated tritiated thymidine in CHX-treated heterokaryons. The optimal duration of exposure to CHX was 1–3 h immediately after fusion, although treatments beginning as late as 9 h after fusion elevated the heterokaryon labeling index. Prefusion treatments with CHX were without a significant effect. These results are consistent with the interpretation that regulatory cell cycle inhibitor(s) which are dependent upon protein synthesis may be present in heterokaryons between senescent and actively replicating cells.

The finite in vitro replicative lifespan of human diploid fibroblastlike (HDFL) cells has been well documented (1, 2). Studies have demonstrated that the proportion of nondividing or slowly cycling cells increases with serial passage until the entire population becomes arrested predominantly in the G_1 phase of the cell cycle (3, 4). The mechanism(s) responsible for limiting the growth potential remains unclear. Rabinovitch and Norwood (5) and Stein and Yanishevsky (6) have proposed that cessation of proliferation or "senescence" results from the constitutive synthesis of an inhibitory factor(s). This hypothesis is based primarily upon observations derived from cell fusion studies carried out in these laboratories. These investigators observed that initiation of DNA synthesis in the young, actively proliferating cell is inhibited in heterokaryons after fusion with senescent HDFL cells, but ongoing DNA synthesis was not inhibited. In addition, Rabinovitch and Norwood (5) demonstrated that inhibition of entry into DNA synthesis occurs if fusion with a senescent cell is complete while the young nucleus is in the G_1 phase of the cell cycle at a point earlier than 3 h before the onset of the S phase. Similar results were observed in parallel studies where actively proliferating HDFL cells were fused with cells rendered quiescent by serum deprivation (5). While the proposal that senescent and quiescent cells elaborate a cell cycle inhibitory factor(s) is a reasonable interpretation of these observations, alternative hypotheses cannot be excluded. For example, it is possible that fusion between actively proliferating low passage and late passage senescent cells results in dilution of factors necessary for cell cycle progression and initiation of DNA synthesis, resulting in passive inhibition of DNA synthesis.

We further investigated these alternative interpretations in studies in which the effect of transient inhibition of protein synthesis on DNA synthetic activity was examined in heterokaryons between old and young HDFL cells. We observed inhibition of entry into DNA synthesis of young nuclei upon fusion with senescent cells to be significantly delayed by brief treatments with protein synthesis inhibitors. These results are interpreted to be consistent with the presence of a regulatory inhibitor of cell cycle function in the senescent cell.

MATERIALS AND METHODS

Cells and Culture Conditions

HDFL cells described in these studies were initiated in this laboratory from primary explants. The sources, population doubling levels, and labeling indices of these strains are described in Table I.

Cells were maintained in Eagle's minimum essential medium (EMEM) with Earle's Salts (Gibco Laboratories, Grand Island Biological Co., Santa Clara, CA), buffered with 26 mM sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories; and Reheis Chemical Co., Div. Armour Pharmaceutical Co., Kankakee, IL), penicillin (100 U/ml), streptomycin (100 μg/ml), and incubated at 37°C in an atmosphere of 5% CO_2 in air. Early passage cells were maintained in an actively cycling state by passaging twice a week. Tests for mycoplasma by aerobic and anaerobic cultures were negative (7).

Cell Fusion Procedure

To facilitate identification of heterokaryons, the early passage, actively proliferating HDFL cells were prelabeled for 24 h with 2.5 μCi/ml [^{3}H]methionine

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 JULY 1982 187–192

TABLE I

Origin and In Vitro Age of Cell Strains Used in These Studies

Strain	Source	No. of population doublings*	[^{3}H]Thymidine-labeling index‡
			%
Early passage			
76-109	Newborn foreskin	14 (70)	94
75-69	Newborn foreskin	7 (58)	87
75-73	Fetal upper arm skin	18 (59)	92
72-166	Adult upper arm skin	8 (58)	90
Late passage			
72-117	Adult upper arm skin	60 (60)	<5
75-69	Newborn foreskin	56 (58)	<5
72-166	Adult upper arm skin	58 (58)	<5

* These strains were used at the indicated population doublings from the time of the initial passage after explanation. The maximum lifespan is shown in parentheses.

‡ The percentage of labeled cells in subconfluent cultures after a 24-h pulse with [^{3}H]thymidine (0.5 μCi/ml).

(L-[methyl-^{3}H]-methionine, sp act 200 mCi/mM; New England Nuclear, Boston, MA) and 0.9-μm diameter latex beads (Polysciences, Inc., Warington, PA). This prelabeling protocol was not observed to affect the proportion of [^{3}H]thymidine-labeled cells in actively cycling HDFL cultures during the labeling periods described in these experiments. The senescent cells were prelabeled for 24 h with 2.0-μm latex beads. At the termination of the prelabeling procedure the cultures were trypsinized, rinsed free of extracellular beads by three successive washes using 1,000-*g* centrifugation, and plated on 25-mm round plastic cover slips (Lux Scientific Corp., Naperville, IL) in 35-mm petri dishes (Corning Glass Works, Scientific Products Div., Corning, NY) at a density of ~12,000 of each cell type per cover slip. This procedure results in a partial synchrony of DNA synthesis over the following 24-h period in young cells. After allowing for overnight attachment (~16 h), the cells were fused by a 1-min exposure to 44% polyethylene glycol (PEG) (1,000 mol wt; Koch-Light Laboratories Ltd., Research Products International, Mt. Prospect, IL) dissolved in serum-free medium containing 10% dimethyl sulfoxide (DMSO; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY), immediately followed by three successive rinses in 10% DMSO-EMEM, and a single wash with medium containing 10% fetal bovine serum. The cultures were then exposed to medium containing cycloheximide (CHX; Sigma Chemical Co., St. Louis, MO) or puromycin dihydrochloride (Sigma Chemical Co.) at the concentrations and durations of exposure indicated, and, following removal of the inhibitors, the cultures were rinsed three times with Hanks' Balanced Salt Solution (HBSS; Gibco Laboratories). The cultures were then pulsed with [^{3}H]thymidine (0.5 μCi/ml, S.A.-6.7 Ci/mM; New England Nuclear) at various times and for various durations after treatment with the inhibitors, depending upon the experimental situation. Details of the various [^{3}H]thymidine pulse schedules are described in the figure legends. At the end of each pulse period, the cover slips were washed three times in phosphate-buffered saline, fixed for 24 h in Bouin's fluid, and processed for autoradiography as previously described (8).

Experiments in which cultures were exposed to the inhibitor before fusion were performed as described above, except that young and senescent HDFL cells were co-cultivated, treated with the inhibitor for various durations of exposure, and rinsed three times with HBSS immediately before fusion.

Experiments involving CHX treatment of only the senescent cells before fusion were accomplished via a Sendai virus-mediated fusion protocol as described previously (9). Briefly, senescent cells were plated at a density of 12,000/25-mm cover slip, treated with CHX for various durations of exposure, and rinsed three times with HBSS before the addition of UV-inactivated Sendai virus (200 HAU) and 12,000 young cells. The cultures were maintained at 4°C for 20 min, incubated at 37°C for 60 min, rinsed once with HBSS, and pulsed with [^{3}H]thymidine (0.5 μCi/ml) for three consecutive 24-h pulse periods. The Sendai virus was kindly provided by Dr. H. Harris, Oxford University, Oxford, England.

The autoradiographs were screened at × 500 magnification under an oil immersion lens. Subconfluent areas of the preparations were analyzed where heterokaryons could be unambiguously identified. Heterokaryons were defined as bi- and multinucleate cells containing ten or more beads of each size and methionine label. Before analysis, all preparations were screened for bead contamination, and those with excessive numbers of extracellular beads and/or inappropriate labeling by the beads in monokaryons were excluded from the analysis. To minimize the possibility of observer bias, randomly selected preparations were analyzed by another individual familiar with the prelabeling system. The percentage of labeled nuclei in the homodikaryons and heterodikaryons were calculated by dividing the number of [^{3}H]thymidine-labeled nuclei by the total number of nuclei multiplied by 100.

Measurements of Amino Acid and Thymidine Incorporation

The kinetics of recovery of protein and DNA synthesis were determined after treatment with CHX and puromycin. In these studies, ~1 × 10^5 cells were plated into 60-mm tissue culture dishes (Corning Glass Works), and, after overnight attachment, some dishes were treated with medium containing CHX or puromycin for 1 h. The medium was then aspirated, the dishes were rinsed three times with HBSS, and medium lacking inhibitors was added. At various times after release, replicate cultures were pulsed for 1 h with [^{3}H]methionine (4 μCi/ml, S.A.-200 mCi/mM, New England Nuclear) for determination of protein synthesis or with [^{3}H]thymidine (10 μCi/ml, S.A.-6.7 Ci/mM, New England Nuclear) for determination of DNA synthesis. Immediately after the pulse, the cells were rinsed three times in ice-cold phosphate-buffered saline, dissolved in 1 ml of 1% (wt/vol) SDS (Bio-Rad Laboratories, Richmond, CA) containing 100 μg/ml carrier DNA (Type 1: calf thymus DNA; Sigma Chemical Co.), and precipitated with 0.5 ml of ice-cold 50% TCA. The precipitate was pelleted, resuspended in 1 ml of 0.3 N NaOH, and re-precipitated with 0.5 ml of 50% TCA two more times before adding 1 ml of 0.1 N NaOH and incubating at 60°C for 10 min. The solution was placed in a scintillation vial in 10 ml of Aquasol (New England Nuclear), with 75 μl of acetic acid, and counted on a Packard Tricarb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). To obtain baseline values of amino acid incorporation in the presence of inhibitor, cultures were exposed to CHX or puromycin simultaneously with [^{3}H]methionine for 1 h, and immediately rinsed and dissolved in SDS.

Cell counts were obtained from duplicate dishes using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

RESULTS

Initial studies were performed to determine the effect of a short postfusion treatment with CHX on the nuclear [^{3}H]thymidine labeling index in heterodikaryons at various times after release from the protein synthesis inhibitor. A concentration was used which inhibits protein synthesis by >98% (10 μg/ml). A representative result from multiple experiments is shown in Fig. 1. As we have observed in previous studies (5), the fraction of [^{3}H]thymidine-labeled nuclei in heterodikaryons in the untreated cultures began to decline after a 3-h delay after fusion, and by 9 hs it was similar to that enumerated in old homodikaryons (Fig. 1*a*). In contrast, the heterodikaryon labeling index in the CHX-treated cultures was elevated throughout the labeling period, while the young and old homokaryon labeling

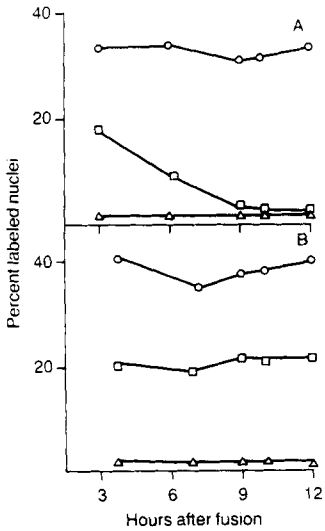

FIGURE 1 Nuclear [^{3}H]thymidine-labeling indices in heterokaryons at various times after fusion, without exposure (*A*) and with exposure (*B*) to CHX. Young, actively proliferating cells (strain 76-109) were fused with senescent fibroblasts (strain 72-166) and pulsed for 1 h with [^{3}H]thymidine (10 μCi/ml) at the times indicated after fusion. The experimental cultures were exposed to CHX (2 μg/ml) for 1 h immediately after fusion, and pulsed for 1 h with [^{3}H]thymidine at the times indicated. The results shown are from a representative experiment. (○) young homodikaryons; (□) heterodikaryons; (△) old homodikaryons.

indices were unaffected by the treatment (Fig. 1 *b*). In the absence of markers the parental origin of the nuclei in the heterokaryons cannot be identified. However, the fraction of heterokaryons with both nuclei labeled provides an estimate of the extent to which thymidine incorporation occurs in the senescent nuclei. In all cell strains examined in this study the proportion of double-labeled heterodikaryons was significantly increased in the CHX-treated cultures (Table II).

To determine whether the elevation in the heterokaryon labeling index observed following postfusion CHX treatment was specifically the result of inhibition of protein synthesis or an action unique to CHX, studies were conducted using puromycin, which has a different mode of action than CHX (10). Dose-response studies carried out with both inhibitors demonstrated that similar results were obtained when the cultures were treated with puromycin after fusion (Fig. 2). An approximate linear increase in the percentage of labeled heterokaryons was observed with a 1-h postfusion treatment, correlating with exponential increases in the concentration of CHX in the range of 0.05 to 2 μg/ml and with puromycin in the range of 1 to 50 μg/ml.

The degree of inhibition of protein synthesis corresponding to these concentrations of inhibitors was determined by liquid scintillation counting of [^{3}H]methionine incorporation into acid-precipitable material (Fig. 3). Increased concentrations of inhibitors resulted in an increased inhibition of amino acid uptake in both young and senescent cultures. A 1-h exposure

TABLE II

Percentage of Heterodikaryons with 0, 1, or 2 [^{3}H]Thymidine-labeled Nuclei

Cell cross (young × senescent)	Without CHX			With CHX		
	0	1	2	0	1	2
76-109 × 75-69*	77	14	9	41	25	34
76-109 × 72-166‡	99	1	0	63	25	12
75-73 × 72-166	90	7	3	59	19	22
75-69 × 75-69	73	7	20	36	17	47
72-166 × 72-117‡	82	10	8	42	20	38

The effect of CHX on the proportion of labeled nuclei in heterodikaryons. Actively proliferating and senescent HDFL cells were fused, treated with 10 μg/ml of CHX for 1 h, and pulsed with 0.5 μCi/ml of [^{3}H]-Thymidine for 24 h after release from the inhibitor. An average of 75–100 heterokaryons was counted for each determination.

* Average values from three separate experiments.

‡ Average values from two separate experiments.

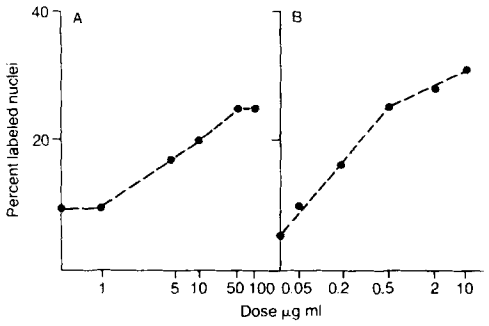

FIGURE 2 The effect of various concentrations of CHX or puromycin on the [^{3}H]thymidine-labeling indices in heterodikaryons between early and late passage cells. Strain 75-73 (PD 18) and 72-117 (PD 60) were fused, treated for 1 h immediately after fusion with puromycin (*A*) or CHX (*B*) at the concentrations shown, then immediately pulsed for 24 h with [^{3}H]thymidine (0.5 μCi/ml), and processed for autoradiography.

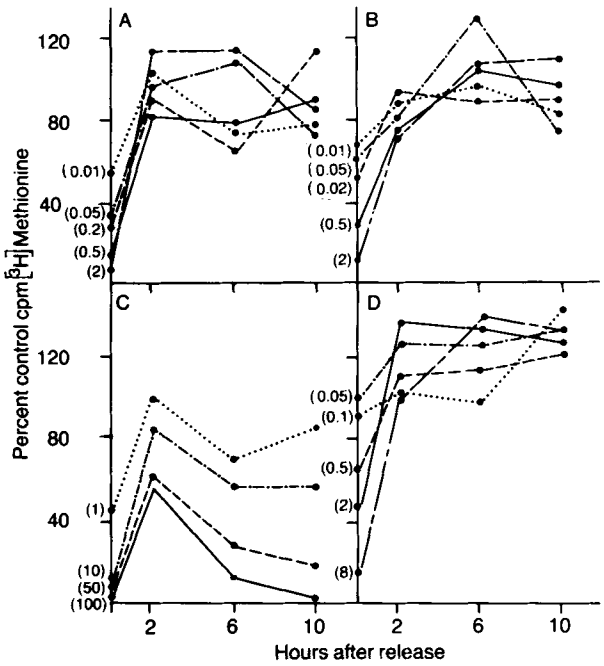

FIGURE 3 Recovery of [^{3}H]methionine incorporation into acid-precipitable material after release from cycloheximide or puromycin in early and late passage HDFL cells. Details of the procedure are described in the text. Concentrations of the inhibitors are shown in parentheses on the vertical axis of each graph. Strains 75-73 (PD 18) and 72-117 (PD 60) were exposed to CHX at 0.01, 0.05, 0.2, 0.5, and 2 μg/ml for 1 h before release from the inhibitor (*A* and *B*, respectively). Strain 75-73 was treated with 1, 10, 50, and 100 μg/ml puromycin (*C*), and Strain 72-117 with 0.1, 0.05, 0.5, 2, and 8 μg/ml puromycin (*D*) for 1 h. All counts were performed in duplicate and were standardized per 10^5 cells before calculating the values for percent control.

to 10 μg/ml puromycin or 2 μg/ml CHX was sufficient to reduce [^{3}H]methionine incorporation by ~90%. Recovery of synthetic activity was observed to be 90–100% of controls within 2 h in both early and late passage cultures. No consistent significant differences were observed in the kinetics or extent of recovery after treatment with CHX at the various concentrations (Fig. 3 *a* and *b*). In contrast, treatment of young cultures with concentrations of puromycin necessary to inhibit [^{3}H]methionine uptake by >90% (50–100 μg/ml) and maximally stimulate DNA synthesis in heterokaryons resulted in a significantly decreased recovery in the incorporation of the labeled precursor, which is in agreement with a previous report (11) (Fig. 3 *c*). A large proportion of cells, exposed to the highest concentration, were judged nonviable by their inability to exclude trypan blue dye. Similar studies carried out with senescent cells at concentrations of puromycin of <10 μg/ml displayed complete recovery of incorporation of [^{3}H]methionine (Fig. 3 *d*).

The kinetics of recovery of protein synthesis as a function of duration of exposure to CHX (2 or 10 μg/ml) in young and senescent cultures was also examined. No differences were observed in cultures exposed from 1 to 24 h (data not shown).

Recovery of DNA synthesis in young cultures as assessed by [^{3}H]thymidine incorporation into acid-precipitable material was also observed to be independent of the concentration of CHX used in these studies (Fig. 4). Within 4 h after release from CHX, the cultures had recovered to within 80% of the values for untreated controls.

To further characterize the effect of inhibition of protein synthesis on the DNA synthetic activity in these heterokaryons, the effect of duration of exposure to CHX immediately after fusion was examined (Fig. 5). Exposure periods of 1 to 3 h resulted in the greatest elevation of the nuclear thymidine in the heterokaryons. Increasingly lowered values were observed with longer exposure periods. Treatment periods of 15 or 30 min produced no elevation in the fraction of labeled nuclei in these cells (data not shown).

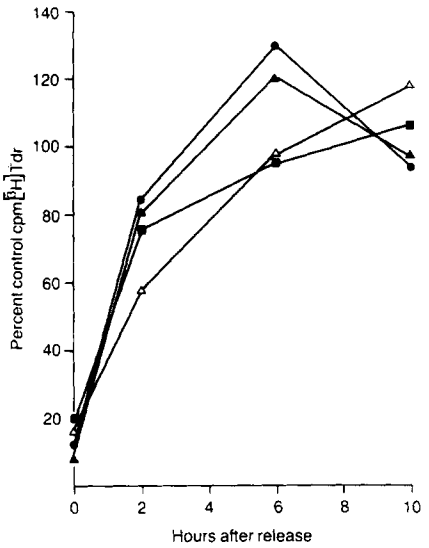

FIGURE 4 Recovery of [^{3}H]thymidine ([^{3}H]Tdr in figure) incorporation after release from various concentrations of CHX. Strain 75-73 at early passage (PD 18) was exposed to 0.2 (■), 0.5 (△), 2 (●), and 10 (▲) μg/ml CHX for 1 h. At the times after release indicated in the figure, cultures were pulsed with 4 μCi/ml of [^{3}H]thymidine for 1 h. All determinations represent the mean value of duplicate counts, standardized to 10^5 cells.

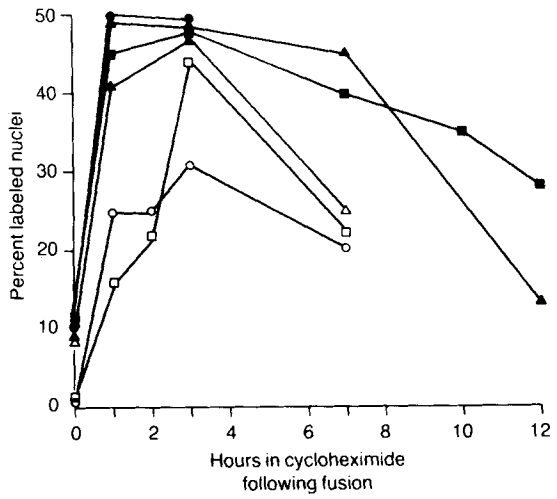

FIGURE 5 The effect of durations of exposure to CHX, immediately after cell fusion, on the [^{3}H]thymidine-labeling indices in heterodikaryons derived from the fusion of a variety of strains of human diploid cells. Actively proliferating cells were fused with senescent cells, treated with 2 μg/ml CHX for various durations of exposure, and pulsed with [^{3}H]thymidine (0.5 μCi/ml) for 24 h immediately after release from the inhibitor. The values plotted at 0 h indicate heterodikaryon labeling indices of untreated cultures. (○) 76-109 × 72-117; (□) 76-109 × 72-166; (△) 75-73 × 75-69; (●) 76-109 × 75-69; (■) 76-109 × 75-69; (▲) 75-73 × 72-166 (young × senescent).

The time of initiation of the CHX treatment in relation to cell fusion proved to be a significant variable. Exposure for 3-h intervals within the first 24 h after fusion resulted in elevations in the [^{3}H]thymidine labeling index in the heterokaryons (Fig. 6 *b*, *c*, and *d*). Stimulation of thymidine incorporation was observed when the treatment was initiated as late as 9 h after fusion. When the protein synthesis inhibitor was introduced at 24 h after fusion for either 3- (Fig. 6 *e*) or 12-h (Fig. 6 *f*) intervals, no increase in the fraction of labeled nuclei in the heterokaryons was observed. Note also that the labeling index remains elevated for at least 24 h after termination of the treatment with the inhibitor.

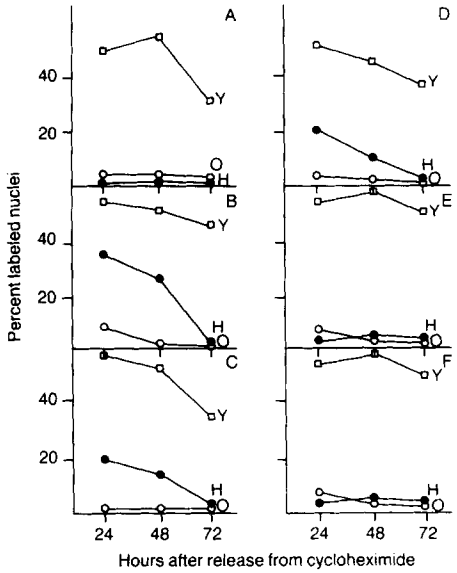

FIGURE 6 [^{3}H]thymidine-labeling index of heterodikaryons treated with CHX at various times after fusion. Strains 76-109 and 72-166 were exposed to 2 μg/ml CHX at 0-3 h (*B*), 3-7 h (*C*), 9-12 h (*D*), 24-27 h (*E*) and 24-36 h (*F*) after fusion, and pulsed with [^{3}H]-thymidine (0.5 μCi/ml) for three consecutive 24-h periods beginning immediately after release from the inhibitor. Control cultures (*A*) which were not treated with CHX were pulsed with [^{3}H]thymidine immediately after fusion. (□, *Y*) young homodikaryons; (○, *O*) senescent homodikaryons; (●, *H*) heterodikaryons.

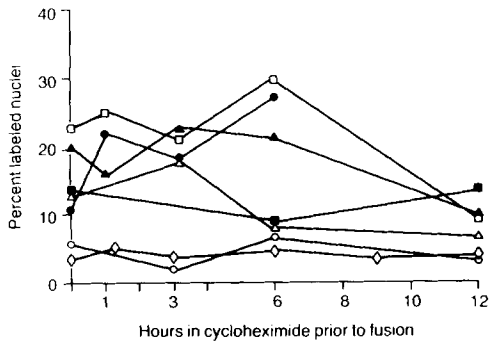

FIGURE 7 The effect of prefusion treatment with CHX of various durations on the heterodikaryon [^{3}H]thymidine-labeling index. Young, actively proliferating, and senescent cells of various strains were cocultivated and exposed to CHX for the durations indicated on the horizontal axis. The cultures were immediately fused after removal of the inhibitor and then pooled with [^{3}H]thymidine (0.5 μCi/ml) for 24 h. In the cross 76-109 × 72-166 (open circles) only the senescent cells were treated with CHX. (△) 72-166 × 72-117; (●) 76-109 × 75-69; (■) 76-109 × 75-69; (▲) 76-109 × 75-69; (□) 75-69 × 75-69; (○) 76-109 × 72-166 (young × senescent).

In contrast to the dramatic increase in the heterokaryon labeling indices observed with postfusion CHX treatments, exposures of both young and senescent cells to the inhibitor for various durations before fusion did not result in significant increases in the percentage of labeled heterokaryons (Fig. 7). Similarly, prefusion treatment of senescent cells alone with CHX for various durations of exposure had no effect on the subsequent heterokaryon labeling index (Fig. 7, open circles).

DISCUSSION

The experiments described here have demonstrated that brief treatments with protein synthesis inhibitors after cell fusion results in a significant but temporary elevation of the nuclear thymidine-labeling index in heterokaryons derived from the fusion of young, actively proliferating, and senescent human diploid cells. The transient nature of this effect (24–48 h) and the observation that introduction of the inhibitors 24 h after fusion fails to stimulate nuclear thymidine incorporation in the heterokaryons suggest that inhibition of protein synthesis permits the young nuclei to progress into the S phase in the hybrid environment but does not stimulate sustained sequential cell cycle activity. In addition, the increased frequency of heterokaryons with both nuclei labeled after treatments with these inhibitors indicates a modest stimulation of initiation of DNA synthesis in the senescent nuclei. Identical results have also been observed in parallel experiments using Sendai virus as a fusogen (results not shown), demonstrating that the stimulatory effects of postfusion treatments with protein synthesis inhibitors were not specific to the polyethylene glycol (PEG)-mediated fusion protocol.

The elevation in the heterokaryon labeling index after treatment with CHX or puromycin correlated with the degree of inhibition of protein synthesis (as measured by the incorporation of [^{3}H]methionine) produced by different concentrations of the inhibitors. Also, treatments of <1 h (15 and 30 min) produced no effect on the [^{3}H]thymidine labeling indices. These results are consistent with the interpretation that a labile factor(s) dependent upon protein synthesis may be involved in the inhibition of DNA synthesis in heterokaryons derived from fusions between senescent and actively replicating human diploid cells. The DNA synthesis-inhibiting activity becomes insensitive to interruption of protein synthesis if the introduction of CHX is delayed by at least 24 h after fusion. This suggests this product(s) of protein synthesis is not required once inhibition of DNA synthesis is completed.

CHX has been shown to produce a reduction in the rate of DNA replication in cultured mammalian cells (12–14). Thus, it is possible that these results could reflect a prolonged residual recovery from the action of the inhibitor on the young nucleus and not a real increase in the fraction of labeled heterokaryons. However, this interpretation appears to be unlikely in view of our observation that recovery of [^{3}H]thymidine incorporation was complete within 3–4 h after release from treatment in actively cycling early passage cells (Fig. 4). This is consistent with the rapid recovery of DNA synthesis reported by other investigators after withdrawal of this inhibitor from cells in early S phase (15–18).

Several investigators have hypothesized the presence of an inhibitor(s) of DNA synthesis which acts to block entry into S phase in senescent cells (5, 6, 19). This idea is further supported by reconstruction experiments between cytoplasts derived from senescent cells and karyoplasts (nuclei) from early passage cells. The reconstructed cells formed from these fusions are incapable of extended replicative activity, which is consistent with the idea that a diffusible cytoplasmic regulatory inhibitor may be present in late passage cytoplasts (20). However, it is unlikely that protein regulatory inhibitors are solely responsible for preventing senescent cells from initiating DNA synthesis. Stimulation of entry into S phase was never observed in senescent monokaryons or homokaryons after release from prolonged treatments with CHX, although recovery of [^{3}H]-methionine uptake was rapid under these conditions. The presence of a positive regulatory factor(s) from the actively replicating parental cells is apparently necessary for the induced stimulation of DNA synthesis in senescent nuclei observed in a small fraction of double-labeled heterokaryons after inhibition of protein synthesis (Table II).

A somewhat surprising observation was that treatment with CHX immediately before fusion had minimal, if any, effect on the post-treatment [^{3}H]thymidine-labeling indices in the heterokaryons. The interpretation of this result is not clear at the present time. However, as indicated in Results, the putative inhibitor(s) must be depressed for at least 1 h. If the half-life of the affector molecule(s) is significantly shorter than 1 h, then functional levels may be restored rapidly enough to achieve inhibition of DNA synthesis in the heterokaryons. Alternatively, a unique fusion specific product which is dependent upon polypeptide synthesis may be produced by the heterokaryons immediately after fusion.

While a number of investigators have postulated the presence of an inhibitor(s) of DNA synthesis to explain the mechanism of inhibition of DNA synthesis in heterokaryons between senescent and low passage, actively dividing HDFL cells, there are other interpretations of this observation. It is possible that initiation factors present in the young cell are diluted below a minimum-required threshold after fusion. The results presented here are inconsistent with the latter hypothesis, which would not predict that inhibition of protein synthesis would elevate the [^{3}H]thymidine-labeling indices in the heterokaryons. However, transient elevations of protein synthesis above pretreatment values following recovery from exposure to CHX have been reported in cultured primary chick cells and in vivo (21, 22). It is possible that activators of DNA synthesis are synthesized in excess immediately after release from inhibition of protein synthesis, resulting in the initiation of DNA synthesis in a larger fraction of heterokaryons. Although we were unable to consistently observe this overshoot of protein synthesis in our monokaryon cultures after CHX treatment, this interpretation cannot be ruled out on the basis of our studies.

In addition to its dramatic inhibitory effect on protein synthesis, CHX has also been shown to inhibit both basal and induced autophagic proteolysis in cultured cells (23–25). Another possible explanation for our results is that CHX treatment produces an increase in the turnover time of the polypeptide initiation factors in the young cell. This might stimulate the heterokaryons to progress through one round of DNA synthesis.

While studies presented in this paper are consistent with the interpretation that an inhibitor(s) is involved in the regulation of cell cycle activity in human diploid cells derived from the skin, clearly this hypothesis can be confirmed only by the isolation and biochemical characterization of this factor(s). This will require the development of suitable bioassays for this factor(s). We are currently attempting to develop an assay system which will detect physiologic inhibition of cell cycle activity by polypeptides. Manual microinjection may provide

a potential technical approach to the development of such a bioassay system for these putative regulatory peptides.

This work was supported in full by National Institutes of Health grant AG 01751. G. C. Burmer is a predoctoral fellow at the Medical Scientist Training Program at the University of Washington (GM 07266).

Received for publication 20 January 1982, and in revised form 12 April 1982.

REFERENCES

1. Hayflick, L. 1965. The limited *in vitro* lifetime of human diploid strains. *Exp. Cell Res.* 37:614–636.
2. Martin, G. M., C. A. Sprague, and C. J. Epstein. 1970. Replicative life span of cultivated human cells. *Lab. Invest.* 23:86–92.
3. Macieira-Coelho, A. 1974. Are non-dividing cells present in aging cell cultures? *Nature (Lond.).* 248:421–422.
4. Yanishevsky, R., M. L. Mendelsohn, B. H. Mayall, and V. J. Cristofalo. 1974. Proliferative capacity and DNA content of aging human diploid cells in culture: a cytophotometric and autoradiographic analysis. *J. Cell. Physiol.* 84:165–170.
5. Rabinovitch, P. S., and T. H. Norwood. 1980. Comparative heterokaryon study of cellular senescence and the serum deprived state. *Exp. Cell Res.* 130:101–109.
6. Yanishevsky, R. M., and G. H. Stein. 1980. On-going DNA synthesis continues in young diploid cells (HDC) fused to senescent HDC but entry into S phase is inhibited. *Exp. Cell Res.* 126:469–472.
7. Kenny, G. E. 1969. Serological comparison of ten glycolytic mycoplasma species. *J. Bacteriol.* 98:1044–1055.
8. Norwood, T. H., and C. J. Zeigler. 1977. Complementation between senescent human diploid cells and a thymidine kinase-deficient murine cell line. *Cytogenet. Cell Genet.* 19:355–367.
9. Norwood, T. H., W. R. Pendergrass, C. A. Sprague, and G. M. Martin. 1974. Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells. *Proc. Natl. Acad. Sci. U. S. A.* 71:2231–2235.
10. Vazquez, D. 1979. Inhibitors of protein biosynthesis. *Mol. Biol. Biochem. Biophys.* 30:103–108.
11. Anderson, K. M., S. Friedel, and J. Baranowski. 1980. Effects of temperature and inhibitors on protein synthesis in a temperature sensitive CHO cell line. A proposed role for protein synthesis intrinsic to cell nuclei in the synthesis of RNA. *Int. J. Biochem.* 12:421–426.
12. Stimac, E., D. Housman, and J. A. Huberman. 1977. Effects of inhibition of protein synthesis on DNA replication in cultured mammalian cells. *J. Mol. Biol.* 115:485–511.
13. Hand, R. 1975. DNA replication in mammalian cells: altered patterns of initiation during inhibition of protein synthesis. *J. Cell Biol.* 67:761–773.
14. Seale, R. L., and R. T. Simpson. 1975. Effects of cycloheximide on chromatin biosynthesis. *J. Mol. Biol.* 94:479–501.
15. Tobey, R. A., E. C. Anderson, and D. F. Peterson. 1966. RNA stability and protein synthesis in relation to the division of mammalian cells. *Biochemistry.* 56:1520–1527.
16. Terasima, T., and M. Yasukawa. 1966. Synthesis of G_1 protein preceding DNA synthesis in cultured mammalian cells. *Exp. Cell Res.* 44:669–672.
17. Highfield, D. P., and W. C. Dewey. 1972. Inhibition of DNA synthesis in synchronized Chinese hamster cells treated in G_1 or early S phase with cycloheximide or puromycin. *Exp. Cell Res.* 75:314–320.
18. Schneiderman, M. H., W. C. Dewey, and D. P. Highfield. 1971. Inhibition of DNA synthesis in synchronized Chinese hamster cells treated in G_1 with cycloheximide. *Exp. Cell Res.* 67:147–155.
19. Smith, J. R., and C. K. Lumpkin. 1980. Loss of gene repression activity: a theory of cellular senescence. *Mech. Ageing Dev.* 13:387–392.
20. Muggleton-Harris, Audrey L., and L. Hayflick. 1976. Cellular aging studied by the reconstruction of cells from nuclei and cytoplasms isolated from normal human diploid cells. *Exp. Cell Res.* 103:321–330.
21. Christopher, C. W., K. Ishikawa, and H. Amas. 1971. Anomalous stimulation of protein synthesis: evidence for translational control in primary cells. *Cell Tissue Kinet.* 4:75–92.
22. Rothblum, L. I., T. M. Devlin, and J. J. Ch'ih. 1976. Regulation of mammalian protein synthesis in vivo: protein synthesis in rat liver and kidney after the administration of sublethal doses of cycloheximide. *Biochem. J.* 156:151–157.
23. Amenta, J. S., M. J. Sargus, and F. M. Baccino. 1978. Inhibition of basal protein degradation in rat embryo fibroblasts by cycloheximide: correlation with activities of lysosomal proteases. *J. Cell. Physiol.* 97:267–284.
24. Amenta, J. S., and S. C. Brocher. 1981. Minireview: mechanisms of protein turnover in cultured cells. *Life Sci.* 28:1195–1208.
25. Kovacs, A. L., and P. O. Seglen. 1981. Inhibition of hepatocytic protein degradation by methylaminopurines and inhibitors of protein synthesis. *Biochim. Biophys. Acta.* 676:213–220.