Clonal Selection in Cultured Human Fibroblasts: Role of Protein Synthetic Errors

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ABSTRACT Protein synthetic error frequency, determined in cell-free extracts as Δleu/Δphe incorporation following poly(U) stimulation, has been found to decrease progressively in several strains of human diploid fibroblasts during their limited replicative lifespan. To explore the basis of this phenomenon, we followed a mass (uncloned) culture of one normal strain at 13 stages of its replicative lifespan. We found a progressive tenfold decline in error frequency that was inversely correlated with passage level ($r = -.93$, $p < .001$). This could not be ascribed to the slow rates of replication associated with fibroblast senescence because slowing of growth by serum deprivation did not change error frequency. Additionally, terminal mass cultures maintained for 16 wk at saturation density to minimize cell selection did not change error frequency over this time. Error frequencies in 12 individual clones purified from the parental culture did not decline on repeated passage, either remaining constant or, in two clones, rising abruptly three- to five-fold after initial assays. Error frequencies of clones showed a weak inverse correlation with growth vigor but not with the maximum doubling number. We conclude that selective pressures favor more vigorously dividing clones with low protein synthetic error frequencies leading to their predominance in mass cultures.

Orgel first suggested (26) that errors introduced into critical information-handling proteins during protein synthesis could lead to positive feedback resulting in an "error catastrophe." This, in turn, would contribute to the extinction of individual cell clones or to the biological aging process in higher organisms. Reconsideration of the nature of the positive feedback loop (27) led to the refinement that errors could eventually stabilize at a new steady state compatible with continued growth and viability. Indeed, Gallant and co-workers (6–8) have shown in *Escherichia coli* that the protein synthetic error frequency induced by streptomycin could stabilize at a level 50 times greater than normal without extinction of growth or increased wastage of cells although the overall cell population grew more slowly.

These observations on bacterial cells are of great interest but, due to substantially different genetic makeup and stability, they may not be applicable to higher forms. For similar reasons, permanent lines of animal cells in culture also have limited utility. On the other hand, human diploid fibroblasts are particularly advantageous for such studies. Much is known about these cells in tissue culture, especially that they are genetically stable and retain an essentially diploid karyotype during their finite replicative life span (3, 11, 19). Therefore, cultured human fibroblasts provide an excellent model for study of the impact of protein synthetic errors on cellular population dynamics over an extended number of cell divisions.

In earlier work, we observed that mass (uncloned) cultures of human diploid fibroblasts from four different normal donors had a lower protein synthetic error frequency at late passage compared to their early-passage counterparts (36). We used the artificial system of poly(U)-directed polyphenylalanine synthesis in which the UUU codon specifies phenylalanine incorporation (25), but where leucine incorporation, coded by UUA, UUG, and CUU, serves as an index of unfaithful protein synthesis due to ribosomal ambiguity (5, 9, 35). Indeed, determinations of protein synthetic error frequency by an independent assay using intact cells confirmed these findings (17). In order to explore the basis of this apparently increased protein synthetic fidelity with continuous cell division, we have carried out detailed studies on one normal cell strain during its limited replicative life span. We present data on synthetic fidelity in

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mass cultures and purified clones at several stages of their replicative lifespan, and in terminal, "postreplicative" cultures in which cell selection is minimized. The results are consistent with the idea that selective pressures favor the growth of clones that synthesize proteins with the highest fidelity whereas errors measured by the poly(U) assay have no apparent effect on ultimate clonal viability.

MATERIALS AND METHODS

Cell Culture

The A2 strain (36) was derived from an anterior forearm biopsy of a normal 11-year-old boy (16). This strain was stored in liquid N_2 at a passage level of 14 mean population doublings (MPD), which includes 10 MPD during the period of skin explantation in primary culture plus 4 MPD during expansion in secondary culture. After reconstitution from storage, cells were serially subcultivated at 37°C to the desired passage level on plastic dishes (58 cm^2, Corning Glass Works, Corning, NY) in Eagle's minimum essential medium supplemented with 15% fetal calf serum ("regular growth medium," RGM) under 95% air, 5% CO_2 (13). Larger quantities of cells were grown in roller bottles (490 cm^2, Corning Glass Works).

Derivation of Clonal Cultures

A confluent culture of A2 at MPD 17 was inoculated in 100-mm dishes at low density (~100 cells/dish). After 10–14 d, colonies ranging in diameter from 0.5 to 1.5 cm were isolated within cloning cylinders and removed by trypsinization, then re-inoculated separately into 35-mm dishes (14). At confluence, the clonal cultures were split into a 100-mm dish and, when they reached confluence a total of 20 MPD was added to each clone's cumulative number of MPD from the time of low-density seeding to this stage. The cells were propagated to late passage by serial subcultivation in dishes and inoculated into roller bottles at mid-passage (45–53 MPD) and late passage (60–70 MPD). Rank of growth vigor was determined by an objective scheme which weighted, respectively, the mean number of days required to attain confluence, the cell density at confluence, and the accumulated debris determined by microscopic examination. Estimates of the maximum in vitro life span of each clone were made by taking the cumulative MPD reached by the culture when it did not become confluent after two twice-weekly refeedings.

Postreplicative Cultures

A confluent mass culture of A2 nearing replicative senescence at 67 MPD was inoculated at a 1:16 split ratio into roller bottles containing 150 ml of growth medium. Upon reaching confluence, after ~16 d, the culture was refed at weekly intervals by adding 40 ml of fresh RGM to the roller bottles. Cultures were harvested for error frequency assays at 0, 6, 10, and 16 wk postreplication. Cells from each harvest were inoculated into dishes for thymidine labeling index over 30 h (4) and protein synthetic rate determinations.

Preparation of Extracts

Extracts were prepared as described (36). Briefly, cells were harvested when 2–3 d postconfluent as determined by microscopic examination, then washed and suspended in hypotonic homogenization buffer (34). After swelling for 30 min, cells were ruptured in a Dounce homogenizer followed by restoration of isotonicity. The lysate was centrifuged at 30,000 *g* for 30 min and the supernatant (S-30) was removed. The S-30 was incubated with 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, and 10 μg/ml creatine phosphokinase for 45 min and then dialyzed against a buffer containing 20 mM Hepes-K^+, pH 7.4, 120 mM KCl, 2.5 mM Mg $(OAc)_2$, and 6 mM β-mercaptoethanol in a spinning dialysis unit for 2 h with four changes of buffer. The S-30 was adjusted to an RNA concentration of 10 A_{260} units/ml and used immediately. The K^+ optimum was 140 mM whereas the Mg^{2+} optimum was 5.5 mM. These optima were re-evaluated at several stages of passage and in various clones and found to remain constant. Unlabeled amino acids were added at final concentration of 10 μM, and 20 μCi/ml [^{3}H]phenylalanine, 160 μCi/ml each of [^{3}H]leucine and [^{3}H]lysine as described (36). The total volume of the incubation mixture was 50–100 μl, and 5–20 μl were removed at each time point and spotted onto glass fiber disks followed by immersion in 5% trichloroacetic acid containing 0.5% casamino acids, heating at 90°C for 10 min and washing with trichloroacetic acid (TCA). Disks were washed with ethanol and diethylether, air dried, and their radioactivity was measured by liquid scintillation counting.

Cellular Protein Synthetic Rate

Cells grown in replicate 60-mm dishes until 2 d postconfluent were trypsinized and the total number of cells/dish was determined on a Coulter electronic counter (Coulter Electronics, Hialeah, FL). The medium was aspirated from other replicate dishes, and fresh growth medium containing 1.0 μCi/ml [^{3}H]phe (specific activity 5.15 Ci/mole) previously equilibrated to 37°C and 95% air, 5% CO_2 was added and the dishes were returned immediately to the incubator. Protein synthesis was measured at 0, 20, 40, and 60 min by removing the labeling medium, rinsing cells twice with ice-cold phosphate-buffered saline, and precipitating the protein with two washes of ice-cold 5% TCA containing 1 mM phenylalanine. The dishes were washed quickly with ice-cold distilled water, and the precipitated protein was dissolved in 0.4 M NaOH/0.4% deoxycholate (DOC). Protein radioactivity was determined by liquid scintillation counting in PCS (Packard Instrument Co., Inc., Downers Grove, IL) made acidic with 20 μl/ml of 1.0 N HCl (to prevent precipitation of NaOH/DOC). Counting efficiency was 35–38%. Protein synthetic rates were determined after adjusting for dilution of labeled phenylalanine by unlabeled (0.2 mM) phenylalanine in the medium and expressed as femtomoles of phenylalanine incorporated per cell.

Cellular Proteolytic Rate

We used a slight modification of the method of Bradley et al. (2). Cells were grown in 60-mm dishes until 2 d postconfluent. The growth medium was aspirated and the cells were pulsed with regular growth medium containing 10% fetal calf serum plus [^{3}H]phenylalanine at 10 μCi/ml. After a 2-h pulse period, the dishes were put on ice and the cells were rinsed five times during 25 min with ice-cold growth medium minus serum containing HEPES 20 mM, pH 7.4, substituted for $NaHCO_3$, and 20 mM phenylalanine. The dishes were placed in a 37°C water bath oscillating at 60 cycles/min through an amplitude of 4 cm. This medium, when withdrawn after 15 min, largely contained [^{3}H]phe that was unincorporated into protein during the pulse. To begin the chase, 3 ml of the same fresh serum-free medium previously warmed to 37°C was added to each dish. The entire volume of chase medium was replaced by fresh chase medium at hourly intervals. Radiolabel released from the cells was measured in an aliquot of the chase medium by liquid scintillation counting of the aliquot directly in PCS. After 8 h, the last aliquot of the chase medium was removed and the cell protein was precipitated with ice-cold 15% TCA containing 20 mM phenylalanine. The precipitated monolayer was washed twice with 5% TCA with 20 mM phenylalanine, rinsed quickly with water, and dissolved in 0.4 M NaOH/0.4% DOC. Labeled protein in this solution was counted in acidified PCS.

The percent proteolysis at time t (t = 1, 2, ... 8) was determined as:

$$100 \times \sum_{n=1}^{t} X_n / \text{Total after 8 h},$$

$$\text{where total} = \sum_{n=1}^{8} X_n + X_i,$$

and where X_n = dpm in chase aliquot at the nth hour and

X_i = dpm in TCA-insoluble material after the eighth hour.

RESULTS

Determination of Error Frequency

To estimate the error frequency of in vitro protein synthesis, we preincubated 30,000 *g* supernatants of homogenized cells augmented with standardized components to "run off" endogenous mRNA and then incubated with [^{3}H]leucine or [^{3}H]phenylalanine with and without addition of poly(U) (reference 36, and see Materials and Methods). Phenylalanine incorporated into hot-acid insoluble material in the absence of poly(U) was very low compared to an approximately thousandfold stimulation of poly(U)-directed phenylalanine incorporation (Δphe) averaging about 20 pmoles/h/10^6 cells (equivalent to about 0.15 nmoles/h/A260 unit). Poly(U)-directed protein synthesis was linear with time for at least 30 min and was inhibited >90% by increasing concentrations of cycloheximide up to 2 mM and >99% by 4 mM puromycin. Similarly, low levels of leucine were incorporated into protein in the

absence of poly(U), and an increment (Δleu) that was small relative to Δphe was always seen after poly(U) addition (see below). To monitor a possible effect of poly(U) on leucine incorporation due to enhancement of endogenous mRNA translatability, we measured the incorporation of [^{3}H]lysine (coded for by AAA and AAC and thus most remote in the genetic code) with and without poly(U). In over 80 experiments poly(U) was found not to affect lysine incorporation substantially [range ± 3% of no poly(U)] and we conclude that Δleu incorporation following addition of poly(U) was entirely related to direct coding by poly(U).

Cell-free extracts were found to be free of RNase activity under conditions optimized for poly(U) translation, showing no degradation of [^{3}H]poly(U) or [^{3}H]UdR-labeled cellular RNA (data not shown). There was also no demonstrable protease activity as no degradation of [^{3}H]poly (phe) or ^{35}S-met-labeled cell proteins was observed (35). This is an important negative finding because proteolysis could mask mistranslation if there were differential degradation of normal and error-containing proteins (10).

Replicating Mass Cultures

To analyze more precisely the trend toward lower error frequency observed at late passage (36), we determined error frequencies in extracts of one normal cell strain, A2, at several stages of its replicative life span. All cells used in this series of experiments were mass cultures subcultivated from a single ampule of A2 cells reconstituted at 14 mean population doublings, and error frequencies were determined sequentially at 13 passage levels from MPD 22 to MPD 74. The results are shown in Fig. 1. Error frequencies declined >10-fold in a progressive manner from 0.27% at MPD 22 to 0.025% at MPD 74. Linear analysis of the data by the method of least squares showed that the line $y = -0.0046x + 74.6$ fits the data with a high correlation ($r = -0.93$) that was significantly different from zero ($p < 0.001$). The rate of decline in error frequency was −0.0046%/MPD.

Measurements of poly(U)-directed protein synthesis ranged widely but did not show any significant trend as a function of passage, whether expressed per A_{260} unit, per mg total protein, or per 10^6 cells. The rate of phenylalanine incorporation directed by poly(U) had a mean of 22.7 pmoles/h/10^6 cells ranging from a low of 0.1 pmoles/h/10^6 cells at 71 MPD to a high of 76 pmoles/h/10^6 cells at 66 MPD. Similarly, residual levels of endogenous mRNA-directed protein synthesis varied considerably in each extract as shown by the incorporation of leucine and lysine in the absence of poly(U). Despite this variation in Δphe, Δleu changed commensurately so that error frequency was remarkably consistent, showing good reproducibility at a given passage level (36), or the progressive downward trend with continuous passage (Fig. 1). Consistently, endogenous leucine incorporation was higher than lysine incorporation by an average of 1.5-fold (data not shown), in keeping with the molar proportions of each amino acid in fibroblast proteins (15). It was noteworthy that the optima for Mg^{2+} and K^+ did not vary from early to late passage of mass cultures or in the clonally pure populations (35).

Slowing of Growth Rate by Serum Depletion

Because cells approaching senescence progressively slow down their replicative cycle, the inverse correlation between error frequency and level of MPD can also be expressed as an inverse correlation between the error frequency and the number of days for mass cultures to reach confluence. That is, error frequency fell as cellular growth rate slowed. To determine whether decreasing growth rate was responsible for the observed decline in error frequency at late passage, the rate of proliferation of early-passage cells we reduced by maintaining them in lower concentrations of fetal calf serum. As seen in Table I, early-passage cells grown in low serum concentrations (2.0–2.5%) required approximately the same time to reach confluence as late-passage cells grown in 15% serum. In two separate experiments, the error frequency of slow-growth cells was identical to that of rapid-growth cells of the same passage level. As before, in vitro synthetic rates were variable, yet error frequencies were consistent and significantly lower in late-passage cells. Protein synthetic rates of intact cells were very similar in these three growth states (Table I).

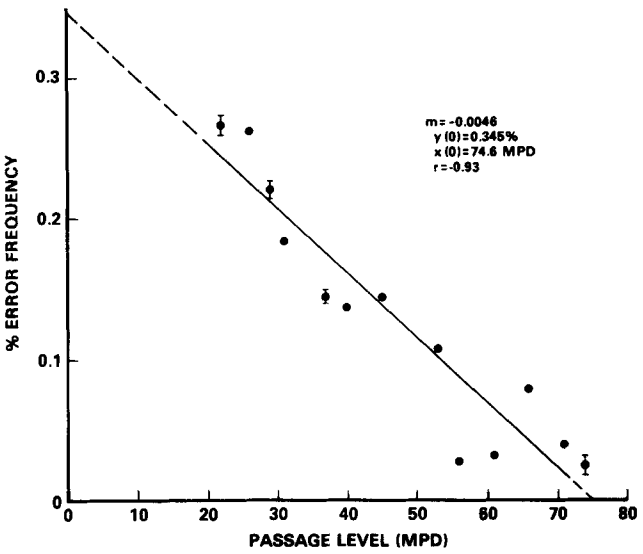

FIGURE 1 Decrease of protein synthetic error frequency as a function of passage in a mass culture of human diploid fibroblasts. A single lot of strain A2 cells was serially subcultivated and assayed for error frequency at 13 passage levels during its replicative life span. Error frequency is expressed as Δleu/Δphe incorporation × 100 after poly(U) stimulation. Least-squares analysis shows the regression line $y = -0.0046x + 74.6$ fits the data with a correlation coefficient of $r = -0.93$. Horizontal bars show the range of error frequencies determined in duplicate.

Postreplicative Mass Cultures

To ascertain whether error frequencies changed over a long period of minimal cell selection, we studied cells under conditions in which replication was drastically reduced. Mass cultures of strain A2 that were terminally senescent at 71 MPD such that they could not grow to confluence, were maintained at saturation density with regular refeedings of fresh medium. Cells were then harvested at 0, 6, 10, and 16 wk after replicative senescence. A small portion of these cells was re-inoculated into fresh medium for [^{3}H]thymidine labeling index (4). Another aliquot of cells was also replated at a 1:4 dilution to confirm that they were at terminal passage because they could not attain confluence after two weekly changes of medium. Not all cells in terminal cultures were devoid of mitotic activity since small foci of growth in the reinoculated cultures could be found after 2 wk in fresh medium. ~10–15% of cells in these

TABLE I

Effect of Serum Limitation on Growth Rate and Error Frequency of Human Fibroblasts

	Passage level (MPD)	Serum %	Days to confluence	In Vivo synthesis*	In Vitro synthesis‡	Percent error frequency (± range)
Experiment 1	30	15	5	ND	39.6	0.36 ± 0.02
	30	2.5	10	ND	52.4	0.33 ± 0.01
Experiment 2	35	10	5	8.4	19.1	0.25 ± 0.04
	35	2.0	15	8.6	16.3	0.27 ± 0.02
	63	15	10	7.6	46.5	0.03 ± 0.005

* Cellular protein synthetic rate was determined as described in Materials and Methods and expressed as fmoles phenylalanine incorporated/h/cell.
‡ Poly(U)-directed phenylalanine incorporation in cell-free extracts (pmoles phenylalanine/h/10^6 cells).
MPD: Mean population doublings
ND—not determined

TABLE II

Error Frequency in Postreplicative Human Fibroblasts

Weeks post-replicative	Cell density*	^{3}H-TdR index	Multinucleated cells	Protein synthetic rate‡	Percent error frequency (± range)
0	10.1	11.1	2	7.54	0.030 ± 0.004
6	10.5	15.3	9	ND	0.048 ± 0.004
10	10.3	13.1	9	6.71	0.004 ± 0.004
16	10.2	9.5	17	7.05	0.023 ± 0.006

ND—not determined
* As grown in roller bottles: 10^3 cells/cm^2
‡ Protein synthetic rate in intact cells—fmoles phenylalanine incorporated/h/cell
Mass cultures of A2 cells at terminal stages of replication (71 MPD) were held in roller flasks with weekly refeeding. At intervals, a portion of cells was harvested and studied as shown.

cultures possessed DNA synthetic activity as shown by [^{3}H] thymidine labeling index (Table II), but there was no significant difference in protein synthetic rate of intact cells between replicating cultures at early or late passage or in postreplicative cells (compare Tables I and II). An increase in the number of multinucleated cells from 2–17% after 16 wk was characteristic (Table II) and has been reported before (23). Thus, some of these postreplicative cells have the ability to carry out semiconservative DNA synthesis and nuclear fission, but seem to be impaired in cytokinesis. The possibility that multinucleation is due to cell fusion is unlikely due to the low spontaneous rate of this process in human fibroblast cultures (12). Error frequencies determined in extracts of these terminal cultures showed some variation (Table II) ranging from a low of 0.004% to a high of 0.048%. However, no consistent trend was observed over the 16-wk period and there was clearly no increase in error frequency under these conditions of sharply reduced cell division and selection.

Clonal Error Frequencies

To compare the effects of cell division on error frequencies of clonally pure cells with those of a mass culture, we established several clonal cultures from the parental strain. Because of the limited replicative capacity remaining after initial clonal selection and propagation to sufficient bulk, error frequency assays of each clone were confined to the final 15–25% of their replicative life span. A brief description of the method is important. Parental cells were reconstituted from the frozen state at MPD 14 and, upon reaching confluence (MPD 17), 100 cells were inoculated into each of five 100-mm dishes. The plating efficiency of this strain at low-density inoculation was ~20%. Of these, only one-half (50 cells) gave colonies large enough for picking. There was much heterogeneity in the colonies that formed, in the size of each colony (0.5–1.5 cm), the number of cells per colony, the size of cells, and the packing density of cells. Of the 50 clones that were picked, most were able to eventually fill at least one 100-mm dish. At this point, 20 MPD were added to the passage level of the cells at seeding to a cumulative 37 MPD (19). Subsequently, most of the clones did not grow well enough to survive the establishment period of a further 8 MPD. Clones that did not reach confluence at 1:8 split after a weekly refeeding were not carried further. Therefore, by the selection criteria used, only 12 clones were established, representing ~2% of the initial number of cells used. Each clone was then subcultivated to terminal passage and several growth characteristics (general growth vigor, time to confluent cell density, cell size, maximum passage number attained) were monitored along with periodic determinations of error frequency until termination of growth.

Clones varied appreciably in error frequency (Fig. 2). For example at MPD 53, error frequencies ranged from 0.026% in clone 10 to 0.40% in clone 18, with the rest of the clones falling within this 15-fold range. The mean clonal error frequency at MPD 53 was 0.17 ± 0.10 (± SD) with a median of 0.12%. The frequency distribution of the log of clonal error frequencies was consistent with a normal distribution (not shown).

Strikingly unlike the mass culture, none of the clones exhibited a significant decline in error frequency on continued cell division (Fig. 2). Three clones (13, 26, 32) were assayed only at one stage. Of the remaining clones, two groups could be identified: group A, comprising seven clones (2, 10, 13, 18, 29, 30, 36), in which the error frequency remained relatively constant through subsequent passage although clones 10 and 29 were only assayed singly at two passage levels; group B, comprising two clones (8 and 14) in which an abrupt increase in error frequency at later passage was observed. It bears emphasis that these two clones were the only cases in which an increase in error frequency was observed with passage among all such determinations on mass cultures or clonal populations. In clone 8 the increases continued from MPD 63 to MPD 66. In clone 14, which exhibited an increase from 0.045% at 59 MPD to 0.25% at 66 MPD, the error frequency remained at the elevated level when this clone was studied 6 wk after termination at 68 MPD (error frequency 0.25%). In general, clonal cultures did not grow as well as mass cultures and contained larger cells with longer doubling times, lower cell densities and more cell debris at confluence, all features of fibroblast senescence (3). None of the clones replicated as long (mean, 63 MPD, range 60–68) as the parental mass culture (mean, 73 MPD) ($p < 0.01$).

It was of great interest to rank error frequency against growth

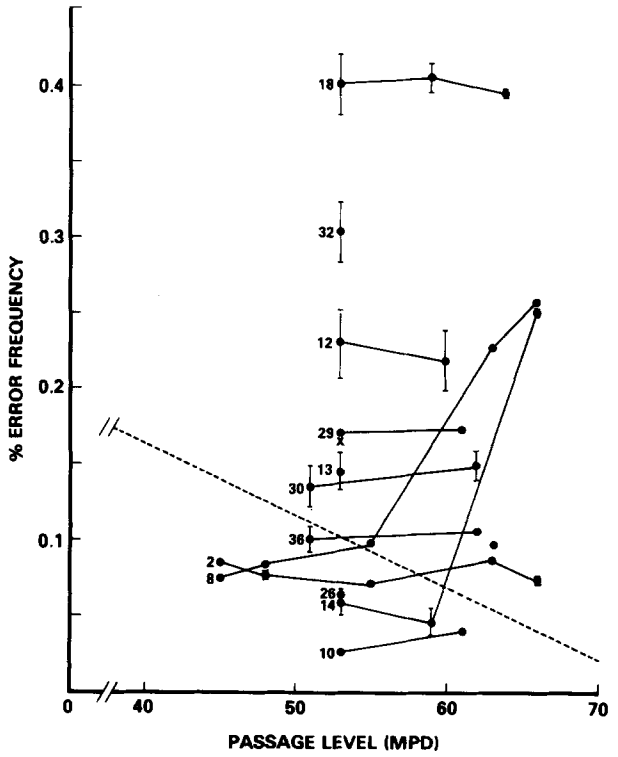

FIGURE 2 Error frequencies in clones of strain A2. 12 clones were selected from the parental mass culture and assayed for error frequency at various passage levels. Shown are the means of duplicate determinations, ± the range indicated as bars, or as single determinations. The mean of the error frequencies of the clones at MPD 53(×) was 0.169 ± 0.103%. The regression line from Fig. 1 is shown as the dashed line.

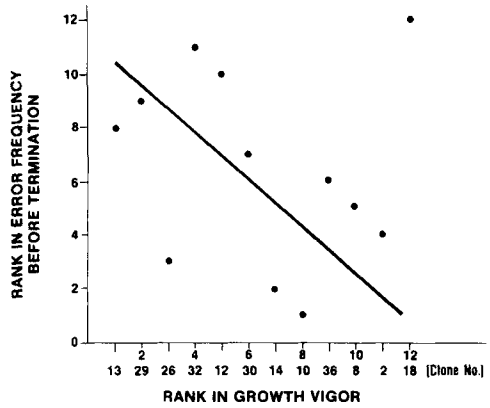

FIGURE 3 Correlation between rank of error frequency and rank of growth vigor. Each clone was ranked according to its relative error frequency (1 = lowest, 12 = highest) and its relative growth vigor (1 = worst, 12 = best) and analyzed by Kendall's rank correlation test (20). Initially determined error frequency was used in all cases because clones ostensibly spent most of their replicative life spans at these levels. The rank correlation coefficient was −0.15, which was not statistically significant. If the outriding clone 18 was omitted, this coefficient was −0.38, which is significant, $p < .05$.

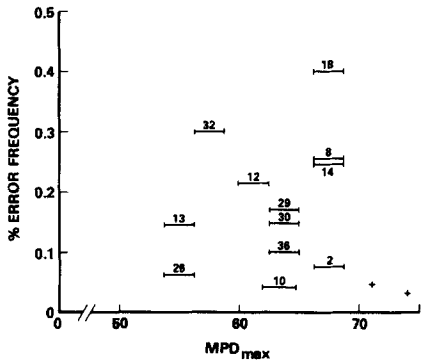

FIGURE 4 Correlation between clonal error frequency and maximum replicative life span. Estimates of MPD_{max} for clonal cultures were made at advanced passage levels when cultures could no longer reach confluence after two weekly refeedings, and are shown as bar graphs ± 2 MPD. Two unique estimates of mass culture MPD_{max} are shown as the symbol (+). When analyzed by linear regression the correlation was not significant whether all 12 clones were included (r = −0.189) or clone 18 excluded (r = −.46).

vigor (Fig. 3). If clone 18 was excluded from calculations, there was a weak but significant inverse correlation ($p < .05$) between the rank of clonal error frequency and the rank of growth vigor derived from several growth characteristics. When a correlation was sought between error frequency and maximum MPD attained (Fig. 4), this did not attain statistical significance even if clone 18 was excluded from calculations.

Clonal in vivo synthetic rates (4.46 ± 1.05 fmoles phe incorporated/h/cell, mean ± SD) were significantly lower ($p < 0.001$) than those of the mass culture (7.36 ± 0.55) at comparable passage levels. There were, however, no differences in the rates of in vivo protein synthesis between clones with high and those with low error frequency. It was also observed that no change occurred in the protein synthetic rate of clone 14 after its increase in error frequency. Clone 10 (lowest error frequency) possessed the lowest cellular protein synthetic rate. However, an overall correlation between error frequencies and in vivo synthetic rates could not be established.

To determine whether error frequency bore a relationship to protein degradation rates in vivo, we measured proteolysis in clone 10 (low error frequency) and clone 18 (high error frequency) and compared it to proteolysis in mass cultures of strain A2 at early and late passage (Fig. 5). Clone 10 showed the greatest proteolysis after 8 h (32%), clone 18 the lowest (28%), with the mass cultures showing intermediate values. The kinetics of cellular proteolysis were complex, but analysis of variance of the data (2-factor design, repeated measures on one factor) revealed that the small apparent difference in proteolytic rate between early and late-passage mass cultures was not significant. However, the difference between clone 10 and clone 18 was significant ($p < .01$).

DISCUSSION

Despite its obvious importance relatively little is known about the dynamics of cell lineages in vivo and in vitro. We have observed a linear 10-fold decline of protein synthetic error frequency in mass cultures of a normal strain of human fibroblasts as they traverse their replicative lifespan. After the cultures reached terminal passage, no further decline in error frequency was seen when they were maintained in an essentially postreplicative state for up to 16 wk. Although some DNA synthesis and nuclear cleavage occurred, there was apparently little cytokinesis and rare mitosis as indicated by the increasing percentage of multi-nucleated cells accumulating in these postreplicative cultures and the failure to fill the vessels to confluent density. It seems clear, therefore, that mass cultures exhibit a decrease in error frequency only during the replicative portion of their lifespan. Thus, postreplicative cells, wherein

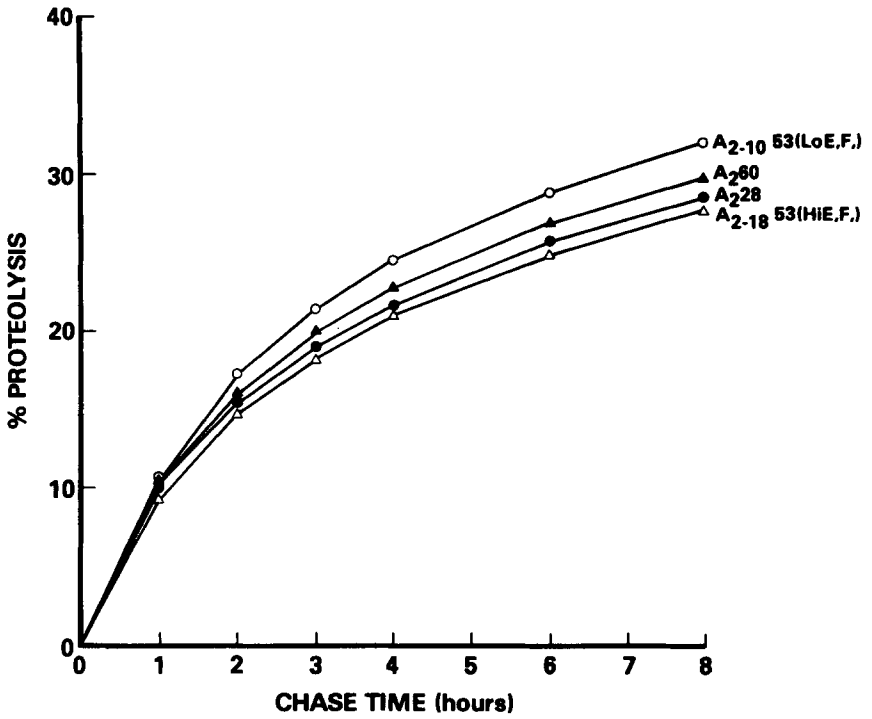

FIGURE 5 Cellular proteolysis in early- (A228) and late-passage (A260) mass cultures and in high and low error frequency clones. Cells were prelabeled with [^{3}H]phenylalanine for 2 h, rinsed thoroughly and incubated in isotope-free medium for times shown. Proteolysis was determined as in Materials and Methods.

clonal selection (see below) is eliminated or at least sharply curtailed, are not subject to further improvements in translational accuracy nor do they suffer error catastrophe as predicted by the Orgel hypothesis (26, 27).

Two possible mechanisms that could account for the passage-related decrease in error frequency are growth rate per se and clonal selection. If the error frequency were growth-related, then slowing the growth rate of early-passage cells to mimic that of late-passage cells should lead to a decreased error rate. In fact, this was not observed (Table I). Additionally, such growth-retarded cells maintained their cellular protein synthetic rates equal to that of rapidly growing cells (Table I). Slower growth in the face of normal synthetic rates thus requires that the proteolytic rate be elevated in the slow-growing cells as indeed it is in late-passage cells (2, 15).

The Clonal Selection Hypothesis

If clones with high error frequency were selectively eliminated from the mass culture, only the progeny of low error frequency cells would survive to late-passage. The result would be a late-passage culture consisting of a mixed population of clones with low error frequency. The elimination of clones with high error frequency from the mass culture could be accomplished in two ways (operating alone or in combination). First, the high error frequency clones could have slow growth rates such that they are diluted out by clones with low error frequencies. Second, the clones with high error frequency could grow as well as low error frequency clones but could have shorter in vitro lifespans. Statistical analysis of the correlation between clonal error frequency and growth vigor (Fig. 3) showed no significant relationship unless clone 18 was omitted. However, there was no correlation between error frequency and MPD_{max} in any case. These findings are consistent with those for *E. coli* in which an increase in protein synthetic errors up to 50-fold higher than normal can slow growth but does not lead to cellular extinction (6–8). Thus, although the error feedback mechanism surely exists, it may not be sufficient to cause an error catastrophe. In group A, clone 18, with the highest error frequency, had the greatest growth vigor and attained approximately the same MPD_{max} as clone 10 with an error frequency 10 times lower. Furthermore, in the two clones of group B, no abrupt decrement in growth vigor was associated with the error increase, nor did lethality occur. Additionally, error frequency did not continue to rise in clone 14 after 6 wk in the postreplicative state.

The constant error frequency of most clones during serial passage is of interest in the context of clonal selection although this hypothesis does not require a constant error frequency throughout a clone's lifetime; error frequency could increase or decrease as long as higher error frequency clones were selected against. The wide range of error frequencies found among clones supports the contention that an early-passage culture represents a broad distribution of individual clonal error frequencies. However, the mean of clonal error frequencies at MPD 53 was 0.165%, whereas the error frequency of the parental mass culture when cells were initially plated to prepare clones was ~0.3%. Because none of the clones declined in error frequency during the period assessed, this bias in the clones further supports the idea of clonal succession based on higher plating efficiency and/or selective advantage of low error frequency clones.

Other data support the concept of clonal succession during in vitro passage of human fibroblast cultures (30), and heterogeneity in growth characteristics of clones has been reported by several others (1, 4, 14, 22, 24, 31). Indeed, it has also been shown that a large proportion of cells in a mass culture possess very limited proliferative capacity (32) and that the maximum MPD of clones which make up the mass culture are bimodally distributed (31, 33). Thus, one subpopulation of clones has low replicative potential whereas the other reaches the higher maximum MPD approximately equal to that of the mass culture.

The increase in error frequency in two clones could result from a mutation altering the fidelity of the translational apparatus. However, this seems unlikely because such a mutation

would have to occur with a relatively high frequency to be observed in two out of nine clones.

The nature of errors measured here must be considered because leu substitution for phe is relatively conservative. Indeed, poly (phe) is predicted to be an α-helix (with some β-sheet toward the C-terminus) that is somewhat stabilized by leucine substitution (R. M. Stroud, personal communication). Thus, such errors would only be relevant to the vigor of a cell if they were to reflect nonconservative errors with a deleterious effect on protein configuration in a fixed proportion. In fact, the error frequency of protein synthesis determined by the Δleu/Δphe method is about one order of magnitude higher than that found by more direct measurements in endogenous proteins (6, 17, 18, 21). Although the exact relation between the Δleu/Δphe values and the other determinations is unknown, the synthesis of aberrant proteins should trigger the proteolytic surveillance system (10). However, proteolysis was not increased in high-error frequency clone 18 or early-passage mass cultures. Moreover, it did not increase in *E. coli* induced to mistranslate proteins leading to two different sets of hydrophobic substitutions (28). In fact, the errors observed here and in other animal and bacterial systems (17, 18, 29) very likely involve codon-anticodon misreading at the third position. Because this is the wobble site, predominantly hydrophobic ↔ hydrophobic and hydrophilic ↔ hydrophilic substitutions are to be expected. It may be, therefore, that detrimental mistranslations are far less frequent than the conservative type measured here and contribute more rarely to cell selection.

A final possibility must be considered that clones compete in mass cultures to allow clonal selection to occur, but that growth as pure clones is not a good predictor of success in competition. This could be tested by studying suitably marked clones with high and low error frequencies, alone and in various mixtures, after several intervals of active replication.

In summary, none of the clones studied showed the decrease in error frequency with passage seen in mass cultures whereas postreplicative cultures showed constant error frequencies. Because the major difference is the clonal heterogeneity of the mass culture coupled to vigorous cell division, it follows that these two factors are preconditions and that clonal selection is the likely mechanism for the observed decrease in error frequency during passage of the mass culture.

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