

Human skin fibroblasts *in vitro* differentiate along a terminal cell lineage

(cell types/stem cell system/cellular aging/two-dimensional gel electrophoresis)

KLAUS BAYREUTHER*, H. PETER RODEMANN, RAJA HOMMEL, KLAUS DITTMANN, MARIO ALBIEZ, AND PAL I. FRAN CZ

Institut für Genetik, Universität Hohenheim, D 7000 Stuttgart 70, Federal Republic of Germany

Communicated by W. Beermann, March 23, 1988

ABSTRACT Secondary mitotic human skin fibroblast populations *in vitro* underwent 53 ± 6 cumulative population doublings (CPD) in 302 ± 27 days. When the growth capacity of the mitotic fibroblasts is exhausted, and if appropriate methods are applied, the fibroblasts differentiate spontaneously into postmitotic fibroblast populations, which were kept in stationary culture for up to 305 ± 41 additional days. Mitotic and postmitotic fibroblast populations are heterogeneous populations with reproducible changes in the proportions of mitotic fibroblasts F I, F II, and F III, and postmitotic fibroblasts F IV, F V, F VI, and F VII. This process makes it evident that the fibroblasts differentiate spontaneously along a seven-stage terminal cell lineage F I-F II-F III-F IV-F V-F VI-F VII. Shifts in the frequencies of the mitotic and postmitotic fibroblasts in mass populations are accompanied by alterations in the [³⁵S]methionine polypeptide pattern of the developing mass populations. The [³⁵S]methionine polypeptide patterns of homogeneous subpopulations of F I, F II, F III, F IV, F V, and F VI isolated from heterogeneous mass populations reveal that the six fibroblast morphotypes studied express their cell-type-specific [³⁵S]methionine polypeptide pattern in the heterogeneous mass populations.

The ontogenetic development of the normal fibroblast cell systems in vertebrate and mammalian organisms *in vivo* and *in vitro* is not fully understood. *In vivo*, a metabolically inactive (1) and a metabolically active (2) fibroblast have been described; in primary *in vitro* cell systems, a differentiation sequence of three mitotic fibroblasts, F I-F II-F III, with distinct cell-type-specific biological and biochemical properties have been described in BN (brown Norway) rat skin (3).

In vitro, normal secondary fibroblast populations have a definite mitotic lifespan, which in some species (e.g., chicken and man) is followed by cellular degeneration (4, 5) and in other species (e.g., mouse and rat) is succeeded by cellular transformation (6, 7). Distinct morphotypes and quantitative and/or qualitative disparities for a multitude of biochemical parameters are recorded for secondary human fibroblasts in early and late passages *in vitro* (8-11). No attempts have been undertaken to correlate the distinct fibroblast cell types with dissimilarities in biochemical parameters in secondary human fibroblast populations at nonidentical passage levels. As a consequence, in biological and biochemical studies of cells, secondary fibroblast populations often have been dealt with like homogeneous nondifferentiating cell systems. Secondary BN rat fibroblast populations *in vitro* have been shown to be composed of three mitotic fibroblasts, the fibroblasts F I, F II, and F III, which exhibit cell-type-specific biological and biochemical dissimilarities (7). The BN rat fibroblasts differentiate along the cell lineage F I-F II-F III; the cell-type

frequency is a function of the cumulative population doubling (CPD) level of the secondary mass populations studied (7).

For secondary fibroblast populations of various species (12-14), postmitotic fibroblast populations have been described to live for longer periods in stationary culture *in vitro*. On the basis of these findings, it has been assumed that the fibroblast cell systems are terminally differentiating stem-cell systems (15, 16). We will provide morphological and biochemical evidence that secondary human skin fibroblasts of the cell line HH-8 *in vitro* differentiate along a terminal stem-cell-like lineage with the mitotic fibroblasts F I-F II-F III, the postmitotic fibroblasts F IV-F V-F VI, and the degenerating fibroblast F VII in three morphologically recognizable differentiating cell compartments of the fibroblast stem-cell system.

MATERIALS AND METHODS

Cell Cultures. Primary skin fibroblast populations of the cell line HH-8 were established from the lower right abdominal region of an 8-yr-old female donor as described (17). After 3 weeks, primary skin fibroblasts were treated with 0.05% trypsin and 0.1% EDTA. For determination of growth rates and CPDs, cells were counted at each transfer in a Fuchs-Rosenthal hemocytometer. For serial subcultivations, secondary HH-8 fibroblasts were transferred once weekly and seeded at a density of 2×10^4 cells per cm^2 up to CPD 40 and at a density of 8×10^3 cells per cm^2 at the CPD 40-53 levels in 55- cm^2 tissue culture dishes (Falcon) in 10 ml of Dulbecco's modified Eagle's medium (DME medium, Microbiological Associates) supplemented with 10% (vol/vol) fetal calf serum (Boehringer Mannheim, lots 139, 182, and 881), penicillin (557 units per ml), and streptomycin (740 units per ml). The cells were incubated in humidified 95% air/5% CO_2 . Culture medium was renewed once weekly.

Transition of Mitotic to Postmitotic Fibroblasts. Late mitotic skin fibroblasts (CPD 50-53) with growth rates (population doublings per day) less than 0.02 for 3 consecutive weeks were gently trypsinized with 0.05% trypsin/0.1% EDTA at room temperature, resuspended in DME medium containing 10% fetal calf serum, and seeded at a constant density of 1.5×10^3 cells per cm^2 . The medium was changed 24 hr later, and cell populations were maintained as stationary postmitotic fibroblast populations for at least 315 days with one medium change weekly.

Preconditions for the reproducible spontaneous transition of mitotic-to-postmitotic fibroblasts are: (i) complete exhaustion of the mitotic capacity of F III; (ii) cautious trypsin treatment of the highly trypsin-sensitive late F III; (iii) uniform distribution at a constant density of 1.5×10^3 cells per cm^2 ; (iv) use of selected charges of fetal calf serum that support

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CPD, cumulative population doublings; BN rats, brown Norway rats.

*To whom reprint requests should be addressed.

differentiation of mitotic fibroblasts to postmitotic fibroblasts (two of nine charges tested); (v) use of selected charges of plastic dishes for tissue culture (Falcon) (3 of 11 charges tested).

Cell-Type-Specific Morphology and Frequencies. At various CPD numbers, mitotic skin fibroblasts were seeded at a density of 10^3 cells per cm^2 as low-density cultures and maintained for 4 days, during which they express their cell-type-specific morphology. Thereafter, they were washed with phosphate-buffered saline (PBS), subsequently fixed with 3.5% paraformaldehyde and 70% ethanol, and stained for 30 sec with 0.1% Coomassie blue and 30 min with Giemsa solution. Postmitotic skin fibroblasts at various times of stationary postmitotic culture were fixed and stained as above. The frequencies of the different mitotic and postmitotic fibroblast cell types were determined as a function of the CPD number or as a function of the duration of the postmitotic stationary culture, by counting at least 4000 cells in random microscopic fields of cells in different tissue culture dishes. Cell types were photographed to document morphological characteristics of the different fibroblast cell types.

[^{35}S]Methionine Polypeptide Pattern. Subconfluent mitotic fibroblast mass populations (CPD 20) and postmitotic fibroblast mass populations (21 days of stationary postmitotic culture) were labeled with 200 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (400 Ci/mmol, New England Nuclear) per ml for 18 hr, as described (18). After labeling, homogeneous subpopulations (50–100 cells) of mitotic fibroblasts F I, F II, and F III and of postmitotic fibroblasts F IV, F V, and F VI were surrounded by silicone-sealed glass rings. Cells were lysed by lysis buffer, and after determination of radioactivity, aliquots of the various samples (containing 500,000 cpm) were analyzed by two-dimensional polyacrylamide gel electrophoresis, as described (18).

RESULTS

Population Dynamics. In 11 experiments, secondary mitotic fibroblast populations of HH-8 *in vitro* underwent 53.6 ± 6 CPD in 302 ± 27 days. When the growth rate was found to be lower than 0.02 for three consecutive passages, the fibroblast populations were transferred for the last time. When appropriate methods were applied, in all experiments the mitotic fibroblasts differentiated spontaneously into postmitotic fibroblast populations, which were kept in stationary culture for up to 305 ± 41 days and terminated thereafter.

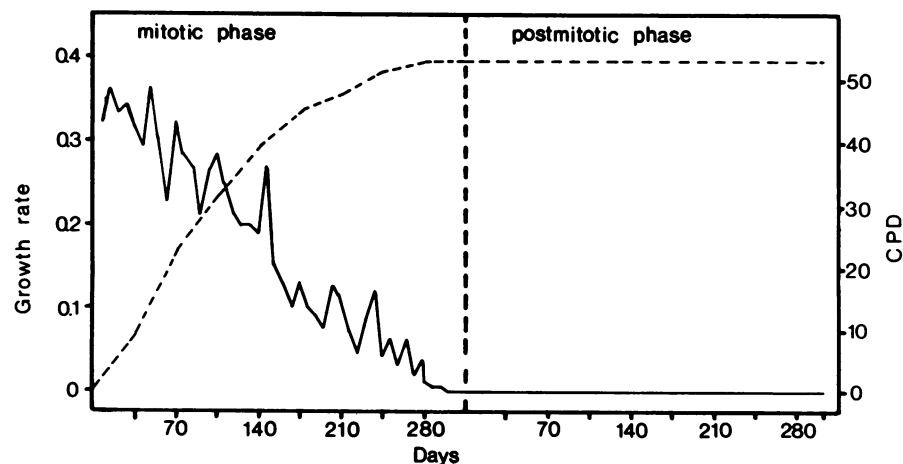


FIG. 1. Population dynamics of human skin fibroblast cell line HH-8. Fibroblast populations were cultured as described, and growth rates (—, population doublings per day) and CPDs (---) were calculated at each transfer of mitotic fibroblast populations or every 14 days by counting the cell number of stationary postmitotic fibroblast populations; 11 experiments gave similar results. The population dynamics of 1 experiment is shown, giving a maximum CPD value of 53.5 in 315 days of the mitotic phase; after transition to stationary postmitotic cultures, fibroblast populations were maintained for an additional 301 days and terminated thereafter.

Fig. 1 shows the characteristic population dynamics of one typical experiment. The maximum lifespan of the postmitotic HH-8 cells in stationary culture has not been determined yet.

Fibroblast Cell Types. The mitotic fibroblast population is made up predominantly of three mitotic fibroblast cell types, for which we propose the names fibroblast I = F I, fibroblast II = F II, and fibroblast III = F III (Fig. 2 A–C). F I is a small spindle-shaped cell (Fig. 2A), F II is a small epithelioid cell (Fig. 2B), and F III is a larger pleiomorphic epithelioid cell (Fig. 2C). The postmitotic fibroblast population is mainly composed of four postmitotic cell types, for which we suggest the terms fibroblast IV = F IV, fibroblast V = F V, fibroblast VI = F VI, and fibroblast VII = F VII (Fig. 2 D–G). Since fibroblasts F IV, F V, F VI, and F VII represent the postmitotic cells of the fibroblast cell lineage, they could also be named fibrocytes F IV, F V, F VI, and F VII. F IV is a large spindle-shaped cell (Fig. 2D), F V is a larger epithelioid cell (Fig. 2E), F VI is the largest epithelioid cell of the fibroblast series (Fig. 2F), and F VII is the degenerating fibroblast (Fig. 2G).

Changes in the Frequencies of Mitotic and Postmitotic Fibroblasts. Mitotic and postmitotic fibroblast populations of HH-8 *in vitro* were observed to be heterogeneous cell populations with reproducible changes in the proportions of the mitotic (F I, F II, and F III) and of the postmitotic (F IV, F V, F VI, and F VII) cell types in the mitotic progenitor fibroblast compartment, the postmitotic differentiating and maturing fibroblast compartment, and the degenerating fibroblast compartment. The spontaneous shifts in the relative number of F I, F II, F III, F IV, F V, F VI, and F VII in the different stages of the development of the fibroblast stem-cell system are shown in Fig. 3 and Table 1. The seven fibroblast cell types acquired their characteristic morphology when seeded in low-density populations. The morphological properties of the cytoplasm and the nuclei could be revealed best when the cells were stained with Coomassie blue and Giemsa solution as described.

[^{35}S]Methionine Polypeptide Pattern. Significant qualitative and/or quantitative differences in the [^{35}S]methionine polypeptide pattern of different mitotic and postmitotic fibroblast populations could be demonstrated by two-dimensional gel electrophoresis. Fig. 4 shows a comparison of the [^{35}S]methionine polypeptide pattern of early-passage mitotic mass populations (CPD 14, Fig. 4A) and early postmitotic mass populations (6 weeks of stationary culture, Fig. 4B). From mitotic to postmitotic mass populations, 120 quantitative

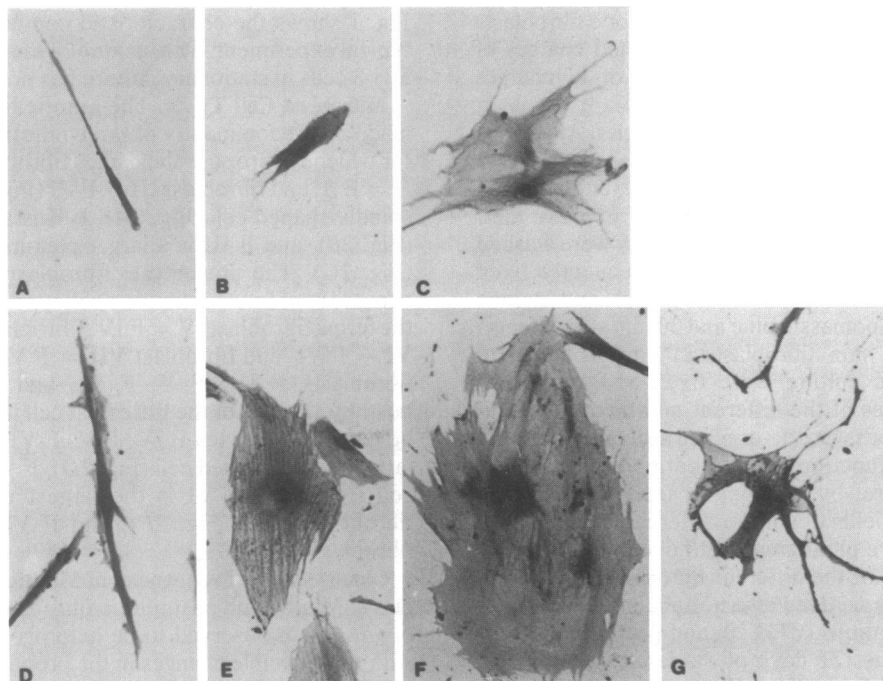


FIG. 2. Mitotic and postmitotic fibroblast morphotypes. (A) Mitotic fibroblast cell-type F I. (B) Mitotic fibroblast cell-type F II. (C) Mitotic fibroblast cell-type F III. (D) Postmitotic fibroblast cell-type F IV. (E) Postmitotic fibroblast cell-type F V. (F) Postmitotic fibroblast cell-type F VI. (G) Postmitotic degenerating fibroblast cell type F VII. ($\times 86$.)

and/or qualitative differentiation-dependent differences appeared mainly in the regions marked by insets (Fig. 4). When homogeneous subpopulations of nearly 95% purity for one mitotic (F I, F II, or F III) and one postmitotic (F IV, F V, or F VI) fibroblast cell type were analyzed, differentiation-dependent and cell-type-specific qualitative and/or quantitative changes in the expression of specific proteins could be observed. Proteins specific for mitotic fibroblasts F I, F II, and F III could be identified in region 3 (Fig. 5A). Although in homogeneous subpopulations of mitotic fibroblasts F I no specific protein could be detected, mitotic fibroblasts F II

were characterized by the differentiation-dependent expression of protein PIIa (32 kDa/ p_1 8.2), protein PIIb (37 kDa/ p_1 7.9), and protein PIIc (40 kDa/ p_1 7.2) (Fig. 5A). Homogeneous subpopulations of mitotic fibroblasts F III expressed protein PIIIa (38 kDa/ p_1 7.0) that was absent in fibroblasts F I and F II (Fig. 5A). Postmitotic fibroblasts F IV were characterized by the appearance of the differentiation-dependent proteins PIVa (33 kDa/ p_1 5.0) in region 7 (Fig. 5B) and PIVb (31 kDa/ p_1 9.2) in region 1 (Fig. 5C), which were not expressed in mitotic fibroblasts F I, F II, and F III. The postmitotic fibroblast F V was characterized by two proteins in region 1, protein PVa

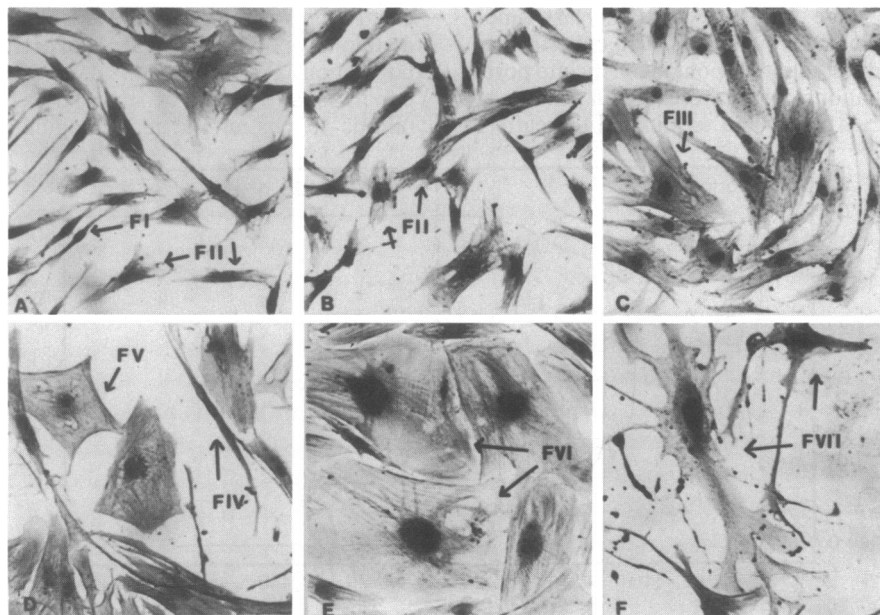


FIG. 3. Cell-type composition of mitotic fibroblast (MF) and postmitotic fibroblast (PMF) populations at different stages of the mitotic and postmitotic lifespan. (A) MF population, CPD 14, predominantly made up of fibroblasts F I and F II. (B) MF population, CPD 30, primarily composed of fibroblasts F II. (C) MF population, CPD 50, predominantly built up of fibroblast F III. (D) PMF populations, 21 days of stationary culture, predominantly made up of fibroblasts F IV and F V. (E) PMF population, 84 days of stationary culture, primarily composed of fibroblasts F VI. (F) PMF population, 280 days of stationary culture, with increasing numbers of degenerating fibroblasts F VII. ($\times 70$.)

Table 1. Changes in the frequencies of mitotic and postmitotic fibroblasts as a function of CPD and duration of stationary culture

CPD (days)	Fibroblast cell type, %						
	F I	F II	F III	F IV	F V	F VI	F VII
	MF populations						
11.6 (35)	18	70	10	1	0.5	0.5	—
22.0 (70)	8	78	12	1	0.5	0.5	—
31.1 (105)	3	78	16	2	0.5	0.5	—
40.3 (147)	1	38	49	10	1	1	—
50.1 (231)	—	1	79	14	3	3	—
	PMF populations						
(7)	—	—	30	18	40	12	—
(21)	—	—	3	31	43	23	—
(42)	—	—	—	16	32	51	1
(84)	—	—	—	4	5	87	4
(140)	—	—	—	—	2	89	9
(280)	—	—	—	—	—	88	12

Cell-type frequencies of mitotic fibroblast (MF) and postmitotic fibroblast (PMF) populations were determined in low-density mass cultures. In seven experimental series, the relative proportions of the different fibroblast cell types were analyzed by identifying the cells by morphological criteria (Figs. 2 and 3). At different time points (CPD level or days in stationary culture shown in parentheses), at least 4000 cells were analyzed in random microscopic fields.

(31 kDa/p_I 9.2) and protein PVb (32 kDa/p_I 9.0), that were absent in fibroblasts of the differentiation states F I, F II, F III, and F IV (Fig. 5C). In postmitotic fibroblasts of the terminal differentiation state F VI, four specific proteins did appear in region 3 that were not expressed in fibroblasts of earlier differentiation states—protein PVIa (33.5 kDa/p_I 8.0), protein PVIb (31.5 kDa/p_I 7.8), protein PVIc (30 kDa/p_I 7.3), and protein PVI d (37 kDa/p_I 7.3) (Fig. 5D). Thus, along the cell lineage F I–F II–F III–F IV–F V–F VI, the expression of specific proteins is sequentially turned on, depending upon the cytodifferentiation state of the fibroblasts studied.

As shown by degradation experiments using ammonium chloride to inhibit intracellular protein degradation (19), the relative abundance of these proteins was the same in the presence and absence of the inhibitor, indicating that they are not degradation products (data not shown).

DISCUSSION

The morphological and biochemical data provided make it evident that secondary human skin fibroblasts of the cell line HH-8 *in vitro* can spontaneously differentiate along a seven-stage terminal cell lineage F I–F II–F III–F IV–F V–F VI–F VII, when the routine cell culture techniques for fibroblasts

are modified and standardized. Shifts in the frequencies of the mitotic and postmitotic fibroblast morphotypes in mitotic and postmitotic mass populations are accompanied by alterations in the [³⁵S]methionine polypeptide patterns of the fibroblast mass populations in various stages of development. The [³⁵S]methionine polypeptide patterns of homogeneous subpopulations of F I, F II, F III, F IV, F V, and F VI isolated from heterogeneous mass populations reveal that the six fibroblast morphotypes studied express their cell-type-specific [³⁵S]methionine polypeptide pattern in heterogeneous mass populations. Thus, the [³⁵S]methionine polypeptide patterns of the heterogeneous mass populations of HH-8 are the sums of the [³⁵S]methionine polypeptide pattern of the distinct fibroblast cell types that make up the mass populations at different stages of development. Methods have been worked out to isolate routinely up to 10⁸ spontaneously arisen or experimentally induced fibroblasts of one cell type. The analysis of a multitude of cell-type-specific biological and biochemical parameters of 95% pure populations of the seven fibroblast cell types has provided additional evidence for the distinct existence of these seven fibroblast cell types (unpublished data). The morphological and biochemical cytodifferentiation of the human skin fibroblasts speaks for a seven-stage cell lineage controlled by seven genetic programs. Analogous seven-stage differentiation sequences have been found in primary and secondary prenatal and postnatal skin and lung fibroblast populations of Valo chickens, C3H mice, BN rats, and humans (3, 7, 20). Similarities in the design of the multistage differentiation sequence in the four species studied make it likely that the fibroblast differentiation sequences described represent parts of a general fibroblast stem-cell system, which in its plan seems to resemble that of the hematopoietic stem-cell system. Since the morphological and biochemical characteristics of the fibroblast stem cells *in vivo* and *in vitro* are not yet known, it remains open whether they can be propagated and induced to differentiate under the cell culture conditions used. Under the modified cell culture conditions described, connective tissues of chicken, mouse, rat, and man give rise to the mitotic committed stem cells or progenitor cells F I, F II, and F III, which proliferate and differentiate along the cell lineage F I–F II–F III in the morphologically recognizable differentiating progenitor compartment. When the mitotic capacity of F III is exhausted, fibroblast F III becomes a member of the postmitotic and maturing compartment in which the cells differentiate along the sequence F IV–F V–F VI. Fibroblast F VI, the terminally differentiated end cell of the fibroblast differentiation sequence, is the cell with the greatest number of proteins synthesized. After a long postmitotic lifespan, fibroblast F VI differentiates to fibroblast F VII, the degenerating fibroblast.

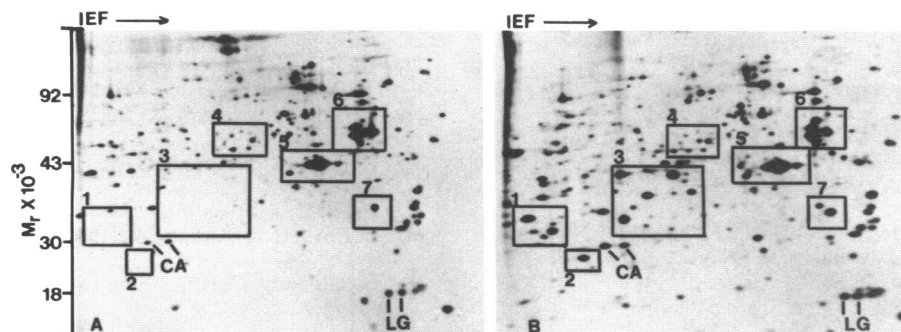


FIG. 4. Mitotic and postmitotic fibroblast mass populations were labeled with [³⁵S]methionine and prepared for two-dimensional gel electrophoresis as described. The first dimension was isoelectric focusing (IEF; pH-gradient 3–10, 7600 V/hr). The second dimension was NaDodSO₄/15% polyacrylamide gel electrophoresis (18). For standardization of fluorography, external radioactive marker proteins were added to the samples: CA, 30-kDa bovine [¹⁴C]carbonic anhydrase (3000 cpm); and LG, 18.3-kDa bovine [¹⁴C]lactoglobuline A (4000 cpm) (18). (A) Mitotic mass population (cell line HH-8), CPD 14. (B) Postmitotic mass population (cell line HH-8), 6 weeks of stationary culture. (Insets) Regions with significant quantitative and/or qualitative differences in polypeptide patterns.

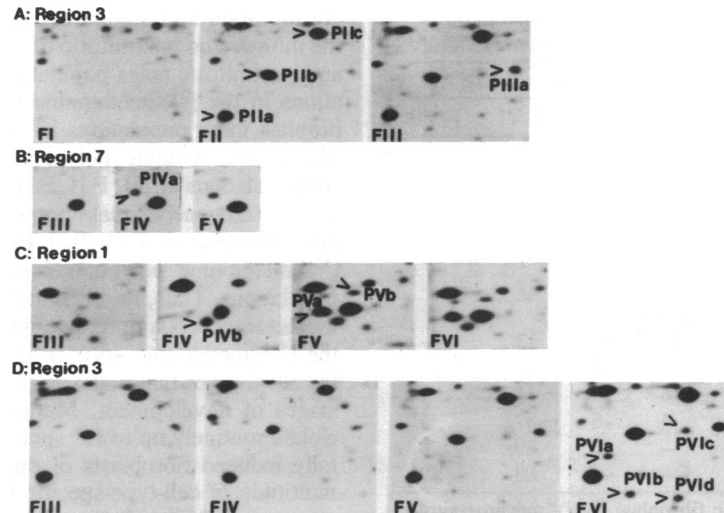


FIG. 5. Homogeneous subpopulations predominantly made up of one of the three mitotic (F I, F II, and F III) and one of the three postmitotic (F IV, F V, and F VI) fibroblast cell types were analyzed by two-dimensional gel electrophoresis (ref. 18, Fig. 4). (A) Region 3 (molecular mass range, 42–30 kDa; pI range, 7.2–8.4). (B) Region 7 (molecular mass range, 35–30 kDa; pI range, 4.9–5.2). (C) Region 1 (molecular mass range, 42–31 kDa; pI range, 9.0–9.4). (D) Region 3 (molecular mass range, 42–30 kDa; pI range, 7.2–8.4).

Human fibroblasts *in vitro* have a finite mitotic lifespan, which is followed by cellular degeneration (5). This limited lifespan has been interpreted as a manifestation of aging and death at the cellular level (5). Various theories concerning the molecular mechanisms of cellular aging and death of fibroblasts have been formulated and tested in *in vitro* fibroblast populations. These theories can be summarized as (i) differentiation theories (15, 21, 22), (ii) error theories (4, 23–26), and (iii) virus theories (27). The data presented in this paper show a morphological and biochemical seven-stage differentiation sequence of cellular aging and death of human fibroblasts *in vitro* in the three morphologically recognizable differentiating cell compartments of the fibroblast stem-cell system. As a consequence, most data from other experiments designed to test the various theories of cellular aging and death of fibroblasts in nondefined fibroblast cell systems should be reexamined and/or reevaluated in the light of the stem-cell-system nature of the fibroblast system.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ba 526/5), the Fritz Thyssen Stiftung, the Breuninger Stiftung, and the Cosmital S.A., CH-1723 Marly. H.P.R. is a recipient of a Heisenberg grant from the Deutsche Forschungsgemeinschaft (Ro 527/2-1).

- Ross, R. (1968) in *Treatise in Collagen*, ed. Gould, B. S. (Academic, New York), Vol. 2, Part A, pp. 1–82.
- Gabbiani, G. & Rungger-Brändle, E. (1981) in *Handbook of Inflammation: Tissue Repair and Degeneration*, ed. Glynn, L. E. (Elsevier/North-Holland Biomedical, Amsterdam), Vol. 3, pp. 1–50.
- Mollenhauer, J. & Bayreuther, K. (1986) *Differentiation* **32**, 165–172.
- Macieira-Coelho, A., Diatloff, C. & Malaise, E. (1976) *Nature (London)* **261**, 586–588.
- Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell Res.* **25**, 585–621.
- Todaro, G. J. & Green, H. (1963) *J. Cell Biol.* **17**, 299–313.
- Kontermann, K. & Bayreuther, K. (1979) *Gerontology* **25**, 261–274.
- Hayflick, L. (1981) in *Cell Death in Biology and Pathology*, eds. Bowen, D. & Lockshin, R. A. (Chapman & Hall, London), pp. 243–285.
- Smith, J. R. & Pereira-Smith, O. M. (1985) in *Handbook of Cell Biology and Aging*, ed. Cristofalo, V. J. (CRC, Boca Raton, FL), pp. 375–425.
- Schneider, E. L. (1985) in *Handbook of Cell Biology and Aging*, ed. Cristofalo, V. J. (CRC, Boca Raton, FL), pp. 425–433.
- Williams, J. R. & Dearfield, K. L. (1985) in *Handbook of Cell Biology and Aging*, ed. Cristofalo, V. J. (CRC, Boca Raton, FL), pp. 433–453.
- Raes, M. & Remacle, J. (1983) *Exp. Gerontol.* **18**, 223–240.
- Razin, S., Pfendt, E. A., Matsumura, T. & Hayflick, L. (1977) *Mech. Ageing Dev.* **6**, 379–384.
- Pereira-Smith, O. M. & Smith, J. R. (1982) *Somatic Cell Genet.* **6**, 731–742.
- Martin, G. M., Sprague, C. A., Norwood, T. H. & Pendergrass, W. R. (1974) *Am. J. Pathol.* **74**, 137–154.
- Bell, E., Marek, L. F., Levinstone, D. S., Merrill, C., Sher, S., Young, J. T. & Eden, M. (1978) *Science* **202**, 1158–1163.
- Rodemann, H. P. & Bayreuther, K. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5130–5134.
- Rodemann, H. P. & Bayreuther, K. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2086–2090.
- Amenta, J. S. & Brocher, S. C. (1980) *Exp. Cell Res.* **126**, 167–176.
- Bayreuther, K., Rodemann, H. P., Kontermann, K., Mollenhauer, J., Franz, P. I., Dittmann, K., Hommel, R., Müller, C. & Müller, G. (1987) *Eur. J. Cell Biol.* **43**, Suppl. 17, abstr. 7, 5.
- Cristofalo, V. J. (1972) *Adv. Gerontol. Res.* **4**, 45–79.
- Holliday, R., Huschtscha, L. J., Tarrant, G. M. & Kirkwood, T. B. L. (1977) *Science* **198**, 366–372.
- Hart, R. W. & Setlow, R. B. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2169–2173.
- Holliday, R. & Tarrant, G. M. (1972) *Nature (London)* **238**, 26–30.
- Orgel, L. E. (1963) *Proc. Natl. Acad. Sci. USA* **49**, 517–521.
- DeDuve, C. & Wattiaux, R. (1966) *Annu. Rev. Physiol.* **28**, 435–492.
- Gajdusek, D. C. (1972) *Adv. Gerontol. Res.* **4**, 201–218.