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GENE REORGANIZATION DURING SERIAL DIVISIONS OF NORMAL HUMAN CELLS

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We have followed during serial divisions of human fibroblasts the presence in chromosomal and extrachromosomal DNA, of two genes that are expressed in fibroblasts, actin and interferon, and of one that is not expressed, globin. The intensity of the blot hybridization of the actin and globin probes with chromosomal DNA diminished during serial divisions of diploid fibroblasts. The interferon gene remained constant throughout the human fibroblast life span. Chromosomal DNA sequences were present in extrachromosomal circular DNA which appeared at the end of the fibroblast life span. The results could explain some functional changes that occur in these cell populations when their division potential declines.

During serial divisions, the genome of human fibroblasts is not stable, it bears various reorganizations at different levels (1) while small molecular weight DNA accumulates (2).

Alterations of the actin cytoskeletal protein were first reported by Bowman and Daniel (3). They observed with scanning electron microscopy that old cells lacked prominent bundles of microfilaments and that this deficiency coincided with reduced motility. This was confirmed by Kelley et al. (4) who found that cell spreading is prolonged in old cells and is correlated with a retarded assembly of actin bundles. Only the alpha- and beta-actin are found in microfilaments of non-muscle cells (5). In order to study the mechanism by which the changes in actin fiber organization occur during cellular senescence, we used the actin gene as probe in blot hybridization experiments with genomic

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and extrachromosomal DNA of human embryonic lung fibroblasts during their in vitro life span. The actin gene is part of a multigene family (6). In parallel we followed the behavior of the beta-interferon gene whose product is known to remain steady in cultured fibroblasts (7), as well as of the globin gene which is not expressed in fibroblasts (8) and is part of a multigene family (9). We show here that chromosomal sequences were present in extrachromosomal circular DNA of human embryonic fibroblasts at the end of their life span.

MATERIAL AND METHODS

Cell culture. The normal human embryonic fibroblast line-(ICIG-7) has been initiated as described elsewhere (10). Cells were grown in Earle's Minimal Essential Medium supplemented with 10% fetal calf serum. They were serially propagated at a 1:2 split ratio.

DNA extractions. The cell monolayer was rinsed three times with cold phosphate-buffered saline, then scraped with a rubber policeman and pelleted by centrifigation during 10 min at 1000 rpm at 4°C. The cell pellets were incubated 2 hr at 60°C in a lysing solution (50mM Tris pH 7.5, 10 mM EDTA, 1% SDS, 100 ug/ml proteinase K). Then deproteinized twice by redistilled phenol and extracted once with chloroform. After ethanol precipitation, the first extraction material was incubated with RNase (10 ug/ml) in 50 mM Tris pH 7.5, 10 mM EDTA, overnight at room temperature, then with proteinase K (50 ug/ml) and 0.1% SDS, 1 hr at 37°C. The DNA was finally reextracted with phenol/chloroform and ethanol precipitated. After lyophilization, DNA concentrations were determined both from the absorbance at 260 nm and chemically according to Burton (11).

Extrachromosomal DNA preparations were obtained according to Hirt (12) and then treated with RNase and proteinase K as before. The partially purified low molecular weight DNA was separated from contaminating genomic DNA by isopycnic ultracentrifugation CsCl (density 1.59 g/ml) containing ethidium bromide. Fractions were collected and ethidium bromide was removed by isopropanol extraction. After dialysis, DNAs were ethanol precipitated.

Probes. The human alpha-globin cDNA probe (JW101) is inserted in plasmid pMB9, the hamster beta-actin cDNA probe (pAct-1) the mouse beta-actin cDNA probe (pAL41), the human beta-interferon genomic probe and Alu probe (BLUR-8) are all inserted in plasmic pBR322. Plasmid DNAs were extracted according to Humphreys et al. (13) and purified by CsCl density gradient centrifugation. The extrachromosomal DNA with a modal size of 5 kb was purified by electroelution according to Girvitz et al. (14). Although the actin probe we used is not of human origin, previous workers have shown that actin coding sequences are highly conserved in warmblooded vertebrates and cross-hybridize to DNA from other species (6)

Blot hybridization. Genomic DNAs (5 ug) and Hirt extracted DNAs (2 ug) were respectively digested with Eco R1 and Bam H1 restriction enzymes for 1 hr at 37°C and migrated on 0.7% horizontal agarose gels in running buffer (89 mM tris-borate, 89 mM boric acid, 2 mM EDTA). Transfer of DNA to nitrocellulose filters was performed according to Southern (15). Prehybridization (1 hr) with (^{32}P)nick-translated plasmid probes ($5x10^7$ to 10^8 cpm/ug) were performed at 42°C in 50% formamid, 5x buffer (1x: 0.15 M NaCl, 15 mM sodium citrate), 5x or 1x Denhart (1x: 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone) for prehybridization and hybridization respectively, 0.1% SDS, 100ug/ml denaturated salmon sperm DNA, and 50 mM sodium phosphate pH 6.8 buffer solution. Dextran sulfate (10%) was added in the hybridization mix. After hybridization, filters were washed twice during 15 min with 2x washing buffer (0.15 m NaCl, 10 mM sodium phosphate bufffer pH 8.5, 1 mM

EDTA), 0.1% SDS at room temperature, twice with 0.1x washing buffer, 0.1% SDS at 50°C for 30 min each and treated with 100 ug/ml proteinase K in 0.1x washing buffer for 30 min at 37°C. Filters were exposed to Fuji X-ray film for 24 hr at -80°C, using intensifying screens. Filters were dehybridized by soaking them in 0.01x buffer (1x: 0.15 M NaCl, 15 mM sodium citrate) during 2 hr at 80°C. Then the dry filters were exposed to X-ray film for several days to check that all the specific hybridization had disappeared. The filters were then rehybridized to another probe.

For evaluation of the size of extrachromosomal circular DNA, plasmids with known sizes were migrated on agarose gels, transfered to nitrocellulose filters and hybridized to the same (^{32}P)labelled plasmids.

Electron microscopy. Spreads were performed according to a modified Kleinschmidt technique (16).

RESULTS

The hybridization pattern of the (^{32}P)labelled alpha-globin, beta-actin and beta-interferon probes with the genomic DNA of human embryonic lung fibroblasts as a function of population doubling level is shown in fig. 1. We chose to hydrolyse the human genomic DNA with the Eco R1 restriction endonuclease since this enzyme does not cut the coding sequences of the alpha-globin genes (17). The intensity of the band at 22.5 kb, corresponding to the two alpha-globin genes (18,19) (fig. 1), decreased at high population doubling level. Identical findings were obtained 27 times with this human embryonic line and with 10 different human fibroblast lines. The Alu highly repeated

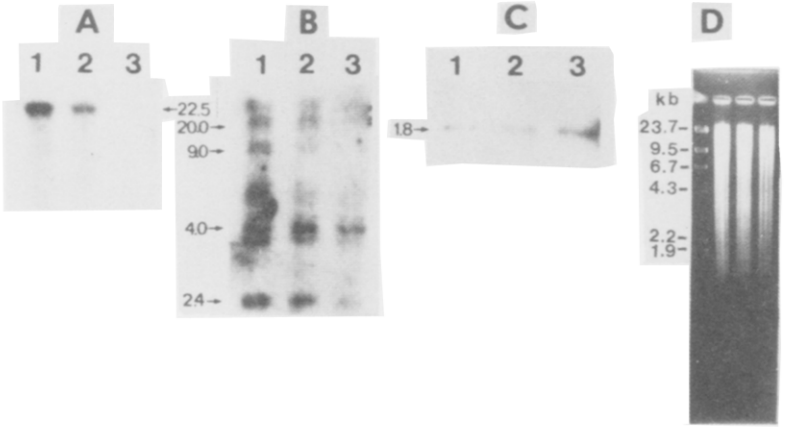


Fig. 1. Hybridization of (^{32}P)labelled alpha-globin (A) and beta-actin (B) probes with Eco R1-digested DNA isolated from human embryonic fibroblasts at doubling 33 (1), doubling 45 (2) and doubling 56 (3). The filters used in these experiments were dehybridized and hybridized again with the beta-interferon probe (C). The numbers to the right and left of the blots indicate length in kilobases (kb) calculated from Hind III digests of lambda-DNA. (D) Ethidium bromide fluorescence of DNA in the gel used for the blot illustrated in (B).

sequence probe BLUR-8 (20) hybridized as a smear with the Eco R1 digested genomic DNA. Control hybridization with the plasmid pBR322 (vector of the beta-actin, beta-interferon and Alu DNA) showed no homology with the DNA of human embryonic fibroblasts. Eco R1 restriction enzyme digests of fibroblast' DNA were also hybridized to a cloned actin cDNA probe from mouse beta-actin (fig. 1B and 1D). The autoradiogram showed multiple bands whose intensity diminished with the DNAs extracted from cells at the 56th doubling as compared to the 33rd, except for a band at 4.0 kb whose intensity remained constant even at the 56th doubling. The ethidium bromide fluorescence of DNA in the gel has the same intensity on the three lanes (fig. 1D) showing that the decline in the hybridization signal is not due to variations in the amount of DNA loaded on the gel. Filters used with these probes were dehybridized as described under Methods and rehybridized with the interferon probe. By contrast the human beta-interferon DNA of 1.8 kb hybridized with identical intensity to the DNA of fetal fibroblasts at population doublings 33, 45 and 56 (fig. 1C). The reverse experiment was also done, i.e., filters formerly hybridized with the beta-interferon probe were dehybridized and hybrized again with the beta-globin probe. A decreased intensity of the hybridization signal with the latter probe could be seen at high passage level.

Undigested DNA from cells at all passages tested migrated to the same extent in the agarose gel, indicating that none of the DNA preparations was degraded prior to the blot analysis.

We examined the DNA from the human' embryonic fibroblasts found in the supernatant fraction obtained after extraction according to Hirt (12). In this study cells were maintained without any use of antibiotics which may lead to amplification of circular extrachromosomal DNA (21). The supernatants obtained from cells at doubling 16 and 59 were centrifuged to equilibrium in CsCl gradients containing ethidium bromide and were collected in four different fractions (1 to 4 from the bottom to the top). DNA from these fractions, undigested or cleaved with Bam H1 restriction enzyme, was hybridized to the alpha-globin and beta-actin probes (fig. 2). In order to identify mitochondrial DNA

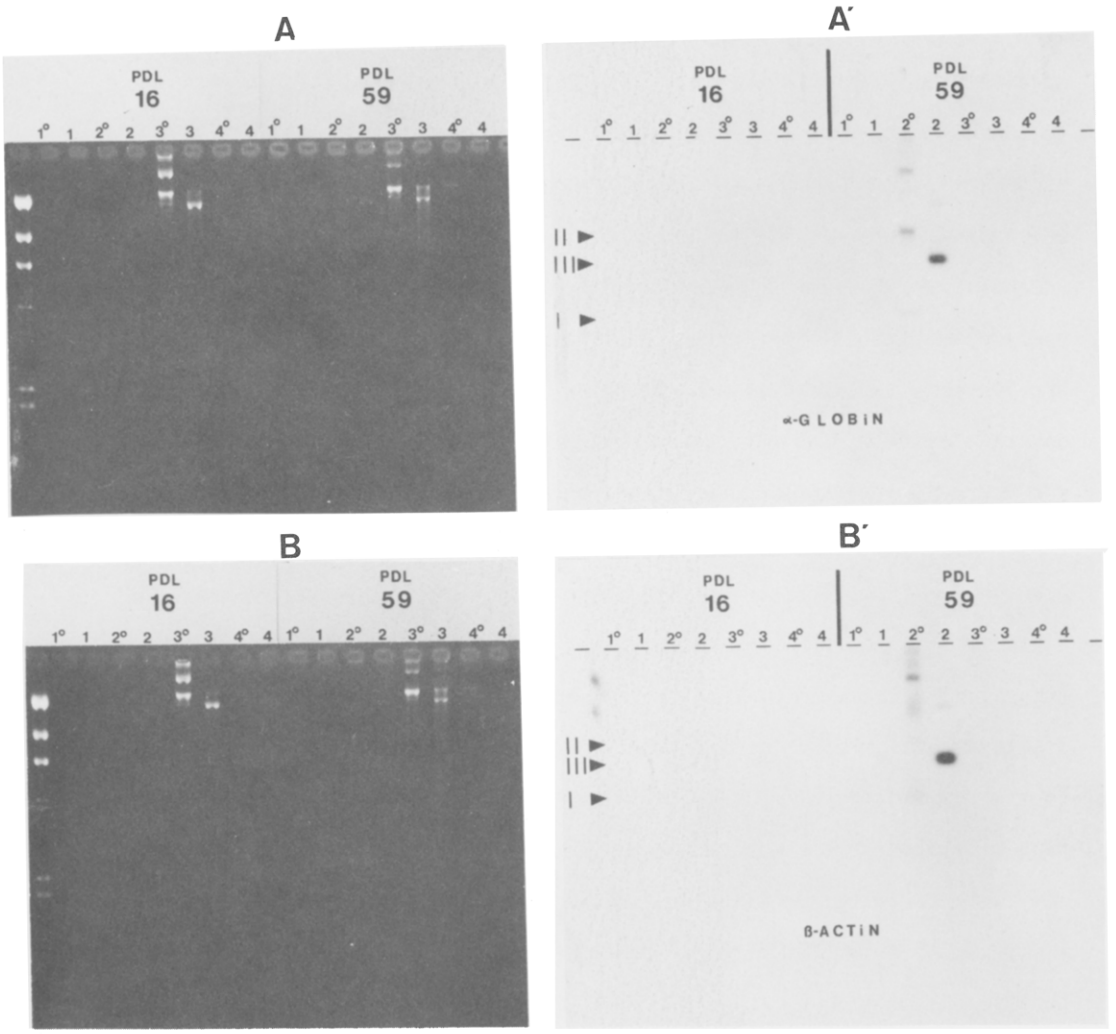


Fig. 2. Identification of alpha-globin and beta-actin probes in extrachromosomal sequences.
A and B: UV fluorogram of DNA banded in CsCl isopycnic gradient.
A' and B': DNA blot hybridization with alpha-globin and beta-actin (^{32}P)-labelled probes to fractions from the CsCl gradient. Undigested DNA (1^0-4^0) and Bam H1 digested DNA (1-4) from cells at the 16th and 59th doubling. I: covalently closed circular DNA; II: nicked circular DNA; III: linear DNA.

from other circular DNA, we chose to hydrolyse the extrachromosomal DNA with Bam H1 enzyme which cleaves mitochrondrial DNA in a single 17 kb fragment which is not the case with the Eco R1 restriction enzyme (22). For DNA isolated from cells at the 16th doubling, the alpha-globin and beta-actin probes hybridized as a very faint band at 5 kb (fraction 2) (fig. 2A' and B'). In old cells (doubling 59) the autoradiogram of the undigested DNA sample from frac-

tion 2^{o} (density 1.62) showed several bands of hybridization with the alpha-globin and beta-actin probes (fig. 2A' and B'). The fastest running band (I) corresponds to covalently closed circular extrachromosomal DNA, the second band (II) contains nicked circular molecules and the other bands, dimers and multimers of the form I. The Bam H1 digested material ran as 5 and 14 kb (doubling 59, fraction 2) (fig. 2A' and 2B'). The interferon probe had no homology with the DNA extracted according to Hirt. In both young (doubling 16) and old (doubling 59) DNA was visible by UV fluorescence after ethidium bromide staining in fraction 3 (density 1.59) (fig. 2A and B). It probably corresponded to mitochondrial DNA since it could be cleaved by Bam H1 in a single fragment of 17 kb (22). This DNA did not hybridize to the globin and actin cDNA probes (fig. 2). The Alu human highly repeated sequence probe BLUR-8, hybridized to the circular DNA present in fractions 2^{o}, 2, 3^{o} and 3 (fig. 3). Identical results were obtained with young cells at the 16th doubling and old cells at the 54th and 60th doublings from another serial culture. Control hybridization with the plasmids pMB9 (vector of alpha-globin) and pBR322 (vector of the beta-actin, beta-interferon and Alu DNA) showed no homology with the cellular DNA extracted according to Hirt.

To see if circular DNA molecules could hybridize to specific bands on a genomic DNA blot, the extrachromosomal DNA from cells at the 59th doubling

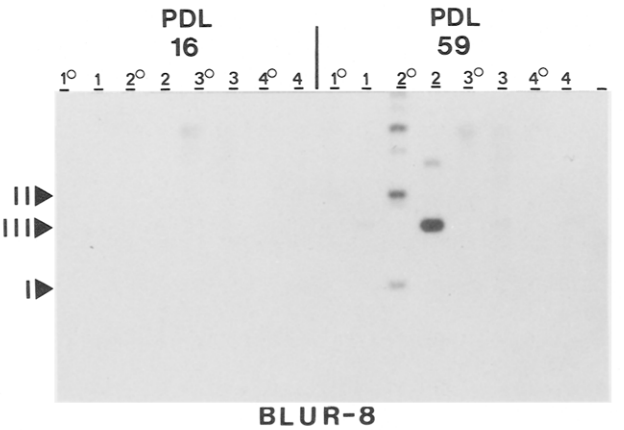
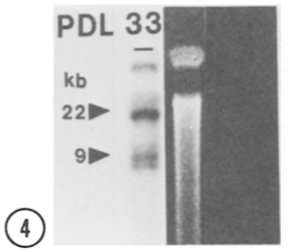


Fig. 3. Identification of BLUR-8 probe in extrachromosomal sequences. See legend to fig. 2.



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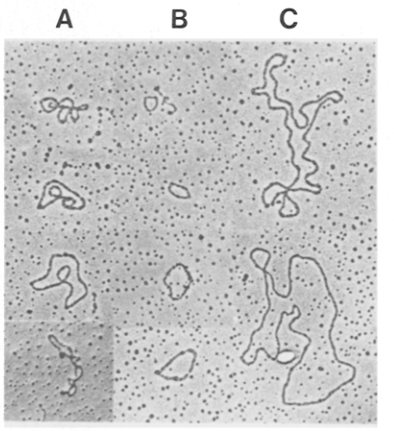

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Fig. 4. Hybridization of (^{32}P)labelled extrachromosomal DNA probe with Eco R1-digested chromosomal DNA isolated from human embryonic fibroblasts (UV fluorogram shown on the right). PDL: population doubling level. Numbers to the left of the figure indicate length in kilobases (kb) calculated from Hind III digests of lambda-DNA.

Fig. 5. Electron micrograph of circular DNA from human embryonic fibroblasts at doubling 59. DNA preparations of 4.91+1.1 kb (A), 1.5+0.4 kb (B) and 17.2+2.4 kb (C) were obtained according to Hirt (12). Length measurements were made relative to the plasmid pBR322 (4361 bp). Magnification x51000.

(fraction 2) was purified by migration on a 0.7% agarose gel and electroelution. This yielded a pure population of circular molecules with a modal size of 5 kb, free of 17 kb mitochondrial molecules. This small size DNA, used as a probe was hybridized to Eco R1 digested genomic DNA from human embryonic fibroblasts (fig. 4). Three main bands could be detected on the autoradiogram at 22, 9 and 8.5 kb. Identical results were obtained using genomic DNA from earlier passage cultures.

DNAs from fractions 2 and 3 were prepared for electron microscopy according to Davis et al. (16). Covalently closed circular molecules could be seen in undigested DNA of fraction 2 from cells at doubling 59 with sizes around 5 kb (figs. 5A and 6C) and between 8 and 14 kb (fig. 6C). In the third fraction of the CsCl gradient, we found molecules of 17 kb in DNA preparations from cells at doubling 16 as well as doubling 59 (figs. 5C, 6B and 6D). Most of the mitochondrial DNA molecules seemed to be nicked into form II probably due to the formamid present in the spreading buffer (50%). Few additional circular molecules ranging between 0.8 and 2.7 kb could be seen at the same density

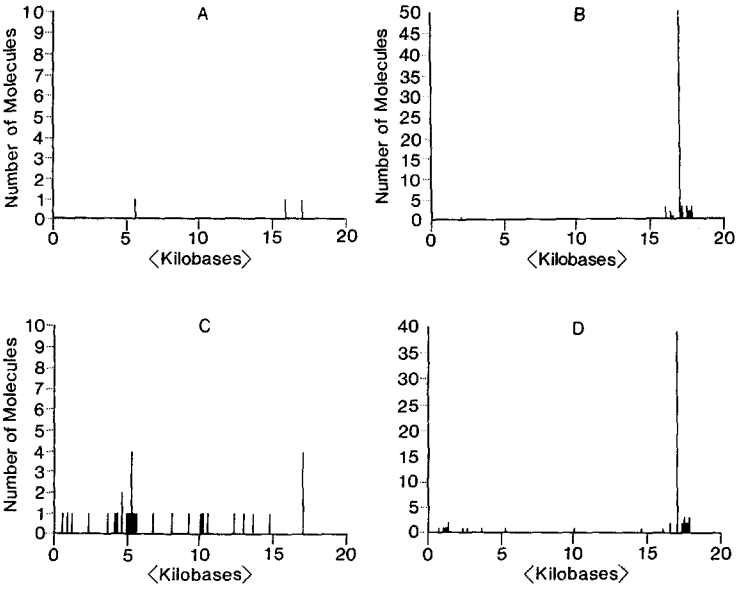

Fig. 6. Computer print out of the size distribution of extrachromosomal circular DNA present in young and old cells.
A: circular DNA from cells at doubling 16 (density 1.62) present in fraction 2 of the CsCl gradient.
B: circular DNA from cells at doubling 16 (density 1.59) present in fraction 3.
C: circular DNA from cells at doubling 59 (density 1.62).
D. circular DNA from cells at doubling 59 (density 1.59).
Data from the same experiment illustrated in fig. 5.

(1.59) in preparation from cells at doubling 59 (figs. 5B and 6D). Small linear fragments of DNA, heterogeneous in size were also abundant in the fractions examined (data not shown).

In order to confirm these results, undigested DNA (50 ug) from cells at the 22nd and 66th doublings were laid on to a 10-40% sucrose gradient buffered with 0.2 M NaCl, 20 mM Tris pH 8.0 and 1 mM EDTA, and centrifuged at 30,000 rpm for 16 hr. Thirty fractions of 0.36 ml each were collected and 70 ul of each fraction were electrophoresed in a 0.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the alpha-globin probe as described above. Hybridization of the alpha-globin probe occurred with fractions from the lower 2/3 of the gradient obtained with DNA from cells of both passages, although this hybridization was weaker with DNA from old cells. Hybridization of the probe with the fractions from the upper 1/3 of the gradient, i.e. small molecular weight DNA, occurred only with DNA from cells at doubling 66.

DISCUSSION

Human fibroblasts after serial divisions in vitro stop proliferating and go through profound morphologic and metabolic changes (23). The results described above suggest that these changes are accompanied by modifications in the structure of some genes. Indeed the hybridization signal obtained with the blobin and actin probes on Eco R1 digested chromosomal DNA was less pronounced in late passage cells. With the actin probe, one band at 4.0 kb had the same intensity in early and late passage cells. This shows that the decreased hybridization was not due to variations in DNA load or hybridizability. The hybridization signal with the beta-interferon gene probe, hybridized with the same intensity on genomic blots of DNA from young and old cells.

The decrease in the hybridization signal could be due to loss of sequences. There is evidence that small molecular weight DNA accumulates during the last stage of these cell populations life span (2) and that most of this DNA is present in a circular form (24, 25). We have found that cDNA from alpha-globin and beta-actin hybridized with 5 and 14 kb circular extrachromosomal DNA from old cells, but did not hybridize with a population of larger circles (17.1 kb) which corresponds probably to mitochondrial DNA and is present in both early and late passage cells. The interferon probe did not hybridize with the circular extrachromosomal DNA. The small circular molecules were purified (mitochondrial-DNA-free) and back hybridized with genomic DNA forming bands at 22, 9 and 8.5 kb (fig. 4). This also suggests that sequences corresponding to the alpha-globin and beta-actin genes are present in the small circular DNA populations and that the latter are heterogeneous. Hence the decrease in the hybridization signals could be due to loss into an extrachromosomal form. Control hybridizations with the vectors alone did not reveal any bands. The fact that the small circular DNA hybridized with an Alu repeat also shows that other chromosomal DNA sequences are present in the circular DNA. Hence it is possible that some of the hybridization between the probes and the extrachromosomal DNA is due to a repetitive sequence common to all probes. In this respect it is pertinent to remind that the alpha-globin gene is included in a 4 kb-long

DNA unit terminated by two Alu family repeats (26) and that some actin genes are also surrounded by repeated sequences included in a 5 kb-long DNA unit (27). This could explain the similarity of hybridization patterns for the probes shown in figs. 2 and 3. Since the back hybridization of the 5 kb purified circles with the genomic DNA did not reveal all the bands corresponding to the beta-actin gene, we assume that some of those genes might be carried on the 14 kb circular DNA which hybridized to the actin probe (fig. 2). We did not attempt to purify those circles to use them as probe on genomic DNA because of the heavy contamination by the mitochondrial DNA.

The presence of highly repeated sequences near the two alpha-globin genes (18, 26) as well as in the vicinity of the actin genes (27) and in circular molecules (28) detected in aging cells (fig. 3) suggests a mechanism by which these circles could be recovered in an extrachromosomal form. This repeated sequence may work as a transposable element. This phenomenon could be similar to that reported by Flavell and Ish-Horowicz (29) who found extrachromosomal circular copies of the transposable element, copia, in Drosophila cells. However, we do not have any evidence that the circular DNAs found in human fibroblasts have the retroviral-like structure of the copia element. The fact that there is no alteration in one of the bands corresponding to the actin gene and in the interferon gene suggests that some parts of the genome are better conserved than others. It is not known if the interferon gene is surrounded by potentially mobile sequences. Cytogenetic studies have shown that predominant fragile sites exist in the human genome (30, 31) and it is possible that the interferon gene is not within these sites. In this respect it is pertinent to remind that the beta-interferon gene product remains unchanged up to the end of the life span of cultured human fibroblasts (7).

Reorganization of the globin genes is probably without implication for these cells physiology since they are not expressed in fibroblasts (8). Alterations of the actin cytoskeletal proteins, however, have been reported (3, 4, 32). Changes in the cell contractile activity, which result from the disorganization of actin filaments have been described in the terminal phase of the

fibroblast life span and may be related with a decreased probability of initiating DNA synthesis (33). These untoward functional changes may be related with the gene reorganization reported herein.

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REFERENCES

1. Macieira-Coelho, A. (1984) Mech. Ag. Dev. 27, 257-262.
2. Puvion-Dutilleul, F., Puvion, E., Icard-Liepkalns, C. and Macieira-Coelho, A. (1984) Exp. Cell Res. 151, 283-298.
3. Bowman, P.D. and Daniel, C.W. (1975) Mech. Ag. Dev. 4, 147-158.
4. Kelley, R.O., Trotter, J.A., Marek, L.F., Perdue, B.D. and Taylor, C.B. (1980) Mech. Ag. Dev. 13, 127-141.
5. Vandekerckhove, J. and Weber, K. (1979) Differentiation 14, 123-133.
6. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J. and Kirschner, M.-W. (1980) Cell 20, 95-105.
7. Komatsu, H., Machara, N., Shimoda, K., Mori, T., Lee, T., Makino, S. and Matsumoto, M. (1981) Arch. Virol. 70, 367-371.
8. Kator, K., Cristofalo, V., Charpentier, R. and Cutler, R.G. (1985) Gerontology 31, 355-361.
9. Efstratiadis, A., Posakony, J.M., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., Deriel, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C. and Proudfoot, N.J. (1980) Cell 21, 653-668.
10. Macieira-Coelho, A. and Azzarone, B. (1982) Exp. Cell Res. 141, 325-332.
11. Burton, R. (1956) Biochem. J. 62, 315-323.
12. Hirt, B. (1967) J. Mol. Biol. 26, 365-369.
13. Humphreys, G.O., Williams, G.A. and Anderson, E.S. (1975) Bioch. Bioph. Acta 383, 457-463.
14. Girvitz, S.C., Bacchetti, S., Rainbow, A.J. and Graham, F.L. (1980) Anal. Biochem. 106, 492-496.
15. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
16. Davis, R.W., Simon, M.N. and Davidson, N. (1971) Methods in Enzymology, vol. XXI, ed. L. Grossman and K. Moldave, p. 413-428, Academic Press, New York.
17. Orkin, S.H. (1978) J. Biol. Chem. 253, 12-15.
18. Lauer, S.H., Shen. C.K.J. and Maniatis, T. (1980) Cell 20, 119-130.
19. Lie-Ingo, L.E., Dozy, A.M., Kan, Y.W., Lopes, M. and Todd, M. (1979) Blood 54, 1407-1416.
20. Rubin, C.M., Houch, C.M., Deininger, P.M., Friedman, T. and Schmidt, C.W. (1980) Nature 284, 373-374.
21. Smith, C.A. and Vinograd, J.J. (1972) J. Mol. Biol. 69, 163-178.
22. Ojala, D. and Attardi, G. (1977) Plasmid 1, 78-105.
23. Macieira-Coelho, A. and Taboury, F. (1982) Cell Tissue Kinet. 15, 213-224.

24. Kunisada, T., Yamagishi, H., Ogita, Z.I., Krakawa, T. and Mitsui, Y. (1985) Mech. Ag. Dev. 29, 89-99.
25. Calabretta, B., Robberson, D.L., Barrera-Saldana, H.A., Lambrou, T.P. and Saunders, G.F. (1982) Nature 296, 219-225.
26. Hess, J.F., Fox, M., Schmid, C. and Shen, C.K.J. (1983) Proc. Nat. Acad. Sc. USA 80, 5970-5974.
27. Soriano, P., Szabo, P. and Bernardi, G. (1982) EMBO J. 1, 579-583.
28. Krolewski, J.J., Shindler, C.W. and Rush, M.G. (1984) J. Mol. Biol. 174, 41-54.
29. Flavell, A.J. and Ish-Horowicz, D. (1981) Nature 289, 591-595.
30. Aula, P. and vonKoskull, H. (1976) Hum. Genet. 32, 143-148.
31. Sutherland, G.R. (1977) Science 197, 265-266.
32. Wang, E. and Gundersen, D. (1984) Exp. Cell Res. 154, 191-202.
33. Macieira-Coelho, A. (1983) Int. Rev. Cyt. 83, 183-220.