Changes in Chromatin Structure during the Aging of Cell Cultures As Revealed by Differential Scanning Calorimetry†

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ABSTRACT: Nuclei from cultured human cells were examined by differential scanning calorimetry. Their melting profiles revealed four structural transitions at 60, 76, 88, and 105 °C (transitions I–IV, respectively). In immortalized (i.e., tumor) cell cultures and in normal cell cultures of low passage number, melting profiles were dominated by the 105 °C transition (transition IV), but in vitro aging of normal and Werner syndrome cells was associated with a marked decrease in transition IV followed by an increase in transition III at the expense of transition IV. At intermediate times in the aging process, much DNA melted at a temperature range (95–102 °C) intermediate between transitions III and IV, and this is consistent with the notion that aging of cell cultures is accompanied by an increase in single-strand character of the DNA. Calorimetric changes were observed in the melting profile of nuclei from UV-irradiated tumor cells that resembled the age-induced intermediate melting of chromatin. It is suggested that aging is accompanied by an increase in single-stranded character of the DNA in chromatin, which lowers its melting temperature, followed by strand breaks in the DNA that destroy its supercoiling potential.

Differential scanning calorimetry of nuclei in physiological salt conditions reveals four structural transitions as the nuclei are heated (Touchette & Cole, 1985). The first, at 55–60 °C (transition I), is complex and essentially represents nonchromosomal components. The next two, at 76 and 88 °C (transitions II and III, respectively), are found not only with nuclei but also with chromatin solubilized by brief nuclease treatment; the 76 °C transition is due to the collapse of the nucleosome, and the 88 °C transition represents the unstacking of bases in the (relaxed) DNA released by the nucleosomal collapse (Almagor & Cole, 1989). The fourth transition, at 105 °C (transition IV), is absent from isolated chromatin but is the dominant transition in nuclei from dividing cells. Transition IV (105 °C) represents the unstacking of bases in topologically constrained DNA since a brief nuclease exposure of nuclei from rapidly dividing cells results in the replacement of transition IV with transition III (88 °C) in equal magnitude (Touchette & Cole, 1985). Since chromatin is thought to be bound to a matrix as loops of nucleosomal strands (Cook & Brazell, 1975) and since the DNA in closed-circle minichromosome forms supercoils when released from nucleosomal constraints (Bradbury et al., 1981), transition IV (105 °C) is most likely the unstacking of bases in supercoiled DNA released by the nucleosomal collapse at 76 °C. Vinograd et al. (1968) reported that supercoiled polyoma DNA melted at 104–107 °C, and it is known that the melting temperature of DNA becomes insensitive to the presence of histones as salt concentrations approach physiological levels [Subirana, 1973; see also Jensen and Von Hippel (1976)].

The major concern of the present series of experiments is the correlation of changes in transition IV with changes in the physiological state of cells. We have already learned that nuclei from differentiated cell cultures (Touchette & Cole, 1985; Almagor & Cole, 1987) and from mature, differentiated tissues (Touchette et al., 1986), and also from nutritionally deprived cell cultures that have lost mitotic potential (Almagor & Cole, 1987), give calorimetric scans that are dominated by transition III (88 °C) rather than by transition IV (105 °C), as was the case for nuclei from rapidly dividing cells. A high ratio of IV to III is not simply a characteristic of DNA replication (Rice et al., 1988) but seems to represent the potential of cells for division (Almagor & Cole, 1987). This raised the possibility that the aging of normal cells in culture (i.e. loss of replication capacity after approximately 50 passages) might be explained by changes in their chromatin structure which could be demonstrated calorimetrically by a loss of transition IV. This possibility was tested, as reported below, with the differential scanning calorimetry of nuclei of cell cultures at different stages of aging. Age (i.e., passage number)-dependent changes in transition IV for cultures of normal human fibroblasts were compared to those for Werner's syndrome fibroblasts which show accelerated aging and also compared to cultured human tumor cells that are immortalized.

Experimental Procedures

Cell Culture. Normal human skin fibroblasts (CRL 1489, CRL 1493) and human tumor cells derived from a tumor of the nasal septum (CCL 30) were obtained from the American Type Culture Collection, Rockville, MD. Skin fibroblasts from a patient with Werner's syndrome (AG 5229) were obtained from the NIA Aging Cell Repository, Camden, NJ. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 units/mL penicillin G, and 100 units/mL streptomycin. Werner syndrome cells were cultured in the presence of 20% fetal bovine serum. All other cells were cultured in 10% fetal bovine serum. The cells were grown in 150 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) at 37 °C under a humidified 5% CO_2 atmosphere.

The cells were received at passage 3 and were subcultured to passages 5 (normal cells) and 9 (Werner syndrome cells) to obtain the quantities of nuclei needed for calorimetric experiments.

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Table I: Calorimetric Measurements of Nuclei from Cultured Human Cells[a]

cell type	enthalpy change (cal/g of DNA) for transition I	II	III	IV
normal skin fibroblasts (1489, passage 5)	2.4	0.6	0.7	2.9
normal skin fibroblasts (1493, passage 11)	2.1	0.6	0.6	3.2
Werner syndrome fibroblasts (5229, passage 13)	1.6	1.1	1.0	2.2
tumor cells (2650)	1.8	0.8	0.6	7.5

[a] Nuclei were isolated and scanned as described under Experimental Procedures. The results represent the mean of at least three experiments.

Measurement of Replication Rate. The cells were passaged with 4 mL of trypsin/EDTA (Gibco). Cellular replication rate was determined as the viable cell count upon subcultivation per time interval between two successive passages.

Isolation of Nuclei. The culture medium was removed, and the layer of cells was washed 3 times in swelling buffer (0.1 M hexylene glycol, 1 mM $CaCl_2$, and 0.06 M Pipes, pH 6.8). The cells were scraped off by using a rubber policeman and were resuspended in swelling buffer for 10 min. Nuclei were obtained after disruption of the cells with 25 strokes in a loose-fitting Dounce homogenizer and centrifugation for 3 min at 1000*g*. The pellet of nuclei was washed 3 times in calorimetry buffer (buffer C: 5 mM Tris, 0.2 mM EDTA, 250 mM sucrose, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.5). All steps were done at 0–4 °C.

Differential Scanning Calorimetry. Samples of nuclei were pelleted by centrifugation for 3 min in an Eppendorf microcentrifuge. The pellets (5–15 mg moist weight) were transferred to aluminum pans which had been previously siliconized to prevent the accumulation of moisture at the sealing surfaces, and the pans were hermetically sealed.

All the calorimetric measurements were done on a DuPont 990 thermal analyzer with a temperature gradient from 25 to 120 °C at a scanning rate of 5 °C/min. Preliminary studies showed that the calorimetric profiles were not very sensitive to the scanning rate. Lowering the scanning rate from 5 to 2 °C/min lowered melting temperatures only about 2–3 °C. At 0.2 M Na^+, the melting temperature of DNA is similarly insensitive to the rate of heating (Kozyakin & Lybchenko, 1989). Data were recorded automatically as a function of the heat absorbed in millicalories per second per 2.54 cm versus temperature. Total enthalpy was calculated by cutting out peaks from tracings of the scans and weighing them. After scanning, sample pans were opened, and the contents were dissolved in 0.1 M NaOH. The amount of DNA in each sample was determined by the absorbance at 260 nm, assuming an extinction coefficient $E^{1\%} = 280$.

UV Irradiation. For irradiation experiments, tumor cells were cultured in petri dishes. Immediately prior to irradiation, the medium was replaced with 10 mL of phosphate-buffered saline, pH 7.4, preheated to 37 °C. The uncovered dishes were placed in a hood (Bellco Glass, Inc., Vineland, NJ) and were irradiated with a germicidal lamp (G30T8, 30 W, General Electric) at a dose rate of 0.02 J m^{-2} s^{-1}.

Results

The nuclei from several types of cultured human cells were examined by differential scanning calorimetry. Typically, as shown in Figure 1 and Table I, the heating of the nuclei revealed four structural transitions with maximal heat uptake at 60 °C (transition I), 76 °C (transition II), 88 °C (transition III), and 105 °C (transition IV); the melting profiles were normally dominated by transition IV. This was similar to the

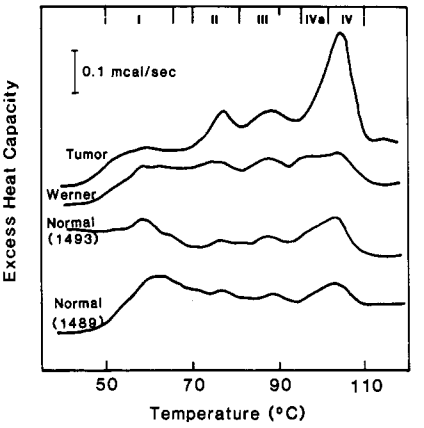

FIGURE 1: Differential scanning calorimetry of nuclei from cultured human cells. Heat absorption curves as a function of temperature are shown for nuclei isolated in buffer C. DNA amounts in the samples were the following: normal 1489 fibroblasts, 0.11 mg from passage 5; normal 1493 fibroblasts, 0.22 mg from passage 11; Werner syndrome cells, 0.15 mg from passage 13; tumor cells, 0.37 mg. The bar indicates 0.1 mcal/s absorbed at a scanning rate of 5 °C/min.

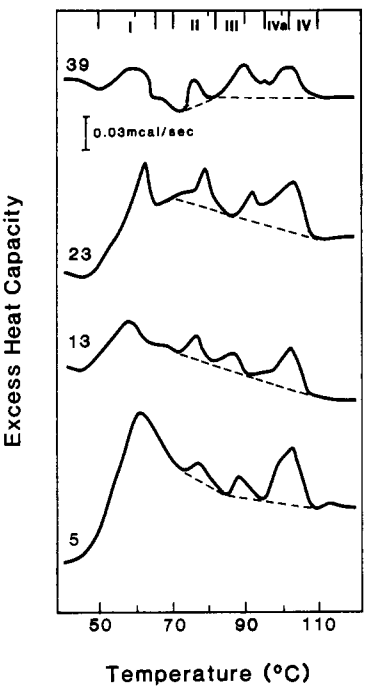

FIGURE 2: Differential scanning calorimetry of nuclei obtained from aging cultured human fibroblasts (1489). Curves of heat absorption as a function of temperature are shown for nuclei isolated in buffer C. The amount of DNA in each sample was 0.11 mg from passage 5, 0.16 mg from passage 13, 0.14 mg from passage 23, and 0.12 mg from passage 39.

scanning calorimetry of nuclei previously reported for other cultured cell lines (Touchette & Cole, 1985; Touchette et al., 1986; Almagor & Cole, 1987). Some of the profiles shown in Figure 1, however, differed from previous observations made with rapidly dividing cells in that the former revealed substantial heat absorption in a temperature range intermediate between that of transitions III and IV. This was especially true for nuclei from Werner syndrome fibroblasts.

Since Werner's syndrome is characterized by accelerated aging, it was of interest to compare, calorimetrically, nuclei from normal and Werner syndrome fibroblasts as a function of passage number, that is, as the cell cultures "aged". The differential scanning calorimetry of nuclei from cultured normal human fibroblasts (1489) after various numbers of passages revealed a progressive loss of transition IV with in-

Table II: Calorimetric Measurements of Nuclei from Cultured Human Tumor Cells after Exposure to UV Irradiation

	enthalpy change (cal/g of DNA) for transition				
treatment	I	II	III (82–95 °C)	IVa (95–102 °C)	IV (102–110 °C)
control	2.0	0.9	1.4	1.9	4.7
UV, 15 min	2.3	1.1	1.9	3.0	2.6
UV, 60 min	2.1	1.0	3.4	3.0	1.6
UV, 120 min	1.7	1.1	4.4	2.4	1.4

creasing amounts of chromatin melting between transitions III and IV (Figure 2). Earlier, we demonstrated in nuclei from rapidly dividing cells that even slight cleavage of DNA by micrococcal nuclease or DNase I resulted in a total loss of transition IV and its replacement by transition III—no intermediates were observed (Touchette et al., 1986). It was also observed (Almagor & Cole, 1987) that the melting temperature for transition IV appeared to decrease when cultured cells lost their potential for mitosis. Therefore, we expected that a progressive destabilization of the form of chromatin that gives rise to transition IV would correlate with aging as well as with the loss of mitotic potential. Figure 2 demonstrates that correlation, showing a dramatic shift in transition IV. This correlation might also be demonstrated by comparison between the aging of normal fibroblasts (e.g., 1489) and that of Werner syndrome fibroblasts. The in vitro aging of the former inhibits their culture after about 40–50 passages while the rapid aging of the latter stops their culture after about 25–30 passages. It may be observed, then, that the profile of scanning calorimetry of nuclei from Werner's syndrome fibroblasts in the 13th passage (Figure 1) showed a proportion of heat absorbed in the region intermediate between transitions III and IV at least comparable to that for normal fibroblasts at the 23rd passage. This observation agrees with the expectation that the progressive destabilization of the high-melting form of chromatin is correlated with aging in cell cultures.

The observed structural changes in chromatin during the aging of cells indicated an accumulation of damage to the DNA, but not one that abruptly destroyed the presumed supercoiling potential, as did nucleases. Therefore, we considered ways in which DNA might be damaged by base modification that would interfere with base pairing and so affect the unstacking of bases as the temperature is raised. In view of previous evidence linking DNA damage and UV irradiation (Lehmann et al., 1977), the calorimetric profiles of nuclei from human tumor cells were studied after various times of exposure of the cells to UV light. These doses and times of exposure would favor the production of pyrimidine dimers while permitting only limited DNA repair. The results of these experiments are presented in Figure 3 and Table II. The change in calorimetric scans as a function of UV exposure is strikingly like that seen for aging cell cultures.

Quantification of the scanning data is problematic because of uncertainties in the base line. The first problem to be mentioned is modest and concerns transition I. Previously, we showed that this transition represents nucleoplasmic rather than chromosomal components and if cytoplasmic contaminants (especially RNA) are present they contribute to transition I. The large magnitude of transition I in some of the calorimetric scans of aged cell cultures reflects the fact that it was difficult to clean nuclei when very limited quantities of cells were available. Usually, however, the calorimetric scans seemed to establish a reasonable base line before transition II was observed.

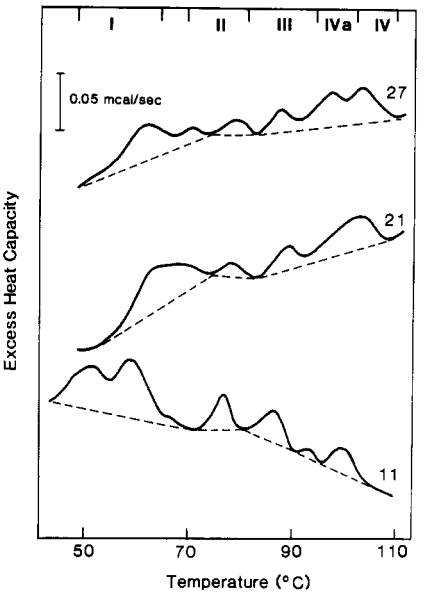

FIGURE 3: Differential scanning calorimetry of nuclei obtained from Werner syndrome cells aging in culture. Curves of heat absorption as a function of temperature are shown for nuclei isolated in buffer C. The amount of DNA in each sample was as follows: lower curve, 0.18 mg from passage 11; middle curve, 0.18 mg from passage 21; upper curve, 0.22 mg from passage 27.

A more serious base-line problem relates to the nature of the instrument used in these experiments. The thermal analyzer depends on a null point balance between the sample pan and a control pan (which contained buffer but no nuclei). In common with other instruments that depend on a null balance, the base lines are extremely sensitive and may drift during the experiment. Where peaks in the final profile are well separated, it is reasonable to suppose that the minima on either side of the peak define the base line. In our previous work on normal and rapidly dividing cells, we defined base lines in this way, and the internal consistency of the results from many scores of experiments supported this approach as a good approximation. Enthalpy changes estimated in this way proved to be proportional to the mass of DNA submitted to calorimetry. In the present experiments, however, the structural transitions observed in the nuclei of aging cell cultures and of UV-irradiated cells were not well separated from each other, in that substantial amounts of heat were absorbed between the originally defined peaks III and IV. Clearly, base lines drawn between the minima on either side of a peak become invalid when one or both of the minima are distant from the true base line. Consequently, we are forced to draw arbitrary, but hopefully reasonable, base lines, as shown in Figures 2–4. The selected base lines were checked against two assumptions: (1) transition II, which we have shown elsewhere to represent the collapse of the nucleosome, ought to have nearly the same magnitude for all samples; (2) the summation of excess heat absorbed between 82 and 110 °C represents the unstacking of bases in the DNA released from the nucleosomes by their collapse and ought to be approximately constant in magnitude in comparing samples. The base lines shown in Figures 2–4 seem to meet these criteria.

Quantification of the calorimetric scans from aging normal human fibroblast (1489) cultures and Werner's syndrome fibroblast cultures is given in Figures 5 and 6, respectively. Evidently, aging of the cultures results in a decrease of material melting in the temperature zone 102–110 °C accompanied by an increase in material melting in the zone 95–102 °C and subsequently a decrease of material melting in the zone 95–102

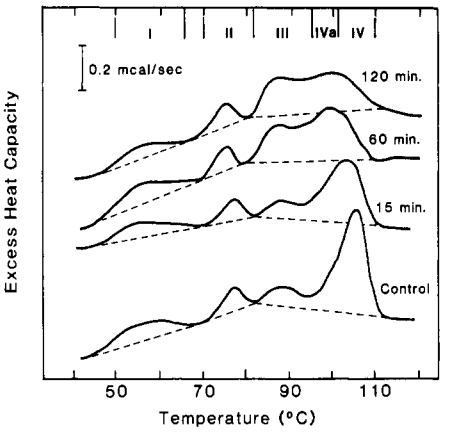

FIGURE 4: Differential scanning calorimetry of nuclei obtained from UV-irradiated human tumor cells. Curves of heat absorption as a function of temperature are shown for nuclei isolated in buffer C. The amount of DNA in each sample was 0.73 mg from control, 0.33 mg from 15-min irradiation, 0.64 mg from 60-min irradiation, and 0.55 mg from 120-min irradiation.

°C with a corresponding increase in transition III (82–95 °C) material. A similar series of shifts characterizes the effects of increasing UV exposure on the calorimetric scans of nuclei from human tumor cells (Figure 4, Table II). This sequence of changing proportions indicates that transition IVa (95–102 °C) represents material kinetically intermediate between transition IV and transition III, and the system could be described kinetically as two consecutive reactions. Since the decrease in enthalpy change for transition IV was exponentially correlated with passage number for both normal and Werner syndrome cells (Figure 7), the two consecutive processes seem to be first order. A similar series of shifts characterizes the effects of increasing UV exposure on the calorimetric scans of nuclei from human tumor cells (Figure 4).

Discussion

Aging of normal cell cultures is characterized by a progressive decrease in their proliferative capacity (Hayflick & Moorehead, 1962) until the cells can no longer be multiplied even though they are quite viable. Although the mechanisms responsible for this phenomenon are not yet understood, it has been suggested that aging is linked to instability in DNA and that DNA structure might be altered in aging cultures (Dell'Orco & Whittle, 1978; Collins & Chu, 1985). A direct test of this notion was made in the present study, by application of differential scanning microcalorimetry to probe the thermal stability of chromatin in intact nuclei from aging cultured cells. Our results indicated that cellular senescence was in fact accompanied by progressive damage to DNA and that it was the high-melting (105 °C) form of chromatin that was lost. It must be emphasized that no such loss of transition IV was observed with cell cultures that did not age, in the sense of losing their potential for cell division. Despite indefinitely large numbers of passages, transition IV was dominant (e.g., the scan for tumor cells in Figure 1) for such immortalized cell lines as HeLa, mouse neuroblastoma, and Chinese hamster ovary cells (Touchette & Cole, 1985; Touchette et al., 1986; Almagor & Cole, 1987). Therefore, the process of aging in cell cultures is strictly correlated with a loss of transition IV.

The nuclei of cultured cells in general exhibited four thermal transitions: I (60 °C), II (76 °C), III (88 °C), IV (105 °C) (Figure 1). Transition IV dominates the melting profiles of nuclei from all types of dividing cells (Touchette et al., 1986; Almagor & Cole, 1987). Transition I for the most part represents the melting of miscellaneous nucleoplasmic components since it was nearly absent from calorimetric scans of isolated

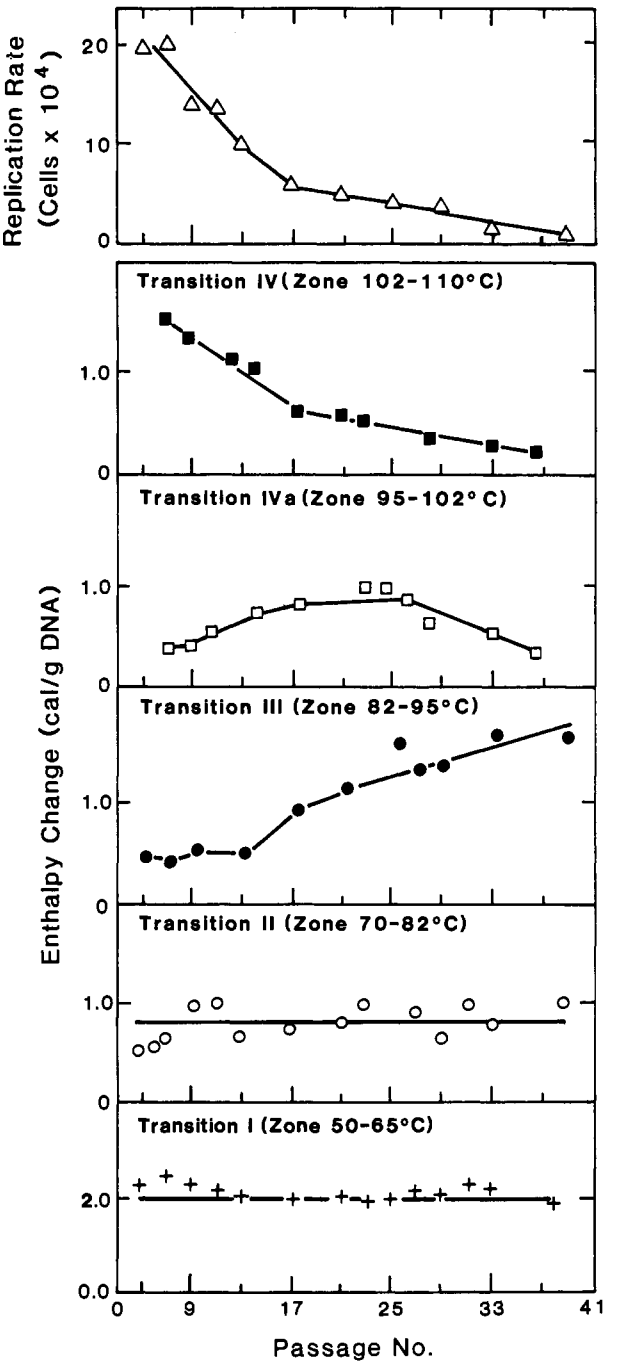

FIGURE 5: Replication rates of normal cultured human fibroblasts (1489) and enthalpy changes in the calorimetric profiles of their nuclei, as a function of passage number. Replication rates of cultured fibroblasts (1489) and enthalpy changes of their nuclei were determined as described under Experimental Procedures (similar results were obtained with cultured normal fibroblast 1493).

chromatin (Touchette et al., 1986). Transitions II, III, and IV are associated with the melting of chromatin in nuclei (Touchette & Cole, 1985; Touchette et al., 1986). Our recent studies on isolated chromatin (Almagor & Cole, 1989) indicated that the nucleosomal core complex collapses at 76 °C (transition II) and the released DNA in its nicked, relaxed form unstacks at 88 °C (transition III). Under the salt conditions used in our experiments, the temperature at which the bases unstack in DNA is insensitive to the presence of proteins. Transition IV, which dominates the profile, seems to represent the melting of a topologically constrained form of chromatin, probably intact supercoiled DNA (Vinograd et al., 1968). In earlier work, we demonstrated that transition IV was associated

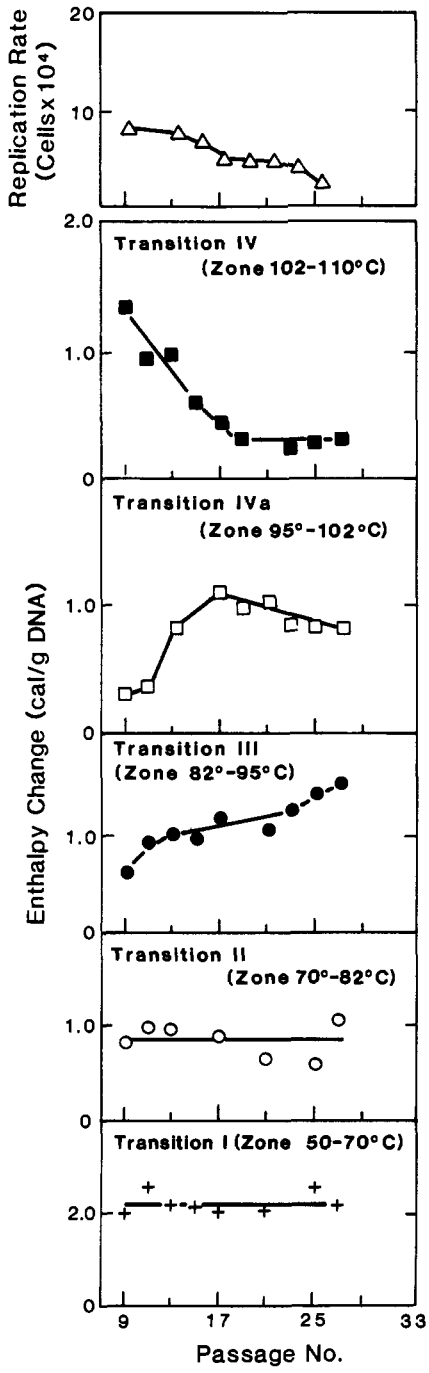

FIGURE 6: Replication rates of Werner syndrome fibroblasts and enthalpy changes in the calorimetric profiles of their nuclei as a function of passage number. Replication rates and enthalpy changes were determined as described under Experimental Procedures.

with cellular mitotic potential in nutritionally deprived cells, and we shoed in cells undergoing terminal differentiation that the complete loss of transition IV corresponded to irreversible loss of cellular capacity to divide (Almagor et al., 1987).

The gradual decrease in magnitude and temperature of transition IV in aging cells observed in our present study can therefore be explained as representing the progressive decrease in their proliferative potential. The exponential correlation between reduction in transition zone IV and cell senescence implies that (a) loss of proliferative capacity as a consequence of aging in vitro corresponds to loss of the structural feature of DNA that gives rise to this high-temperature structural transition and (b) the rate with which the structure represented by transition IV is lost is proportional to the amount of DNA that possesses that structure. Since the loss of transition IV

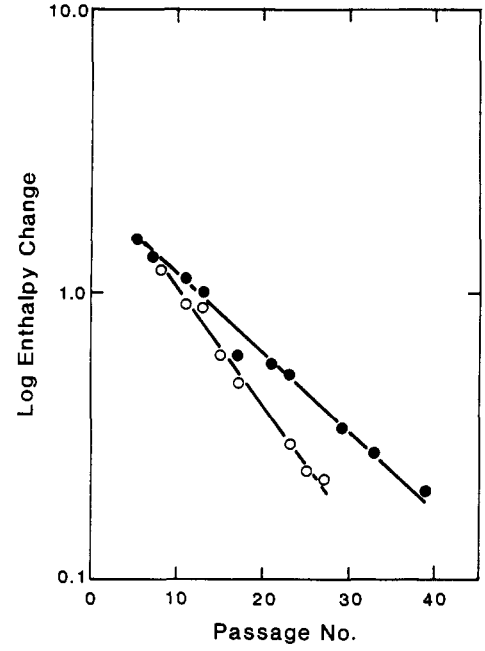

FIGURE 7: Log enthalpy change of transition IV as a function of passage number for normal (●) skin fibroblast and Werner's syndrome (○) cells.

during aging is not accompanied directly by an equal gain in transition III, the loss would not appear to be simply the conversion of intact, supercoiled DNA to the nicked, relaxed form. Transitions III and IV both represent the melting of double-stranded DNA, however, and so we suggest that the transient destabilization of DNA is the result of increasing the single-strand-like character of the DNA without cleavage of the phosphodiester backbone. Indeed, this suggestion is consonant with the report of Collins and Chu (1985), who found a 3-fold increase in the number of S1-sensitive sites of DNA in aging WI 38 cultured human cells.

To test our suggestion, human tumor cells (which lost their proliferative control and are considered immortalized) were subjected to UV irradiation, which, under the conditions used, introduces intrastrand pyrimidine dimers (Bohr et al., 1986), loss of bases, and partial unstacking of the double strands. The calorimetric profile of nuclei from irradiated cells was similar to that of nuclei from aged cells. Transition IV zone decreased in magnitude, but transition III zone was not changed until late in the UV exposure period when excision repair would be expected; instead, the chromatin melted at intermediate temperatures after short exposures to UV. This similarity to the effects of aging implies that DNA damage caused by UV irradiation and damage by the aging process are analogous in nature. It should be noted that increased UV sensitivity with in vitro aging was observed by Mayer et al. (1987).

In addition to strand breakage, which would produce a gain in transition III at the expense of transition IV, UV irradiation damages DNA in numerous ways (Hanawalt et al., 1979) that could weaken base pairing and so might give rise to transition IVa. Marmur et al. (1961) showed a broadening of the melting curve of DNA and a lowering of the T_m after its exposure to UV light. The modifications that could lower the T_m are of many kinds: various base modifications, pyrimidine dimer formation, depurination, and depyrimidization. The magnitude of the effects of these modifications has been estimated in some cases. For example, thymine dimer formation lowers the T_m of DNA about 0.8 °C for every percent of base damage (Rahn & Patrick, 1976), and depurination decreases the T_m about 3 °C for each percent of base damage (Ullman

& McCarthy, 1973). Although comparisons between systems are unreliable, a very rough estimate might be made of how much change might have been expected in our experiments. Our longest UV exposure was for 1440 erg/mm^2. Ljungquist et al. (1974) estimated about 0.01% bases depurinated at 100 erg/mm^2, and Setlow and Carrie (1963) estimated about 0.01% of bases formed thymine dimers at the same exposure. From these two major forms of base damage, we might have expected about a 5 °C lowering in the average melting temperature. Although this estimate is extremely rough, it is gratifying that the lowering of melting temperature we observed was indeed about 5 °C. Many of the modifications just mentioned would probably be included as the "singlestrandedness" that Mitchell et al. (1986) observed in DNA as a result of its exposure to UV irradiation, and this again would correlate with the increase of single-strandedness during aging (Collins & Chu, 1985).

In view of these results, it was of interest to learn whether accelerated aging is manifested differently than normal aging in the calorimetric profile of nuclei from cultured cells. Werner's syndrome is an autosomal recessive disorder that is generally characterized by an apparent acceleration of many of the processes associated with aging (Thannhauser, 1945; Epstein et al., 1966; Salk et al., 1981). Skin fibroblasts from patients with Werner's syndrome have reduced life spans and a lengthened mean population doubling time when grown in vitro (Salk et al., 1981; Bauer et al., 1986). Therefore, it was of interest to compare the calorimetric profile of chromatin from aging Werner's syndrome cells to that of senescent normal cells. As expected, Werner's syndrome cells exhibited reduced proliferative capacity, and this seems to be reflected in a lesser amount of DNA melting in the 102–110 °C zone when compared to normal cells of similar early passage number, and a greater amount melting between 92 and 102 °C. Moreover, the subsequent rate of decrease in transition IV (102–110 °C) in Werner's nuclei was more rapid than that of normal cells (Figure 6). Although Werner's syndrome may represent specific pathology that leads to premature death (Salk, 1982; Epstein, 1985) and the limited in vitro proliferative capacity of Werner syndrome cells, which qualifies it as a segmented progeroid syndrome, may not be caused directly by accelerated deterioration of their chromatin, it is clear that accelerated aging is accompanied by changes in chromatin structure.

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