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DNA degradation in terminally differentiating lens fiber cells from chick embryos

(chromatin subunits *in vivo*/nuclear degeneration/lens cell differentiation/nuclease digestion/gel electrophoresis)

DAVID W. APPLEBY* AND SOHAN P. MODAK

Unité de Biologie du Développement, Institut Suisse de Recherches Expérimentales sur le Cancer, 1066 Epalinges, Lausanne, Switzerland

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ABSTRACT **During the terminal differentiation of lens fiber cells, nuclear DNA is known to accumulate free 3′-OH ends and is progressively lost from the nucleus. Toward the end of this process, nuclei undergo pycnosis and disappear. The size of the DNA in the epithelia and in early and late stages of fiber cell development was examined by electrophoresis on nondenaturing agarose/polyacrylamide gels. Low molecular weight DNA of discrete sizes appears only at the final stages of nuclear degeneration in central fiber cells and persists after the disappearance of the nuclei. These low molecular weight DNA fragments appear as multiples of a monomeric unit and are similar to the fragments produced by the digestion of epithelial cell nuclei by micrococcal nuclease. The data indicate that in lens fiber nuclei the double-strand breaks *in vivo* affect the chromatin during nuclear degeneration, and the data suggest that the DNA of these cells is organized into chromatin composed of discrete subunits.**

After 4 days of chick embryogenesis the primitive lens of the eye is already formed as an autonomous organ system which is topographically subdivided into actively proliferating lens epithelium and nondividing lens fibers (1). Throughout the subsequent development some of the peripheral epithelial cells stop dividing and become protodifferentiated fibers giving rise to the annular pad (2–4) that joins the lens fiber area along its equator. Annular pad cells are continuously engulfed by the fiber area so that the latter population grows in cell number (4). Thus, the youngest fiber cells are located at the periphery and the oldest are in the center along the optical axis (3, 4). At 8 days of embryonic life the central fiber cell nuclei begin to undergo pycnosis and degenerate, and subsequently they disappear (3). This phenomenon progressively affects increasing numbers of fiber cells in a cylindrically symmetrical fashion (3, 4). At 13 days 1% of fiber cells contain pycnotic nuclei (S. P. Modak, unpublished data), and these progressively disappear while more peripheral fiber nuclei repeat the process (3). By the time the embryo hatches, slightly over half of the fiber mass contains nonnucleated cells.

During their differentiation, fiber cell nuclei lose DNA (3), and this has been correlated to increased appearance and accumulation of free 3′-OH ends in the DNA that act as initiators for terminal deoxynucleotidyl transferase, indicating extensive strand scission (5–7). The highest numbers of strand breaks were associated with DNA in the most advanced stages of nuclear pycnosis (5–8). That the DNA in fiber cell nuclei degrades, as indicated by our cytoenzymological studies (5–8), has been confirmed by sedimentation on alkaline sucrose gradients of lysates of double-labeled epithelial explants differentiating *in vitro* (9). However, nothing is known about the mode of participation of single- and double-strand breaks, nor is it known whether the breaks occur in chromatin randomly or at specific sites.

In this paper we describe results of experiments designed to achieve an understanding of this process at the molecular level. The central fiber (CF) cells, peripheral fiber (PF) cells, and epithelial tissue (EP) cells of 8-, 13-, 15-, 17-, 19-, and 21-day embryonic lenses have been isolated and the size of DNA present in these three cell populations has been determined by gel electrophoresis. We have found that low molecular weight (M_r) DNA molecules in the CF of 15- to 21-day embryonic lenses exist in a series of discrete molecular weights, occurring in a pattern that resembles in many respects the chromatin subunit pattern of DNA sizes. These low-M_r DNA molecules are not detectable in PF and EP cells at all ages examined or in CF cells of 8- and 13-day embryonic lenses.

METHODS

Tissue Preparation and Storage. Fertile eggs of Hubbard chickens were incubated in a forced-draft, continuous agitation incubator at 37° and 98% relative humidity for 8, 13, 14, 17, 19, and 21 days. Lenses of any given age were washed three times in either Tyrode solution (10) or culture medium (Eagle's minimal essential medium + 15% chicken serum) and dissected in batches of 10 to 15. The lens capsules with adhering epithelia were removed with tungsten needles. The fiber masses were rinsed into solution A (per liter: 85.7 g of sucrose, 0.2 g of KCl, 1.0 g of glucose, 1.0 g of $NaHCO_3$, 0.05 g of NaH_2PO_4) and individually placed on a glass petri dish. The excess solution was removed and the masses were immediately frozen on solid CO_2. The center of each lens was punched out with an 18-gauge hypodermic needle cut perpendicular to its long axis and sharpened from its outside surface. This punch produced a section of fiber tissue 1 mm in diameter cylindrically symmetrical about the optical axis. These tissue sections (CF) and the remaining fiber mass (PF) were then immediately refrozen in solution A. Occasionally these sections were refrozen in culture medium. The results from the two methods of storage were the same. It was found important to store the dissected tissues in a very small volume of storage medium, because during the thawing procedure some of the low-M_r DNA is released in the supernatant and lost if the latter is discarded. The EP was scraped from the lens capsules and similarly frozen on CO_2. All samples were then stored in a liquid N_2 vapor freezer at −140° until needed.

Sample Preparation. Tissue was thawed at 4° and combined in a Dounce homogenizer in solution A and EDTA (0.05–0.1 M), sodium dodecyl sulfate (1–2%), and proteinase K (200–400

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Abbreviations: CF, central fiber; PF, peripheral fiber; EP, epithelial tissue; M_r, molecular weight; SV40, simian virus 40; bp, base pairs.

* Present address: Department of Biology, University of Pennsylvania, Philadelphia, PA 19102.

μg/ml) (Merck) were added. The final lysate volume was 30–50 μl. Tissue was immediately homogenized and digested for 1 hr at 37°. Just prior to loading on the gel, sucrose was added to a final concentration of 10%. In some cases samples were treated with KCl (1 μl, 2 M, 15 min, on ice) in order to remove sodium dodecyl sulfate. For purification of polymeric nucleic acids (DNA and RNA) the lysates from CF and PF were extracted with phenol, precipitated with ethanol, and recovered by centrifugation. In some cases samples were digested with RNase A.

Gel Electrophoresis. Mixed agarose/polyacrylamide gels (11) were made in slabs (12 × 12.5 × 0.25 cm) using Tris/acetate buffer (40 mM Tris/20 mM Na acetate/2 mM EDTA, pH 7.8, adjusted with acetic acid). Electrophoresis was carried out at 80 V. In the case of whole cell lysates and with some phenol-extracted samples, gels were treated with 250 ml of RNase A (25 μg/ml, 37°, 30 min) prior to staining. Gels were stained in ethidium bromide (2 μg/ml, 20 min) and photographed, using UV transillumination and Polaroid film (type 107 C for positives) and Kodak film (no 6142) for negatives.

Digestion of EP Nuclei with Micrococcal Nuclease. EP nuclei were prepared and digested with micrococcal nuclease by a modification of the method of Marshall and Burgoyne (12). EP (15-day, derived from 90 to 120 lenses) was thawed and combined in a 2-ml homogenizer in solution A on ice and centrifuged (1500 rpm, 10 min, 4°, MSE clinical centrifuge). The pellet was resuspended in 1 ml of buffer A1 (12). The tissue was homogenized with three strokes of the pestle and the nuclei were pelleted (2000 rpm at 4°, 5 min). Pellets were resuspended in 1 ml of buffer A4 (12) supplemented with 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) (Sigma). The nuclei were again pelleted and resuspended in 170 μl of the same buffer and immediately treated (37°) with three units of micrococcal nuclease (PL Biochemicals) after adding 2 μl of 0.085 M $CaCl_2$. Controls were the same without enzyme. Aliquots (20 μl) were taken at various times and lysed immediately by adding them to mini centrifuge tubes (400-μl volume) containing 2 μl of EDTA (0.5 M), 2 μl of proteinase K (1 mg/ml), and 1 μl of sodium dodecyl sulfate (10%) and digested (1 hr, 37°). Sucrose was added to 10% and the samples were loaded onto a gel, run, and processed as before.

Molecular Weight Estimations. Gels were calibrated by running digests of simian virus 40 (SV40) DNA with *Hin*dIII or *Alu* I restriction enzymes as markers. SV40 DNA was a gift from P. Beard and G. Carmichael. The *Hin*dIII and *Alu* restriction enzymes were obtained from New England Biolabs (Waverly, MA). The reactions were stopped by either sodium dodecyl sulfate or EDTA and these marker solutions were stored in the freezer (−20°). Because we do not claim to know exact molecular weights and the exact amount of DNA in lens tissue samples, we placed the marker DNA in wells adjacent to sample tracks. The uncertainty introduced into the measurements by this method is on the order of 1–2% (as determined by running alternate marker and sample wells and determining the variation in migration of bands between marker wells). So for each gel all marker tracks were plotted as log (band M_r) versus migration (in cm from the origin), and the points were fit with a least square line. This line was used to interpolate molecular weights and number of base pairs of average M_r 652 for bands in the sample tracks.

RESULTS

The nondividing lens fiber cell population grows centripetally by addition of annular pad cells at the periphery. The oldest fiber cells are always situated at the center. Analysis of CF cells during the development would thus allow understanding of biochemical processes affecting the genome during the terminal differentiation, i.e., prior to, during, and after the nuclear degeneration and disappearance. Fig. 1 illustrates a 19-day embryonic lens sectioned parallel to the optical axis. The topography of CF, PF, and EP cells is shown. Lens cells are mononucleate and we have only depicted the general morphology and localization of nuclei in different lens regions. At 19 days the CF region consists of anucleate fiber cells while the PF section is made up of the complete spectrum of fiber cell types, from new cells at the outermost edge to middle fibers with elongated nuclei and cells having pycnotic nuclei (spindle-shaped and small dark nuclei). The outer lens surface is layered with EP consisting of cells from both epithelium and the annular pad.

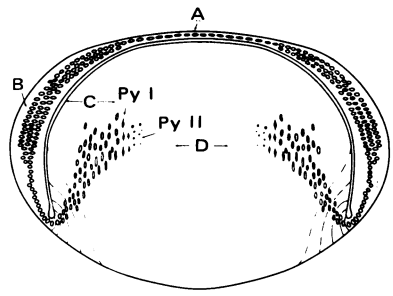

FIG. 1. Section of a 19-day chick lens. Cellular outlines are not shown but general distribution and morphology of nuclei are indicated. (*A*) Lens epithelium, (*B*) annular pad (*A* + *B* = EP), (*C*) nondegenerating fibers (PF), (*D*) nonnuclear central fibers (CF). Py I, early stages of nuclear pycnosis; Py II, advanced pycnosis.

We have obtained migration of DNA from EP, PF, and CF by loading whole cell lysates and phenol-extracted, RNase A-treated samples on gels. With whole cell lysates, technical problems arose, such as sample aggregation at the origin, appearance of opacity during exposure to UV light, etc. Subsequently we used phenol-extracted samples. This procedure eradicated both of these problems. The general result using the two methods is the same: at later stages of differentiation (15–21 days) in the CF region we see some DNA at high molecular weights (≥10,000 base pairs) and some DNA at low molecular weights (100–900 base pairs) (Fig. 2). The low-M_r DNA is grouped into at least four classes (Fig. 2 *B* and *C*), resembling in some respects the monomer through tetramer pattern obtained by treating chromatin (15) or nuclei (16) with micrococcal nuclease. The low-M_r bands are not detectable in the 8- and 13-day fibers (Fig. 2*B*) as analyzed here and in all PF (Fig. 2*A*) and Ep (not shown) sections studied. With this general pattern seen in both whole cell lysates and in phenol-extracted material, the results from the two methods of analysis differ in detail. Fig. 2 *A–C* provides examples of these points. Fig. 2 shows DNA from CF and PF regions from 13-, 15-, 17-, and 19-day lenses electrophoresed as whole cell lysates and as phenol-extracted DNA. In Fig. 3 we show microdensitometer scans of the negatives of electrophoretograms shown in Fig. 2 *B* and *C*. (Because the scans are taken from different gels the absolute migrations cannot be compared.) Notice that there are at least four bands in each gel and that the band morphology is highly variable in CF whole cell lysates (Fig. 2*B*) but not in phenol-extracted samples (Fig. 2*C*).

We have assigned molecular weights to each peak of Fig. 3 and all other samples run, as described in *Methods*. These numbers are converted to base pairs and given in Table 1. If we group the numbers for whole cell lysates and for phenol-extracted samples, it would appear that there is a decrease in size

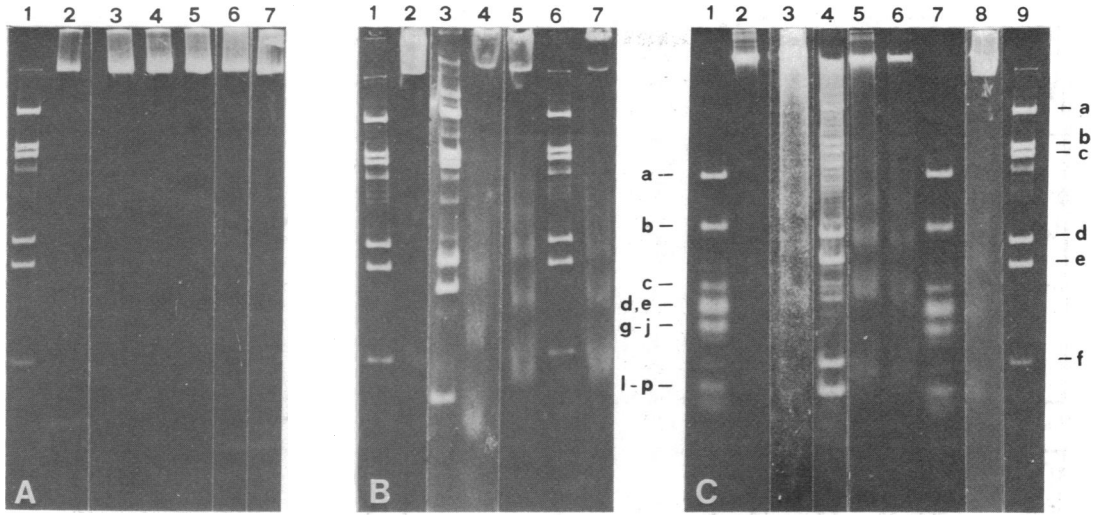

FIG. 2. Gel electrophoresis of DNA from 13- to 21-day lens fiber regions PF and CF. The composite plate shows photographs of electrophoretograms. Molecular weights of SV40 DNA *Hin*dIII and *Alu* restriction fragments were calculated using the data of Yang *et al.* (13, 14) and assuming the length of SV40 DNA to be 5200 base pairs. (*A*) All samples were run in the same gel and rearranged according to lens age. Track 1, SV40 DNA *Hin*dIII fragments; 2, phenol-extracted DNA from 15 total fibers of 8-day lens; 3–7, phenol-extracted DNA from 15 PFs of ages 13–21 days. Notice that there is no low-M_r DNA in any of the sample tracks. (*B*) Whole cell lysates of CF DNA from lenses of 13–19 days. All gels were treated with RNase A from electrophoresis, prior to staining. Track 1, SV40 DNA *Hin*dIII fragments; 2, 13-day CF; 3, SV40 DNA *Hin*dIII partial digest; 4, 15-day CF; 5, 17-day CF; 6, SV40 DNA *Hin*dIII fragments; 7, 19-day CF. Notice that 15- to 19-day CFs contain 4–5 low-M_r DNA bands. The band seen in 13-day CF is due to sodium dodecyl sulfate–ethidium bromide complex. (*C*) Phenol-extracted DNA from 13- to 21-day CF (30–70 sections). Track 1, SV40 DNA *Alu* fragments: a, 738 base pairs (bp); b, 577 bp; c, 338 bp; d–e, 283 bp; g–j, 240–224 bp; l–p, 161–140 bp. Track 2, 13-day CF; 3, 15-day CF; 4, SV40 DNA *Hin*dIII fragments—the extra low-M_r fragment in this sample is from an SV40 spontaneous mutant; 5, 17-day CF; 6, 19-day CF; 7, SV40 DNA *Alu* fragments; 8, 21-day CF. Track 9, SV40 DNA *Hin*dIII fragments: a, 1754 bp; b, 1161 bp; c, 1105 bp; d, 542 bp; e, 439 bp; f, 206 bp.

of all classes of DNA that accompanies the development of the lens from 15 to 19 days. This decrease in size is equal to or larger than the observed difference between determinations of CF DNA from a given age. For example, the trimer peak from phenol-extracted samples changes from 531 ± 14 (uncertainties are given as SD) base pairs (15 days) to 502 ± 11 base pairs (17 days) to 489 ± 10 base pairs (19 days). It is interesting to note that, compared to 17 and 19 days, in 21-day samples the size of low-M_r DNA is somewhat great.

We digested EP nuclei with micrococcal nuclease and examined the sizes of DNA obtained on gels. Fig. 4 represents a summary of these experiments. The scans of the time points are shown in Fig. 4. We should point out that there is no digestion of the EP DNA detectable by this method unless enzyme is added to the reaction mixture with EP nuclei (data not shown). In the inset of Fig. 4 we have plotted the number of base pairs in the various peaks as a function of time and provided an extrapolation to zero time. This value is 195 ± 5 base pairs for the monomer and 400 ± 10 base pairs for the dimer. We see that during digestion with exogenous enzyme the sizes of all classes of DNA decrease by 50–100 base pairs. This decrease may be due either to trimming of the oligomers by the enzyme or the existence of a range of repeat lengths in the chromatin of EP nuclei, or to both effects.

In order to see if two cycles of freezing and thawing of the tissue and handling activate endogenous nucleases and give rise to the observed bands, we performed cell self-digestion experiments. In such experiments damage by our handling procedure would cause either the amount of DNA appearing in the low-M_r bands to increase or the position of the bands to change (for example, see refs. 17 and 19). In the first experiment three samples composed of 13-, 15-, and 17-day CF sections were incubated on ice for 10, 20, and 40 min and then lysed with sodium dodecyl sulfate/EDTA/proteinase K and electrophoresed. In the second experiment 15-day CFs were incubated for 20, 40, and 50 min on ice in culture medium and in solution A and then lysed and extracted with phenol (the time between thawing and lysis for the experiments reported in Table 1 was always less than 10 min). In both experiments all bands were present in the correct abundance and there was no

Table 1. Size distribution of low-M_r DNA in chick lens central fibers*

Polymerization state	Whole cell lysates				Phenol-extracted DNA			
	15-day	17-day	19-day	21-day	15-day	17-day	19-day	21-day
Monomer	185 ± 6 (4)	171 ± 14 (3)	177 ± 7 (3)	189 ± 5 (4)	181 ± 10 (2)	182 ± 2 (3)	174 ± 9 (3)	191 ± 6 (2)
Dimer	335 ± 27 (4)	310 ± 11 (3)	307 ± 9 (3)	346 ± 20 (4)	347 ± 20 (4)	339 ± 4 (3)	323 ± 5 (3)	371 ± 20 (2)
Trimer	516 ± 21 (4)	477 ± 24 (3)	463 ± 11 (3)	473 ± 30 (4)	531 ± 14 (4)	502 ± 11 (3)	489 ± 10 (3)	508 ± 1 (2)
Tetramer	687 ± 34 (4)	617 ± 17 (3)	611 ± 30 (3)	637 ± 30 (4)	744 ± 1 (3)	694 ± 30 (3)	649 ± 9 (2)	732 ± 3 (2)
Pentamer	—	770 ± 30 (2)	812 ± 80 (3)	781 (1)	—	859 (1)	—	—

Molecular weights of DNA molecules present in various samples were determined by calibrating each gel run (see Fig. 2) with DNA fragments of known molecular weight. The migration distances of SV40 restriction fragments were measured from the microdensitometer scan of the gel negative (Fig. 3). The data were plotted as log (base pairs) calculated assuming length of intact SV40 DNA as 5200 base pairs *versus* migration distance from origin. The points were fit by a least squares straight line and the migrations of the unknown bands were interpolated to give the molecular weights of the DNAs.

* Sizes are given as mean number of base pairs, ±SD. Numbers of determinations are given in parentheses.

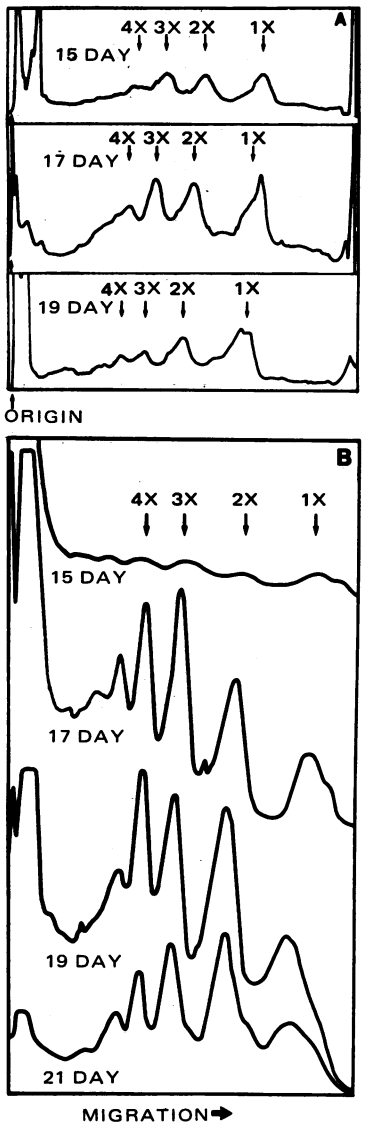

FIG. 3. Microdensitometer scans of negatives of gels from (*A*) whole cell lysates or (*B*) phenol-extracted samples of CF derived from 15-, 17-, 19-, and 21-day embryonic chick lenses. Arrows indicate positions of monomer (1X), dimer (2X), trimer (3X), and tetramer (4X). Because samples were not run on the same gel, the size determinations were carried out separately, using SV40 DNA *Alu* or *Hin*dIII restriction fragments described in Fig. 2.

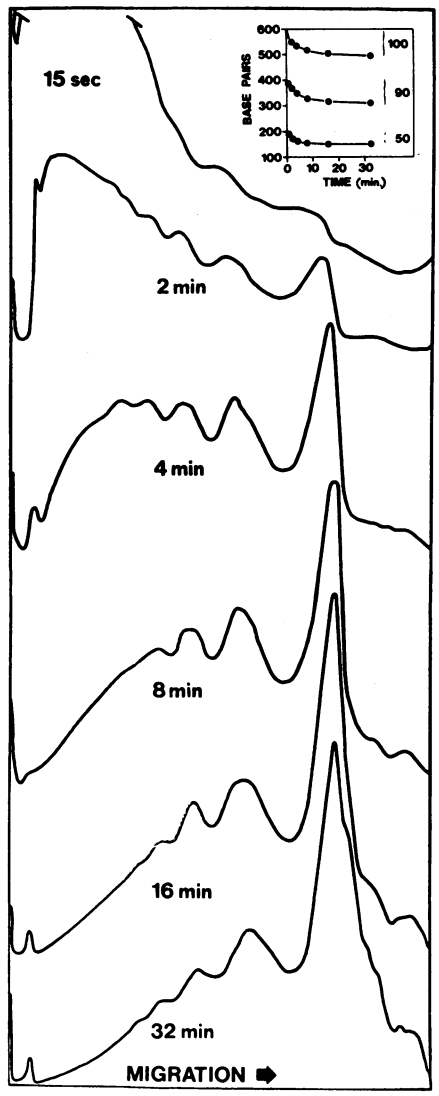

FIG. 4. Micrococcal nuclease digestion of EP nuclei. EP nuclei were digested with micrococcal nuclease for the indicated times and lysates were electrophoresed. The gel was calibrated using SV40 DNA *Hin*dIII restriction fragments. Microdensitometer tracings of digests are shown; note the time-dependent decrease in the amount as well as size of DNA protected by chromatin subunits. With time, size of DNA in individual subunits also decreases. (*Inset*) Sizes of monomer, dimer, and trimer DNA subunits were estimated as described in the legend of Table 1 and are plotted. The fitted curve was extrapolated to the ordinate to obtain the size of DNA (bp) in chromatin subunits at zero time. During digestion mono-, di-, and trimer DNA decrease in sizes by 50, 90, and 100 bp, respectively.

change in band position as incubation progressed (data not shown). Thus, the bands we observe from 15-, 17-, and 19-day tissue probably do not arise from our handling procedures.

DISCUSSION

The data presented here provide characterization of a series of low-M_r DNA molecules that exist in terminally differentiating chick lens fiber cells. Low-M_r DNA of discrete size classes has been previously observed in irradiated cells (19), but we do not know of any other reports of such molecules coming from cells undergoing their normal differentiation.

The four bands in the series have molecular weights corresponding to integral multiples of a unit length. If the molecular weights of Table 1 are plotted versus the number of subunits, we find that for each age the points fall on a straight line (within the limits of error reported), and this line goes through the point (0,0). The slope of this line decreases with age so that the number of base pairs in DNA per subunit decreases from 180 (15 days) to 171 (17 days), to 160 (19 days). In transcriptionally inactive populations of chicken erythrocytes the repeat length of DNA in chromatin is uniform (17) and a similar situation may exist in CFs, which also contain transcriptionally inactive cells (18). The uniform decrease in the size of DNA in CF chromatin subunits suggests that histones in the nucleosome slide along the DNA during the digestion *in vivo*, causing a reduction in the length of DNA between nucleosomes. Similar plots of the data on digestion of EP with micrococcal nuclease *in vitro* show that the number of base pairs of DNA per subunit decreases from 195 at the earliest time point to 168 at the latest time point; these data are consistent with the above model. On the other hand, we cannot exclude the possibility that EP

chromatin may also contain several different DNA repeat sizes, because, during the digestion *in vitro*, the length of DNA does not decrease proportionately; 50 base pairs for monomer, 90 base pairs for dimer and 100 base pairs for trimer (Fig. 4 *inset*). The apparent discrepancy may be due to the fact that, although CF cells are ontogenetically related to EP (1–4), the latter population is heterogenous and composed of cells in different phases of the cell cycle (2) and with different levels of transcriptional activity (18). So, DNA from EP nuclei digested with micrococcal nuclease resembles in its gross features the pattern of DNA degradation seen *in vivo* in CF cells.

The fact that fiber cells arise from EP cells (1–4) justifies a comparison of the chromatin subunit structure and repeat distance of these two cell types. For instance, the size of monomeric DNA in 15- to 19-day CF can be extrapolated to that of digests *in vitro* of EP DNA with micrococcal nuclease. Clearly, the digestion *in vivo* proceeds very slowly due to either a very low level of nuclease activity or a peculiar state of the lens fiber chromatin. From such an extrapolation we find, that low-M_r DNA fragments are produced in CF beginning $13\frac{1}{2}$ days of embryogenesis.

In summary, we have established that the DNA molecules that we see are present in at least four subgroups and the average sizes of the molecules in each group are integral multiples of a monomer unit. These low-M_r DNAs probably arise as a result of the autodigestion of nuclei in the CF. The nuclear degeneration and disappearance is a normal process in these cells (3) and therefore we are observing an *in vivo* digestion of chromatin. The chromatin subunit structure for the CF cells, which one may infer from our data, is qualitatively similar to that found in other organisms and cell types (12, 15–17, 19–21). Finally, the data presented here represent direct evidence that during nuclear degeneration in these cells double-strand breaks affect the DNA. Initially, the nuclear degeneration occurs in the CF and as the nuclei disappear the phenomenon affects younger cells centripetally (3, 4). Thus we would expect to find low-M_r DNA in PFs with increasing embryonic age. In practice, however, we do not (Fig. 2A), almost certainly because in PF the degenerating nuclei constitute a very small proportion of the total. Recently, we have fractionated PF into middle fibers (immediately adjacent to CF) and the outer fibers, and we find low-M_r DNA of the size distribution described in this manuscript in middle fibers at stages corresponding to those when nuclear degeneration occurs in these cells (S. P. Modak, unpublished results). On the other hand, the significance of our finding that some high-M_r DNA persists in CF after the loss of nuclei is intriguing and needs further investigation.

Earlier, we have shown that, during nuclear degeneration in lens fibers, the primer activity of DNA for exogenous calf thymus DNA polymerase (7, 8) and terminal deoxynucleotidyl transferase (5–7) increases; these results were considered to be strongly indicative of extensive strand scission (5–7). The present data give a biochemical proof to that assertion. However, it is not yet clear whether the low-M_r DNA fragments are produced by direct double-strand breaks or as a consequence of overlapping single-strand breaks. From our cytoenzymological studies (5–8) we also concluded that, well before the onset of nuclear degeneration, differentiating fiber cell nuclei accumulate free 3′-OH ends in their DNA. However, it is not yet clear whether the DNA breaks appear randomly or at specific sites. Experiments using alkaline gel electrophoresis (22) may provide answers to some of these questions.

Note Added in Proof. The double- and single-strand size of DNA from 19-day CF was determined by two-dimensional electrophoresis in horizontal 1.6% agarose slab gels (S. P. Modak, unpublished), using Tris/acetate (11) and NaOH/EDTA (22) buffers, respectively, in the first and second dimensions. We found that single-strand breaks occur between nucleosomes in high-M_r chromatin ($\geq$10,000 bp), and some of these convert into double-strand breaks, giving rise to the monomer- through pentamer-size DNA fragments. Similar results are obtained for chick embryo liver chromatin digested with micrococcal nuclease (S. P. Modak, unpublished data).

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