# Nucleosome Organization during Germ Cell Development in the Sea Cucumber *Holothuria tubulosa*†

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ABSTRACT: Conformational changes that occur in chromatin from developing germ cells of the echinoderm *Holothuria tubulosa* have been probed with micrococcal nuclease. The results indicate that the extent of DNA degradation to acid-soluble nucleotides is highest in chromatin at the early stages of gonad growth, being drastically subdued in the mature sperm cell. Production of nucleosomal particles also varies with development, involving at least 70% of the chromatin at the final stage of maturation, whereas in immature germ cells it remains much lower. In contrast, electrophoretic analysis for DNA size has shown that the average nucleosome repeat length, about 227 base pairs, does not change throughout the maturation process. However, kinetics of the enzyme reaction have revealed that, although brief digestion of chromatin from both immature gonads and sperm yields comparable series of higher oligomers, extensive digest patterns differ widely. Sperm chromatin, highly protected, releases a 275 base pair intermediate fragment, wholly absent in immature gonads. The 145 base pair core released in both chromatins is not further digested in sperm. In comparison to sperm chromatin, that of immature germ cells is much more susceptible to fragmentation, yielding the usual set of smaller subnucleosomal fragments. These data suggest the induction of differential accessibilities of chromatin DNA with maturation, which is not accompanied by displacement of the histone complement. The histone variants present in this species may well be instrumental in the process.

Eucaryotic chromatin seems to be organized into nucleosome particles, each containing a DNA segment of unit size tightly bound to a histone core. These complexes appear evenly interspersed along the strands by stretches of DNA, thereby determining a periodic nucleosome repeat length [see reviews by Kornberg (1977), Felsenfeld (1978), Klug (1978), and Chambon (1978)]. Within this repeat two distinct sites have been recognized as regards micrococcal nuclease susceptibility: a relatively resistant region which correlates with the nucleosome core and that of the much more nuclease-sensitive DNA linker (Sollner-Webb & Felsenfeld, 1975; Axel, 1975). Recently accumulated evidence has revealed the existence of interspecies size differences of the repeat unit (Spadafora et al., 1976a; Compton et al., 1976; Lohr et al., 1977a; Thomas & Thompson, 1977) which has been explained on the basis of a variable DNA content of the linkers since nucleosome cores appear to be rather constant in this respect. This variability in turn has been associated with histone H1 heterogeneity which, aside from being the least conserved among histone fractions, is presumed to preferentially interact with the linker DNA region (Noll, 1976; Morris, 1976). Several attempts have been made to corroborate this assumption using systems undergoing differentiation because of the chromatin transitions involved in the process (Lipps & Morris, 1977; Prince et al., 1977; Parish et al., 1977; Stalder & Braun, 1978). However, these studies have been mainly restricted to lower organisms, and the resulting information has not been precise enough since in most cases histone composition was not completely determined. In addition, although it has been found that the nucleosome repeat varies during erythropoiesis (Weintraub, 1978), the variation reported may well arise from the unusual circumstance of the partial replacement of histone H1 by fraction H5 known to occur in erythrocyte nuclei. To unambiguously ascertain whether there exists a strict correlation between variability of the DNA repeat and histone modifications, the study of a system undergoing known histone transitions during development should be considered.

Our work has been involved in the analysis of chromatin from the germinal tissue of the echinoderm *Holothuria tubulosa*. Ultrastructural studies have demonstrated that spermatogenesis in this organism is rather simple (Pladellorens & Subirana, 1975). Furthermore, during sperm maturation there is no bulk replacement of the histone complement, while transitions are restricted to the presence of two groups of lysine-rich histones (Phelan et al., 1972) plus the appearance of a minor histone variant in the maturing sperm (Subirana, 1970; Cornudella, 1977). We have also shown that a differential nuclease susceptibility is generated during development in this organism (Rocha & Cornudella, 1976). We report here the detailed assessment of nuclease digestion products of chromatin at various stages of development, introducing the distinction between the extent of enzyme-dependent DNA degradation and the release of nucleosomal particles. Analyses for DNA size have indicated that the average nucleosome repeat length remains unaltered during maturation. We have also found that the extensive digestion pattern of sperm chromatin is remarkably stable, giving rise to an oligomeric particle not present in immature germ cells. Inference is made about the ability of histones, particularly lysine-rich species, to induce changes in nucleosome other than to specify the DNA linker length.

## Materials and Methods

*Cell Fractionation and Preparation of Chromatin.* Male specimens of *H. tubulosa* were collected on a fortnight basis during maturation and moved to the laboratory in cold sea water. Gonads were excised immediately before use, pooled, and chilled in several volumes of ice-cold 0.25 M sucrose, 50 mM Tris-HCl[1] (pH 7.4), 25 mM KCl. Cells were fractionated as described previously (Rocha & Cornudella, 1976) with some

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid; OD, optical density; $NaDodSO_4$, sodium dodecyl sulfate; bp, base pairs of DNA.

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modifications. Briefly, pooled gonads were minced and homogenized in 3 volumes of the above buffer containing 5 mM DTT and 0.5% Triton X-100 (v/v) in a MSE homogenizer at 5000 rpm for 1 min, filtered through flannelette, and centrifuged at 1000*g* for 10 min. Crude nuclear pellets were then washed and resedimented 3 times in 10 volumes of the same buffer made 1 mM in $PhCH_2SO_2F$ (2-propanol was used as the solvent) and lacking detergent. The purified nuclear pellets were next resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM $PhCH_2SO_2F$, 20 mM $Na_2EDTA$, followed by centrifugation at 3000*g* for 10 min, and then 2 more times in the same buffer, omitting the chelating agent. Nuclei were forced to swell with these repeated hypotonic washes. Examination under the phase-contrast microscope revealed that the size of the nuclei increased by about two-thirds upon swelling, but nuclear breakage was actually minimal. A concomitant increase in pellet volumes was noticed. This behavior is wholly consistent with the well-documented resistance of *Holothuria* nuclei to disruption (Subirana, 1970; Rocha & Cornudella, 1976). The swollen pellets thus obtained were finally resuspended by five complete cycles with a wide-bore 10-mL glass pipet in appropriate volumes of 10 mM Tris-HCl (pH 8.0), 0.1 mM $PhCH_2SO_2F$ (digestion buffer) to yield 20 $OD_{260}$/mL. The adjustment was based on the absorbance at 260 nm of aliquots diluted 100-fold with 1 M NaCl (Camerini-Otero et al., 1976). The contribution due to scatter was routinely checked by measuring the visible-light scattering of the suspensions at 310 nm, and it was systematically found to be less than 5% of the absorbance at 260 nm.

These suspensions are referred to in the text as chromatin preparations, and they were used immediately.

*Nuclease Digestion and Quantitation of Released Products.* Chromatin samples in digestion buffer were made 0.1 mM in $CaCl_2$ and incubated at 20 °C with 100 $A_{260}$ units/mL of micrococcal nuclease (Sigma) for various times with the exception of extensive incubations which were carried out at 37 °C. Reactions were halted by chilling on ice for 30 min after addition of 100 mM $Na_2EDTA$ (pH 7.0) to 1 mM. The extent of solubilization was assessed by acid solubility according to Axel (1975). Aliquots (1 mL) of the chromatin digests were centrifuged at 12000*g* for 10 min, and the supernatants were retained. The resulting pellets were resuspended in a small volume of digestion buffer supplemented with 1 mM $Na_2EDTA$, briefly vortexed, and resedimented. Both supernatants were pooled and considered as the nuclease-released fraction, while the final pellets were designated the undigested chromatin fraction. The former was further differentiated into two subfractions, that of the DNA degraded to soluble oligonucleotides and that of the enzyme-liberated nucleosome oligomers, by precipitating the supernatants with an equal volume of cold 2 M $HClO_4$–2 M NaCl in an ice bath for 30 min, followed by centrifugation at 12000*g* for 15 min. The resulting pellets, as well as those corresponding to undigested chromatin fractions, were then resuspended in 1 M $HClO_4$–1 M NaCl, and the DNA was completely hydrolyzed by heating at 70 °C for 20 min. This step was repeated once more after centrifugation of the samples, and finally both supernatants were combined. The absorbance at room temperature of all the acid extracts was measured at 260 nm and expressed as percent of the total hyperchromic $A_{260}$ of hot-acid hydrolysates from starting chromatin samples which had been similarly precipitated with perchloric acid in the cold and recovered by centrifugation prior to enzyme addition. The value of trace amounts of absorbing material (less than 1–2%) present in supernatants from the cold-acid precipitation of these untreated chromatin samples was deducted from those corresponding to the cold-acid extracts of the nuclease-released fractions so as to obtain the true enzyme-dependent soluble absorbance.

*DNA Extraction and Gel Electrophoresis.* Remaining portions of the chromatin digests were centrifuged at 12000*g* for 10 min. The pellets were washed as described above in digestion buffer made 1 mM in EDTA. The combined supernatants, containing the solubilized chromatin fragments, were treated with 100 μg/mL proteinase K (Merck) overnight at 37 °C in the presence of 1 M NaCl. After incubation, samples were brought to 1% in $NaDodSO_4$ and deproteinized by three successive extractions, first with an equal volume of saturated phenol (10 mM Tris-HCl, pH 8.0)–chloroform–isoamyl alcohol (48:24:1) (Sollner-Webb et al., 1976) and then twice with chloroform–isoamyl alcohol (24:1). Aqueous phases were made 0.3% in NaCl, and the DNA was precipitated with 2.5 volumes of ethanol upon standing overnight at −20 °C. DNA was next recovered by centrifugation in a swinging-bucket rotor at 12000*g* for 20 min. The precipitates were washed and resedimented twice with cold 70% ethanol–0.3% NaCl, dried, and finally dissolved in 1 mM Tris-HCl (pH 8.0), 0.1 mM $Na_2EDTA$ at about 5 μg/mL. Samples were stored frozen at −20 °C until use.

Analytical electrophoresis of DNA fragments isolated from chromatin digests was performed at room temperature on vertical 2% agarose slab gels 18 × 16 cm and either 1.5- or 3-mm thick, casted, and run in the Tris–borate–EDTA buffer system of Peacock & Dingman (1968). Thawed DNA samples were supplemented with glycerol to 10% final concentration, bromophenol blue (0.05%) was added as the tracking dye, and 10-μL aliquots (about 20 μg of DNA) were loaded into each slab well. Electrophoresis was conducted at 100 V until the marker dye had migrated 14 cm (about 4–5 h). Gels were next stained in the dark with ethidium bromide in water (2 μg/mL), transilluminated with a black-light lamp, and photographed on Kodak Plus-X film through a UV haze filter (Kodak Wratten 2B) and a Kodak 23A red filter. To better resolve DNA fragments smaller than 200 base pairs, DNA samples were electrophoresed on 6 and 10% polyacrylamide gels [acrylamide–bis(acrylamide) ratio = 19:1] in the same buffer system as described above. Gels were preelectrophoresed at 100 V overnight at 4 °C. DNA samples were next loaded and slab gels run at 200 V at 4 °C for 4–5 and 2 h, respectively. Gels were visualized with ethidium bromide as described above except for 10% acrylamide slabs that were stained in 0.005% Stains-all (Eastman) in formamide–$H_2O$ (1:1) overnight and destained in running water for a minimum of 3 h (Soller-Webb & Felsenfeld, 1977). For DNA size measurements, photographic negatives of ethidium bromide stained gels were scanned on a Joyce-Loebl microdensitometer.

*Nucleosome Repeat Length Determination.* DNA fragments isolated from nuclease digests of Ehrlich-Lettre ascites chromatin were calibrated and used as the standard set to determine the length of the DNA bands in 2% agarose gels. Except for the increase in the detergent concentration to 1% in the lysis step and the addition of 1 mM $PhCH_2SO_2F$ thereon, ascites nuclei were fractionated according to Faiferman et al. (1973), and the chromatin was prepared as described here for *Holothuria* chromatin. Tumors were maintained in female Swiss albino mice by weekly intraperitoneal transfers. Previously sized *Bacillus subtilis* restriction nuclease fragments of λdv 1 DNA (Steinmetz et al., 1978), electrophoresed in parallel, were used as mobility markers. A calibration curve was constructed by plotting the

logarithm of the length in base pairs of the restriction fragments vs. migration distance (midpoint of bands). A least-squares procedure was used to determine the size of the ascites DNA fragments by linear regression analysis (Morris, 1976). The size of the DNA fragments produced upon nuclease digestion of *Holothuria* chromatin was in turn evaluated in a similar way by comparison with the standard set of ascites DNA fragments. The same calculations were carried out to determine the length of the DNA fragments resolved in 6% acrylamide gels but using PM2 *Hae*III restriction fragments of calibrated size as the DNA marker set (Noll, 1976). DNA repeat lengths were expressed as the quotient of oligomer size and the corresponding band number. The average nucleosome repeat at each digestion interval was computed from the slope of the regression line obtained from the respective data (Johnson et al., 1976).

## Results

The reproductive activity and seasonal changes of gonad growth in *H. tubulosa* have been already described (Cornudella, 1977). The annual maturation cycle is rather simple, involving several well-defined stages which start with a midwinter activation of the resting gonad, followed by a growth period, a breeding phase, and the final shedding of mature gametes (late summer) with recession to the resting stage. The overall temporal pattern of the maturation process is highly reproducible and synchronous; therefore, it appears particularly suitable to the purpose of probing chromatin transitions that take place during development.

*Quantitation of Nuclease-Released Products.* We have previously observed after gentle EDTA lysis of the digests that chromatin fragments produced by micrococcal nuclease are freed in soluble form with no soluble material trapped in the sediments, even on prolonged digestions (Rocha & Cornudella, 1976). Here we have followed nuclease action on chromatin at various stages of development by the extent of solubilization, assessing the products released at each digestion interval by the perchloric acid assay (Axel, 1975) on the basis of their differential solubility (Clark & Felsenfeld, 1974). The kinetics of nuclease solubilization of chromatin from immature gonads (early growth period) and that of ripe sperm are compared in Figure 1. DNA in chromatin from growing immature gonads appears to be easily degradable to soluble nucleotides while production of cold acid-precipitable products is kept at a low value, displaying a gradual trend to decrease with the digestion process. On the other hand, the overall enzyme access to chromatin appears to be limited since even at long digestion intervals about 40% of total chromatin remains undigested regardless of the enzyme concentration used. This resistance to enzyme action may result from a tight nucleohistone organization in chromatin from immature germ cells which would hinder nuclease access. It is worth noting the above-mentioned low tendency of these nuclei to swell in hypotonic media, reflecting a compact structural arrangement of chromatin. It is unlikely that the decrease with digestion of acid-precipitable particles in the solubilized chromatin fraction can be accounted for by the divalent cation dependent tendency of nucleosomes to aggregate (Whitlock & Simpson, 1976; Sanders & Hsu, 1977). Divalent cations were held absent throughout cell fractionation and subsequent isolation procedures since it has been observed that they enhance the mentioned nuclear compactness (Rocha & Cornudella, 1976). Furthermore, digestions were carried out at low ionic strength according to Clark & Felsenfeld (1974) and were quenched in large EDTA excess. In contrast, sperm chromatin offers much greater accessibility as revealed by both the extent and

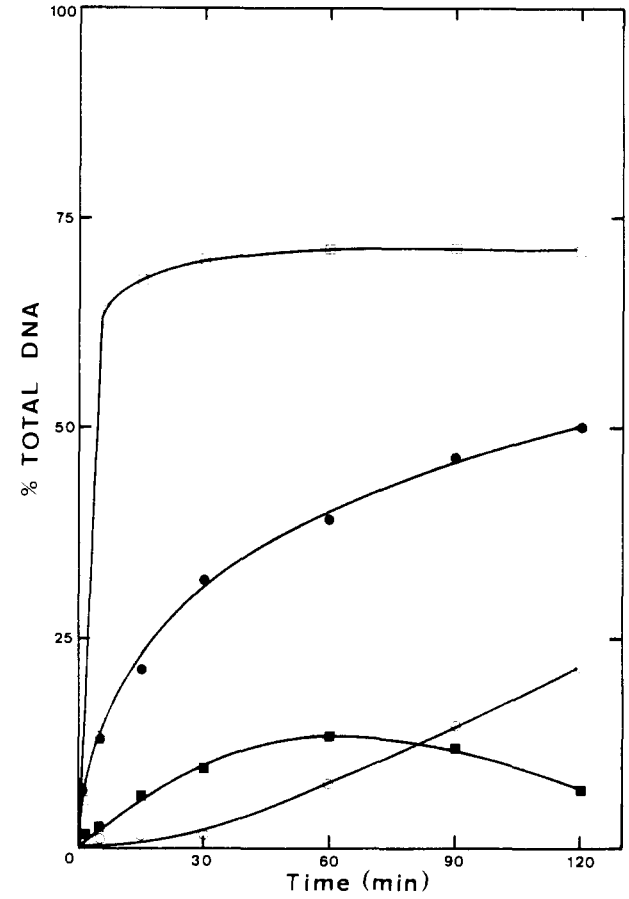


FIGURE 1: Time course of micrococcal nuclease solubilization of *Holothuria* chromatin from growing immature gonads and ripe sperm. Solubilized portions of the chromatin digests were fractionated by precipitation in the cold with 2 M $HClO_4$–2 M NaCl and centrifugation. The resulting pellets were completely hydrolyzed by heating at 70 °C in the perchloric acid solution. Absorbance at room temperature of both the soluble fractions and the hot-acid extracts was read at 260 nm and referred at each digestion interval to the total hyperchromic absorbance of hot-acid hydrolysates of comparable nondigested chromatin aliquots. Growing immature gonad chromatin digests: (●) cold acid-soluble; (■) cold acid-precipitable DNA products. Ripe sperm chromatin digests: (○) cold acid-soluble; (□) cold acid-precipitable DNA products.

rate of release of cold acid-precipitable products. These fragments appear much more stable as judged by the low extent of acid solubility with time.

The contrasting behavior of chromatin toward nuclease in this organism during development argues for differences in the structural arrangement of the deoxyribonucleohistone complex, namely, a tight organization in the immature gonad that hinders nuclease access to DNA and a more relaxed conformation in sperm. On the other hand, the distinctive susceptibility to degradation displayed by both chromatins implies the existence of modifications in the fine structure of the nucleohistone itself. These dissimilarities can be tentatively attributed to the actual distinctions between these chromatins: the exclusive presence in ripe sperm of a rather basic, minor histone-like variant, named $\phi_0$ by analogy to sea urchin histones (Palau et al., 1969), and the existence of an unusual group of lysine-rich histones.

*Comparison of DNA Electrophoretic Patterns.* Agarose gel electrophoretic patterns of DNA fragments from nuclease digests of Ehrlich-Lettre ascites chromatin are shown in Figure 2. *B. subtilis* restriction fragments of λdv 1 DNA run in parallel were used for calibration purposes, and subsequently, ascites fragment sizes were determined by linear regression

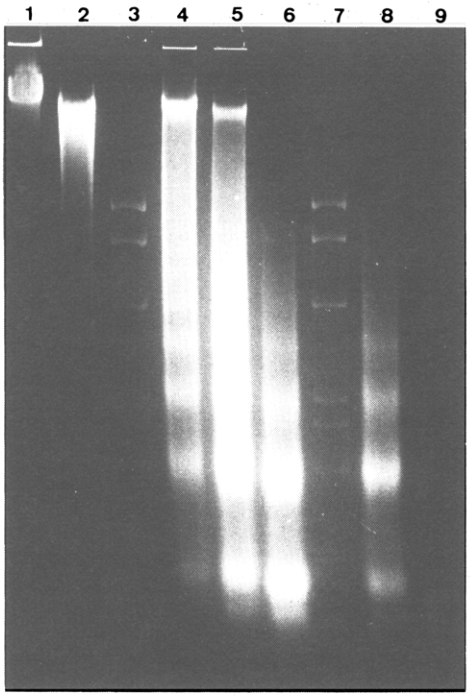


FIGURE 2: Agarose (2%) slab gel electrophoretic patterns of DNA fragments from Ehrlich-Lettre ascites chromatin digested with micrococcal nuclease for various times. Percentages of acid solubility correspond from the leftmost slot to the following: (1) 0.7; (2) 1.2; (4) 1.7; (5) 2.5; (6) 4.0; (8) 14.0; (9) 30.8%. *B. subtilis* restriction fragments of λdv 1 DNA were run as mobility markers (slots 3 and 7). Ascites fragment sizes were computed from their respective migration distances (midpoint of bands) by using the equation of the standard calibration line graphed for the logarithms of the restriction fragment lengths in base pairs vs. their mobility and adjusted by a least-squares procedure. Ascites fragments from a mild digest (1.7% acid solubility) were subsequently used as the standard set in agarose gels. Electrophoretic mobility is from top (−) to bottom (+).

analysis. The average DNA repeat was computed from data corresponding to very mild digestion intervals and was found to be about 188 base pairs in length. This value is in close agreement with those reported for related tumor cells (Compton et al., 1976). The shortening of oligomer lengths with digestion time and the shift toward lower oligomers are apparent as reported elsewhere. The sizes of the monomer fragment and the nucleosome core DNA averaged 190 (±4) and 145 (±3) base pairs, respectively, in close correspondence with those found in Ehrlich carcinoma chromatin (Bakayev et al., 1977). DNA fragments from early digests were used as a standard set in all subsequent agarose electrophoreses.

DNA fragments produced during the course of the digestion of chromatin from both immature germ cells and ripe sperm were compared electrophoretically as shown in Figure 3. At early digestion intervals, comparable DNA patterns appear consistently as regards migration of higher oligomers. At longer digestion intervals, oligomeric DNA bands from immature gonads appear to be susceptible to further degradation as deduced from the increasing mobility and their final absence from the patterns. This behavior contrasts with the persistent presence of equivalent DNA bands from sperm chromatin which are visible even at long-time digestion intervals. In addition, the breadth displayed by monomer DNA bands from immature gonad chromatin has no counterpart in the corresponding bands from ripe sperm chromatin. The latter exhibits instead three well-defined bands in the monomer region of the gels which are presumed to correspond from bottom to top to nucleosome core, monomer DNA, and a component longer than the monomer fragment. This assignment is corroborated upon increasing resolution of this region on 6% acrylamide slab gels and determining the size of these components. The minor band breadths noticed on sperm oligomers may be considered as reflecting a peculiar resistance to exonucleolytic trimming (Noll, 1974) of the sperm DNA linker, which may also impose a certain degree of uniformity upon the linker length, counterbalancing the band spreading due to linker heterogeneity as reported by Martin et al. (1977).

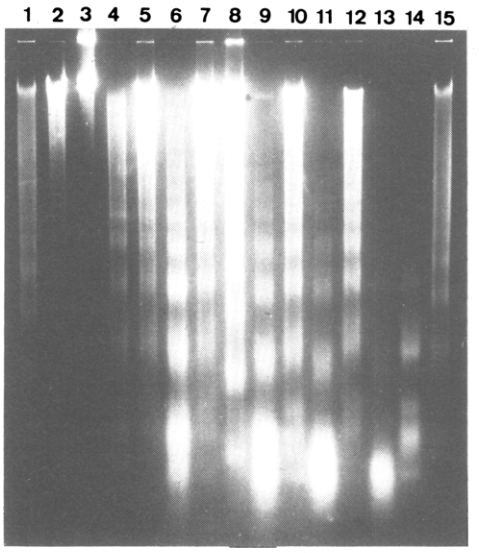


FIGURE 3: Agarose (2%) slab gel electrophoretic patterns of DNA fragments from micrococcal nuclease digests of *Holothuria* chromatin of 1, 5, 15, 30, 60, and 120 min, respectively. Chromatins from immature gonads (slots 2, 4, 6, 9, 11, and 13) and ripe sperm (slots 3, 5, 7, 10, 12, and 14) were digested for the indicated intervals, and aliquots of the isolated DNA were alternately loaded from the leftmost slot in the order of increasing digestion times. Previously calibrated ascites DNA fragments (1.7% acid solubility) were used as the standard set (slots 1, 8, and 15) for calibration. Note that migration of DNA fragments from immature gonads and sperm is similar and clearly patent for bands higher than the trimer. In turn, *Holothuria* oligomeric fragments are markedly larger than those from ascites chromatin. Electrophoresis is from top (−) to bottom (+).

*Determination of DNA Repeat Lengths.* DNA fragments resolved on 2% agarose gels were compared with a previously calibrated standard set of ascites DNA fragments, and their sizes were determined by linear regression analysis (Materials and Methods). Repeat lengths were measured for each multiple in the patterns higher than the dimer as the quotient of size and band number. The slopes of the regression lines obtained by plotting fragment sizes as a function of band order at each digestion time were considered the average repeats. Table I summarizes the values calculated for DNA fragments produced during digestion of chromatin from both immature gonads and sperm at comparable time intervals. It can be seen that intermediate oligomer repeats (trimer to pentamer) in both chromatins are somewhat larger than those of higher oligomers. Since all DNA fragments resolved in 2% agarose gels fell within the linear portion of the calibration curves, underestimated measures for larger fragments can be discounted. It therefore seems reasonable to think of a tendency for the higher components to be preferentially digested (Lohr et al., 1977b). It can also be observed that DNA repeats tend to decrease with the course of digestion and that this trend is markedly less pronounced in those from sperm chromatin, particularly at longer digestion intervals. This difference becomes even more manifest in the dimer repeat which is reasonably constant in sperm but which decreases steadily with digestion in chromatin from immature gonads. It should be

Table I: Nucleosome Repeat Lengths[a] of Chromatin from Sea Cucumber Developing Germ Cells

| | repeat lengths (base pairs) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | growing gonad: digestion time (min) | | | | | mature sperm: digestion time (min) | | | |
| oligomer | 1 | 5 | 15 | 30 | 60 | 5[b] | 15 | 30 | 60 |
| heptamer | 230 | 227 | 220 | 220 | | 231 | 226 | 223 | 220 |
| hexamer | 230 | 229 | 226 | 222 | 219 | 231 | 230 | 227 | 220 |
| pentamer | 244 | 231 | 230 | 224 | 218 | 233 | 229 | 228 | 222 |
| tetramer | 240 | 238 | 229 | 222 | 212 | 235 | 237 | 236 | 224 |
| trimer | 242 | 240 | 224 | 213 | 203 | 232 | 231 | 231 | 224 |
| dimer | 234 | 224 | 211 | 198 | 180 | 223 | 223 | 221 | 215 |
| av DNA repeat[c] (±SD) | 237 ± 6.0 | 224 ± 6.8 | 224 ± 7.1 | 224 ± 5.7 | | 234 ± 5.2 | 226 ± 5.7 | 225 ± 6.1 | 222 ± 3.9 |
| weighted-av[d] DNA repeat (±SD) | 227.2 (±6.0) | | | | | 227.9 (±5.1) | | | |
| correlation coeff ($r$) | 0.9899 | | | | | 0.9960 | | | |

[a] Repeats were determined by dividing fragment lengths in base pairs by the respective oligomer number and are mean values derived from four 2% agarose slab gels independently measured. DNA sizes were evaluated by linear regression analysis using previously sized ascites DNA fragments as the standard set. [b] No electrophoretically detectable DNA fragments were released from sperm chromatin digested for less than 5 min. [c] Average values at each digestion interval were calculated as the slope of the corresponding regression lines. [d] Weighted-average repeats were computed from all repeat values reported in Table I exclusive of those from 60-min digestion of immature gonad chromatin which are largely affected by enzyme degradation.

Table II: Lower DNA Fragment Sizes[a] from Immature Gonad and Ripe Sperm Chromatin Digests

| | fragment sizes (base pairs) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | gonad DNA: digestion time (min) | | | | | sperm DNA: digestion time (min) | | | | |
| component | 5 | 15 | 30 | 60 | 120 | 5 | 15 | 30 | 60 | 120 |
| intermediate | | | | | | 303 | 291 | 264 | 266 | 252 |
| monomer subspecies[b] | 242 | 235 | 228 | | | 244 | 234 | 221 | 227 | |
| | | 210 | 208 | 199 | | | | (193) | (194) | 191 |
| | | 184 | 183 | 167 | 162 | | | | | |
| core | | 158 | 159 | 144 | 141 | 158 | 156 | 151 | 153 | 147 |

[a] DNA fragments were resolved on a 6% polyacrylamide gel, and their sizes in base pairs were determined by regression analysis using PM2 *Hae*III restriction fragments as the standard set for calibration. [b] The values in parentheses refer to those subspecies that appear as a small shoulder on the monomer peaks of the densitometer tracings.

emphasized that the acid-solubility measurements are inadequate to compare the extent of digestion of chromatin from different developmental stages, due to the low proportion of acid-soluble DNA found in sperm (see Figure 1). Assessment of acid-soluble DNA release rates may be misleading since the latter are very sensitive to digestion conditions (Greil et al., 1976).

Average DNA repeats appear to adjust for the individual deviations observed at each digestion interval and point to a close correspondence among the values computed from both chromatins. Hence, they strongly suggest that the DNA repeat length does not change throughout germ cell development. Due to the rather complex nature of the kinetics of the nuclease reaction, it is hard to unambiguously establish true repeats (Rill et al., 1977). Therefore, it appeared reasonable for comparative purposes to contrast both chromatins, by deriving a weighted-average repeat length from all values obtained throughout the course of digestion examined. As suspected, an identical value was found for chromatin from both growing gonads and mature sperm. This result argues that the differential chromatin susceptibility to nuclease during the maturation process does not necessarily imply a change in the DNA repeat length.

*Electrophoretic Pattern of Lower DNA Fragments.* Polyacrylamide gels (6%) were used to resolve lower DNA fragments produced over the digestion course, and their sizes were derived from a calibration curve of PM2 *Hae*III DNA restriction fragments. Figure 4 shows that the DNA pattern from digested sperm chromatin contains a smaller number of fragments which are clearly larger in size than those from immature gonad chromatin. In addition, sperm chromatin releases with digestion a fragment intermediate between the dimer and monomer DNA positions which is never detected on DNA patterns of growing gonads (Figure 5). These dissimilarities are better illustrated by the sizes of the entire set of fragments produced (Table II). All sizes gradually decrease as digestion proceeds, reflecting a clear tendency to become constant at late time intervals. The estimated size of the distinct DNA intermediate fragment from sperm, averaged from all measured values, is of about 275 base pairs in length. Monomer DNA, which appears on the scans as a discrete peak at the first time interval, splits into three poorly defined subcomponents during the digestion (see Figure 5) and finally resolves again to a single component measuring the smallest size. This behavior parallels that reported for the yeast monomer (Lohr et al., 1977b) although the recurring phase frequency of about 25 base pairs observed between successive subcomponents appears somewhat larger. Both chromatins exhibit similar nucleosome core sizes with a consistent trend to become stabilized at a common value of 140–145 base pairs in length. On the other hand, subcore DNA fragments are hardly seen on sperm chromatin digests (Figure 6), whereas immature gonads exhibit a characteristic set of small sizes which are close to those described elsewhere (Axel et al., 1974; Bellard et al., 1976) for various chromatin types.

By adding the size of the intermediate DNA fragment found in sperm chromatin to that of the respective DNA core at each digestion interval, a value close to the corresponding dimer size is obtained (see Table I). Hence, it seems reasonable to think of different nicking sites in the nucleosome dimer which could give rise either to two symmetrical monomers or to a nucleosome core plus a monomer with a longer DNA linker

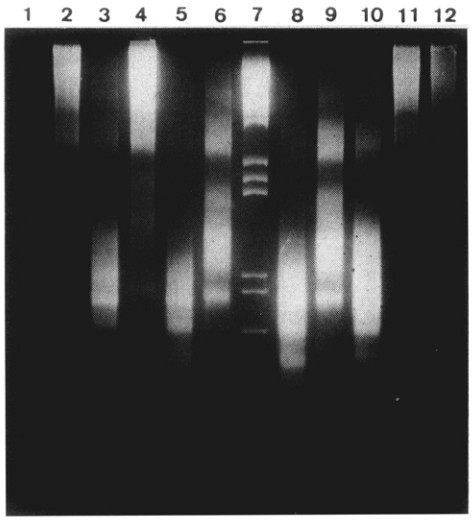


FIGURE 4: Comparison of DNA fragment patterns on 6% polyacrylamide slab gel electrophoresis of middle (30 min) and late (1 and 2 h) digestion intervals of chromatins from immature gonads (slots 1, 3, and 5) and ripe sperm (slots 2, 4, and 6). DNA samples were alternated in order of increasing time intervals. Slots to the right half of the slab contained respectively a twofold load of the same samples but in reverse order, starting with immature gonad DNA from 2-h digestion and ending in the rightmost slot with sperm DNA from 30-min digestion. PM2 *Hae*III restriction fragments (slot 7) were run as migration markers. Note that the DNA band present in all sperm digests, particularly visible at late intervals, migrates in a position very close to that of the restriction fragment K (272 bp). Densitometer tracings of the photographic negative of the ethidium bromide stained gel are shown in Figure 5. Electrophoresis is from top (−) to bottom (+).

attached. The significance of these alternative possibilities is not clear; however, it seems to be a reflection of the resistance of chromatin to enzyme action. These results further support the notion that chromatin DNA appears singularly protected from degradation in sperm cells.

## Discussion

The results presented here on the assessment of the micrococcal nuclease digestion of chromatin from developing germ cells of the echinoderm *H. tubulosa* suggest that the nucleohistone complex is tightly packed during the growth stages, thus effectively hindering enzymatic access to developing chromatin. However, those chromatin regions actually accessible appear highly sensitive to further degradation to acid-soluble nucleotides. In contrast, sperm chromatin is easily converted into nucleosome oligomers, which in turn are much more resistant to further enzyme degradation. This differential susceptibility to nuclease seems to be a common trend among chromatins from related developing systems. Thus, sea urchin embryo chromatin appears to be rather resistant to enzyme fragmentation (Spadafora et al., 1976b), although the readily accessible regions are easily degraded to soluble nucleotides. Almost all embryo chromatin is rendered acid soluble on prolonged digestion. In opposition, the extent of sperm chromatin solubilization is much lower, and the chromatin appears to be wholly organized in stable components. Chromatins of *Physarum polycephalum* amoebas and plasmodia behave similarly (Stalder & Braun, 1978). The former is much more degradable by nuclease than plasmodial chromatin, even lacking a stable nucleosome core. Chromatin from logarithmically growing cells of both *Paramecium aurelia* and *Tetrahymena pyriformis* (Prince et al., 1977) also appears very nuclease sensitive as judged by both the rapid change in repeat patterns with the digestion course and the band

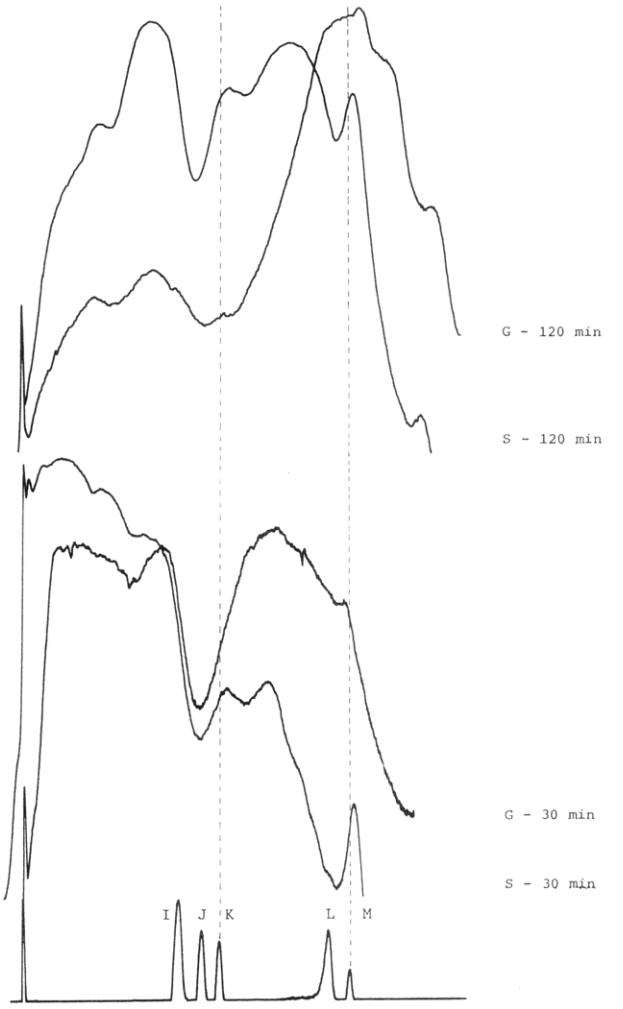


FIGURE 5: Densitometer tracings of DNA fragments resolved on a 6% polyacrylamide slab gel (Figure 4) from nuclease digests of immature gonads (G) and ripe sperm (S). Scans have been carefully aligned for the respective origins and superimposed at identical digestion intervals (times indicated to the right) to emphasize the appearance on sperm digests of a band intermediate between the dimer and monomer positions. The scan across the bottom corresponds to PM2 *Hae*III restriction fragments (I–M) electrophoresed in parallel on the same slab gel. Migration is from left to right.

broadness displayed by the monomer and nucleosome core as compared to chromatin from stationary cells.

Apparently, the gross behavior of chromatin from *Holothuria* immature germ cells seems rather perplexing. However, the overall observed resistance may be explained by assuming a tight superstructural organization at the onset of maturation which would efficiently hinder enzyme access. This external conformation, reflected to some extent in the observed nuclear compactness, might well exist along with an intrinsically "weak" fine structure of chromatin. This weakness would determine the rapid rate of degradation of those nucleosome particles located in the regions first entered by the enzyme which, on prolonged digestion, could progressively degrade the bulk of the chromatin. Although we consider this explanation valid, there is no direct evidence supporting the requirement for such structural arrangement during development.

The opposite behavior of the sperm chromatin may be accounted for, provided that basic features of the sperm cell are considered. The histone complement is maintained during spermatogenesis in *Holothuria*, with inclusion of an unusual group of lysine-rich histones as well as a histone H1 species resembling the more usual types (Phelan et al., 1972). Both

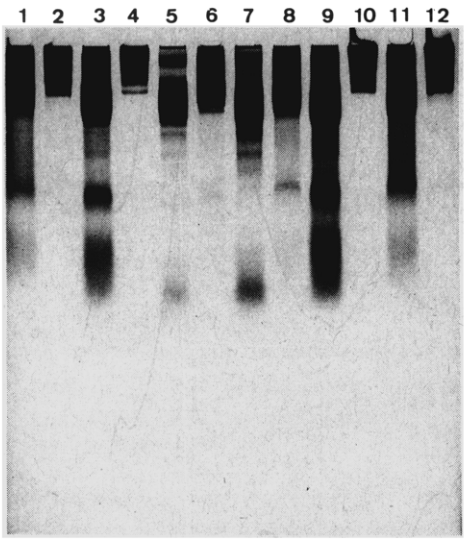


FIGURE 6: Comparison of DNA fragment patterns on 10% polyacrylamide slab gel electrophoresis of middle (30 min) and late (1 and 2 h) digestion intervals of chromatins from immature gonads (slots 1, 3, and 5) and ripe sperm (slots 2, 4, and 6). DNA samples were alternated from the leftmost slot in order of increasing digestion times. The slots to the right contained a twofold volume of the same samples loaded in reverse order. The slab was stained in Stains-all, destained in running water for 3 h, and photographed. Note that chromatin digests from immature gonads exhibit a set of small fragment sizes similar to those described previously for various chromatins (Axel et al., 1974), which are not present in sperm digests but for a diffuse band at the latest digestion interval. Migration is from top (–) to bottom (+).

H1 groups are rather similar except for a significant increase in the number of arginine residues in the unusual type, although the ratio of total basic to acidic amino acids is not altered. In sperm only, another species which appears is a basic minor histone named $\phi_0$ (Figure 7). In histone $\phi_0$, the total number of lysines and arginines amounts to 33 residues, with a lysine/arginine ratio of about 0.65 (amino acid composition not shown). Interaction of the novel H1 species with nucleosomal DNA may be expected to be similar, although somehow strengthened due to the increase in the arginine content. Histone $\phi_0$, by contrast, may exert a stronger influence on the nucleosome array. The insertion of $\phi_0$ into the chromatin fiber may be reasonably regarded as involving an opening up of the chromatin superstructure to allow for positioning of the incoming protein. Such conformational relaxation would indirectly favor nuclease access. This assumption is supported by the low extent of chromatin condensation in *Holothuria* sperm, which is reflected in a 20-nm diameter of the fiber, comparable to that found in somatic nuclei (Pladellorens & Subirana, 1975). In addition, it is consistent with the much greater susceptibility to nuclease found in sperm chromatin (Figure 1). On the other hand, the actual presence of histone $\phi_0$ in sperm nucleosomes (unpublished experiments) can be presumed to modify to some extent their fine structure and therefore affect nuclease action. The data suggest that there should exist a dependence between the distinctive features of sperm nucleosomes and the additional presence in *Holothuria* sperm chromatin of the basic $\phi_0$ species. It is reasonable to think that this dependence is expressed in a reinforcement of the histone–DNA interaction rather than in a gross rearrangement of the nucleohistone complex. A gross rearrangement would imply alteration of the DNA repeat during maturation. In actuality, no change in the repeat length has been detected throughout germ cell development.

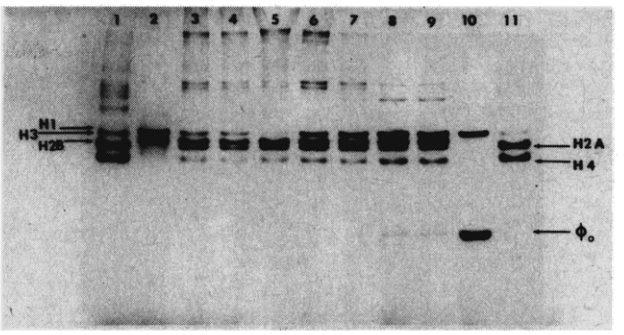


FIGURE 7: Electrophoretic separation of the various histone species from *Holothuria* germ cells at different stages of maturation. Whole histones were prepared from isolated nuclei by acid extraction with 0.25 N HCl. The crude acid extracts were filtered through 0.45-$\mu$m Millipore filters, dialyzed against 0.1 M acetic acid, and lyophilized. Single histones and histone fractions used as controls were extracted from sperm chromatin according to the procedures of Johns (1964). The histone samples were dissolved in 10 M urea, 0.1 M acetic acid, 5% 2-mercaptoethanol, and 10% glycerol and characterized by electrophoresis in acid–urea–polyacrylamide slab gels (Panyim & Chalkley, 1969) containing 12% acrylamide and 1.5 M urea. About 40 $\mu$g of whole histones was loaded onto the slab wells. Control histone fractions, as well as individual histones, were applied to the wells in 10-$\mu$g amounts. Pyronine Y was used as the tracking dye. Electrophoresis was carried out at 100 V until the dye had migrated 8 cm (about 5–6 h). Gels were next stained for 2 h in 0.1% amido black in 7% acetic acid–20% ethanol and destained by diffusion in the same solvent. The histone patterns correspond to the following: resting gonad (slot 3); activation stage (slot 4); early and late growth period (slots 5 and 6); breeding phase (slot 7); maturing and ripe sperm (slots 8 and 9). The following histone fractions are shown: H3/H2A/H4 fraction (slots 1 and 11); H1 and H2B (slot 2); H1 and $\phi_0$ (slot 10). Note the appearance of histone $\phi_0$ in the sperm cells. Electrophoretic migration is from top (+) to bottom (–).

The positioning of this protein in the nucleosome may be correlated with the results reported here for sperm chromatin. It may be visualized in two alternative ways: either near the DNA linker region or directly on the intracore DNA. The simultaneous presence in both sites is unlikely due to the low proportion of $\phi_0$ in the sperm histone content as reported by Subirana (1970). Furthermore, it is presumed that the interaction of $\phi_0$ with DNA may well be cooperative with that of the unusual histone H1. Therefore, it would further protect those specific DNA linkers to which the latter is attached, in turn enhancing nucleosome stability. The lower extent of DNA degradation detected in sperm chromatin as compared to that found for immature gonad chromatin correlates with this suggestion, as does the smaller number of subnucleosomal fragments seen on electrophoretic DNA patterns of sperm digests. The proposed cooperative effect might simply involve covering the internucleosome DNA stretch not interacting with histone H1. Specific DNA linker sites effectively protected from enzyme access and with a discontinuous distribution would become established, hence giving rise to different DNA fragments upon nuclease digestion. This assumption can be correlated to the values reported on Table II. Thus, if we consider a 460-bp dimer derived from the average sperm repeats (Table I) at early digestion intervals (5 and 15 min), the oligomer DNA length either correlates with the sum of the corresponding average sperm core size (157 bp) and that of the intermediate fragment between dimer and monomer positions (297 bp) or else correlates with twice the average monomer size. The latter value might be slightly overestimated due to less trimming of DNA linker trails on mild digestion. Similar correspondence is apparent at moderate digestion intervals (30 and 60 min). Therefore, it can be inferred that either intermediate fragments plus corresponding cores or sets

of two regular monomer fragments may alternatively arise from sperm dimers.

The existence of distinct linker regions as regards nuclease recognition, specified by the lysine-rich histones and the $\phi_0$ species, appears to explain the alternative release of DNA fragments. Those intercore regions associated with the unusual H1 and $\phi_0$ would give rise to particles having longer DNA linker tails and averaging 275 bp in total length (Table II) plus adjacent cores. The model of the asymmetrical binding of H1 to the nucleosome (Hayashi et al., 1978) helps to illustrate how $\phi_0$ might bridge the portion of the linker region not covered by H1, consequently compelling the enzyme to cut close to the contiguous core. In this respect, it is worth noting that the linker region between neighbor nucleosomes as derived from the average repeat (235 bp) measured at the briefest digestion of chromatin from both immature gonads and sperm, and assuming a 140 base pair limit core, appears to be about 95 base pairs long. This value is a good approximation of the sum of the contents of lysines and arginines (99 residues) in both the unusual H1 and $\phi_0$; thereby, it further supports the proposed correspondence between basic residues in lysine-rich histones and DNA linker length (Noll, 1976). Although there is a good correlation among the values reported, more detailed quantitation of the levels of both H1 groups during maturation in *Holothuria* gonads is needed as well as the precise location of $\phi_0$ in the nucleosome to unambiguously confirm these predictions.

The longer intermediate particle is considered to be a monomer having longer DNA tails rather than a dimer composed of two closely packed cores as seems to be the case for yeast chromatin (Lohr et al., 1977b), since it is much more nuclease sensitive than the other oligomeric fragments, as indicated by the drastic size variation observed during digestion. At late digestion intervals, the fragment resolves to a size significantly smaller than that required for two stacked cores. In addition, the existence of such a closely packed fragment should be an intrinsic property of the structure of "native" chromatin, and therefore it should be independent of the process of development. On the contrary, the results presented here indicate that a fragment of these characteristics can only be observed in ripe sperm chromatin, and it is wholly absent from immature gonad chromatin. This specificity strongly suggests that the appearance of this fragment may arise from distinctive interactions of the sperm histones with nucleosomal DNA.

The evidence reported here concerning nucleosome organization in developing germ cells of *Holothuria* suggests that differential susceptibilities to nuclease emerge in chromatin during development. In addition, it argues that variation in genetic activity may not necessarily imply a concomitant change in the DNA repeat length. The presence of lysine-rich histone variants and other histone species may modify DNA accessibility without altering the periodicity of the nucleosome distribution in chromatin.

## Added in Proof

After this manuscript was submitted for publication, Keichline & Wassarman (1979) reported similar observations for chromatin from developing sea urchin embryos. It appears that the DNA repeat length remains the same throughout sea urchin development. On the other hand, the only change detected in chromatin during embryogenesis is a decrease in the extent of DNA degradation by nuclease. In addition, sea urchin sperm chromatin appears to be particularly stable to enzyme digestion. These results further support a correlationship between DNA accessibility and the presence of unique histone variants in chromatin.

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# Distribution of Repair-Incorporated Nucleotides and Nucleosome Rearrangement in the Chromatin of Normal and Xeroderma Pigmentosum Human Fibroblasts†

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ABSTRACT: The distribution of UV-induced DNA repair synthesis in chromatin was measured in normal human fibroblasts and in xeroderma pigmentosum (XP) fibroblasts which are partially deficient in excision repair. With normal cells we investigated the effects of hydroxyurea and UV dose on the initial distribution of nucleotides incorporated during repair synthesis and the subsequent changes in distribution during nucleosome rearrangement [Smerdon, M. J., & Lieberman, M. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4238]. These cells were pulse-labeled with [$^3$H]dThd for 30 min immediately after irradiation and chased for varying times with unlabeled dThd. The initial distribution (0 chase time) of repair-incorporated nucleotides in chromatin indicated that most of these nucleotides are initially staphylococcal nuclease sensitive, and this distribution was unaffected by either the presence of 10 mM hydroxyurea or the amount of damage (3–40 J/m$^2$). The rate at which these repair-incorporated nucleotides became increasingly nuclease resistant was also unaffected by hydroxyurea or UV dose. These data lead to two conclusions: (1) under our conditions hydroxyurea has no measurable effect on either the initial distribution of repair-incorporated nucleotides or the subsequent rate of redistribution of these nucleotides during nucleosome rearrangement; (2) if nucleosome rearrangement is *induced* by damage and/or the repair process, then this induction is a "local event". Cells from XP complementation groups C and D were irradiated with 3 J/m$^2$ UV, pulse-labeled for 60 min, and chased for varying times. In both groups, the initial distribution (0 chase time) indicated that most of the repair-incorporated nucleotides are initially staphylococcal nuclease sensitive. Electrophoretic data demonstrated a concurrent underrepresentation of repair synthesis in core DNA. With increasing chase time, the distribution of repair-incorporated nucleotides became more uniform in both groups of XP cells. Thus, both the initial enhanced sensitivity of repair-incorporated nucleotides to staphylococcal nuclease and the subsequent changes in sensitivity of these nucleotides during nucleosome rearrangement reported for normal cells occur in at least two complementation groups of XP cells.

It is rapidly becoming apparent that the constraints placed upon DNA in chromatin (Kornberg, 1977; Felsenfeld, 1978) play a significant role in the distribution of excision repair synthesis in mammalian cells. Many of the nucleotides incorporated by repair synthesis, induced by either chemical damage (Bodell, 1977; Tlsty & Lieberman, 1978) or UV[1] (Cleaver, 1977; Smerdon et al., 1978; Smerdon & Lieberman, 1978a,b), are rapidly digested by both staphylococcal nuclease and DNase I. Results from our laboratory indicate that, in fact, almost all repair-incorporated nucleotides are initially sensitive to staphylococcal nuclease and are present at only very low levels in core DNA. Furthermore, our results from pulse-chase experiments indicate that the repair-incorporated nucleotides become more staphylococcal nuclease resistant during the chase period and suggest that nucleosome rearrangement occurs in the regions of repaired DNA[2] (Smerdon

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[1] Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; XP, xeroderma pigmentosum; UV, ultraviolet radiation.

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