THE LENGTH AND DISTRIBUTION OF ACCESSIBLE DNA REGIONS IN HUMAN CHROMATIN IN SITU

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SUMMARY: The structure of chromatin in intact nuclei has been investigated by studying the kinetics of DNA degradation with exogenous nucleases. We find that human chromatin in its native state consists of relatively inaccessible DNA regions alternating with accessible regions of essentially uniform length. The length of the accessible regions, as calculated from two independent sets of data, is about 50 base pairs.

INTRODUCTION: In recent years much has been learned about the relationship of the histones to chromatin structure. Four of the five major mammalian histones have been shown to form an octomer (1) which interacts as such with DNA, forming a discrete structure (1,2) which has been called a nucleosome. Nucleosomes have been visualized with the electron microscope (3) and have been isolated after deoxyribonuclease treatment of chromatin (4). The DNA in the nucleosome is relatively protected from nucleases, since progressive degradation of isolated chromatin leads to a discrete "unit" size of DNA (5) corresponding to that found in the isolated nucleosome (4,6). A protected DNA unit (about 150-205 base pairs) is also obtained when chromatin is degraded *in situ*, *i.e.* in intact nuclei (7,8); this indicates that inaccessible (herein called nucleosomal) DNA units separated by nuclease-accessible DNA regions are present even when the chromatin is in its undisturbed native state. It is important to establish whether the sizes and distribution of these regions *in vivo* are the same as those which have been observed *in vitro*. This report is concerned with this question. We find that the nucleosomes of intact, intracellular human chromatin are separated by about 50 base pairs of accessible DNA, and that this spacing is approximately uniform *in situ*. A preliminary report of these findings was presented in March, 1975 (9). The structural and functional implications of these observations are considered elsewhere (10).

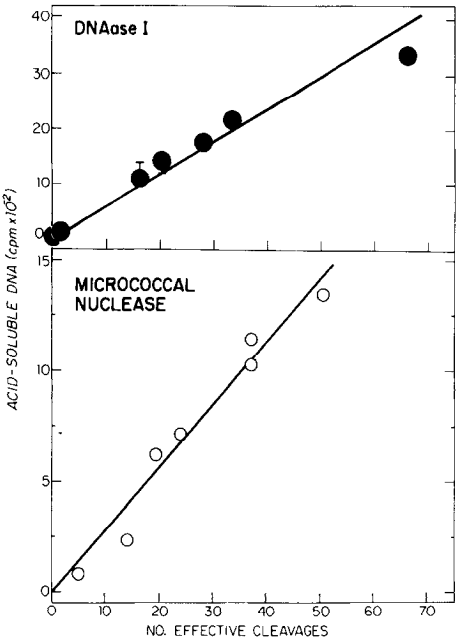
Several earlier publications, all based on electron microscopy and utilizing isolated chromatin, have reported much larger (up to 1500 base pairs), much smaller or extremely varied inter-nucleosome spacings (4,11,12,13). These discrepancies probably arise from distortions produced during preparation of specimens for electron microscopy or from artifacts during chromatin preparation. In most cases the chromatin had been subjected to various degrees of shear as part of the preparation procedure. As Noll *et al.* (8) have pointed out, the shear used to solubilize chromatin distorts its structure, thereby changing its accessibility to nucleases. Thus the large and variable spacings observed probably result from the pulling out of DNA from the ends of some of the nucleosomes. Loss of some histone during the preparation is probably an additional factor; Oudet *et al.* (12) have shown that the spacing in reconstituted chromatin is

proportional to the histone/DNA ratio, as would be expected. No doubt these experimental artifacts also contribute to the smaller protected DNA unit size (about 100-120 base pairs) and the large amount of acid-soluble DNA produced by nuclease treatment of chromatin in solution (4,5) rather than in situ. An additional problem may arise from a tendency of nucleosomes to aggregate (14), thereby making it impossible to visualize their spacing.

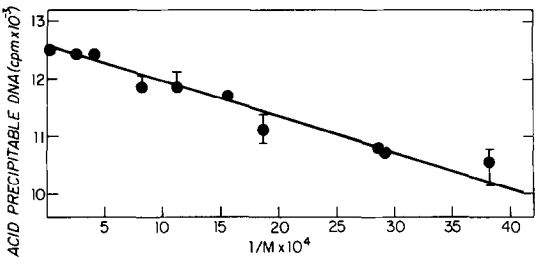
The problem of shear is circumvented in the electron-microscopic work of Griffith (14), which appeared simultaneously with the first presentation of our work (9). The isolated SV40 nucleoprotein studied by him is so small as to be, in all likelihood, exempt from the shear artifact. When conditions were chosen to disaggregate the native structure into the "string of beads" configuration, the inter-nucleosomal distance measured in vitro by Griffith was essentially the same as our in situ value, obtained by an entirely different method. Another type of in vitro study has also given a similar value (15). We feel that the convergence of the two approaches strengthens the conclusion that there is a fixed distance of about 50 base pairs between nucleosomes in situ.

METHODS: A normal human lymphocytic cell line, WiL_2, was labeled during logarithmic growth for about 1 generation with ^{14}C-thymidine (0.25 µCi/ml, 49 mCi/mM). The cells were washed, suspended in 0.15 M NaCl - 0.01 M Tris pH 7.8 - 0.001 M $MgCl_2$ and lysed by treatment with 0.1% Nonidet-P40 for 15 minutes at 0° C. Nuclei were isolated by centrifugation for 5 minutes at 430 xg and suspended in 0.15 M NaCl - 0.01 M Tris pH 7.1 to a concentration of 2 x 10^7 per ml. The nuclei were almost entirely free of cytoplasmic contamination, as judged by methyl green-pyronin staining. For nuclease treatment, 1 ml of fresh nuclear suspension was incubated at 25° C for 30 minutes with a fresh solution of pancreatic DNAase I (0-45 µg/ml) (Worthington, low RNAase grade) and 0.01 M $MgCl_2$, or with micrococcal nuclease (0-30 u./ml) (Worthington) and 0.01 M $CaCl_2$. Varying the time of incubation with a fixed concentration of nuclease gave similar results. Nuclei remained intact for several days under these conditions. The degradation was terminated and the nuclei lysed by diluting the 1.02 ml reaction mixture to 5 ml with 0.02 M Tris pH 8.3 - 0.02 M EDTA - 3% sodium lauroyl sarcosinate (Sarkosyl) at 0° C. The DNA in the lysate is stable indefinitely at -20° C.

RESULTS AND DISCUSSION: Samples of nuclei isolated from ^{14}C-thymidine-labeled, cultured normal human lymphocytes, which contain negligible endogenous deoxyribonuclease activity under the conditions employed, were treated with various concentrations of an exogenous nuclease so that any accessible DNA in the nuclei was degraded to various extents. The reaction was then terminated in each sample, the nuclei lysed, and the molecular weight of the DNA determined by sedimentation. From the molecular weight data one can calculate for each sample the number of effective cleavages that have occurred per molecule, i.e. the number of events resulting in a decrease in molecular weight. This quantity is $(M_o/M - 1)$, where M_o is the initial, and M the final, molecular weight. We then determine the amount of DNA which has been converted to acid-solubility for each effective cleavage. Fig. 1 shows that this



Legend to Figure 1. In situ degradation of DNA to acid-solubility, as a function of the number of effective cleavages per molecule. Degradation results from varying degrees of treatment of intact nuclei with an exogenous deoxyribonuclease. An effective cleavage is defined as an event or set of events which results in a decrease in the molecular weight of the macromolecule. The number of effective cleavages is calculated as $M_o/M - 1$, where M_o is the molecular weight of a relatively undegraded reference sample (for the sample chosen, M_o = 13,400 base pairs) and M is that after further degradation. The actual M_o chosen does not alter the conclusions reached. Molecular weights were determined as in Table I. Closed circles: Nuclei were treated with DNAase I (0-45 µg/ml) and then lysed as described in "Methods." Then 50 µl aliquots of the lysates were precipitated with TCA and counted as described in Table I. The amount of acid-soluble DNA produced was calculated as $AP_o - AP$, where AP_o is the acid-precipitable radioactivity in the reference sample (12,660 cpm). From three to six samples of each lysate were counted and averaged, and the range of each calculated value is shown by a vertical line wherever it exceeds that indicated by the size of the circle. Open circles: Nuclei were treated with micrococcal nuclease (0-30 units/ml) and lysed as described in "Methods." The acid-soluble (AS) DNA in 50 µl aliquots of the lysates was determined directly by filtering the TCA-precipitated lysates, collecting the acid-soluble filtrate and drying an aliquot of it in a counting vial for 4 hrs. under an infra-red lamp; the residue was dissolved in 0.1 ml water to which 10 ml of a dioxane-based scintillation fluid was added. The counts were corrected for background (about 28 cpm), aliquot volume and quench, and the acid-soluble counts in the reference sample (AS_o = 35 cpm) were subtracted. Each point represents the average of three determinations.



Legend to Figure 2. Determination of the length of an accessible DNA region in chromatir Cells were labeled, the nuclei isolated, treated with micrococcal nuclease (0-30 units/ml and lysed as described in "Methods;" the molecular weight (in terms of base pairs) and acid-precipitable counts of the DNA were determined as in Table I, which contains the data on which this curve is based. Each point represents an average of 3-6 determinations; the range is indicated by the size of the circle or the vertical bar. The length of an accessible DNA region, calculated from the slope and intercept of this curve as described in the text, is 50 base pairs.

value is constant, since the curves are linear. We conclude that every time an effecti cleavage occurs in an accessible region of DNA, the entire accessible region is degrade to acid-solubility (16). Otherwise, cleavage would leave accessible DNA "tails" which would be vulnerable to degradation upon more extensive nuclease treatment, resulting in the production of an increasing amount of acid-soluble DNA per cleavage as the number of cleavages increased; this would result in upward curvature in Fig. 1, which is not observed. Thus the acid-soluble DNA produced per cleavage can be translat into the number of base pairs in a nuclease-accessible region. These regions lie between nucleosomes (4,7,8).

We can also deduce from the linear curves in Fig. 1 that nucleosomes are more or less uniformly spaced. If there were both large and small inter-nucleosome regions the larger ones would be attacked first, on the average, and the curves would tend to be non-linear.

The determination of the size of an accessible DNA region is shown in Fig. 2 (the direct data from which the curve is derived is presented in Table I). From this curve, obtained with micrococcal DNAase, we calculate a size of 50 base pairs, as follows: The fraction of DNA degraded to acid-solubility as the molecular weight drops from any arbitrary value M_o to M is $(AP_o - AP)/AP_o$ (where AP indicates the amount of acid-precipitable DNA after degradation, and AP_o before, measured as cpm); the number of base pairs degraded per DNA molecule is $(AP_o - AP)M_o/AP_o$ (where M_o is expressed as base pairs). The number of base pairs degraded per effective cleavage, shown above to be the number of base pairs per accessible region (R), is therefore

(1) $$\frac{(AP_o - AP)M_o}{AP_o} \Big/ \left(\frac{M_o}{M} - 1\right) = R$$

Rearranging,

(2) $$AP = -R\ AP_o\left(\frac{1}{M}\right) + \frac{AP_o\ (R + M_o)}{M_o}$$

This equation gives a straight line when AP is plotted vs 1/M. Dividing the intercept by the slope gives

TABLE I

DEGRADATION OF DNA IN INTACT NUCLEI BY MICROCOCCAL NUCLEASE

Nuclease Concentration (units/ml)	Acid-Precipitable DNA(cpm)* (Av. of 3-6 samples)	Sedimentation Constant**	Molecular weight(base pairs)†
0	12,520	28.6	27,642
1	12,460	14.3	3,727
2	12,440	12.3	2,413
3	11,880	9.7	1,215
5	11,850	8.7	887
10	11,710	7.8	646
15	11,120	7.3	534
20	10,790	6.3	349
25	10,750	6.3	349
30	10,550	5.7	261

*The amount of acid-precipitable radioactivity in DNA from 50 µl of lysate (corresponding to 2 x 10^5 nuclei) was determined by placing the samples on Whatman GF/B glass fiber filters held in individual compartments of an especially designed tray, and immersing the tray in 5% TCA at 0° C for 20 minutes. The filters were then washed by shaking the tray gently in 1% TCA for 10 minutes; after repeating the TCA wash the tray was shaken in three changes of water for 3 minutes each. We have shown that there is complete removal of acid-soluble material under these conditions, and complete retention of acid-precipitable material. The filters were dried, oxidized in an Intertechnique "Oxymat" sample oxidizer, and counted in the Intertechnique ^{14}C scintillation fluid.

**Sedimentation constants were measured by layering 0.5 ml of lysate on 4.8 ml of a 4-24% sucrose gradient containing 0.1 M NaCl - 0.01 M EDTA - 0.01 M Tris pH 7.8 - 0.5% Sarkosyl; the sample was centrifuged for 150 minutes at 40,000 rpm, 24° C, in the Spinco SW 50.1 rotor. Fractions were collected from the top of the tube, using a Buchler Auto-Densi-Flow apparatus, and were precipitated and washed as described above*. The dried filters were counted in a toluene-based scintillation fluid. The sedimentation constant was determined from the distance traveled down the tube, after calibrating the system with RNA's and enzymes of known S. The sedimentation constants are reproducible and the effect of the initial layer is negligible in the molecular weight range studied, as shown by the fact that the same constants are obtained under altered, separately-calibrated conditions.

†Molecular weights were calculated from sedimentation constants using the equation of Studier (17), and then converted to base pairs.

$$\frac{AP_o\ (R + M_o)}{M_o}\left(\frac{1}{R}\ \frac{1}{AP_o}\right) = \frac{1}{M_o} + \frac{1}{R} \quad .$$

Since $1/M_o$ is very much smaller than 1/R, the former can be neglected and R can be calculated from the slope and intercept of the curve in Fig. 2.

Using pancreatic DNAase I the calculated length of accessible DNA regions between nucleosomes is about 63 base pairs. Our data for DNAase I (Fig. 1) are less extensive than our analogous data for micrococcal nuclease, and in addition may give an estimate on the high side for the following reason: at the highest nuclease concentrations utilized here we have found that DNAase I, but not micrococcal nuclease, produces some internal single-strand breaks in nucleosomal DNA (shown by sedimentation before and after heat-denaturation); it is therefore possible that occasionally more than one inter-nucleotide bond is broken at these internal sites, thereby producing acid-soluble nucleotides but no effective cleavage. We therefore feel that the micrococcal nuclease data is more reliable and that the length of the inter-nucleosome region in human chromatin is close to 50 base pairs. This size is similar to that measured by Griffith (14) in electron micrographs of SV40 nucleoprotein, namely 39±8 base pairs. Likewise, both studies indicate approximately uniform spacing of nucleosomes. Knowing the length of inter-nucleosomal DNA, the size of nucleosomal DNA can be estimated as about 150 base pairs (18).

At much higher nuclease concentrations than those utilized for the figures the molecular weight drops below 150 base pairs and we have observed as much as 60% acid-solubility. Under these conditions the data do not fall on the linear curves of Figs. 1 and 2 but are too high in acid-solubility. Clearly this is due to the ability of nucleases to attack the "protected" DNA within the nucleosome when the nuclease concentration is sufficiently high. But in the nuclease range studied here the linearity observed in Figs. 1 and 2, plus the lack of intra-nucleosomal nicks with micrococcal nuclease, indicate that the degradation studies presented here (at least those utilizing micrococcal nuclease) refer only to degradation of DNA between nucleosomes.

These observations on chromatin structure raise the question of the functional significance of the regularly-spaced accessible and inaccessible DNA regions in chromatin. We discuss this subject in another report (10), which also considers the question of whether or not the accessible DNA region is structurally a part of the nucleosome.

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16. The endonucleases employed appear to make repeated, random single-strand DNA scissions which eventually result in an effective cleavage when two scissions on opposite strands are located within a few nucleotide pairs of each other. Once an accessible DNA region is attacked the nuclease molecule apparently tends to remain in that region, probably due to diffusional restraints imposed by chromatin structure.

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18. The size of the histone-protected DNA unit *in situ* has been v iously measured as 150-205 base pairs. It would be possible to refine this esi nate, given the reasonable certainty that the intervening accessible DNA reg: ns are 40-50 base pairs long, if one had a reliable value for the stoichiometric ratio of DNA to histone in mammalian chromatin. This can be calculated from the mammalian weight ratio of DNA to histone (which has not been accurately established) and the histone molecular weights (19,2) as about 155-185 base pairs of DNA per histone oligomer. Other methods for calculating this value give rather different results. For example, the number of nucleosomes per SV40 DNA molecule has been determined as 21±1 by electron microscopy (14), and is indirectly indicated to be 24±2 based on Keller's (20) determination of the number of superhelical turns in SV40 DNA I and the demonstration by Germond *et al.* (21) that there is one superhelical turn per nucleosome. Thus 22 is the most probable number of nucleosomes per SV40 DNA molecule. The molecular weight of SV40 DNA, however, is not accurately known; measurements range from 2.9 - 3.6 x 10^6 (14,22). These data indicate that there are probably 200-250 base pairs per histone oligomer, although the range of uncertainty is considerably larger. Alternatively, one can use the electron-microscopically measured contour lengths of SV40 DNA and nucleoprotein and the diameter of the nucleosome (14) to calculate that there is 1 nucleosome per 193-266 base pairs (210-229 base pairs, using average values for the various measurements), assuming that the lengths of the measured samples are the same as their lengths in solution. These three different calculated estimates can be reconciled only by assuming that there are approximately 190-200 base pairs of DNA per histone oligomer in chromatin. Thus, subtracting the 40-50 accessible base pairs, there are probably about 150 base pairs in the protected DNA of the nucleosome. This agrees with the lower end of the directly-measured size range.

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