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The effects of salt concentration and H-1 depletion on the digestion of calf thymus chromatin by micrococcal nuclease

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ABSTRACT

We have removed histone H1 specifically from calf thymus nuclei by low pH treatment, and studied the digestion of such nuclei in comparison with undepleted nuclei. By a number of criteria the nuclei do not appear damaged. The DNA repeat-length in nuclear chromatin is found to be the same (192 ± 4 bp) in the presence or absence of H1. These experiments demonstrate that the core histone complex of H2A, H2B, H3, and H4 can itself protect DNA sequences as long as 168 bp from nuclease. Our interpretation is that this represents an important structural element in chromatin, carrying two full turns of superhelical DNA. Depending on conditions of digestion this 168 bp fragment may be metastable and is normally rapidly converted by exonucleolytic trimming to the well-known "core-particle" containing 145 bp. Larger stable DNA fragments observed in digestion of H-1 depleted nuclei appear to arise from oligomers assembled from 168 bp cores in close contact exhibiting trimming of 0-20 bp at the ends. Electrophorograms of undepleted nuclear digests reveal oligomer bands in several size classes, each corresponding to one or more combinations of 168 bp particles, H1-protected spacers of about 20 bp length, and particles with ends trimmed to varying degrees.

INTRODUCTION

According to the "core and spacer" model (1-3), the universal unit of chromatin structure is the core particle which contains about 140 bp (more recent estimates (4) would be near 145 bp) of DNA and the octamer of inner histones. The spacer is held to be heterogenous in length, with the average length differing from cell type to cell type, leading to the now well-recognized variance in the "repeat length" of various chromatins as defined by micrococcal nuclease digestion (5, 6).

The picture is so complete as to be almost cozy. However,

for some time a number of bothersome problems have continued to reappear. Noll and Kornberg (7) pointed out the transient stability of larger DNA fragments during the digestion. Similar evidence for somewhat stable particles containing DNA larger than 145 bp has been reported a number of times (8-12). Such reports, however, have been complicated in many cases by the presence of H1 in at least some of the particles.

We wished to ask the question as to whether the inner histones alone could protect more than 145 bp of DNA. Thus we had to work with chromatin depleted of H1; ideally the depletion would be completed in situ. Learning of the technique of Cole et al (13, personal communication) for H1 removal at low pH, we utilized this method to prepare calf thymus nuclei free of H1. Suspecting that the mode of action of micrococcal nuclease might be sensitive to salt concentration in the digestion mixture, we have employed variation in this parameter to produce quite varied patterns of digestion in both H1-depleted and undepleted (native) nuclei.

MATERIALS AND METHODS

Isolation of Nuclei: Thymus tissue was removed from calves during slaughter and cooled by immersion in iced 0.05 M Tris HCl, pH 7.2 containing 0.2 mM phenylmethylsulfonylfluoride (PMSF). After removal of connective tissue and blood vessels it was sliced into 20 mm thick strips, frozen in liquid nitrogen and stored at -80°C. Blocks of frozen tissue weighing 10 - 30 g were removed from storage, re-immersed in liquid nitrogen, then sliced as thin as possible on a chilled glass plate. Periodically the tissue flakes were transferred to cold buffer (Wray & Stubblefield [14]) containing 0.2 mM PMSF. The slurry was homogenized 15-30 sec. at the lowest possible speed in a Virtis homogenizer. About 50% of the nuclei were released from the tissue fragments by this process. Large lumps of unbroken cells and tissue debris were removed by pouring the homogenate first through four layers of cheesecloth, then vacuum-filtering through a single layer of Miracloth. The filtrate containing

free nuclei and small cytoplasmic debris was centrifuged through two cycles of 7 min at 280 g. Nuclei purified by this method were homogenous, free of visible cytoplasmic debris and wholly intact.

Extraction of nuclei: Histone H1 was removed from intact nuclei using the technique of Cole et al (13), but eliminating pre-treatment with Triton X-100. Nuclei were extracted with buffer consisting of 0.25 M sucrose, 0.05 M glycine, 0.025 M KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM PMSF, pH 2.2. Two extractions of 15 min each with periodic agitation sufficed to remove virtually all H1 without removing significant quantities of any other protein (cf. Figure 1A). Careful scrutiny of overloaded gels reveals traces of proteins which electrophorese slightly faster than H1 and which remain attached to nucleoprotein. This mater-

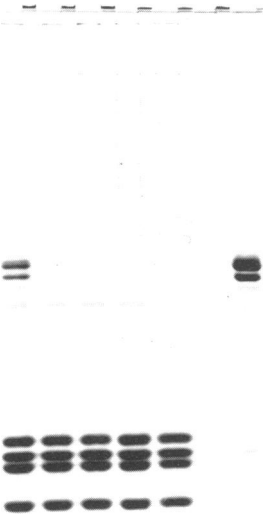

Figure 1A. SDS-gel of the total protein content in (left to right): native nuclei; H-1 depleted nuclei after digestion at 0, 0.1, 0.3, 0.45 M NaCl; and in the extraction supernatant.

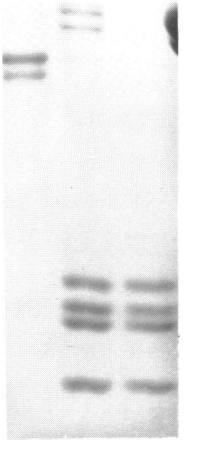

Figure 1B. SDS-gel of the proteins contained in H-1 depleted nuclei (right) whole nuclei (center) and in a 5% perchloric acid extract of H-1 depleted nuclei (left,heavily loaded).

ial shares with H1 the solubility in 5% perchloric acid (cf. Figure 1B). We conclude this represents a fraction of all the subclasses of histone H1 which binds more tightly to DNA and is modified in such a way as to remain insoluble in extraction buffer. This "acid resistant H1" is present in all preparations in a constant small percentage, and is resolved as 3 bands spaced identically to the H1 bands on SDS gels. Note that this material appears in preparations in which there was no evidence of proteolysis. Furthermore, the "acid resistant H1" was never observed in more than trace amounts; the extracted nuclei can be considered essentially free of H1.

Acid solubilities: Measurements of acid soluble and acid insoluble DNA were performed as described by Lohr et al (6) with additional provisions for independent measurement of total DNA.

Time-course of digestion: Micrococcal nuclease (Worthington) was assayed by the method of Heins et al (15). Native and H1-depleted nuclei were digested at concentrations of A_{260}= 50 with 200 units micrococcal nuclease per ml at 37°C. The buffer in all cases was 0.1 mM PIPES, 1 M hexylene glycol, 1 mM $CaCl_2$, pH 7.0 (Wray and Stubblefield [14]). The ionic strength of this buffer was about 3 mM. Higher ionic strengths were achieved by addition of solid NaCl. Aliquots of nuclear suspension were withdrawn at selected times, chilled and brought to a final EDTA concentration of 10 mM. Part of each aliquot was used for the determination of acid solubility. The DNA of the rest was extracted by standard procedures (16) and analyzed by gel electrophoresis (Lohr et al [6]). DNA sizes were determined by interpolation from a semi-logarithmic calibration plot of the size of PM2-fragments vs. distance of migration. The PM2 fragment sizes have been re-calibrated by coelectrophoresis with sequenced SV40 fragments (R. T. Kovacic, unpublished results).

RESULTS

Activity of micrococcal nuclease in salt: The digestion of chromatin with micrococcal nuclease has to be performed under non-optimal conditions for the enzyme insofar as pH and Ca^{++} concentration are concerned. Optimal conditions for hydrolysis

of DNA are pH 7.5 to 11 and 10 mM Ca^{++} (17). In contrast, we digested the nuclear chromatin at pH 7.0 in presence of 1 mM Ca^{++}. In order to allow a comparison of the enzymic activity on double-strand DNA and chromatin we assayed the enzyme with calf thymus DNA at 1 mM Ca^{++}, pH 7.0, at various concentrations of NaCl using the method of Heins (15), with supplementary measurements by the method of Dirksen and Dekker (18). Measurable rates of reaction at 0.45 and 0.65 M NaCl were achieved by increasing the amount of enzyme ten to hundred-fold. We found the activities unaltered by varying the enzyme concentration over a three-fold range at every ionic strength. The results are presented in Table I.

Digestion time-courses of H1-depleted nuclei; effect of ionic strength: Digestions were performed in Wray-Stubblefield buffer with the addition of varying concentrations (0 to 0.45 M) of NaCl. Figure 2 (left) shows scans of the typical band patterns of the DNA content of aliquots taken at various time-points during digestion. At low ionic strengths and early times oligomers up to tetramers are discernible. With increasing duration of digestion the higher bands are lost leaving only monomer and submonomer sizes after 120 min.

At higher ionic strengths (0.2 M NaCl, data not shown, and 0.35 M NaCl, cf. Figure 2) even higher oligomers (up to hexamers) can be distinguished. The individual bands are preserved longer

Table I. Effect of NaCl Concentration on Activity of Micrococcal Nuclease on DNA.

NaCl conc. (M)	Activity (%)	
0.044	100	(100)
0.100	63.9 ± 5	(60.8)
0.200	23.2 ± 5	(19.0)
0.300	7.0 ± 1.2	
0.450	2.5 ± 0.6	
0.650	0.4 ± 0.3	

The activity of micrococcal nuclease on double-strand DNA was measured by the assay of Heins et al. (15) at the various ionic strengths indicated. The maximum deviations of three independent measurements from their means are indicated. Values in parentheses were obtained by a kinetic assay published by Dirksen & Dekker (18).

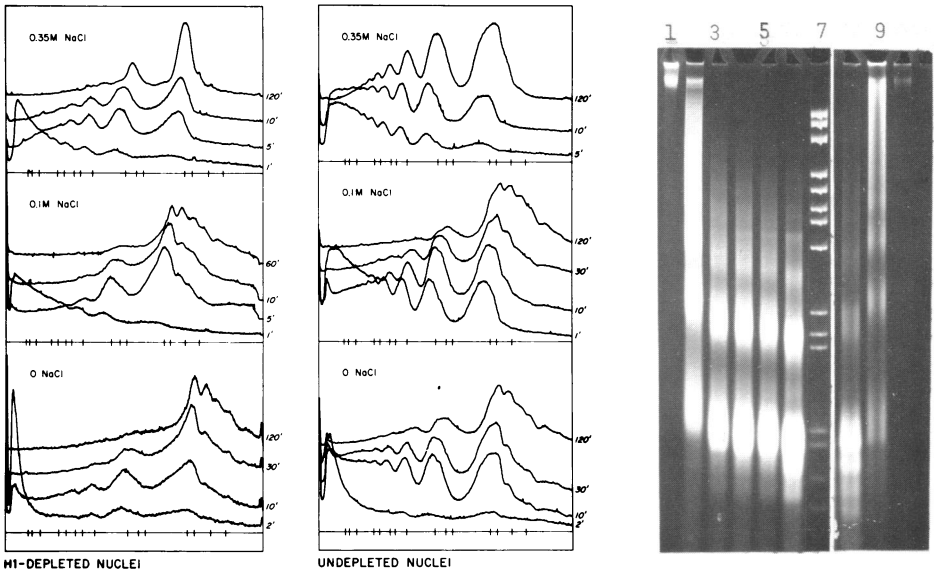

Figure 2: Effects of H-1 depletion and ionic strength in digestion medium on digestion of calf thymus nuclei. Left: Gel scans of DNA fragments obtained by digestion of H-1 depleted and undepleted (native) nuclei at times and added NaCl concentrations indicated. The tic marks indicate the positions of co-migrating PM2-Hae III fragments of sizes (left to right): 1810, 1600, 1270, 885, 833, 683, 631, 339, 301, 273, 166, 148, 122 and 100 bp. Right: Photograph of ethidium bromide stained gel showing DNA from: H1-depleted nuclei digested in 0.35 M NaCl for 0, 10, 120, 240 min (cols. 1-4); 240 min with extra enzyme added at 120 min (col 5); overnight at 37° (col 6); PM2-Hae III fragments (col 7); undepleted nuclei digested without added salt for 120, 10, and 0 min (cols 8,9,10).

and appear more symmetrical. The most prominent feature is the virtual absence of submonomer sizes at 0.35 M NaCl, even after extensive digestion. In contrast, DNA lengths smaller than about 130 bp are observed early in digestion at low ionic strength. A comparison of the gel patterns at 30 min (0 NaCl), 10 min (0.1 M NaCl) and 120 min (0.35 M NaCl) shows clearly that this suppression of digestion to submonomer sizes is not due to a differing extent of digestion since in all three cases the higher order bands are diminished to a comparable degree and the fractions of acid soluble DNA are very similar for these timepoints

(cf. Figure 4).

Scans of denaturing DNA gels including a selection of timepoints taken at various ionic strengths (data not shown) are virtually superimposable with scans of non-denaturing gels. Thus, there is no detectable single-strand nicking by micrococcal nuclease at any ionic strength monitored.

Due to the limited number of timepoints shown in Figure 2, individual bands seem to undergo a gradual shift in average size with increasing time at any ionic strength. When numbers of individual experiments are analyzed with very careful size determination of every peak or shoulder discernible in gel scans a more complex and subtle pattern is revealed. We find in each oligomer class a limited number of subclasses with a very small statistical variability in size. These same subclasses appear in most scans. With the continuation of digestion the staining intensity of the gels at these discrete positions varies, indicating a shift of mass between adjacent size subclasses. Most important, the same size subclasses are visible at the various salt concentrations monitored.

To make this fact more obvious, we have plotted all peak and shoulder positions from as many as six different scans at each ionic strength as a function of time of digestion (cf. Figure 3A). The points are arranged on a number of parallel lines. The resolution into reproducible subclasses is most clearly seen for monomers and dimers and at low ionic strength. Here, intermediate points between the lines are virtually absent at times longer than 2 min. The position of the main peak, as indicated by the filled points in Figure 3A is shifted down stepwise from line to line with increasing digestion. In parallel experiments the main peaks may appear in either one of two very similar bands at equivalent degrees of digestion, as indicated by multiple filled points at particular times.

This pattern of distribution of DNA size subclasses undergoes various changes upon increase of the NaCl concentration: In general, the number of subclasses observed for each oligomer class and the lifetime of each subclass is largest at 0.35 M NaCl and smallest at 0.1 M NaCl. Furthermore, the decrease in the size of the main peak is fastest at 0.1 M NaCl. However,

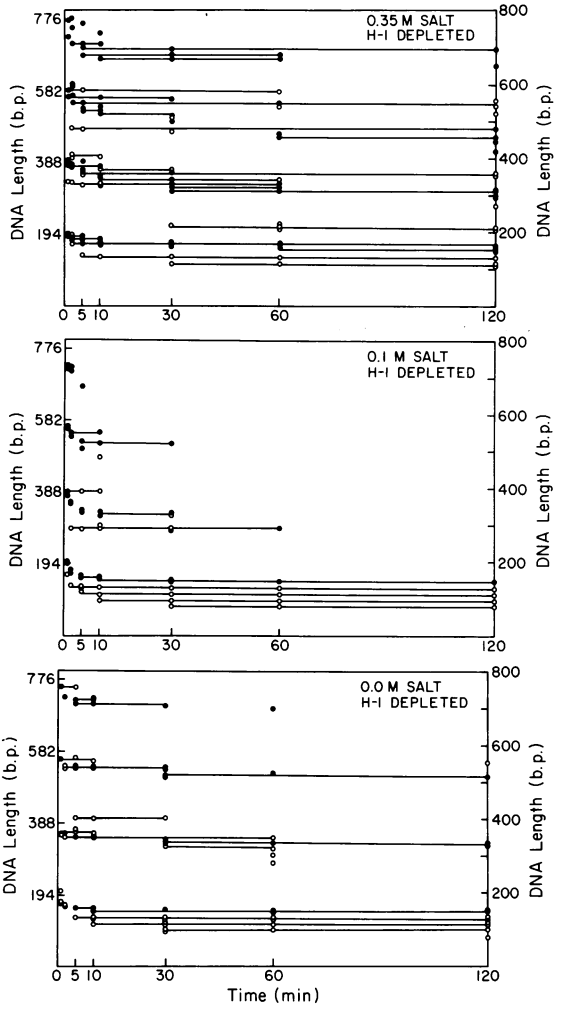

Figure 3A: Sizes (in bp) of peaks and shoulders observed in scans of polyacrylamide gels like those shown in Figure 2. The results of 6 independent experiments with H-1 depleted nuclei are shown. In each case, filled points represent the major peak observed for each oligomer in one or more experiments; the open points correspond to shoulders or minor peaks. Straight lines connect any three or more points which are indistinguishable (less than ±2% different) in size.

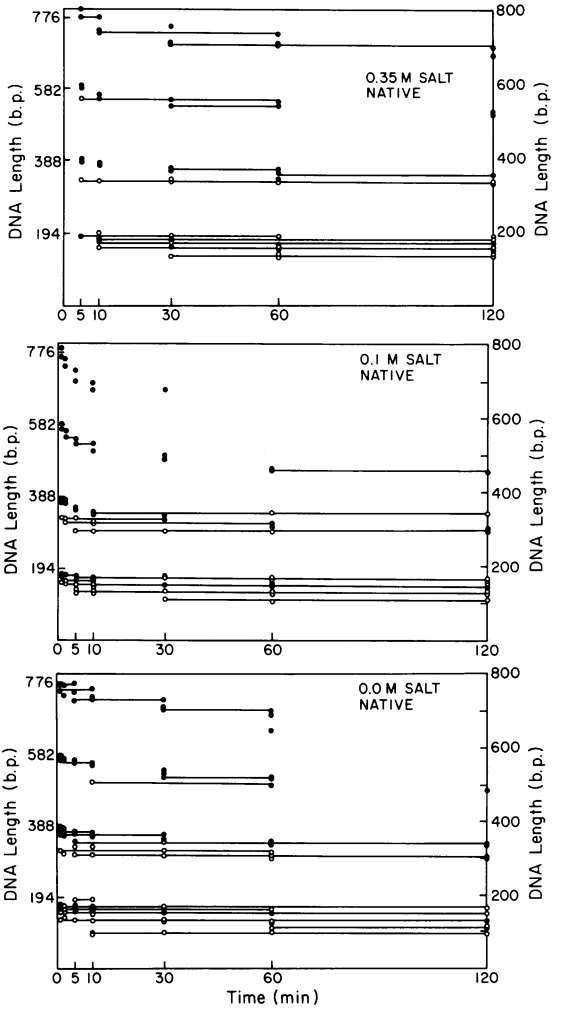

Figure 3B. Same as Figure 3A, but with nuclei that have not been depleted of H-1.

the larger oligomers can be followed for long digestion periods only at high salt, as can be seen from Figures 2 and 3A. A very large monomer DNA (about 216 bp) can also be detected only at 0.35 M NaCl; a possible dimer of this size, (> 400 bp), shows up in both zero and 0.35 M NaCl.

Determination of repeat length and core size in H1-depleted nuclei: One may use the stabilizing effect of salt concentrations higher than 0.3 M to evaluate the repeat length for H1-depleted calf thymus chromatin. We measured the mean length of monomers through pentamers after 1 min of digestion at 0.35 and 0.45 M NaCl. While this method will slightly underestimate the repeat length, it avoids the uncertain extrapolation of sizes to zero times. Evaluating both the difference between adjacent peaks and the ratio of size over degree of polymerization (6), we arrive at 192 ± 4 bp as a result of 26 determinations. The highest mean monitored on one set of gels (10 values) is 194 bp with an average variation from the mean of 1.3 bp.

We also determined the largest monomer size of extended lifetime (persisting to 2 hrs of digestion) appearing in digestions of H1-depleted nuclei. At zero and 0.1 M NaCl the mean size of that class is 156 bp, while it is 168 bp at 0.35 M NaCl (cf. Figure 3A). In the latter case this class represents the main monomer peak through most of the initial 60 min of digestion and it is still a major component at 120 min. A similar finding was made in the presence of 0.45 M NaCl (data not shown). This indicates that even in absence of H1, DNA pieces as long as 168 bp can be protected from digestion by micrococcal nuclease, under appropriate digestion conditions.

Digestion time-courses with native nuclei at various salt concentrations: For purposes of direct comparison we digested undepleted calf thymus nuclei under the same conditions used for H1-depleted nuclei. Scans of the DNA contained in aliquots taken during digestion at various salt concentrations are also presented in Figure 2. The series of oligomeric bands is more strongly developed than in H1-depleted nuclei, particularly at low ionic strengths. In some cases DNA's from oligomers up to heptamers are resolved. Otherwise the effect of increasing NaCl concentrations is the same as found before: a general reduction in rate of digestion, and a reduced formation of submonomer sizes at 0.35 M NaCl.

We also determined the positions of the various peaks and shoulders in the scans from several experiments as done before. Figure 3B shows a plot of the data as a function of

time of digestion and ionic strength. As found with H1-depleted nuclei the points are arranged on a number of parallel lines; they correspond to a series of separate size subclasses which are in part identical with the ones found in experiments with H1-depleted nuclei. The stepwise shift downward in the main peak position, as indicated by the filled points in Figure 3B, is retarded in the presence of H1, irrespective of the ionic strength. This can best be seen by comparing the major dimer and trimer peaks at 120 minutes in Figures 3A and 3B. Furthermore, only in native nuclei are trimeric and tetrameric bands detectable at low ionic strengths after long digestion.

Acid solubility in digestions of H1-depleted nuclei: As the production of small nucleosomes and subnucleosomal fragments depends on the ionic strength so does the yield of acid soluble material. Figure 4, right side, shows the percentage of acid soluble DNA as a function of time of digestion monitored at various salt concentrations under otherwise identical conditions. The results are the following: First, the very low ionic strength of the digestion buffer alone (3mM) is not optimal for digestion. Instead the highest rate of solubilization in our assays is found with 0.1 M NaCl added. Second, salt

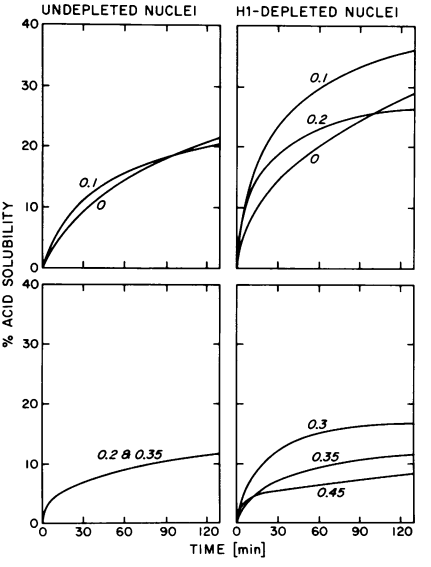

Figure 4. The time dependence of the production of acid-soluble DNA by micrococcal nuclease digestion. Native and H-1 depleted nuclei were digested as explained in Figure 2. The curves are graphical interpolations from 2 or 3 independent series of measurements at each salt concentration. Solubilities were measured at 1,2,5,10,30,60, and 120 min. The average deviations from the means at any time were approximately ±1.2% acid solubility.

concentrations higher than 0.1 M reduce the rate progressively. Third, salt inhibition is most pronounced after an initial 6% solubilization (cf. rates for 0.35 and 0.45 M NaCl in Figure 4, right). Above 0.3 M the shapes of the curves in the region monitored make an asymptote at about 50% acid solubility (19,20) appear unlikely. In fact, addition of an equal amount of fresh enzyme after 120 min does not produce significantly more digestion after 4 h at 0.35 M NaCl (Figure 2, right). The acid solubility at this salt concentration reaches 14% after 12 h at 37°C with a total enzyme concentration of 400 units/ml. This corresponds quite closely to the value expected (~13%) if only the material between 192 bp and 168 bp is solubilized.

Acid solubility in digestions of undepleted nuclei: The course of production of acid soluble material by digestion at low ionic strength differs between native and H1-depleted nuclei. Figure 4 (left) shows that at salt concentrations below 0.35 M the initial rate in native nuclei is approximately half of that in depleted nuclei. A very similar result, based on kinetics of monosome production (rather than acid solubility) has been reported by Cole et.al.(13). Furthermore, increasing the ionic strength of the digestion medium to 0.1 M or 0.2 M does not significantly increase the rate, as was observed with depleted nuclei (cf. Figure 4, right). At 0.35 M NaCl acid soluble oligonucleotides are produced at about the same rate in both undepleted and depleted nuclei, and again the limiting value appears to be lower than 50%.

DISCUSSION

The method of depleting nuclei of histone H1 which was used here may, at first, seem likely to destroy chromatin organization. However, several lines of evidence suggest that this is not the case. First, H1-depleted nuclei have the same appearance in phase contrast microscopy as native ones. In particular, they conserve a pattern of separation into euchromatin and heterochromatin and are not obviously distorted or swollen. Second, only one class of proteins (H1) is removed from the nuclei by the low pH and this is removed

in a reproducible way. No other proteins are extracted in varying amounts as would be indicative of a partial destruction of chromatin. Third, digestion of H1-depleted nuclei with micrococcal nuclease shows basic similarities with the native nuclei in production of DNA fragments and of acid soluble oligonucleotides. Most important, the repeat length determined for H1 depleted nuclei (192±4 bp) is the same as for native nuclei (cf Figure 3) and agrees well with that reported in the literature for calf thymus (10,21). This result should be contrasted with the effect of removing lysine-rich histones from chromatin by 0.6 M NaCl. In the latter case, the repeat patterns and kinds of oligomers obtained are markedly changed (4,21).

We turn now to the effects of NaCl concentration on the digestion. At low ionic strengths ($\leq$ 0.2 M) the values of acid solubility paralleling the digestion of H1-depleted nuclei supports the picture arising from the DNA gels: Even early in the time-course DNA pieces smaller in length than the core size are formed. Concomitant with this rapid degradation of nucleosomes a pronounced early formation of nucleotides or very short oligonucleotides is to be expected. The rather uniformly curved time-courses of acid solubilities at salt concentrations below 0.2 M conform with this expectation.

In contrast, at higher salt, most clearly shown at 0.35 M and 0.45 M NaCl, the digestion is slowed down drastically after an initial 30 min when about 6% of the total DNA is solubilized. The DNA gel at this stage shows a series of peaks at 172, 345, 530 and 690 bp (data not shown). Most important, however, is the fact that the monomer peak does not contain any sizes smaller than 137 bp and even the relative amount of material as small as 150 bp is insignificant. Both results are compatible with the idea that at this point in digestion easily accessible DNA regions in chromatin have been cleaved extensively and solubilized to some degree. Thus, chromatin is reduced to a series of nucleosomal monomers and oligomers with little or no degradation into the core DNA, but with little spacer DNA remaining either. At 60 min of digestion under the same conditions this series of oligomers shows the sizes 168, 331, 505 and 675 bp, leading to exactly 168 bp for the repeating units of the

structures surviving at this point. In the absence of H1-protected spacers this must correspond to a series of closely arranged particles, each containing 168 bp.

The time-course of acid solubility paralleling the digestion of undepleted nuclei is not as simple to interpret. It is known that isolated nucleosomes containing H1 precipitate at salt concentrations between 40 mM and 150 mM (22-24). This aggregation has been correlated with changes in the binding properties of H1 and the microscopic appearance of chromatin fibers (25,26). If similar events take place *in situ* as *in vitro* one might expect the time-courses of digestion to be complicated by salt-induced modulations of the interaction between DNA, (most likely spacer DNA) and histone H1, which are not encountered in H1-deficient nuclei.

In order to better define the mechanism of the salt effect on the digestion, we investigated further the apparent similarities between the salt-dependent kinetics of formation of acid soluble DNA and the generation of monomers and sub-monomers in H1 depleted nuclei. We determined the fraction of DNA in the monomer band as a function of time and salt concentration by comparing the area between 137 bp and 240 bp to the total area under each scan. The limits were chosen such that dimers and submonomers were excluded (cf. Figure 2). With the figures for acid solubility corresponding to each scan we constructed the plot presented in Figure 5. It shows that the higher the salt concentration, the lower is the acid solubility corresponding to the maximum relative fraction of monomers. In other words, the cleavage of accessible spacers at low ionic strength is accompanied by a larger production of very small oligonucleotides and also with more internal degradation in the core particles than at higher ionic strength (cf. Figure 2).

It has been pointed out previously (7, 27) that micrococcal nuclease shows two, normally inseparable, activities on chromatin, the production of double-strand cuts and "end-nibbling". The latter term is used to describe exonucleolytic action on the newly produced ends. The same authors have postulated that the ratio of the two activities is temperature dependent with end-nibbling disfavored at lower temperatures. We propose here that

an increase in ionic strength also favors endonucleolytic action over "end nibbling." This seems to us the easiest way to interpret the findings in Figure 5, and it is in accord with results of micrococcal digestions of DNA (28).

To test this hypothesis it would, of course, be desirable to have existing theories about the action of micrococcal nuclease extended to chromatin. The enzyme has been described as an exclusive endonuclease when acting on core particles at low concentrations (0.1 mM) of divalent ions (11, 29). In contrast, under conditions similar to the ones used here, the exonucleolytic action of micrococcal nuclease was regarded responsible for the fact that its digestion fragments are not necessarily multiples of 10 b in length (30). On the basis of the results presented above we favor the view that the enzyme degrades the DNA of excised nucleosomes from the ends, in a manner analogous to that suggested for exonuclease III (31). The present results can be explained on this basis if a predecessor-product relationship (3) and the existence of certain stop positions for internal degradation are assumed. The first of such positions must be located about 10 bp (yielding the very stable core particle with 145 bp) and 20 bp away from both ends of the 168 bp DNA.

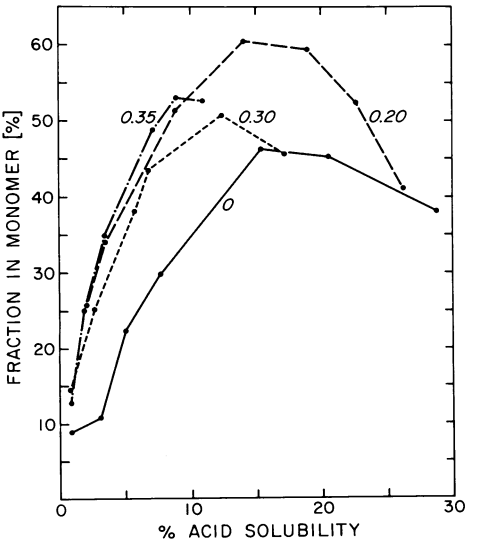

Figure 5. Production of monomers by digestion of H-1 depleted nuclei as a function of concomitant acid solubility. Fractions of monomers were calculated from the areas under scans like those in Figure 2. Monomers are defined as comprising DNA sizes between 137 bp and 240 bp.

In the following we will expand the interpretation of band sizes to include digestion patterns arising from nuclei which have not been depleted of H1. We want to show that the idea of 168 bp being originally associated with the core histones (rather than about 145 bp) plus a series of well defined degradation products of these full cores, as found for H1-depleted nuclei, is compatible with these results as well. The effect of H1 on DNA band patterns produced after digestion is to preserve monomer peaks of sizes even longer than about 168 bp (see Figure 3). Under conditions where in the absence of H1 the species with about 145 bp prevails, we find a well-developed shoulder at 176 bp in presence of H1. One interpretation of this result postulates particles containing about 20 bp of spacer DNA in interaction with an H1 molecule and a core trimmed to the first internal halt on the free end of the DNA (168 bp + 20 bp - 10 bp). This idea of H1 occupying about 20 bp of spacer DNA adjacent to the full core with 168 bp only seemingly contradicts an earlier finding that monomers lose H1 when the peak size shifts from the 168 bp class to the 145 bp class (7). In theoretical considerations based on the above idea and taking various possible pathways of degradation into account, it was shown that the class around 140 bp is the longest one that should be wholly devoid of H1 (32). Classes with longer DNA sizes are expected to consist of a mixture of particles containing and lacking H1. Thus, such classes of particles will carry H1, but in less than stoichiometric amounts.

Simpson (12) has recently shown that treatment of chicken erythrocyte nucleosomes with 0.12 M salt leads to precipitation of a class of particles he terms "chromatosomes." These particles each contain about 160 bp of DNA and one molecule of H1 (or H5). The supernatant particles contain no lysine-rich histones, and have mainly DNA of about 140 bp, "with some tailing to larger sizes" (12). DNase I digestion of chromatosomes indicates that at least some of these particles have the DNA symmetrically wrapped about the histone core, although not all features of the digestion pattern could be explained in this way. The significance of our studies, in

relation to this work, is the demonstration that DNA as large or larger than that in the chromatosome can be protected from micrococcal nuclease, even in the absence of lysine rich histones, if digestion conditions are properly chosen. Thus, protection of DNA beyond the 145 bp core is not necessarily dependent on H1 or H5.

Differences in size can also be seen for the dimers produced in the presence and absence of H1. At the same degree of digestion in terms of acid solubility (about 10%) dimer peaks appear at 312 bp (H1-depleted nuclei) or 336 bp (native nuclei) although the bands start out at the same position at early times (cf. Figure 3). In our model, 312 bp corresponds to two 168 bp DNA lengths trimmed back by about 10 bp on each end. Assuming the same kind of trimming in the case of native nuclei the length of 336 bp would account for the remnants of two 168 bp particles and an intervening 20 bp spacer which is protected by H1. On the other hand, this size also corresponds to two full 168 bp lengths. In the time-course itself, it is not possible to distinguish between these two kinds of dimers. But by the following reasoning we conclude that the 336 bp peak is in fact heterogeneous: A 336 bp species appears very early in digestion (when little or no degradation of spacers has occured) (cf. Figure 2, top). Since this is the smallest separable dimer species at this time we conclude that it corresponds to two adjacent 168 bp particles. It should, thus, exhibit a similar stability during digestion as the whole dimer peak in H1 depleted nuclei. In contrast, the bulk dimer material containing spacer DNA and H1 is transferred into the 336 bp class only by subsequent degradation. It will then gradually bury the remnants of the spacerless dimer. From the magnitude of the 336 bp shoulder in relation to the total dimer peak in the same scans at early times, the amount of spacerless dimers can be estimated to be very small. The probability of spacerless trimers should then be exceedingly small. It is, therefore, no surprise not to find a strong trimer band persisting upon digestion of H1 depleted nuclei (cf. Figure 2, top). Moreover, the higher oligomer

sizes produced from native nuclei are all compatible with the interpretation that they arise from a number of 168 bp particles separated by one or more H-1 bearing spacer regions.

There is no direct evidence that such stable 168 bp particles exist in chromatin from other sources. However, DNA lengths larger than ~140 bp in nucleosomes without H1 have frequently been reported (7-12). This suggests that a conclusion may be generally valid which we can draw for calf thymus only at the moment: A particle carrying 168 bp of DNA may be of fundamental significance in chromatin structure. We cannot rule out, of course, the possibility that these particles carried H1 (as Simpson's chromatosomes) before H1 depletion. Nevertheless, the DNA length of 168 bp was still protected, even after H1 removal.

It might be thought that the protection of 168 bp DNA was a consequence of some higher order structure preserved in nuclei, and thus involve histones from adjacent nucleosomes. We do not believe this to be the case, for we have observed the same results upon digestion of chromatin, isolated from lysed nuclei (date not shown).

Recognition of this requires a significant reorientation in our thinking about chromatin structure. The "core particle" that has been the object of so much study is now viewed as a particularly stable intermediate in the digestion of chromatin. We feel that, to avoid confusion, the term "core particle" should be retained for the 145 bp entity, but we must not overemphasize its importance. It is clear that the interactions of DNA with histones are strongest in the 145 bp domain, and that the additional 23 bp are much more susceptible to nuclease digestion under some circumstances. However, the fact that those conditions which inhibit exonucleolytic attack on the DNA (low temperature, high salt) lead to the stabilization of the 168 bp particle attests to its significance. In particular, in distinguishing between "spacer" or "linker" DNA and that bound to the histone octamer, we should note that under many conditions the "linker" should be redefined as that DNA in the repeat in excess of 168 bp, rather than 145 bp.

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References

1 Sollner-Webb, B., and Felsenfeld, G., (1975) Biochemistry 14, 2915-2920
2 Van Holde, K.E., Shaw, B.R., Lohr, D., Herman, T.N., and Kovacic , R.T., (1975) Proc. Tenth FEBS Meeting, G. Bernardi, F. Gros, Eds. Vol. 38, North Holland - American Elsevier, 57-72
3 Shaw, B.R., Herman, T.M., Kovacic, R.T., Beaudreau, G.S., and Van Holde, K.E. , (1976) Proc. Natl. Acad. Sci. USA 73, 505-509
4 Tatchell, K., and Van Holde, K.E., (1978) Proc. Natl. Acad. Sci. USA 75, 3583-3587
5 Compton, J.L., Bellard, M., and Chambon, P., (1976) Proc. Natl. Acad. Sci. USA 73, 4382-4386
6 Lohr, D., Corden, J., Tatchell, K., Kovacic, R.T., and Van Holde, K.E., (1977) Proc. Natl. Acad. Sci. USA 74, 79-83
7 Noll, M., and Kornberg, R.D. (1977), J. Mol. Biol. 109, 393-404.
8 Simpson, R.T. and Whitlock, J.R., Jr. , (1976) Nucleic Acids Res. 3, 117-127
9 Whitlock, J.P., Jr., and Simpson, R.T., (1976) Biochemistry 15, 3307-3314
10 Todd, R.D. and Garrard, W.T., (1977) J. Biol. Chem. 252, 4729-4738
11 Sollner-Webb, B., Melchior, W. and Felsenfeld, G., (1976) Cell 8, 333-347
12 Simpson, R.T. (1978) Biochemistry 17, 5524-5531
13 Cole, R.D., Lawson, G.M., and Hsiang, M.W. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 253-263

14 Wray and Stubblefield (1970) Expt. Cell Research 59, 469-478
15 Heins, J.N., Tanguchi, H. & Anfinsen, C.B. (1966) Procedures in Nucleic Acid Research, eds. Cantoni, J. and Davies, D. (Harper and Row, New York), 79-84
16 Britten, R.J., Graham, D.E. & Neufeld, B.R. (1974) Methods Enzymol. 29, 363-418
17 Afinsen, C.B., Cuatrecasas, P. & Tanguchi, H. (1971) The Enzymes, ed. Boyer, P.D. (Academic Press, New York), 3rd ed., Vol. IV, 177-204
18 Dirksen, M.L. & Dekker, C.A. (1960) Biochem. Biophys. Res. Commun. 2, 147-152
19 Clark, R.J. & Felsenfeld, G. (1971) Nature 229, 101-105
20 Clark, R.J. & Felsenfeld, G. (1974) Biochemistry 13, 3622 3628
21 Steinmetz, M., Streeck, R.E., & Zachau, H.G. (1978) Eur. J. Biochem. 83, 615-628
22 Olins, A.L., Carlson, R.D., Wright, E.B. & Olins, D.E. (1976) Nucleic Acids Res. 3, 3271-3291
23 Wittig, B. & Wittig, S. (1977) Nucleic Acids Res. 4, 3901-3917
24 Gaubatz, J.W. & Chalkley, R. (1977) Nucleic Acids Res. 4, 3281-3301
25 Renz, M. & Day, L.A. (1976) Biochemistry 15, 3220-3227
26 Renz, M., Nehl, P. & Hozier, J. (1977) Proc. Natl. Acad. Sci. USA 74, 1879-1883
27 Noll, M., Thomas, J.O. & Kornberg, R.D. (1975) Science 187, 1203-1206
28 Kacian, D.L. & Spiegelman, S. (1974) Anal. Biochem. 58, 534-540
29 Camerini-Otero, R.D., Sollner-Webb, B. & Felsenfeld, G. (1976) Cell 8, 333-347
30 Whitlock, J.P., Jr. (1977) J. Biol. Chem. 252, 7635-7639
31 Riley, D. & Weintraub, H. (1978) Cell 13, 281-293
32 Van Holde, K.E. & Weischet, W.O. (1978) in "The Cell Nucleus" Vol. IV.(H. Busch, ed.) Academic Press, New York, 75-105.