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Heterogeneity of chromatin fragments produced by micrococcal nuclease action

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ABSTRACT

Digestion of calf thymus chromatin with micrococcal nuclease produces a mixture of apparently well defined nucleoprotein fragments which have been partially resolved by sedimentation on linear (5-20%) sucrose gradients. Sedimentation patterns reveal a predominent peak at the 11S position, three slower components, which have not previously been reported, at the 3.4S, 5.3S and 8.6S positions, and three faster components at the 17S, 22S and 26S positions. DNA isolated from the 3S to 12S region of gradients has been resolved on polyacrylamide gels into nine to ten discrete components ranging from 47 to 156 base pairs in length. A nearly identical pattern of small DNA products was obtained from chromatin digested in intact nuclei. These data suggest that chromatin contains either several types of subunits or predominently a single type of subunit which can be asymmetrically cleaved at any one of four or more sites.

INTRODUCTION

Considerable evidence now indicates that much DNA in chromatin is compactly folded into small, discrete nucleoprotein complexes or "subunits". Early studies by Clark and Felsenfeld (1), Rill and Van Holde (2) and Sahasrabuddhe and Van Holde (3) demonstrated that nucleases acting on chromatin release approximately half of the DNA in the form of small, compact, nuclease-resistant nucleoprotein fragments while the remaining DNA is readily digested to acid-soluble oligonucleotides. Oosterhof et. al. (4) and Noll (5) have resolved these protected nucleoprotein fragments into a spectrum of components by sedimentation on sucrose gradients. Additional studies in several laboratories have established convincingly that short regions of DNA protected from nucleases by proteins are repeated at closely spaced intervals along chromatin fibrils. Estimates of the length of this basic repeat range from approximately 120-200 base pairs (4,5,6,7,8), as will be discussed in detail in a later report.

The subunit structure inferred from the above studies has been confirmed by electron microscopy as micrographs of chromatin fibrils streaming from

ruptured nuclei (9,10) and of SV40 virus replication complexes (11) have revealed roughly spherical "nu bodies" or "beads" approximately 70-100Å in diameter separated by thin, short filaments. Individual "nu bodies" have been observed in fractions of chromatin obtained after sonication (12) and nuclease digestion (13).

The above observations have generally been interpreted as indicating that a single type of subunit is repeated at fairly regularly spaced intervals along chromatin fibrils. We wish here to present data which reveals that the spectrum of nucleoprotein and DNA fragments produced by nuclease action on chromatin is more complex than was previously supposed and raises the possibility that chromatin may contain a number of "subunits".

MATERIALS AND METHODS

a) Preparation of Nuclei and Chromatin

Calf thymus nuclei were prepared by a modification of the method of Blobel and Potter (14). Previously quick-frozen tissue was homogenized in 0.25 M sucrose, 10mM $MgCl_2$, 0.034M Tris, 0.05M $NaHSO_3$ (added just prior to use, final pH = 7.0). After filtration the homogenate was mixed in a centrifuge tube with two volumes of 2.3M sucrose in the above ionic medium plus 0.7% Triton X-100, underlayered with the above 2.3M sucrose solution lacking Triton, and centrifuged for 2 hr. at 25,000 rpm in the International SB-110 rotor. Nuclear pellets were washed once in homogenization medium and quick-frozen, if necessary, after addition of one-third volume of glycerol.

Chromatin was prepared from fresh or frozen nuclei by washing once in 20mM EDTA, 0.45M Tris, 50mM $NaHSO_3$, pH 7.0; and then five times in digestion buffer (1mM cacodylic acid, 2×10^{-5}M $CaCl_2$, adjusted to pH 6.5 with solid Tris). All operations were carried out at 5° or on ice. After the initial tissue homogenization nuclei and chromatin pellets were dispersed by gentle hand action with a teflon-in-glass homogenizer to minimize shearing.

b) Micrococcal Nuclease Digestion of Chromatin

Micrococcal nuclease obtained from Worthington was of the highest specific activity available. Unlike DNase II preparations, preparations of micrococcal nuclease contained no protease activity detectable in our experiments. At pH 6.5 the endogenous thymus chromatin protease is also relatively inactive as no histone is degraded after incubation with nuclease for 2 hr. under the conditions described below. Digestions were performed immediately after chromatin was prepared from nuclei. Samples were diluted to give a

final DNA concentration of 200 to 400 μg/ml, preincubated at 37° for 10-15 min., then innoculated with 1 μg of nuclease per 100 μg of DNA. Digestion was terminated by addition of 1/10 volume of 11mM EDTA, 11mM cacodylic acid, pH 6.5. Portions of the digests (0.2 to 0.5 ml) were layered onto cold sucrose gradients and the remainder, used later for DNA isolation and chemical analysis, was quick-frozen and stored at -20°.

c. Velocity Sedimentation in Sucrose Gradients (5-20%) was carried out as described previously (4) except that the sucrose solutions contained 1mM EDTA, 1mM cacodylic acid, pH 6.5.

d. Electron Microscopy of Isolated DNA was performed as described previously (4).

e. Electrophoresis of DNA on Polyacrylamide Gels

Total digests or fractions were digested with pronase (Calbiochem) in 1% sodium dodecyl sulfate for 3 hr. at 37°, extracted three times with phenol plus chloroform (1:1, v/v), extracted twice with ether, and the DNA precipitated with ethanol. Electrophoresis was carried out at 5° on 3½ or 6% polyacrylamide gels as described by Loening (15). Gels were stained with ethidium bromide or toluidine blue and photographed or scanned with a Gilford spectrophotometer equipped with a gel scanner attachment.

RESULTS

Unsheared chromatin gels are rapidly solubilized by nucleases. Sedimentation profiles of calf thymus and chicken erythrocyte chromatin samples digested for various lengths of time with micrococcal nuclease (Figures 1 and 2, respectively) reveal that this solubilization process is accompanied by the rapid production of a characteristic spectrum of small nucleoprotein fragments plus acid soluble oligonucleotides. In terms of absorbance the predominent small product at all digestion times has a sedimentation coefficient of 11S±0.5S. Three additional larger fragments with sedimentation coefficients of 17S, 22S and approximately 26S are particularly well resolved in samples digested for moderately long times. Noll (5), using isokinetic gradients, has similarly resolved these four components in rat liver chromatin digested with micrococcal nuclease. Evidence from several laboratories has suggested that the 11S nucleoprotein fragments represent the chromatin "subunit" and correspond closely to the "beads" or "ν-bodies"

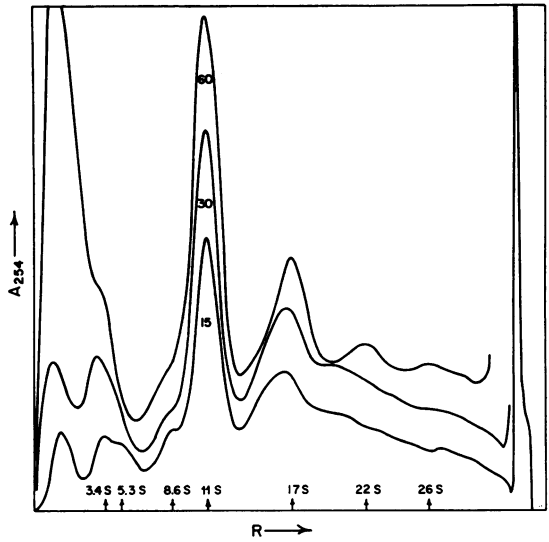

Figure 1: Sucrose density gradient (5-20%) sedimentation profiles of calf thymus chromatin digested with micrococcal nuclease for the number of minutes indicated. Centrifugation was for 10.5 hr at 40,000 rpm in an International SB-283 rotor maintained at 6°. The baselines of the curves are offset.

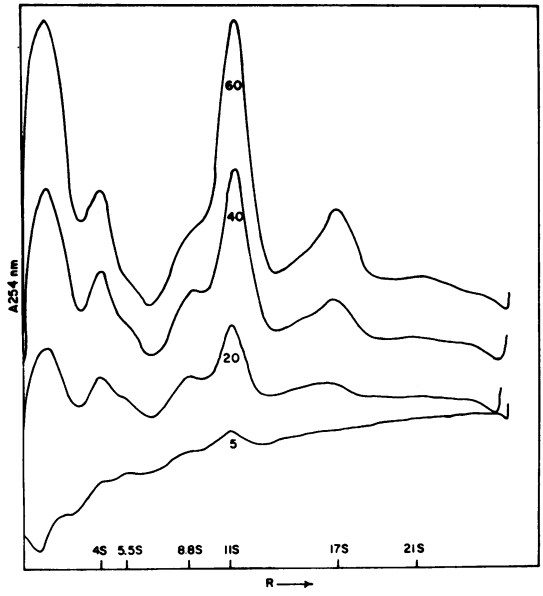

Figure 2: Sucrose density gradient (5-20%) sedimentation profiles of chicken erythrocyte chromatin digested with micrococcal nuclease for the number of minutes indicated. Centrifugation was for 11 hr at 40,000 rpm in an International SB-283 rotor maintained at 6°. The baselines of the curves are offset. Chicken erythrocyte chromatin was prepared according to the method of Axel et al (17).

observed by electron microscopy (4,5,12,13). According to this view the larger fragments would represent dimers, trimers and tetramers of the subunit plus perhaps some extended intersubunit DNA.

Examination of sedimentation profiles of briefly digested chromatin reveals that the above fragments are not the only small nucleoprotein products. At least three fragments sedimenting more slowly than 11S are rapidly released from chromatin, i.e., there is a shoulder on the 11S peak at the 8.6S position and two slower sedimenting peaks at approximately the 3.4S and 5.3S positions. As shown most clearly in Figure 2, these components appear concurrently with the 11S component, before significant amounts of acid soluble oligonucleotides are produced. As the digestion proceeds these three peaks become partially obscured by the increased production of non-sedimenting oligonucleotides and 11S material. Nonetheless, these fragments apparently are not further degraded but instead increase in quantity roughly in parallel with the 11S component. This conclusion is substantiated by analyses of time course of the fragmentation of chromatin DNA described below.

Previous studies in this laboratory (4) have shown that moderate digestion of calf thymus chromatin with DNase II produces a mixture of 11S and faster sedimenting fragments similar to that reported here. The length of DNA isolated from 11S fragments produced by micrococcal nuclease digestion was measured with the electron microscope and compared to the length of DNA found in 11S fragments produced by DNase II. As shown in Figure 3, 11S fragments produced by either enzyme contained DNA with a median length of 400 Å which corresponds to approximately 120 base pairs of B form DNA.

One major difference between the results obtained with these two enzymes should be noted. Our earlier experiments were hampered by the presence of protease activity in DNase II which caused a rapid degradation of histone H1 (F1) and slower degradation of H3 (F3). Under these conditions the nuclease digestion reaches a relatively stable end point when virtually all fragments larger than 11S have disappeared, approximately half of the DNA is found in 11S fragments, and the remaining DNA stays near the meniscus. No material precipitates from solution. In contrast, micrococcal nuclease digestion effectively terminates when up to approximately 50% of the original DNA is reduced to acid soluble products and the remaining undigested chromatin rapidly precipitates from solution. Sedimentation profiles of samples removed at the onset of precipitation reveal that the 17S and 22S fragments remain intact even at this late stage in the digestion process. As will be described in a later report, digestion of chromatin with DNase II under conditions which

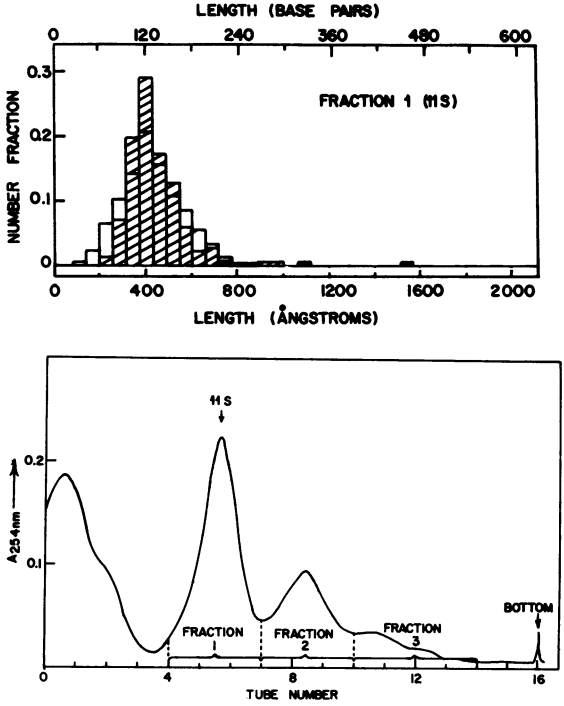

Figure 3: (Bottom) Sucrose density gradient profile of calf thymus chromatin digested for 55 min with micrococcal nuclease. Centrifugation was for 16 hr at 32,000 rpm in the SB-283 rotor maintained at 6°. The length of DNA isolated from fraction 1, containing 11S fragments, was measured with the electron microscope. (Top) Frequency diagram (open bars) of the lengths of DNA pieces found in fraction 1. These data are superimposed upon the previously reported (4) frequency diagram (dashed bars) for the lengths of DNA pieces isolated from a similar 11S fraction produced by DNase II digestion of calf thymus chromatin.

virtually eliminate proteolysis yields a complete spectrum of nucleoprotein fragments very similar to that obtained with micrococcal nuclease, even after long digestion times when the sample begins to precipitate. Apparently histones H1 and/or H3 bind to DNA regions in the 17S and larger fragments which become accessible to nuclease after these histones are degraded.

The time course of the fragmentation of chromatin DNA by micrococcal nuclease was examined by electrophoresis of DNA isolated from whole digests on polyacrylamide gels. As shown in Figure 4, a characteristic series of eleven to twelve small DNA fragments is produced after very brief treatment with nuclease, before significant amounts of acid-soluble oligonucleotides appear. The lengths of DNA within these discrete bands were assigned by

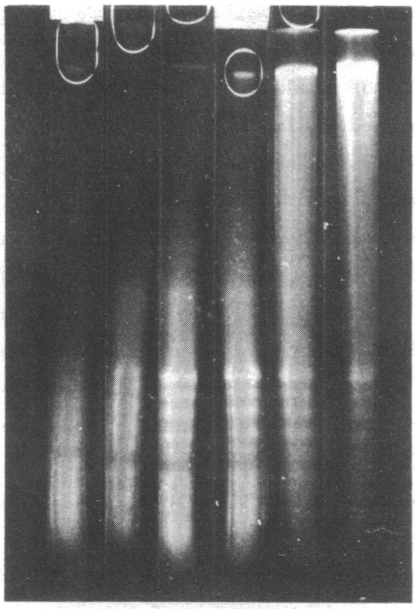

Figure 4: Electrophoretic patterns of DNA isolated from unfractionated chromatin samples digested for various lengths of time with micrococcal nuclease. Electrophoresis was performed on 3½% polyacrylamide gels. Gels were stained with toluidine blue and photographed with Polaroid film, hence stained bands on the gels appear as light bands on the figure. Digestion times (right to left) are 5,10,25,40,60, and 90 min, respectively.

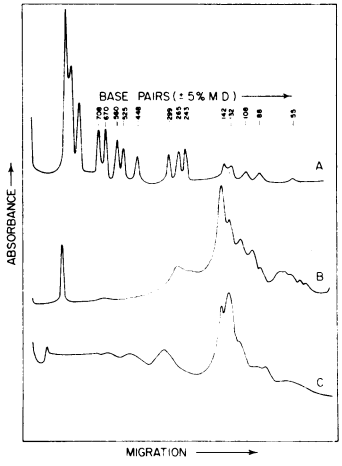

Figure 5: Densitometer scans of electrophoretic patterns of (A) endonuclease Hae III fragments of PM2 DNA, (B) DNA isolated from calf thymus chromatin digested for 40 minutes with micrococcal nuclease, sample electrophoresed concurrently with the PM2 fragments shown above, (C) DNA isolated from Chinese hamster ovary nuclei digested for 10 minutes with micrococcal nuclease. The scale in C is slightly different from the scales of A and B. Electrophoresis was performed on 11 cm gels of 3½% polyacrylamide. Gels were stained with toluidine blue and scanned at 540 nm. The region in the pattern of (B) from 160 to 47 base pairs was resolved into ten Gaussian components. The lengths and relative mole percents, respectively, of these components are: 156 BP, 11.0%; 137 BP, 11.1%; 133 BP, 11.4%; 96 BP, 10.9%; 80 BP, 10.6%; 67 BP, 12.4%; 62 BP, 12.0%; 56 BP, 8.3%; 51 BP, 6.6%; and 47 BP, 6.5%.

comparing their mobilities to the mobilities of fragments of bacteriophage PM2 DNA cleaved by endonuclease R Hae III (from Haemophilus aegyptius) as is shown in Figure 5. Two bands are observed at the 263 and 200 base pair positions, five well resolved bands at the 156, 132, 113, 96, and 88 base pair positions and four or five less well resolved bands in the 45 to 70 base pair range. An identical pattern of DNA fragments was also obtained from chicken erythrocyte chromatin digested with micrococcal nuclease (data not shown). The same length assignments were obtained when DNA from whole digests and the PM2 DNA fragments were denatured and electrophoresed on 6% gels containing formamide. The yields of the 263 to 45 base pair DNA fragments increase with increasing digestion time at close to equal rates until late in the digestion process when the bands at and above the 156 base pair position usually diminish in intensity. Axel et al. (17) have reported very similar data for the digestion of duck reticulocyte chromatin with micrococcal nuclease.

It is important to note that densitometer scans of stained gels yield essentially a weight fraction distribution of molecular species. In terms of the frequency of occurrence of a particular size class of protected DNA in chromatin the number fraction of the different species is a more relevant quantity. Gel scans such as that shown were resolved into Gaussian curves using a DuPont Curve Resolver. Integrated areas were assumed to represent weight fractions and were converted into number fractions using the length assignments given in Figure 5. Such an analysis shows that all of the DNA fragments 156 base pairs long and smaller are present in nearly <u>equimolar</u> amounts in moderately digested samples (see legend in Figure 5B). There are clearly several possible inaccuracies and uncertainties in this analysis, for example careful observation of long gel patterns indicate that a few bands in the 90 to 135 base pair range may be doublets, and more discriminating data are required. Despite these reservations it appears that each of these protected DNA regions occurs with nearly equal frequency along the chromatin fibril.

There is a rather striking similarity between the densitometer scans of these DNA gels and the sucrose density gradient profiles of whole digests. (For example, compare Figure 5B with the gradient patterns of the 10 min. digest shown in Figure 1.) Electrophoretic analyses of DNA isolated from pooled peak fractions of sucrose gradient eluents has established that, in fact, there is a good correlation between the relative sedimentation rates of the nucleoprotein fragments and the lengths of DNA which they contain.

Figure 6 shows the manner in which gradient fractions of nucleoprotein fragments were pooled and the relative electrophoretic mobilities of the major DNA fragments which they contained. All fractions contained two or more major DNA components of relatively discrete length which are indicated in the figure. This result suggests that either each of the peaks in the gradients contain more than one type of nucleoprotein fragment or that each fragment contains more than one piece of DNA as has been suggested by Weintraub (18). Very little DNA larger than 70 base pairs was found in the 3S to 6S fraction (B). Fractions C, D, and E, corresponding to the 8.6S, 11S and 17S fragments, respectively, contained low but significant amounts of DNA both smaller and larger than the major species shown. This would be expected from the relatively poor resolution achieved on these gradients which were overloaded to maximize yields. Histones were found in all of the nucleoprotein fractions. Larger yields and better resolution must be obtained before the histone and DNA contents of these fragments can be specified more precisely.

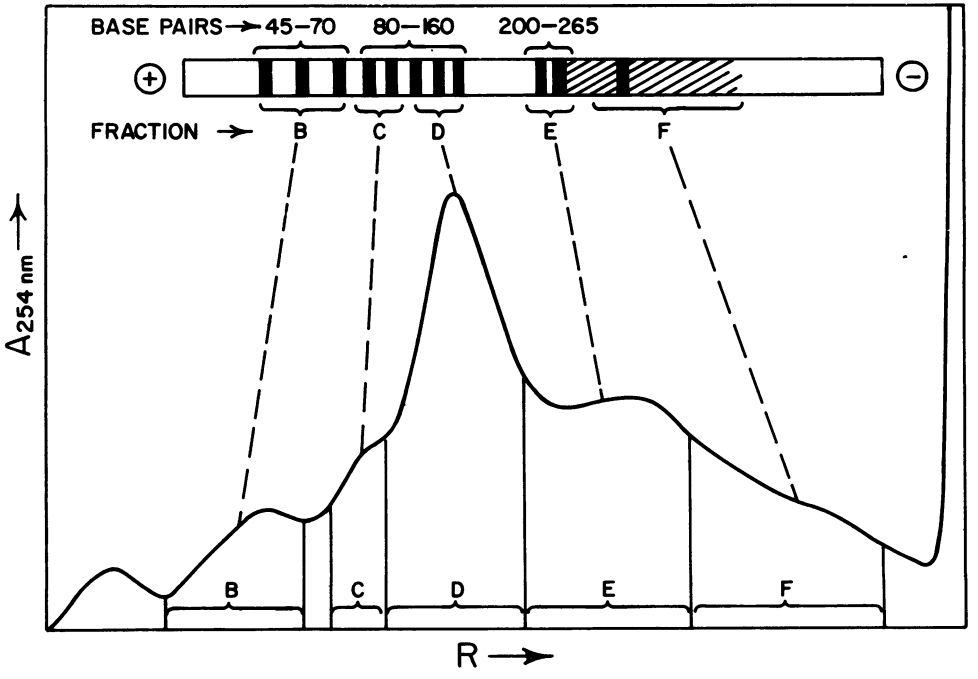

Figure 6: Principle location of specific DNA fragments within nucleoprotein fragments of calf thymus chromatin separated by sedimentation of digested chromatin on linear (5-20%) sucrose density gradients and fractionated as shown.

DISCUSSION

We conclude from these results that there are more "pieces" to the puzzle of chromatin "subunit" structure than has generally been supposed. To avoid semantic ambiguities in this discussion the following definitions have been adopted. Resolvable nucleoprotein or DNA products of nuclease digestion are called fragments, components or fractions. Small local regions of DNA associated with a unique group of proteins in a geometrically defined complex in intact chromatin fibrils are termed subunits. The precise relationships between isolated fragments and true subunits remain to be established. The term "repeating unit" is reserved to refer to a particular linear arrangement of one or more subunits repeated along a significant portion of chromatin fibrils. These are features of what may be called the "primary" level of chromatin structure, ie., the short range folding of DNA and distribution of proteins along the unit chromatin fibril. Higher levels of order may occur under physiological conditions, perhaps through specific subunit-subunit interactions.

A number of models of chromatin structure can be constructed based on the data presented. Early evidence mentioned previously led to the proposal that most chromatin DNA is organized into a single type of repeat unit consisting either of identical subunits containing 200 base pairs of DNA joined by a few nuclease-susceptible bases (5,19) or smaller identical subunits containing on the order of 140 base pairs of DNA joined by about 40 base pairs of susceptible "spacer" DNA (13). The 11S nucleoprotein fragment generally has been assumed to correspond to this subunit and to be closely related to the "nu bodies" observed by electron microscopy (12,13).

It is not obvious how a repeating arrangement of identical subunits, containing on the order of 200 base pairs of DNA, can be cleaved to produce at similar rates nearly equimolar amounts of nine or more discrete DNA fragments that range from approximately 50 to 156 base pairs in length and are contained in four (or more) distinctly different nucleoprotein complexes. Reconciliation of these data with the identical subunit model requires some fairly stringent assumptions about the internal structure of the units. One possible model is suggested by noting that the sums of lengths of appropriate pairs of DNA fragments (88+67, 96+62, 118+56, 132+51) are between 155 base pairs, the size of the largest DNA piece found in 11S fragments, and 183 base pairs, close to the size reported for the repeat unit (5,19,21). This observation is consistent with a subunit containing four specific internal cleavage sites located asymmetrically towards one or both ends of

the unit DNA. Cleavage at one (or perhaps two) of these sites could produce two different "sub-monomer" size nucleoprotein fragments. Additional cleavage within these two new particles would have to be prohibited since neither the particles smaller than 11S nor the size classes of DNA which they contain disappear after prolonged digestion.

Weintraub (18) has proposed a very similar model based on the properties of "sub-monomer" size nucleoprotein particles (termed "ST particles") obtained after treatment of Staphylococcal (micrococcal) nuclease-digested chromatin with trypsin. One important difference between Weintraub's data and ours should be emphasized. Sedimentation profiles of nuclease-digested chromatin show that "sub-monomer" size nucleoprotein fragments are produced in the absence of proteolysis, whereas "ST particles" were observed by their migration on polyacrylamide gels only after trypsinization of digested chromatin. It seems likely that the "ST particles" are derived, at least in part, from preexisting 3.4S, 5.3S and 8.6S fragments since both sets of fragments contain essentially the same size spectrum of DNA pieces.

It must be noted that Noll et al. (19) have suggested that production of DNA fragments smaller than their proposed monomer size of 200 base pairs is an artifact of methods of chromatin preparation which normally involve mechanical shearing. To test this possibility we have examined the DNA products of nuclease action on intact nuclei. Nuclei isolated from Chinese hamster ovary cells grown in culture were gently suspended in buffered isotonic sucrose containing 10 mM calcium chloride and digested with micrococcal nuclease for various lengths of time. The reaction was stopped by adding lysing buffer containing detergent and EDTA and DNA was extracted after pronase digestion by the phenol-chloroform method. Electrophoresis of these DNA samples on 1.4% agarose gels reveals patterns of multiple bands essentially identical to those reported by Hewish and Burgoyne (6) and Noll (5) as is shown in Figure 7A. As the digestion proceeds the upper bands diminish in intensity, the mobilities of the centers of the remaining bands increase slightly (as measured with a densitometer) and the leading edge of the fastest moving "monomer" band spreads significantly. Analyses of DNA fragments on large pore gels alone can be misleading as is illustrated in Figure 7B where the same samples were run on 3½% polyacrylamide gels. The gel containing DNA isolated from nuclei digested for only 90 sec. is particularly informative. Clearly on a weight basis most of the DNA in this sample is in the "monomer", "dimer", "trimer", etc. size range, yet below the "monomer" band one can see a spectrum of smaller fragments similar to that shown previously for calf thymus chromatin digests. The

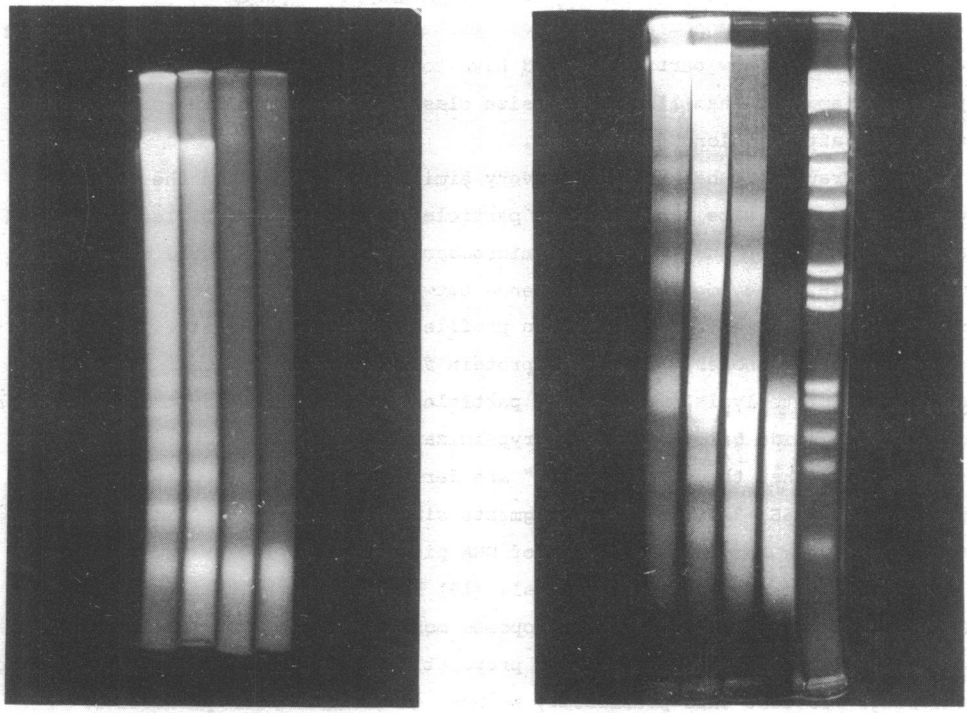

Figure 7: Electrophoretic patterns of DNA isolated from Chinese hamster ovary nuclei digested for various lengths of time with micrococcal nuclease. Electrophoresis was performed on 11 cm gels of (A) 1.4% agarose and (B) 3½% polyacrylamide. Digestion times (l to r) were 20 sec, 90 sec, 10 min, 60 min. Gel on right is PM2 DNA treated with Hae III endonuclease. Lengths assignments for the PM2 fragments are shown in Fig 6A. Gels were stained with ethidium bromide and photographed with Plus X film, hence fluorescent bands appear as light bands in the figures. A densitometer scan of the electrophoretic pattern of DNA from the 10 minute digest run on a 3½% gel is shown in Figure 6C.

similarity between DNA fragments obtained from nuclei and chromatin is further illustrated in the gel scans shown in Figures 5B and 5C. The center of the "monomer" band lies in the 120 to 140 base pair region as is shown by comparison with the gel pattern of PM2 marker DNA fragments included in Figure 7B, in good agreement with our electron microscope measurements of the length of DNA in the 11S nucleoprotein particles from chromatin digests.

Our data, and related data of Axel et al. (17) and Weintraub (18), can also be interpreted in terms of a many subunit model. Non-identical subunits could result from subtle chemical or conformational differences between

structurally similar units as has been suggested by Weintraub (18). Alternatively, chromatin may contain a repeating unit, larger than that generally proposed, made up of several non-identical subunits which yield the 11S and smaller fragments after nuclease digestion. Such proposals are not inconsistent with the data on which the single subunit model is generally based. For example, the "monomer", "dimer", "trimer", etc. DNA bands observed when DNA from whole nuclear digests is electrophoresed on polyacrylamide gels are much broader than those obtained upon electrophoresis of the monodisperse PM2 DNA fragments. This heterogeneity would not be expected if cleavage occurred between very closely spaced identical subunits but could arise from cleavage between non-identical subunits containing the small lengths of DNA reported above or from random cleavage of a 30 to 50 base pair spacer region between identical subunits. Measurements of the diameters of the globular subunits observed in electron micrographs are rather disperse. Furthermore, the thin and extended "spacer" regions observed between globular units in spread chromatin preparations may be complexed with protein and partially protected from nuclease.

Additional characterization of individual chromatin fragments appears to be required before a definitive model of chromatin structure can be accepted.

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