Structural repeat units of Chinese hamster ovary chromatin. Evidence for variations in repeat unit DNA size in higher eukaryotes.

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ABSTRACT

DNA lengths in the structural repeat units of Chinese hamster ovary (CHO) and chicken erythrocyte chromatin were compared by analyzing the sizes of DNA fragments produced after treatment of nuclei with staphylococcal nuclease. The repeat length of CHO chromatin (173 ± 4 BP) is about 20 base pairs (BP) smaller than that of chicken erythrocyte chromatin (194 ± 8 BP). Repeat lengths of rat liver and calf thymus chromatin were found to be about 10 BP shorter than that of chicken erythrocyte chromatin. Thus significant variations occur in repeat units of chromatin of higher eukaryotes. These variations occur in the lengths of "spacer" (or "internucleosomal") DNA segments, not in "core particle" (or "nucleosomal") DNA lengths. The concept of spacer regions and the possible influence of H1 histones is discussed.

INTRODUCTION

Considerable evidence now indicates that histones form specific complexes that cover and fold discrete lengths of DNA at fairly regular intervals along chromatin fibrils.^{1,2} Electron micrographs show these histone-DNA complexes as 80Å to 110Å diameter particles, termed "nu-bodies"³ or "nucleosomes,"⁴ separated by short stretches of thin fibrils. Chemical evidence for a repeating structure was initially derived from the observation that both staphylococcal nuclease and an endogenous nuclease digest rat liver chromatin in isolated nuclei, producing a series of discrete DNA fragments that are about 200 base pairs (BP) and multiples of 200 BP long.^{5,6} These and other results led Kornberg⁷ to suggest that chromatin contains a fundamental repeating unit consisting of eight histones--a tetrameric (H3)₂(H4)₂ complex and two copies each of histones H2A and H2B--associated with 200 BP of DNA. Histone H1 was presumed to be associated with the repeating unit, but not involved in its formation.

A somewhat different view of the repeat unit was obtained from studies of nuclease-resistant portions of chromatin by Van Holde and co-workers⁸⁻¹³ and others.¹⁴⁻¹⁶ The predominant nucleoprotein is a compact particle ($S_{20,w} \approx 11S$) containing slightly more total histone than DNA (by weight), roughly equimolar amounts of all histones except H1, and a single piece of DNA about 140 BP long. (Earlier estimates indicated an average size of about 110-120 BP.⁸⁻¹⁴) Electron micrographs of 11S particles showed spherical objects virtually identical to the spherical particles observed in whole chromatin spreads while "dimer" units appeared as two particles connected by a short, thin strand.¹⁰ Thus Van Holde and co-workers⁹ proposed that the repeat unit consists of a globular "core particle" (an octameric histone complex of two copies each of histones H2A, H2B, H3 and H4 that serves as a nucleus or "core" about which 140 BP of DNA is wrapped) plus a short (30-60 BP), more extended and accessible "spacer" region of DNA.

More detailed analyses have confirmed that the initial nuclease cleavages in chromatin from several sources produce DNA fragments that average about 200 BP long and multiples of this length. Nucleoprotein fragments containing near 200 BP lengths of DNA also contain all five histones.⁶ These particles are rapidly degraded further, predominantly to particles lacking histone H1 (and H5 in erythrocytes), and with a DNA length reduced to about 140 BP.^{12,13,16-20} Thus the octameric complexes of histones appear to be associated predominantly or entirely with 140 BP regions of DNA and are separated by shorter regions associated with histones H1 and H5.²¹

The assignment of H1 and H5 to spacer regions is of special interest in view of the well-known differences between these lysine-rich histones and other histones. Most higher organisms contain at least three subfractions of H1 differing in charge, sequence and, often, in molecular weight. Further diversity is introduced by in vivo side chain modifications. The H1's are also the least evolutionarily conserved of the histones and exhibit a certain degree of tissue and species specificity. We might expect, then, to find species and perhaps tissue variations in spacer lengths due to variations in the size and

intramolecular charge distributions of the H1 histones as well as to other factors such as non-histone protein binding. In fact, the size of the repeat unit in some primitive eukaryotes is considerably lower, being ~160 BP in *S. cerevisiae* (baker's yeast)²²⁻²⁴ and ~170 BP in *Neurospora*.²⁵ A repeat length of about 190-200 BP has been generally accepted for higher eukaryotes although a rather limited number of species have been examined in detail.^{4-6,12,13,16-20,26-28} The sizes of these DNA fragments have most commonly been determined by electrophoretic comparisons with sized restriction nuclease fragments of a viral DNA. Unfortunately in comparing results from different laboratories, it is difficult to determine if apparent small species variations in repeat sizes are real or result from differences in the source or sizing of the calibration fragments.

As described below, a detailed comparison of gel patterns of DNA from CHO, chicken erythrocyte, rat liver and calf thymus nuclei digested with staphylococcal nuclease shows that there are significant variations in repeat lengths of these higher eukaryotes. In these and other species variations in repeat length appear to result from variations in the spacer regions and not in the core particles.

MATERIALS AND METHODS

Growth of Chinese Hamster Ovary (CHO) Cells. CHO cells were grown to late log phase in roller bottles (11 x 22 cm) on Ham's F-10 medium containing 10% serum (equal amounts of calf serum and fetal calf serum). Bottles were briefly rinsed in cold 0.01 M tris, 1 mM EDTA (adjusted to pH 7.8 at 25° with HCl) to remove free metaphase cells, then washed by gentle agitation with fresh tris-EDTA solution at 4° for 10 min when at least 90% of the cells (2×10^8 cells) were detached. Cells were collected by centrifugation at 800 x g for 5 min.

Isolation and Digestion of CHO Nuclei.

Method 1: Cells were resuspended in 10 mM tris, 1 mM EDTA, 4 mM MgCl₂ (adjusted to pH 7.8 at 25° with HCl), allowed to swell for 5 min, then broken with a Dounce homogenizer. An equal volume of the above buffer containing 1% Triton X-100 was added and after 5 min nuclei were collected by centrifugation. Nuclei

were further washed two times with a solution of 0.3 M sucrose, 10 mM CaCl_2 , 5 mM tris (adjusted to pH 7.3 at 25° with HCl), then resuspended in 4 ml of the same solution. One milliliter aliquots of the nuclear suspension (about 1×10^8 nuclei) were digested for various lengths of time with 200 units of staphylococcal nuclease (Worthington) at 37°. Digestion was terminated by making the suspension 1 M in NaCl, 25 mM in EDTA and 0.25 M in tris-HCl (pH 8.0 at 25°).

Method 2: Cells were washed two times in 0.34 M sucrose containing the polyamine stabilizing buffer described by Hewish and Burgoyne⁵ (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM tris, adjusted to pH 7.4 at 20° with HCl). The final pellet of cells was resuspended in 5 ml of the above solution and disrupted with a Dounce homogenizer. Nuclei were collected by centrifugation, washed two times with the above solution, then resuspended in 4 ml of this solution plus 1 mM EDTA, 0.2 mM EGTA, 10 mM MgCl_2 and 1 mM CaCl_2 . One milliliter aliquots (about 1×10^8 nuclei) were digested with nuclease as described above.

Method 3: Cells were dispersed in 5 ml of 0.25 M sucrose in TKMC buffer (0.05 M tris, 25 mM KCl, 5 mM MgCl_2 , 1 mM CaCl_2 , adjusted to pH 7.5 at 20° with HCl) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), then disrupted with 15 strokes of a Dounce homogenizer. Nuclei were collected by centrifugation and washed once with the above solution containing 0.5% Triton X-100. Pelleted nuclei were gently suspended in 7 ml of homogenization solution, mixed in a 40 ml polyallomer tube with 3 volumes of 2.1 M sucrose in TKMC buffer plus 0.1 mM PMSF, underlayered with 6 ml of the above 2.1 M sucrose solution and centrifuged in an International SB-110 rotor at 25,000 rpm for 2 hr (4°). The nuclear pellet was then washed three times in the digestion buffer described by Sollner-Webb and Felsenfeld¹⁹ (0.25 M sucrose, 0.1 mM CaCl_2 , 1 mM tris-HCl (pH 8.0 at 25°)). The final nuclear pellet was suspended in sufficient digestion buffer to yield a final DNA concentration of 500 $\mu\text{g/ml}$, estimated by measuring the absorbance at 260 nm of an aliquot diluted 10-fold with 2.2 M NaCl, 5.5 M urea, assuming that one $A_{260 \text{ nm}}$ unit = 50 μg of DNA. Aliquots of the nuclear suspension

were digested at 37° with micrococcal nuclease at a concentration of 0.5 nuclease units/50 µg DNA. Digestion was terminated as described above.

Isolation and Digestion of Chicken Erythrocyte, Rat Liver and Calf Thymus Nuclei. Blood and tissues were obtained immediately after slaughter and were used fresh (erythrocytes) or were quick-frozen in liquid nitrogen (liver and thymus). Erythrocytes were washed several times by repeatedly suspending in 0.15 M NaCl, 10 mM EDTA (pH 6.5 at 25°) and centrifuging at 800 x g for 5 min until the upper, whitish, "buffy coat" was thoroughly removed. Washed erythrocytes were suspended in 0.25 M sucrose in TKMC buffer containing 1 mM PMSF and disrupted with a Dounce homogenizer. Nuclei were isolated, purified and digested with micrococcal nuclease (5 units/50 µg DNA, 37°) by Method 3 described above. Rat liver and calf thymus were finely minced in 0.25 M sucrose in TKMC buffer plus 1 mM PMSF and homogenized with a motor-driven, teflon-in-glass homogenizer. The homogenate was filtered and nuclei were collected by brief centrifugation. Nuclei were further purified and digested with micrococcal nuclease (5 units/50 µg DNA for calf thymus, 1 unit/50 µg DNA for rat liver) according to Method 3 described above for CHO nuclei.

Isolation of DNA. Digests of nuclei were incubated with 25 µg/ml ribonuclease A (Worthington) for 1 hr at 37°, sodium dodecyl sulfate (SDS) was added to a final concentration of 1%, then 500 µg/ml of pronase (Calbiochem, B grade, nuclease-free) was added and incubation was continued for an additional 4 hr. Solutions were extracted twice with phenol-chloroform (1/1,v/v), once with ether, and the DNA precipitated by addition of two volumes of ethanol. After chilling overnight at -20° the DNA was collected by centrifugation and redissolved in 1 mM EDTA, 10 mM tris-HCl (pH 8.5 at 25°).

DNA Gel Electrophoresis. DNA was electrophoresed on 16 cm gels of 1.4% agarose, 3.5% polyacrylamide and/or 6% polyacrylamide as described by Loening.²⁹ Electrophoresis was carried out at room temperature with a constant current of 2 ma/gel. Gels were stained for at least 3 hr with ethidium bromide or with

"Stains-all" (Eastman). Ethidium stained gels were photographed under blacklight illumination through red and haze filters using Plus-X film (Kodak) and negatives were scanned with a Joyce-Leobl Mark III scanning densitometer. Gels stained with "Stains-all" were scanned using a Gilford spectrophotometer.

DNA sizes were determined using the 16 fragments of phage PM2 DNA cleaved with endonuclease R Hae III (from *Haemophilus aegyptius*). The sizes of these fragments have recently been redetermined by electrophoretic comparisons with Hind and Hae III fragments of SV 40 DNA and Hind fragments of lambda (λ) DNA (R.T. Kovacic and K.E. Van Holde, private communication). These most recent sizes (e.g., see Figures 1 and 2) are about 10 to 15% higher than previously reported values.^{12,13,15} An internal "marker," either a single Hae III fragment of PM2 DNA (fragment K, 265 base pairs) or commercial t-RNA (Calbiochem, yeast soluble RNA, A grade) was added to all samples and the PM2 standards. Migration distances were converted to relative electrophoretic mobilities (REM), relative to the distance of migration of the internal marker, to compensate for differences in gel lengths and conductivities. Calibration curves for each electrophoresis run were obtained by plotting log BP (base pairs) versus REM.

RESULTS

Digestion of Chinese hamster ovary chromatin in situ with staphylococcal nuclease was conducted on nuclei isolated by three methods, including two methods used by other investigators,^{5,19} that differ considerably in the concentrations and types of monovalent and divalent cations present in nuclear wash solutions and digestion media. As observed with nuclei of other higher eukaryotes, digestion of CHO nuclei with staphylococcal nuclease produces a series of fairly discrete DNA fragments of lengths that are integer multiples of a basic repeat size. Sizes of "monomer" through "pentamer" DNA fragments determined in different digests of CHO nuclei are summarized in Table 1. The sizes of the monomer through pentamer DNA decrease with increasing digestion time due to cleavages near the ends of the units, and, perhaps, to variations in the size of the spacer

TABLE I. Sizes of DNA Fragments from Digests of CHO Nuclei

Prep. Method	Digest. Time	Length, in Base Pairs, of DNA in Band:				
		5	4	3	2	1
(1)	20 sec	867±20*	676±13*	499± 3*	346±9*	158±4 ⁺
	90 "	823±35*	670±12*	499± 1*	338±6*	157±1*
	10 min	811±13 ⁺	653±17 ⁺	473±11 ⁺	316±4 ⁺	156±4 ⁺
	60 "	----	----	----	----	146±1*
(2)	20 sec	890	698	543	358±3*	198
	90 "	865	687	503	347±4*	178
	10 min	778	651±31 ⁺	482±14 ⁺	324±7 ⁺	160±2 ⁺
	60 "	----	----	475± 3	308±1 ⁺	151±1 ⁺
(3)**	8 min		682	507	329	163
	Trial 1 16 "		664	500	316	158
	32 "		671	494	309	161
	64 "		---	462	290	157
(3)**	Trial 2 8 min		705	515	321	160
	16 "		688	495	318	163
	36 "		665	485	305	159
	64 "		---	---	286	155

* Average of two determinations using 1.4% agarose and 3½% or 6% polyacrylamide gels.

⁺ Average of three determinations using all three of the above gel types. Single values reported from Methods 1 and 2 were determined on 1.4% agarose, single values reported for Method 3 were determined on 6% polyacrylamide.

** Samples were incubated with 0.5 units of nuclease/50 µg DNA. No measurable small fragments were observed for digestion times less than 8 min.

regions (also see the Discussion). The median repeat length must therefore be estimated either from the lengths observed at very short digestion times, or from the differences in sizes between N-mer and (N-1)-mer DNA fragments at a given digestion time (assuming that the rate of degradation from the ends is independent of the size of the units).

The lengths of dimer through pentamer species of CHO DNA, averaged for all of the samples digested for the briefest lengths of time, are: 343 ± 12 ($2 \times 172 \pm 6$), 513 ± 13 ($3 \times 171 \pm 4$), 687 ± 11 ($4 \times 172 \pm 3$) and 879 ± 12 ($2 \times 176 \pm 2$) BP. Averaging these values yields a repeat size of 173 ± 4 BP.³⁰ The differences in sizes between bands N and N-1, for dimer through tetramer species, averaged for all samples run (i.e., all digestion times and all gel patterns) is 172 ± 13 BP, in excellent agreement with the above estimate. (Pentamer and

monomer data were excluded from this average since the pentamer band is not accurately sized on 6% gels and the monomer band splits into several components after moderate digestion.) Because of the agreement between these two methods and the fact that very little digestion occurred in all of the most briefly digested samples (no acid-soluble oligonucleotides were produced and less than 10% of the DNA was reduced to pentamer size or smaller) it is reasonable to conclude that significant degradation from the ends of the units did not occur in these samples and that 173 ± 4 BP is an accurate measure of the repeat length.³¹ Size data were not extrapolated to zero digestion time because this method is not generally valid, as is illustrated below for the digestion of chromatin in nuclei from the other sources. Within experimental error, no differences were noted in the repeat size in CHO nuclei prepared by the three methods.

The CHO repeat length falls significantly below the 185 to 205 BP range of repeat sizes reported for rat liver^{6,18,26} and chicken erythrocyte^{13,19} chromatin (Shaw et al.¹³ have reported a repeat length of 180 BP for chicken erythrocyte chromatin. This value must be increased by about 10% because of revisions in the sizes of the Hae III fragments of PM2 DNA used as calibration standards, as discussed in the Methods.) To verify the smaller CHO repeat size, nuclei were isolated from CHO cells, chicken erythrocytes, rat liver and calf thymus, by nearly identical procedures, and digested in the same medium (0.25 M sucrose, 0.1 mM CaCl₂, 1 mM tris-HCl, pH 8.0). The results of two digestion experiments with chicken erythrocyte nuclei and single digestions of rat liver and calf thymus nuclei are summarized in Tables II and III.

The sizes of the DNA fragments from chicken erythrocyte nuclei did not decrease with increasing digestion time except in the most extensively digested sample, indicating that clipping from ends was not a significant early degradation mechanism. The average sizes of dimer through tetramer fragments, excluding only data from the most digested sample, were 744 ± 13 ($4 \times 186 \pm 3$), 577 ± 8 ($3 \times 192 \pm 3$), and 379 ± 8 ($2 \times 190 \pm 4$) BP. The average difference in size between trimer and dimer DNA is 199 ± 8 BP,

TABLE II. Sizes of DNA Fragments from Digests of Chicken Erythrocyte Nuclei

Trial	Digest. Time	% [*] Acid Sol.	Length, in Base Pairs, of DNA in Bands			
			4	3	2	1
1 ⁺	15 sec	~0	740	581	390	200
	30 "	~0	722	568	385	173
	1 min	~0	720	569	380	160
	2 "	1	735	565	372	158
	4 "	2	740	565	370	156
	8 "	6	745	575	372	153
	16 "	11	743	585	365	136
	32 "	18	720	565	345	138
	64 "	30	-	-	-	137
2	1 min	-	773	591	392	171
	4 "	-	767	582	387	170
	8 "	-	759	586	378	165

* Determined from A_{260nm} after an aliquot of the digest (chilled) was made 0.5 M in HClO₄ and 0.5 M in NaCl and centrifuged.

+ Samples were incubated with 5 units of nuclease/50 µg DNA.

TABLE III. Sizes of DNA Fragments from Digests of Rat Liver and Calf Thymus Nuclei

Tissue	Digest. Time	% Acid Sol.	Length, in 4	Base 3	Pairs, of 2	DNA in Band: 1
Calf						
Thymus*	15 sec	0	-	532	337	163
	30 "	0	725	549	348	160
	1 min	0	752	550	347	161
	2 "	1	707	535	338	161
	4 "	5	715	530	330	159
	8 "	10	684 ⁺	515	319	155
	16 "	17	645	499	310	152
	32 "	27	627	470	297	136
	64 "	38	-	-	277	137
	128 "	51	-	-	263	135
Rat						
Liver ⁺	15 sec	0	740	563	370	190
	30 "	0	730	565	363	182
	1 min	3	735	563	360	178
	2 "	7	715	558	358	173
	4 "	8	722	547	342	170
	8 "	11	725	547	340	168
	16 "	12	715	536	333	166
	32 "	19	695	528	325	142
	64 "	29	-	508	313	138
	128 "	33	-	-	283	138

* Samples incubated with 5 units of nuclease/50 µg DNA.

+ Samples incubated with 1 unit of nuclease/50 µg DNA.

but the average difference between the tetramer and trimer band is only 167 ± 10 BP. We feel that this latter result can be safely disregarded because the 6% gels are not very accurate in this size range and the positions of the tetramer peaks were difficult to determine precisely because of their low magnitudes. With this exception the above data indicates that the size of the structural repeat in chicken erythrocyte chromatin is 194 ± 8 BP.³² The differences between DNA sizes in CHO and chicken erythrocyte nuclear digests were verified directly in the coelectrophoresis experiment shown in part in Figure 1. DNA samples isolated from chicken erythrocyte and CHO nuclei digested for three different time intervals were electrophoresed individually and in combination. A comparison of the individual patterns indicated that all of the CHO bands move faster than the corresponding chicken erythrocyte DNA bands by the expected amount. Mixing of the samples yielded bands that were shifted and broadened with respect to the bands of the individual digests.

The repeat length in rat liver chromatin, estimated as above from an average of the dimer through tetramer DNA sizes from the three briefest digests, (9 values total), is 185 ± 2 BP. (Table III.) No regular decreases in the sizes of these units were noted in these samples, again excluding degradation from ends of spacer regions. The average difference between tetramer and trimer, and trimer and dimer DNA sizes for all digestion times is also 185 ± 12 BP. This value (185 BP) is identical to the value reported by Axel,¹⁸ but is lower than the value of 205 ± 15 BP reported by Noll,⁶ presumably due to differences in calibration standards. These data indicate a difference of about 10 BP between the sizes of the structural repeats in rat liver and chicken erythrocyte chromatin although some caution is required because of the small data set and limitations of precision.

Data on DNA fragments from the calf thymus digests is less consistent than that from rat liver, but also indicates a repeat size about 10 BP smaller than that found in chicken erythrocytes. In this case the size of the repeat calculated from the average of the dimer through tetramer DNA fragments in the four briefest

Figure 1: Comparison of electrophoretic patterns of DNA from digests of CHO and chicken erythrocyte nuclei. Top to bottom: CHO nuclei digested for 8, 16, 32 and 64 min; chicken erythrocyte nuclei digested for 1 min; mixture of 16 min CHO digest and 1 min chicken erythrocyte digest. Samples were electrophoresed on 16 cm gels of 6% polyacrylamide. Sizes calculated for DNA in these bands are listed in Table I (method 3, trial 2) and Table II (trial 2). The two other chicken erythrocyte digests listed in Table II were coelectrophoresed with CHO digests. In all cases the chicken erythrocyte DNA fragments migrated slower than the CHO DNA fragments (not shown). Arrows indicate peaks due to added yeast soluble RNA. Migration positions of the Hae III fragments of PM2 DNA are given along the abscissa.

digests is 177 ± 5 BP and the average difference between tetramer and trimer, and trimer and dimer DNA is 187 ± 13 BP.

A comparison of the variations, with increasing digestion, of the size distributions of near "monomer" length DNA fragments

from these different sources is also informative. Figures 1 and 2 show electrophoretic patterns of DNA from digests of CHO and rat liver nuclei and Table IV summarizes the lengths determined for all peaks or distinctive shoulders observed in the 120 to 200 BP range for representative time courses of the digestion of all four types of nuclei. The "monomer" DNA is generally initially observed as a broad and relatively symmetric band, with a median size close to the repeat length. The slowly migrating side of this band then sharpens, the peak maximum shifts to 160-170 BP, and the distribution splits, with a second sharp band appearing in the 140-150 BP position. Gradually the ~160 BP band diminishes, the ~140 BP band becomes predominant and appears to decrease slightly in size to 135-140 BP. At this

Figure 2: Electrophoretic patterns of DNA from digests of rat liver nuclei. Digestion times (bottom to top) were 15 sec, 4 min, 16 min, 64 min and 128 min. Samples were electrophoresed on 16 cm gels of 6% polyacrylamide. Arrows indicate the positions of yeast soluble RNA.

stage significant amounts of nine discrete fragments smaller than 140 BP also appear. The appearance of these smaller fragments is generally attributed to cleavages at discrete sites within the core particles.^{13,15,18,19,33} In all of the cases examined here (Table IV) and in the digestion of yeast, mouse myeloma and crab nuclei (unpublished observations) the largest "sub-core" fragment has been sized at 123-127 BP, i.e., about 10-12 BP smaller than the core particle size. Gel patterns of DNA from relatively late digests of nuclei of all types appeared very similar, indicating that the structural features of chromatin determining the internal cleavage sites are also similar in all eukaryotes.

Variations in the method of preparing and digesting CHO nuclei did not significantly alter the general progress of the digestion or the spectrum of discrete fragments 140 BP and smaller observed near the digestion limit (data not shown). Differences in the overall rate of digestion of the various CHO preparations were observed that can be attributed to the pH and

TABLE IV. Appearance of Major DNA Fragments
Between 120 BP & 200 BP

Digest. Time	DNA Lengths (BP)*			Digest. Time	DNA Lengths (BP)		
Chick Ery.				Rat Liver			
15 sec	<u>200</u>			15 sec	<u>190</u>		
30 "	184	<u>157</u>	139	30 "	<u>182</u>		
1 min	200	<u>160</u>	135	1 min	<u>178</u>		
2 "	182	<u>158</u>	135	2 "	<u>173</u>		
4 "		<u>156</u>	146	4 "	<u>170</u>	152	
8 "		<u>153</u>	138	8 "	<u>168</u>	150	
16 "		154	<u>136</u>	16 "	166	<u>147</u>	
32 "		152	<u>138</u>	32 "	160	<u>142</u>	
64 "		155	<u>136</u>	64 "	155	<u>138</u>	122
128 "		147	<u>137</u>	128 "		<u>138</u>	125
CHO				Calf Thymus			
8 min		<u>163</u>	141	15 sec	<u>191</u>	163	
16 "		158	<u>138</u>	30 "		<u>160</u>	141
32 "		161	<u>141</u>	1 min		<u>161</u>	141
64 "		157	<u>137</u>	2 "		<u>161</u>	142
				4 "		<u>159</u>	142
				8 "		<u>155</u>	140
				16 "		152	136
				32 "		<u>148</u>	<u>136</u>
				64 "		<u>137</u>	<u>123</u>
				128 "		<u>135</u>	<u>123</u>

* The predominant DNA size is underlined.

Ca^{++} concentration dependence of the enzyme activity. As noted in previous reports,^{18,19} the progress of the digestion of nuclei is markedly different from that of unsheared chromatin gels prepared by conventional methods. Digestion of chromatin does not generally produce distinctive oligomer DNA fragments larger than trimer size, even in the earliest stages. Instead, core particle (140 BP) and sub-core (<140 BP) size DNA fragments are produced at nearly equal rates and the larger DNA appears as a broad smear with small maxima near dimer, trimer and, occasionally, tetramer size. Recently we have observed that if nuclei are lysed by brief homogenization in dilute EDTA solution (0.1 mM EDTA, pH 6.5), centrifuged, and the pellet resuspended in 0.1 mM CaCl_2 , 1 mM tris-HCl (pH 8.0), the resulting "chromatin" is digested by staphylococcal nuclease in a manner very similar to the digestion of chromatin in intact nuclei. These results suggest to us that the higher selectivity of staphylococcal nuclease for spacer regions in nuclei or in chromatin only briefly exposed to chelating agent may reflect the degree of extension and remaining pattern of folding of the fibrils maintained by divalent cations. Isolated chromatin does not assume a fully extended state until divalent ions are thoroughly removed. Once fully extended, the fibrils may not reassume their original folded configuration, even though Ca^{++} is added back prior to digestion, thereby allowing access of the nuclease to cleavage sites within the core particles as well as in spacer regions.

Significant differences were noted in the rate of digestion of nuclei from the four sources, isolated and digested under identical conditions (using the same solutions), but using different enzyme concentrations (see Tables I, II, III). The rate of digestion follows the order: CHO > rat liver >> calf thymus > chicken erythrocyte, which is also the order of decreasing transcriptional activity and increasing heterochromatin content of these cells. The rate of digestion of rat liver and CHO nuclei is roughly five times that of chicken erythrocyte nuclei. Similar results were obtained in a second series of experiments; however, further work is required to determine the relationship between nuclease accessibility and

the degree of chromatin activity or condensation.

DISCUSSION

Nuclease digestion studies have led to the concept that histones are organized along DNA in chromatin into identical or nearly identical repeated structural units. Previous reports indicated that repeat units from several higher eukaryotes contain about 200 base pairs of DNA--a 140 BP region tightly folded in association with an octameric histone "core" complex, forming a globular "core particle" and a 40 to 60 BP region associated primarily with H1 histones. In many respects this model must be regarded as an oversimplification, reflecting the basic first order structural features of chromatin, but lacking the detail necessary for explaining the obvious functional and higher order structural diversity of chromatin. For example, Weintraub and Groudine³⁵ have recently shown that globin and other active genes in chicken erythrocyte nuclei and in isolated 11S core particles are preferentially attacked by DNase I, a result that is inconsistent with an identical subunit model.³⁶

That other variations in this basic structural theme occur is demonstrated by the findings that the repeat units in some lower eukaryotes such as yeast²²⁻²⁴ and *Neurospora*³⁵ are relatively small (160-170 BP) whereas in another lower eukaryote, *Tetrahymena*, the repeat size is about 200 BP.³⁷ The present study demonstrates that significant differences in the apparent repeat length also occur in chromatin from higher eukaryotes. In both higher and lower eukaryotes these variations in repeat length appear to occur in the spacer regions and not in the length of DNA associated with the core particles.

Several lines of evidence suggest that certain concepts of the spacer regions should be further refined. As noted here and elsewhere, the sizes of monomer, dimer, trimer, etc. DNA fragments decrease with increasing digestion time. The rate of decrease, in our hands, depended on the source of the nuclei and, to some extent, on the preparation method. This size decrease has generally been attributed to cleavages from ends of initially identical spacer regions. Additionally, the significant breadth of these bands has been attributed to random cleavages within spacer regions, implying that all spacer DNA is

equally accessible to nuclease. One alternative possibility, suggested by the fact that near dimer (2x140) BP and trimer (3x140) BP length DNA fragments persist at the presumed digestion limit, is that spacer regions throughout chromatin are not uniformly the same length. A part of the decrease in DNA sizes would in this case be due to the increased probability of cleavage in long versus short spacers, assuming that several sites are available in each. Electron microscopic observations of variations in interparticle distances support this possibility, but are not definitive because of errors due to viewing perspective and the potential for unraveling part of the structure during grid preparation. Also consistent with this suggestion is the reported isolation of dimer particles lacking H1 by Varshavsky et al.²⁰

In addition, all of the DNA in spacer regions does not appear to be equally accessible to staphylococcal nuclease. One preferred cleavage site occurs about 20 BP outside of the core particle in many, if not all, repeat units, as is evident from analyses of the time course of the digestion where one observes that the initially broad "monomer" band rapidly decreases in length and splits into two species 160 and 140 BP long. Both of these discrete DNA species have been observed in the digestion studies reported here and from other laboratories, including the studies of the digestion of yeast²⁴ and *Neurospora*²⁵ nuclei, indicating that this cleavage site external to the core particle as well as the cleavage site adjacent to the core particle has been evolutionarily conserved, despite variations in median spacer length.

Considerable potential for modulation of the basic subunit structure of chromatin exists through the complex patterns of side chain modifications of all of the histones, the molecular diversity of histone H1 (and other histones to a lesser extent) and the possible influence of specific non-histone proteins. In particular, the tissue and species heterogeneity of H1 histones and the 1:2 molar ratio of H1 to each of the core histones mandate an intrinsic non-identity and asymmetry of spacer regions and provide a rational basis for expecting heterogeneity of spacer lengths and accessibility to nuclease. (Noll²⁵ and

Morris³⁸ have suggested that differences in H1 histones may account for the small spacers found in *Neurospora* and *Aspergillus* chromatin; however, there is no intrinsic reason to restrict this argument to very diverse species.) In general one must consider the possibility that the "spacer" regions may also be structured in specific but variable ways, depending on the H1 species (or other proteins) bound. Such specific variability could be important in allowing for diversity in the higher order folding of local regions of chromatin fibrils relating to the functions of these regions. Specific evidence for the structuring of spacer regions by H1 and H5 histones will be presented in a later report (Nelson, Oosterhof and Rill, in preparation).

Note added in preparation: While this manuscript was being reviewed, Compton et al.³⁹ reported a comparison of the sizes of the repeat unit in chromatin from several sources including cultured CHO cells (177 BP), rat liver (196 BP) and chicken erythrocytes (207 BP). The differences between these repeat lengths are consistent with the results reported here although the absolute values are somewhat higher due to discrepancies in the calibration procedures. The repeat length in calf thymus chromatin was not reported by Compton et al.

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31. The lower estimate of the uncertainty (± 4 BP) is cited since both methods yield essentially identical values of the repeat size and the latter method, involving measurement of a small difference between two larger numbers, is necessarily less precise.

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32. This repeat size (194 BP) is the average of the 189 BP and 199 BP estimates of the two methods. The uncertainty cited includes the mean deviation of these values from the average (± 5 BP) plus an expected minimum uncertainty in the precision of these methods (± 3 BP). This uncertainty is admittedly somewhat arbitrary, but should encompass a large enough range to include the true value.
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