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Chemical evidence that chromatin DNA exists as 160 base pair beads interspersed with 40 base pair bridges.

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ABSTRACT

Digestion of rat liver nuclei by an endogenous endonuclease generates double-stranded DNA fragments which are initially about 205 base pairs long, as reported previously by Hewish and Burgoyne. As digestion proceeds, the average size of these fragments is reduced to about 160 base pairs. Electrophoresis under denaturing conditions shows that these DNA fragments contain single strand nicks at ten base intervals. Fifteen bands, 10-150 bases, are clearly resolvable. DNA fragments of 160 to 200 nucleotides are not resolved as distinct species. The results suggest that the chromosomal subunit contains both a 160 base-pair DNA segment, in a conformation susceptible to single strand nicking at ten base intervals, and a forty base-pair DNA segment in a conformation more uniformly susceptible to endogenous endonuclease activity. This chemical evidence agrees with morphological observations suggesting that chromatin has a "bead and bridge" structure.

INTRODUCTION

Recent evidence suggests that the eukaryotic genome consists of repeating subunits, each containing a histone core and a segment of the DNA fiber. Felsenfeld has recently reviewed the electron microscopic observations, the results of nuclease digestion experiments, and the studies of histone-histone interactions which led to this concept[1]. Features common to most models of the chromatin subunit[2-5] include both a 180-205 base pair segment of DNA and a protein core; these are associated as particles, called ν bodies[6] or nucleosomes[7]; the composition of the protein core of such particles may be a histone octomer, two each of H2A, H2B, H3 and H4.

We have attempted to determine whether all of the DNA of the chromatin subunit is contained within the nucleosome. Morphological observations of SV-40 DNA-histone complexes at low ionic strength reveal nucleoprotein "beads" connected by "bridges" of (possibly protein-free) DNA, indicating that some of the DNA is not complexed within the nucleosome under these conditions. Such bridges are not observed when these complexes are visualized at an ionic strength of 0.15[8]. Several groups have interpreted the results of nuclease digestion studies in terms of such a bead and bridge model for the chromosome

9-11. On the other hand, Noll *et al.*,[12] have suggested that all the DNA of the chromatin subunit is in the nucleosome form, complexed with histone, and that other structures, such as "bridges", are artifacts of preparation.

Our results indicate that both micrococcal nuclease and rat liver endogenous endonuclease initially cleave nuclear DNA at 200-205 base pair intervals and that, as digestion proceeds, these fragments are reduced to relatively stable 150-160 base pair pieces. The endogenous endonuclease also makes single strand nicks at ten base intervals; only fifteen such fragments (10-150 bases) are clearly resolved, while longer fragments (160-200 bases) migrate as a broad smear on polyacylamide gels. These results suggest that, at low ionic strength, chromatin DNA exists in at least two conformations. First, a segment containing 150-160 base pairs is in a relatively nuclease resistant "bead" and has a conformation with single strand scission sites at ten base intervals. Second, a segment containing 40-50 base pairs makes up the "bridge" and exists in a conformation more uniformly susceptible to degradation by endogenous or micrococcal nuclease but relatively resistant to DNase I.

EXPERIMENTAL SECTION

Rats were exsanguinated and liver nuclei isolated essentially by the procedure of Hymer and Kuff[13], involving washing in and sedimenting from 0.25 M sucrose, 3 mM $CaCl_2$, 10 mM Tris-Cl, pH 8.0, containing 1% (v/v) Triton X-100. The nuclei were washed free of detergent in this buffer lacking Triton and washed once with the same buffer containing 1 mM $CaCl_2$. After adjusting to a DNA concentration of 1 mg/ml (assuming A_{260} = 20 for 1 mg DNA/ml in 1% sodium dodecyl sulfate), nuclei were either used immediately or frozen at -20° C . Fresh or frozen nuclei produced identical results. For digestion with the endogenous endonuclease, the nuclear suspension was made 10 mM in $MgCl_2$. Digestions were carried out at 37° C with gentle stirring.

Micrococcal nuclease (E.C. 3.1.4.7) was obtained from Worthington Biochemical Corp., their grade NFCP. For these digestions nuclei were suspended in 0.25 M sucrose, 1 mM Tris-Cl, pH 8.0, 0.1 mM $CaCl_2$. Digestions were carried out at 37° C with gentle stirring for varying times at various enzyme concentrations, as indicated. At the conclusion of all digestions, the samples were made 25 mM in EDTA and 1% in sodium dodecyl sulfate and extracted once with an equal volume of phenol saturated with 0.1 M Tris-Cl, pH 8.0, 0.01 M EDTA. After addition of NaCl to a concentration of 0.1 M, DNA was precipitated from the aqueous phase by addition of two volumes of absolute ethanol and storage at -20° overnight. The DNA was dissolved in sample buffer at concentrations of about 2-4 mg/ml, based on the original DNA content.

Electrophoresis was performed in a slab gel apparatus using 1.5 mm thick polyacrylamide gels, with a migration distance for bromphenol blue tracking dye of about 12 cm. Gels were formed using Tris-EDTA-borate buffer (TEB)[14] and contained 6% Cyanogum 41 with 6 M urea. Preelectrophoresis was carried out at 20 mA constant current for 0.5 - 1.5 hours. Samples were applied in 0.2 x TEB with 20% sucrose or 0.2 x TEB containing 10 M urea. The relatively small DNA fragments studied in these experiments are completely denatured in 10 M urea at room temperature. Furthermore, neither renaturation of denatured DNA nor denaturation of native DNA occurs when these fragments are electrophoresed in gels containing 6 M urea (data not shown). Therefore, we are able to observe the electrophoretic behavior of both native and denatured DNA fragments on a single polyacrylamide gel. Electrophoresis at a constant current of 20 mA required about 1.75 hours. Staining was carried out overnight in 0.005% Stains-All in 50% (v/v) formamide. Gels were destained in running water and photographed using Polaroid Type 55 P/N film. Negatives were scanned with a Joyce-Loebl densitometer.

Double-stranded DNA standards for gel electrophoresis were fragments from an *Haemophilus aegyptus* restriction endonuclease (Hae III) digest of SV-40 DNA, having sizes of 1465, 820, 550, 370-300 (quintet), 220 and 165 base pairs[15]. We are indebted to Drs. Tikva Vogel and Maxine Singer for the SV-40 DNA and to Dr. Ron Reeder for performing the nuclease digestion. Phenylalanyl t-RNA, 77 nucleotides in length, was a gift from Dr. George Rushizky.

RESULTS

An endogenous endonuclease[16,17] and micrococcal nuclease[18] each digest rat liver nuclear DNA to fragments which are multiples of about 200 base pairs. These fragments are generated rather early in the course of digestion, and, as digestion proceeds, the amount of "monomer" DNA fragments increases (Fig. 1). In addition, the average size of the monomer DNA band decreases during digestion, from about 200 base pairs at early stages to 150-160 base pairs after longer digestions. This decrease is seen both when the endogenous endonuclease and when micrococcal nuclease is used. Since this decrease in size probably results from continued nuclease activity, the size of the initial cleavage fragment was estimated by determining the size of the DNA in the monomer band after various times of digestion and extrapolating to zero time (Figure 2). This analysis suggests that the initial cleavage of rat liver nuclear DNA by both the endogenous endonuclease and micrococcal nuclease is at intervals of 205 base pairs.

The initial 205 base pair cleavage product is rather rapidly digested to

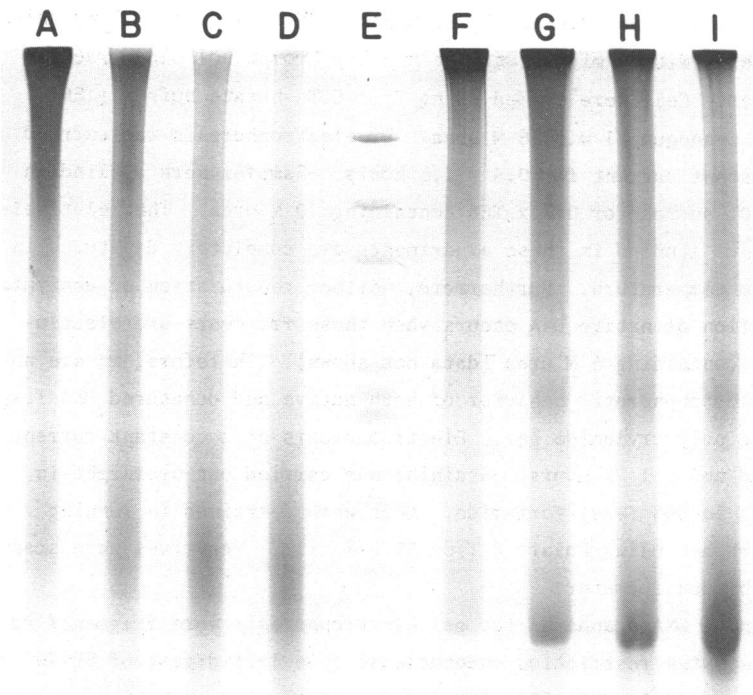

Figure 1

Polyacrylamide gel electrophoresis of DNA samples from digestion of rat liver nuclear DNA *in situ* by endogenous endonuclease (A-D) and micrococcal nuclease (F-I). Endogenous endonuclease digestions were for 30 min (A), 60 min (B), 90 min (C) or 120 min (D). Micrococcal nuclease digestions were for 2 min at 37° with enzyme concentrations of 20 U/ml (F), 50 U/ml (G), 100 U/ml (H) or 200 U/ml (I). DNA was isolated, dissolved in 0.2 x TEB containing 20% sucrose and electrophoresed on 6% polyacrylamide gels containing 6 M urea. Column E contains the SV-40 Hae III standard DNA fragments.

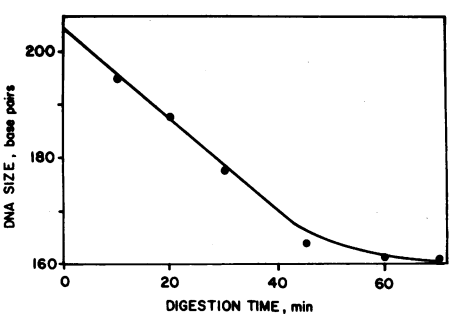

Figure 2

The size of the monomer DNA fragment in endogenous endonuclease digests of rat liver nuclear DNA at different times of digestion. The size of the mid-point of the monomer DNA band was estimated by comparison with the standards. Extrapolation to zero time of digestion shows that the initial cleavages made by endogenous endonuclease are at about 205 base pair intervals.

the 160 base pair fragment, which is much more resistant to further nuclease digestion. Thus, after one hour of digestion, the endogenous endonuclease has converted essentially all the DNA on the gel to 160 base-pair fragments, and the patterns observed after two or four hours of digestion are quite similar to that after one hour. We also find, as noted by others previously[10], that micrococcal nuclease digestion of rat liver nuclei generates a relatively stable 150-160 base pair intermediate between "monomer" DNA and the smaller fragments which characterize a micrococcal nuclease limit digest. Thus, results using both enzymes suggest that a 150-160 base-pair sequence within the 205 base pair DNA repeat exists in a relatively nuclease resistant state.

Electrophoretic analysis under denaturing conditions of the DNA fragments generated by endogenous endonuclease digestion supports the idea that a 150-160 base pair sequence has a conformation which is different from the remainder of the original 205 base-pair fragment. Such analyses show that the monomer DNA fragments contain single strand nicks at regular intervals. Electrophoresis reveals a series of fifteen bands of single-stranded DNA, three of which migrate faster than bromphenol blue in 6% polyacrylamide gels containing 6 M urea (Figure 3). The fifteen bands migrate as multiples of a unit length, with electrophoretic mobilities linearly related to the logarithm of band number (Figure 4). Coelectrophoresis with phenylalanyl t-RNA as standard suggests that these bands are multiples of ten nucleotides. Furthermore, these bands have the same mobilities as the single strand DNA fragments produced by DNase I digestion of rat liver nuclei (Figure 5). Noll[19] has previously shown that these DNase I fragments contain multiples of ten bases.

Densitometric scans of gels of endogenous endonuclease DNA fragments reveals that there are no cleanly resolved bands containing DNA longer than 150 bases (Figure 6). This observation is true at all stages of digestion, including short times where the bulk of the double-stranded DNA fragments are longer than 400 base pairs. We have never observed discrete bands of single-stranded fragments longer than 150 nucleotides as the products of any endogenous endonuclease digestion. Single-stranded DNA fragments of 160 bases and longer (up to 200 bases) appear as a broad smear. This finding is not due to a lack of resolving power; at least 25 bands are clearly discernible when DNase I digests are coelectrophoresed under denaturing conditions with these endogenous endonuclease digests. Our data thus imply that within the monomer DNA fragment there is a 160 base pair segment of DNA susceptible to single strand scission at intervals of 10 bases. The remainder of the 200 base pair monomer DNA fragment is cleaved more randomly, suggesting that this portion has a different structure.

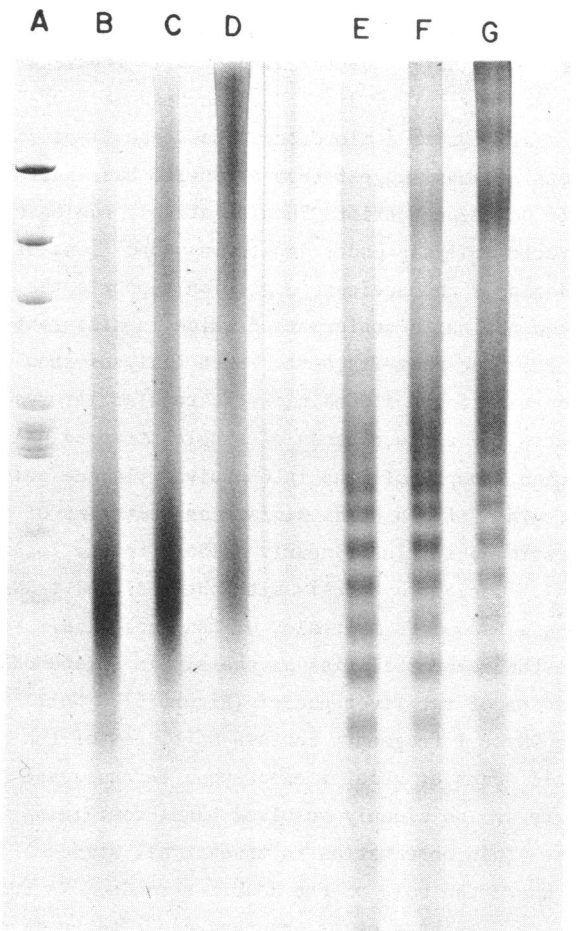

Figure 3

Polyacrylamide gel electrophoresis of DNA samples from digestion of rat liver nuclear DNA *in situ* by endogenous endonuclease analyzed under native (B-D) and denaturing (E-G) conditions. Digestions were carried out for 30 min (D,G); 60 min (C,F); or 120 min (B,E) and the DNA was isolated. Samples B-D were dissolved in 0.2 x TEB containing 20% sucrose. Samples E-G were dissolved in 0.2 x TEB containing 10 M urea. Column A contains the standard SV-40 Hae III fragments. Electrophoresis was carried out on 6% polyacrylamide gels containing 6 M urea.

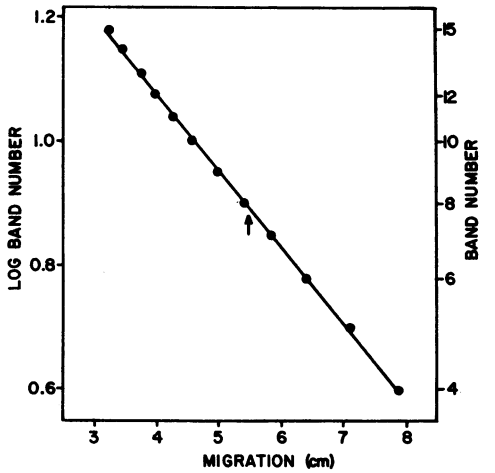

Figure 4

Mobilities of the single-stranded DNA fragments produced by endogenous endonuclease digestion of nuclear DNA. Electrophoretic mobilities of single-stranded DNA fragments such as those shown in Figure 3E or 3F are plotted *vs* the logarithm of the band number. Three bands which migrate faster than bromphenol blue are not included. The arrow indicates the migration position of phenylalanyl t-RNA (77 bases).

DISCUSSION

Our results indicate that the three nucleases most used to probe the subunit structure of chromatin have markedly different substrate specificities. Micrococcal nuclease initially cleaves both DNA strands at 200 base-pair intervals, then continues to digest these fragments to a length of 150-160 base pairs. DNase I produces single strand nicks at 10 base intervals. The endogenous endonuclease of rat liver appears to have properties in common with both of these enzymes. Like micrococcal nuclease, it initially generates 200 base-pair DNA fragments, which are further digested to 160 base pairs. The major finding of this work is that this endonuclease, like DNase I, also produces single strand nicks in chromatin DNA at 10 base intervals. This observation is analogous to the results found by Noll[19] on digestion of nuclear DNA by DNase I. Indeed, the products of the endonuclease digestion have the same mobilities as fragments generated by digestion of rat liver nuclear DNA by DNase I. Certain differences are apparent, however, between our results, and those previously detailed by Noll.

First, the single-stranded fragments generated by the endogenous nuclease yield bands of intensities which suggest that all the susceptible sites have

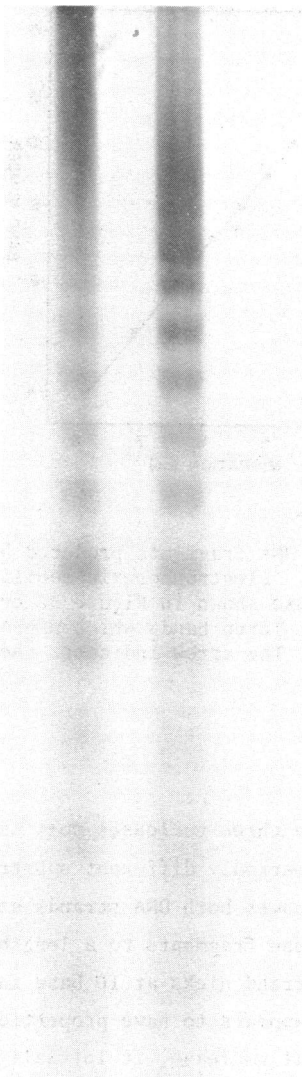

Figure 5

Polyacrylamide gel electrophoresis of DNA samples from digestion of rat liver nuclear DNA *in situ* by DNAse I (left track) and endogenous endonuclease (right track), analyzed under denaturing conditions. DNase I digestion was with 100 U enzyme/ml for 2 min at 37°. Endogenous endonuclease digestion was for 60 min at 37°. DNA samples were analyzed on 6 M urea containing 6% polyacrylamide gels after dissolution in sample buffer containing 10 M urea.

an equal probability of being nicked. In contrast, the fragments generated by DNase I digestion yield bands of varying intensities (data not shown and[19]), suggesting that DNase I cleavage sites within the chromatin subunit might not be equally accessible.

These nucleases also differ in the number of discrete bands of DNA fragments which they generate. Noll[19], using DNase I, observed 22 bands at 10 base intervals. Similarly, we see in DNase I digests up to 25 discrete bands which migrate as multiples of 10 bases. In contrast, the endogenous endonuclease, generates only 15 clearly resolved bands containing multiples of 10 bases. At all stages of digestion, the single-stranded DNA fragments 160-200 bases in length migrate as a smear of heterogeneous material, in marked contrast to the smaller fragments, 10-150 bases in length, which migrate in discrete bands. These differences between the products produced by endogenous endonuclease and DNase I do not derive from differences in method of isolation of the DNA fragments or their analysis, both being identical. Further, the similarity in ionic conditions for the two digestions, the presence of 1 mM $CaCl_2$ in the endogenous endonuclease digestions vs .1 mM $CaCl_2$ being the sole difference, makes it unlikely that the substrate DNA differs for the two enzymes. We conclude that, at low ionic strength, rat liver chromatin DNA contains a 160 base pair segment susceptible to nicking by the endogenous endonuclease at 10 base

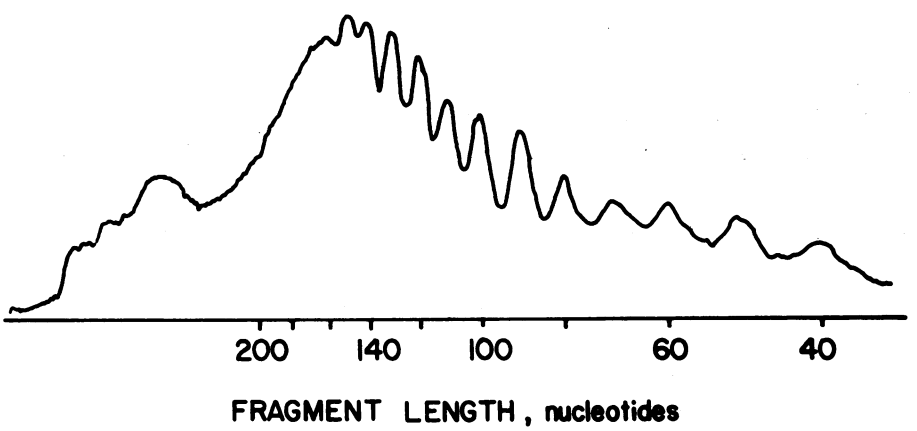

Figure 6

Densitometric scan of the single-stranded DNA fragments of endogenous endonuclease digestion of nuclear DNA. The negative of a photograph of a gel equivalent to that shown in Figure 3F was scanned in a Joyce-Loebl densitometer. The ordinant is linear with optical density.

intervals. This is the same length of DNA as is contained within the relatively stable, nuclease-resistant, double-stranded monomer fragments generated by either micrococcal nuclease or the endogenous endonuclease.

If each chromatin subunit contained only a 160 base pair DNA segment, then the initial cleavage products of both micrococcal nuclease and the endogenous endonuclease ought to migrate as multiples of 160 base pairs, not as multiples of 205 base pairs. Furthermore, the endogenous endonuclease ought to generate single-stranded fragments longer than 160 bases. Since the experimental evidence does not support these predictions, we conclude that the 160 base pair segment represents only a portion of the DNA of the chromatin subunit.

The results suggest that the chromatin subunit contains, in addition to the 160 base pair segments, a 40-50 base pair length of DNA with the following properties: it is digested relatively rapidly by micrococcal nuclease and the endogenous endonuclease of rat liver, and contains a site where these enzymes make their initial cleavages; it is not nicked at regular intervals by the endogenous endonuclease; it is relatively resistant to nicking by DNase I. These properties are quite different from those of the 160 base-pair segment. The relative susceptibility of the 40 base-pair segment to micrococcal nuclease and the endogenous endonuclease suggest that it is in a more extended and/or protein-free conformation. Furthermore, the lengths of these segments are in good agreement with the measurements of Griffith[8] who observed "beads" containing 160-170 base pairs of DNA, connected by shorter DNA "bridges", about 40 base pairs in length.

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