A tenfold decrease in ionic strength caused the compact unit fiber to relax into a flexible string of 21 beads, each 110 Å in diameter. The beads were joined by bridges roughly 20 Å in diameter and 130 Å long.

These measurements indicate that when the DNA of the minichromosome is complexed with histone proteins, the DNA is condensed sevenfold to form a 2100-Å length of the typical unit fiber. This condensation appears to occur in blocks, each block or bead containing 170 base pairs of DNA and each bridge about 40. At physiologic salt concentrations these 21 beads condense sequentially to form a 2100-Å loop, and a repeat of roughly 100 Å can be seen along the fiber axis (Fig. 1). In solutions of low salt concentration the beads are exposed uniformly spaced about the circle. The short bridges have a diameter close to that of the bare duplex and constitute less than 20 percent of the total DNA. Thus, most of the histone protein must reside within the beads.

In a study of water-swollen nuclei by electron microscopy, linear arrays of 70-Å spherical bodies were reported (*13*). Although smaller, they would appear to be related to the beads described here. A 100- to 110-Å repeat along the axis of oriented chromatin fibers has been detected by x-ray crystallography and is dependent on the integrity of the histone-DNA complex and lost on stretching of the chromatin (*14*). It seems likely that the repeating unit corresponds to the 110-Å blocks. Digestion of chromatin fibers with certain nucleases has revealed sensitive regions spaced roughly 200 base pairs apart (*15*). These bridges described here are separated by about 200 base pairs and should correspond to the nuclease sensitive regions. Indeed, nuclease digestion of the analogous polyoma nucleoprotein complex has yielded fragments of DNA consisting of 200 base pairs (*16*).

This work has exploited a defined natural chromatin to measure the unit fiber packing ratio and to verify other key predictions of the Kornberg model. The encapsidation of the SV40 minichromosome (*17*) illustrates the great flexibility of the unit fiber.

DNA in the unit fiber exists in two states revealed by electron microscopy. Bead DNA is highly condensed and masked from the action of a duplex-specific nuclease (*15*). Bridge DNA is flexible, less condensed, and cleaved by this nuclease. The SV40 minichromosome provides a unique structure for studying the factors that determine bead and bridge (*18*) formation and for ascertaining whether they are specified by the DNA sequences they contain (*19*).

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9. SV40 minichromosomes were purified in the following manner. CV-1 monkey cell cultures were harvested 36 hours after a high multiplicity infection with SV40 RH911. Nuclei were purified by differential centrifugation in a buffer containing 0.15*M* NaCl, 10 m*M* tris · HCl (*p*H 7.4), and 10 m*M* $NaHSO_3$. The nuclei were then suspended in buffer (including 0.25 percent Triton X100) and incubated for 5 minutes at 37°C. The SV40 minichromosomes contained in this extract were purified by sucrose velocity sedimentation in this buffer (*5*).
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17. The most tightly twisted forms of the SV40 minichromosome loop are roughly spherical particles 400 Å in diameter which are capable of fitting within the virus capsid. It is possible that the flexible nature of the bridge regions provides a mechanism for the generation of more condensed nucleoprotein fibers during metaphase.
18. Electron micrographs of the beaded forms show open, relaxed contours. Deproteinization, however, frees tightly supercoiled DNA molecules, suggesting that the duplex is partially melted, primarily within the beads, releaving roughly 42 supertwists.
19. This may be approached by investigating the bead or bridge location of various restriction nuclease sites or by studying the reannealing behavior of the nuclease-generated 200 base pair fragments.
20. Single side-band strioscopy is a term to denote the partial darkfield condition; a conical darkfield aperture in the condenser system and an off-centered objective aperture were used.
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Preparation of Native Chromatin and Damage Caused by Shearing

Abstract. *Chromatin prepared by a method involving limited nuclease digestion contains the same repeating structure as chromatin in the nucleus, whereas chromatin prepared by conventional methods involving shear does not.*

The finding of a tetramer of the arginine-rich histones (*1*) has led to a model of chromatin structure based on a repeating unit about 100 Å in diameter, comprising eight histone molecules surrounded by 200 base pairs of DNA (*2*). One line of support for this model comes from studies of the digestion of chromatin by nucleases. The DNA from rat liver nuclei digested with an endogenous nuclease gave a regular pattern of bands on polyacrylamide gels corresponding to multiples of a unit size (*3*). This same pattern of bands was observed when nuclei were digested with purified micrococcal nuclease, and the unit size was shown to be about 205 base pairs (*4*).

Since the nuclease digestion studies were done on whole nuclei, whereas the model involves only histones and DNA, the question arose whether chromatin extracted from the nuclei would give similar digestion results. We found that, when extracted by conventional methods, it does not. We now show that this result is due to damage caused by shearing, and we describe a method of extracting chromatin whereby the native structure is better preserved.

The first requirement in most studies on chromatin is to obtain the material in homogeneous solution. In the simplest of conventional methods, nuclei are lysed in hypotonic buffer, and the viscous mass of chromatin is converted to a homogeneous solution by shearing in a motor-driven homogenizer (*5*). In our alternative to this procedure, the chromatin is first cut into large fragments by digesting the nuclei with micrococcal nuclease and then the nuclei are gently lysed in hypotonic buffer, giving a homogeneous solution; we refer to this as the nuclease method.

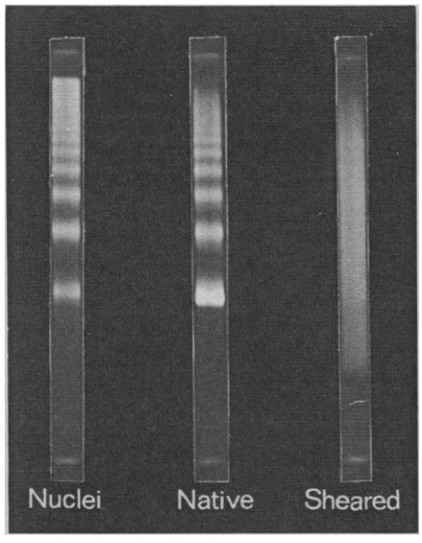

Fig. 1. Comparison of chromatin in nuclei with chromatin prepared by the nuclease method (native) and chromatin prepared by shearing. Nuclei, native chromatin, and sheared chromatin were digested with micrococcal nuclease, and digestion was terminated by the addition of ethylenediaminetetraacetate (NaEDTA) to 5 m*M*, sodium dodecyl sulfate (SDS) to 1 percent (weight to volume), and NaCl to 1*M*. Each digest was extracted with an equal volume of a mixture of chloroform and isoamyl alcohol (24:1). The aqueous phase was reextracted, dialyzed against water at 2°C, and lyophilized; the lyophilized material was dissolved in sample buffer, and volumes containing 3 to 4 μg of DNA were subjected to electrophoresis on 2.5 percent polyacrylamide tube (6 mm in diameter) gels (*19*) for 2 hours at 5 ma per tube at room temperature; the gels were stained with ethidium bromide (*12*).

On further digestion with micrococcal enzyme, chromatin prepared by the nuclease method gives the same pattern of bands on a gel as chromatin in nuclei (Fig. 1), whereas chromatin prepared by conventional methods gives no bands but only a smear. By this criterion, which we refer to as the micrococcal test, chromatin prepared by the nuclease method is native, whereas chromatin prepared by methods involving shear is not.

The nuclease method and procedure for further digestion with micrococcal nuclease were as follows. Nuclei were prepared from rat liver and suspended in 0.34*M* sucrose–buffer A (0.44 ml per gram of liver, resulting in about 1.5×10^8 nuclei per milliliter) as described (*3*). The suspension (1 ml) was made 1 m*M* in $CaCl_2$ and digested with micrococcal nuclease (15 unit/ml) (Worthington) for 30 seconds at 37°C. Digestion was terminated by chilling on ice and addition of 0.02 ml of 0.1*M* NaEDTA, *p*H 7. The nuclei were centrifuged for 5 minutes at 4000*g*, lysed by suspension in 1 ml of 0.2 m*M* NaEDTA, *p*H 7, with the use of a Pasteur pipette, and centrifuged for 2 minutes at 4000*g*. The opalescent supernatant showed an absorbance at 260 nm of 24, corresponding to about 75 percent of the DNA in the starting nuclei. The fragments of native chromatin in the supernatant contained DNA about 95 percent in the size range of 3,000 to 30,000 base pairs, with a weight average of about 9000 base pairs [as determined by comparison with EcoRI fragments of bacteriophage λ DNA (*6*) in 0.6 percent agarose gels (*7*)]. These sizes are the largest that can be obtained in homogeneous solution. Smaller

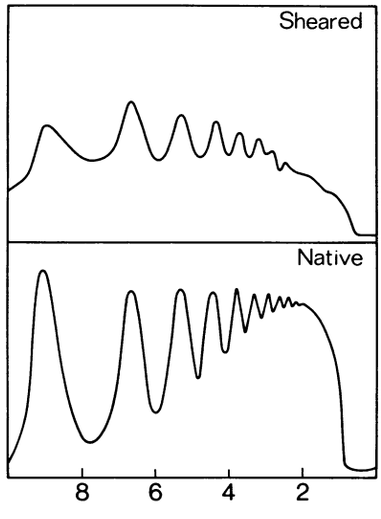

Fig. 2. Effect of shear on native chromatin. Native chromatin was prepared by the nuclease method, diluted ($A_{260} = 10$), homogenized (resulting in a weight average DNA of about 4000 base pairs), further digested with micrococcal nuclease (75 unit/ml) for 30 seconds at 37°C, and the DNA extracted and analyzed in a polyacrylamide gel as described in Fig. 1. The negative of the gel photograph was scanned with a Joyce-Loebl microdensitometer to obtain the tracing labeled "sheared." The same procedure was followed except that the homogenization step was omitted to obtain the tracing labeled "native."

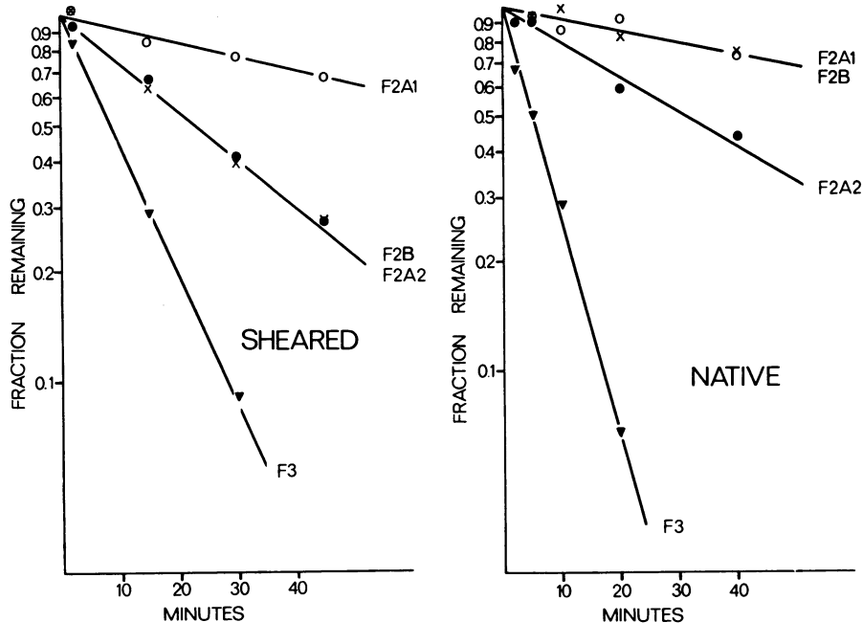

Fig. 3. Time course of tryptic digestion of native and sheared chromatin. Native and sheared chromatin, prepared as described in the text, were diluted ($A_{260} = 8.5$) and digested with trypsin (8.5 μg/ml) (Worthington, Biochemical Co., twice recrystallized) at 23°C. Portions were taken at intervals, mixed with pancreatic trypsin inhibitor (provided by Dr. D. Dyckes) in twofold excess by weight over the trypsin, freeze-dried, and subjected to electrophoresis on 18 percent SDS polyacrylamide slab gels (30 cm long) prepared according to Laemmli (*20*) with modifications (*21*). The gels were fixed for 1 hour in a mixture of methanol, acetic acid, and water (5 : 1 : 5), stained for 30 minutes in 0.1 percent Coomassie brilliant blue in the same solvent, destained in a mixture of 5 percent methanol and 7 percent acetic acid, and scanned with a Joyce-Loebl microdensitometer. The peaks were sufficiently well resolved and constant in width that peak heights could be used to construct the curves shown above.

sizes may be obtained by more extensive digestion of the nuclei, for example, a weight average of about 1600 base pairs by digestion with nuclease (150 unit/ml) for 30 seconds (the unit-sized fragments in this preparation contain DNA of 150 to 200 base pairs, as mentioned later). A narrower range of sizes may be obtained by fractionation on a sucrose gradient (*4*). For the gel in Fig. 1 labeled "native," the preparation containing DNA in the size range 3,000 to 30,000 base pairs ($A_{260} = 24$) was further digested in a solution consisting of 5 m*M* tris·acetate, *p*H 7.8, 20 m*M* ammonium acetate, 0.4 m*M* $CaCl_2$, and 0.2 m*M* NaEDTA with 300 units of micrococcal nuclease per milliliter for 30 seconds at 37°C.

Sheared chromatin was prepared by centrifuging nuclei (1 ml), lysing in 0.2 m*M* NaEDTA, *p*H 7 (2 ml), and homogenizing for 90 seconds at full speed in a Sorvall Omnimixer, resulting in a DNA of weight average size about 4000 base pairs. This sheared chromatin ($A_{260} = 17.5$) was digested as described above for the further digestion of native chromatin except that the nuclease concentration was 200 unit/ml.

Direct evidence that shearing is the factor in conventional methods responsible for damage to the native structure is given in Fig. 2. Chromatin containing DNA of weight average size 9000 base pairs was prepared by the nuclease method and subjected to the micrococcal test before and after shearing. Densitometer traces of the gels show a high background after shearing. On the other hand, chromatin containing 1600 base pairs of DNA, isolated by sucrose gradient fractionation (*4*) of material prepared by the nuclease method, does not give a high background after shearing. Thus, two observations implicate shear in damage to chromatin: the high background on gels, and the fact that only chromatin above a certain size is affected.

If shearing affects the state of the histones in chromatin as well as the state of the DNA, then shearing may alter the pattern of digestion of chromatin by proteases as well as by nucleases. Experiments on the tryptic digestion of native and sheared chromatin reveal a striking difference in the case of histone F2B, which is digested in parallel with F2A1 in native chromatin and several times faster, in parallel with F2A2, after shearing (Fig. 3). This change in relative rates is wholly reproducible, although the actual values of the rates after shearing vary from one preparation to another. The order of digestion of the histones is compatible with results of earlier workers (*8*), but not with the report by Weintraub and Van Lente (*9*) that F2A1 is digested in parallel with F3. As is shown in Fig. 3, we find widely different rates of digestion of F2A1 and F3.

The effect of shearing is readily understood in terms of the model of chromatin structure (*2*) mentioned above. In this model, DNA running the length of a chromatin fiber is tightly coiled or folded around a histone core. Shearing, which stretches a chromatin fiber to the point of breaking covalent bonds in the DNA, will inevitably break the noncovalent bonds involved in coiling or folding. In this pulling out of the folded structure, the DNA may even be partly stripped from the histone core.

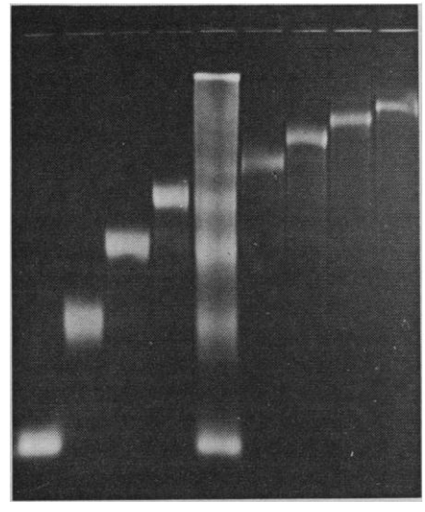

Fig. 4. Analysis of the DNA of native chromatin under denaturing conditions. Native chromatin was prepared by the nuclease method (with 450 units of nuclease per milliliter for 15 seconds), and the DNA was extracted and subjected to electrophoresis on a polyacrylamide gel as described in Fig. 1 except that the acrylamide concentration was reduced to 2 percent, 0.5 percent agarose was added, and the gels were subjected to electrophoresis for 3 hours. The first eight bands were cut out of the gel, and the DNA was eluted, concentrated, and subjected to electrophoresis in a 99 percent formamide–4 percent acrylamide slab gel (*12, 22*) for 19 hours at 70 volts; the gel was destained overnight in 1 m*M* NaEDTA, *p*H 7, before photography. The lanes of the gel contained, from left to right, original double-strand DNA of lengths 200, 400, 600, and 800 base pairs, whole DNA extract of native chromatin, and original double-strand DNA of lengths 1000, 1200, 1400, and 1600 base pairs.

Chromatin prepared without shearing and judged native by the micrococcal test is not necessarily identical with chromatin in the nucleus. For example, chromatin prepared by the nuclease method may have single-strand breaks where the micrococcal enzyme has cut across one rather than both DNA strands at potential cleavage points between 200 base-pair repeating units. The frequency of such single-strand breaks may be estimated by eluting the double-strand DNA from bands of a gel such as that labeled "native" in Fig. 1, and analyzing this DNA in a gel under denaturing conditions. The results of such an experiment, for a chromatin preparation whose DNA had a weight average size of 800 base pairs, are shown in Fig. 4. There is no visible evidence of single-strand breaks, the lengths of single-strand DNA in the bands (200, 400, 600 bases, and so on) being identical with the lengths of the original double-strand DNA (200, 400, 600 base pairs, and so on). However, overexposing the gel photograph reveals additional weak bands at all multiples of 200 bases up to the main bands. The combined intensities in the weak bands in each lane are found by densitometry to be 2, 3.5, 11.5, 23, 28, 25, and 31 percent of the intensity in the main bands at 400, 600, 800, 1000, 1200, 1400, and 1600 bases, respectively. This is consistent with random single-strand breaks occurring at a frequency of 0.04 ± 0.004 per site. Thus micrococcal nuclease cleaves chromatin mainly by cutting across both DNA strands, as was suggested previously (*10*). This is in marked contrast with the action of the endogenous nuclease in rat liver nuclei which gives a high proportion of single-strand breaks (*11*).

Another way in which fragments of chromatin prepared by the nuclease method may differ from chromatin in nuclei is by loss of DNA from the ends. For example, unit-sized fragments in chromatin prepared by brief digestion (see above) contained DNA of 150 to 200 base pairs, whereas after digestion for 20 times longer they contain DNA of 100 to 150 base pairs. Such loss of DNA may alter the native structure since the entire 200 base pairs in a repeating unit is held to be associated with histone [a suggestion (*2*) borne out by the finding (*12*) of a pat-

tern of deoxyribonuclease 1 cleavage sites along the whole length of the 200 base pairs].

The DNA lost from the ends of chromatin fragments during digestion of nuclei with micrococcal nuclease appears in acid-soluble form. On prolonged digestion a plateau is reached, similar to that reported for digestion of sheared chromatin (*5, 13*) in which a limit of about 50 percent of the DNA becomes acid soluble. A study of the limit digest of sheared chromatin has led Van Holde *et al.* (*14*) to a model of chromatin structure in which 100-Å units containing 110 to 120 base pairs of DNA [the size of DNA in chromatin fragments isolated (*15*) from the limit digest] alternate with 240-Å spacer regions. This differs fundamentally from the model mentioned above (*2*) in which the 100-Å units contain 200 base pairs of DNA and are joined directly to one another. Our study of prolonged nuclease digestion (*16*) casts doubt on the significance of the limit at 50 percent of acid-soluble DNA, and thus calls into question any model based on the properties of the limit digest. Convincing, direct evidence of a 100-Å unit containing 200 base pairs of DNA has recently been obtained by Griffith (*17*).

Although chromatin prepared by the nuclease method suffers occasional single-strand breaks and some loss of DNA from the ends, it contains the same repeating structure as chromatin in the nucleus whereas chromatin prepared by shearing does not. Of course we have tried only one of the commonly used methods of shearing, and it may be argued that other methods are less harsh, but we suspect that any shearing sufficient to break covalent bonds in the DNA will have a deleterious effect. Some workers have preferred nuclease digestion to shearing in the preparation of chromatin (*18*) but most studies have been done on sheared material and it seems unavoidable that many of these studies will need to be repeated.

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Insulin-Induced Augmentation of Lymphocyte-Mediated Cytotoxicity

Abstract. *Physiologic concentrations of insulin enhance the ability of cytotoxic lymphocytes to injure target cells. The effect of insulin closely resembles the action of cholinomimetics and guanosine 3′,5′-monophosphate upon this system. Since both insulin and cholinomimetics elevate intracellular concentrations of guanosine 3′,5′-monophosphate, a common mode of action is suggested.*

After sensitization with an allograft, the mouse develops thymus-derived (T) lymphocytes that are selectively cytotoxic in vitro for cells bearing donor alloantigens (*1*). By means of a similar in vitro system, rats immunized by allografts have been demonstrated to develop cytotoxic lymphocytes that appear to be T lymphocytes (*2*) since the cytotoxic cells do not adhere to glass bead columns (*3*) nor do they bear easily detectable surface immunoglobulin (*4*). Intimate contact between viable sensitized lymphocytes and target cells is a prerequisite for cytotoxicity [lymphocyte-mediated cytotoxicity (LMC)]. Increases in the concentration of adenosine 3′,5′-monophosphate (cyclic AMP) inhibit LMC (*2, 4, 5*), whereas cyclic AMP depletion (*4*), exogenous 8-bromoguanosine 3′,5′-monophosphate (*4*), or cholinergic agonists (*2, 4, 6*) that elevate levels of guanosine 3′,5′-monophosphate (cyclic GMP) in lymphocytes enhance cytotoxicity via an effect upon the attacking and not the target cells (*4, 6*). Recent evidence indicates that mononuclear leukocytes bear specific receptors for insulin (*7–9*). Furthermore, stimulation of tissues (*10, 11*) with insulin has been reported to result in elevated intracellular levels of cyclic GMP. Since previous studies indicated that enhancement of LMC should result from elevated levels of cyclic GMP in effector cells (*4*), the effect of physiologic concentrations of insulin upon LMC was studied. The augmented LMC reported here, resulting from an action of insulin upon the effector cell, supports and extends the implications of previous studies which indicated a crucial role for cyclic nucleotides in regulating LMC (*2, 4, 6*).

The agents listed were obtained from manufacturers: bovine crystalline insulin and atropine sulfate (Sigma Chemical, St. Louis); RPMI-1640 medium and fetal calf serum (Grand Island Biological, Grand Island, New York); fluorescein isothiocyanate and unabsorbed guinea pig complement (Nutritional Biochemical, Cleveland); deoxyribonuclease (Pentex Biochemical, Kankakee, Illinois); and ^{51}Cr-labeled sodium chromate (Nuclear Chicago, Chicago). Streptozotocin was a gift from P. Schein, National Institutes of Health, Bethesda, Maryland.

The cytotoxic action of alloimmune splenic lymphocytes on thymocytes bearing alloantigens to which they are sensitized was determined by a previously described modification (*2, 4*) of the technique of Brunner *et al.* (*12*). In brief, splenic lymphocytes were har-