Higher Order Structure in a Short Repeat Length Chromatin

JAMES ALLAN, DON C. RAU, NERINA HARBORNE, and HANNAH GOULD
Department of Biophysics, King's College, London, England; and Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20205

ABSTRACT Polynucleosomes from calf brain cortical neurone nuclei have an average repeat length of <168 base pairs. The ability of this material to adopt higher order structure has been assessed by various physical techniques. Although containing on average less DNA per nucleosome than is required to form a chromatosome, this short repeat length chromatin folded in an H1 dependent manner to a structure with properties similar to those observed for longer repeat length chromatins such as that of chicken erythrocyte (McGhee, J. D., D. C. Rau, E. Charney, and G. Felsenfeld, 1980, *Cell*, 22:87-96). These observations are discussed in the context of H1 location in the higher order chromatin fiber.

The essential components of the eucaryotic chromatin fiber are DNA and histones: only these constituents are required to maintain the fundamental structural properties of this material (23). The core histones H3, H4, H2A, and H2B associate to form an octameric complex (17) on the surface of which are wound about two turns of DNA to form a nucleosome (16). The histone octamers tend to be regularly distributed along the DNA chain. The fifth histone, H1, apparently binds close to the point where the DNA enters and leaves the core histone complex (2, 7, 36). This latter protein confers on the chromatin fiber the ability to adopt a higher order of folding (3, 30, 36).

The DNA in the chromatin fiber exists in two distinct states. About 146 base pairs of DNA are tightly associated with the core histone complex in each nucleosome (16, 32); this length has been found to be invariant in all eucaryotic chromatins studied to date (23). By contrast, the other DNA component that serves to link or separate adjacent core histone complexes and is termed the linker DNA, varies extensively in length (13, 18, 23). As the average of the combined lengths of the linker and core DNAs provides the repeat length of a chromatin, this property will vary only as a function of the linker DNA length.

The shortest documented repeat length occurs in the chromatin of the fungus Aspergillus nidulans and amounts to only 154 base pairs (13), whereas the longest repeat is reserved for the chromatin of the sea urchin sperm and constitutes 248 base pairs (18, 31). The lengths of linker DNA in these two chromatins are therefore ~10 and 100 base pairs, respectively. Although most chromatins tend to have repeat lengths between 180 and 200 base pairs (13, 18), there are so many exceptions both above and below these values (13, 18) that the concept of an archetypal chromatin repeat length is probably not valid.

Variation in repeat length is not limited to chromatins derived from different species. Indeed, distinct repeat lengths can be found to exist between chromatins isolated from separate tissues within the same animal (13, 18, 37), between different cells within the same tissue (37, 42), within the same cell at different stages of development (5, 15, 31, 34, 39), and even between resolvable chromatin domains within the same cell (14).

There is a growing body of evidence in the literature suggesting that a change in nucleosome repeat length within a developing or differentiating cell may be correlated with a switch in its genetic expression. Examples include an increase in the chromatin repeat length during erythropoiesis (39), a reduction in cortical neurone chromatin repeat length during differentiation (8, 10, 15), and a reduction in the repeat length of sea urchin chromatin during the earlier stages of embryogenesis (5, 31). In some of these instances a causal relationship between nucleosome repeat length and histone composition have been suggested (5, 39).

Changes in overall repeat length could substantially alter the structure of the folded or unfolded chromatin fiber, for even very small variations in the length of the spacer DNA will alter the relative orientation of adjacent nucleosomes. An example is the parallel or antiparallel configurations that have been proposed for the short repeat length chromatin of yeast (20, 35). Furthermore, it has been suggested (E. N. Trifonov, Weizmann Institute of Science, Israel, personal communication) on the basis of a computer study that particular linker DNA lengths, and therefore chromatin repeats, may not be permitted, because they could lead to steric interference between adjacent nucleosomes.

The majority of models that describe the chromatin higher order fiber (24, 36, 41) have been based on the properties of relatively long repeat length chromatins such as those found

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 APRIL 1984 1320-1327
© The Rockefeller University Press · 0021-9525/84/04/1320/08 $1.00

in rat liver (199 bp), chicken erythrocyte (212 bp), and *Drosophila* (200 bp) nuclei. Although some of these proposals consider the influence of repeat length on chromatin properties such as H1 location (24), fiber diameter (41), and handedness of the higher order supercoil (41), few experimental facts concerning such potential relationships are available in the literature. The study by McGhee et al. (25), in which the orientation of nucleosomes within the higher order chromatin fiber is demonstrated to be independent of repeat lengths between 178 and 248 base pairs, is a recent exeption.

The nature of the chromatin higher order fiber in a very short repeat length chromatin (<168 bp) has not been described in any detail. In the present study we have investigated such a chromatin, isolated from calf brain cortical neurones, and have assessed in particular the capacity of this material to form higher order structure.

MATERIALS AND METHODS

Nuclei Preparations: Neuronal and glial populations of nuclei from the cerebral cortex of freshly killed bovine calves were prepared essentially as described by Thompson (38), but incorporating our previously described modifications (12). By microscopy, the isolated neuronal nuclei were 75% pure. In many instances a second discontinuous sucrose gradient fractionation was included in an attempt to improve the neuronal nuclei purity. Although this additional step produced another substantial fraction of apparently pure glial nuclei the resulting neuronal preparations were not enriched further than above. Nuclei from chicken erythrocytes were prepared as previously described (1).

Chromatins: "Native" polynucleosomes of defined size were prepared from erythrocyte and neuronal nuclei essentially by the same procedure (1). For the neuronal nuclei micrococcal nuclease digestion was carried out for 12 min at an enzyme concentration of 250 U/ml, which is half the concentration used to digest the erythrocyte nuclei. Size fractionation of the mildly digested neuronal chromatin was carried out by sucrose gradient sedimentation in the Beckman SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA) employing conditions equivalent to those used for the zonal fractionation of the erythrocyte chromatin (1).

The procedures used to deplete native chromatin of linker histone (1) and to reconstitute these depleted chromatins with calf thymus H1 (3) have been described previously.

Nuclease Digestion and DNA Electrophoresis: Both calf nuclei preparations were digested, at a DNA concentration of 1 mg/ml, with micrococcal nuclease. The reaction was at 37°C in 5 mM Tris/HCl, pH 7.5, 80 mM NaCl, 1 mM $CaCl_2$, 0.2 M sucrose, and the enzyme concentration was 146 U/ml. Digestion conditions for isolated polynucleosomes have been described previously (3), as have been the methods for subsequently isolating the DNA and its analysis in agarose or acrylamide gels (3).

Other Methods: We have previously described in detail the procedures employed for the preparation and analysis of chromatins by analytical ultracentrifugation, electric dichroism, and electron microscopy (3, 4).

RESULTS

Repeat Length Determinations

Neuronal nuclei isolated from the cerebral cortex of calves using the method described by Thompson (38) were digested with micrococcal nuclease and the purified DNA products analyzed by electrophoresis in 1% agarose gels. A typical analysis is shown in Fig. 1, which also includes, for comparison, a similar digest of cortical glial nuclei isolated from the same preparation. The lengths of DNA in each of the protected neuronal chromatin fragments, for each time point in the digestion, were determined by reference to the marker DNA's and the resulting relationships between band number and size were used to estimate the repeat lengths (27). In Fig. 2*a* such an analysis is shown for the 0.5-min glial sample and the 2.0-min neuronal sample, which provided repeat lengths of 194 and 168 base pairs, respectively.

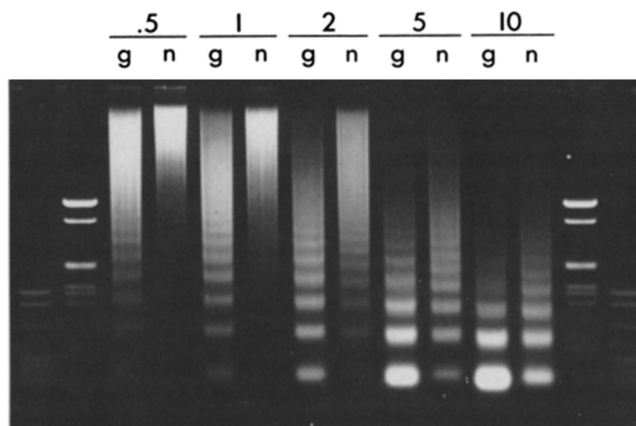

FIGURE 1 1% agarose gel electrophoresis of the DNA products derived from micrococcal nuclease digests of neuronal (*n*) or glial (*g*) nuclei. The reaction times in minutes are indicated. The restriction markers were an Hae III digest of PM2 DNA and an Hpa II digest of PBR322 DNA.

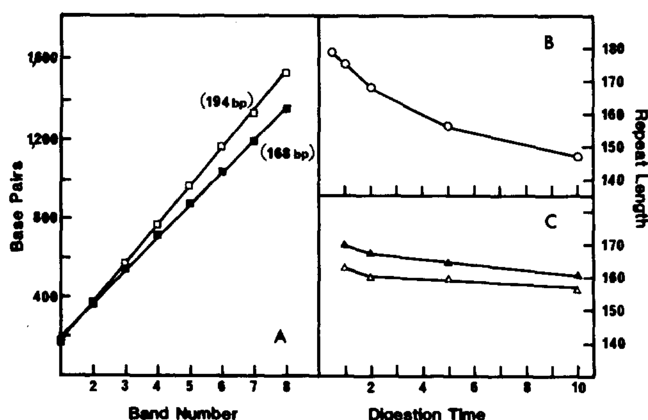

FIGURE 2 (*A*) The relationship between band number and band size for neuronal and glial chromatins is presented. The data was derived from the 0.5 min (glial) (□) and 2 min (neuronal) (■) analyses, shown in Fig. 1, which were considered to be equally digested. The numbers in parentheses give the chromatin repeat lengths (base pairs) derived from the slopes of the two lines fitted to the data by least squares analysis. (*B*) The repeat lengths of neuronal nuclear chromatin, determined from the data in Fig. 1, is shown as a function of digestion time (minutes). (*C*) The variation in repeat length, as a function of digestion time, for two isolated neuronal polynucleosome preparations is presented. The weight average size for each fraction was 10 (▲) and 42 (△) nucleosomes.

The repeat length of the neuronal nuclear chromatin was not constant throughout digestion. This is illustrated in Fig. 2*b* where this property of the chromatin is plotted as a function of digestion time. Initially the repeat length was close to 180 base pairs but was reduced during digestion to ~147 base pairs. At the earliest stages of digestion (0.5 and 1.0 min) only a very small fraction (<10%) of the chromatin contributed to those bands that were used to determine the repeat length (bands $n = 1–8$) and therefore only a very small amount of this chromatin can have repeat lengths in this range. The other three digestion points are presumably a better reflection of the repeat length of neuronal chromatin and here values range from 168 to 147 base pairs.

Contaminating glial nuclei do not appear to have posed a problem in the neuronal digests. There are two indications from the data in Figs. 1 and 2 that suggest that our 25% figure

for the extent of contamination (Materials and Methods) may be an overestimate. Firstly, under our digestion conditions, glial nuclear chromatin was digested 2–3 times faster than neuronal nuclear chromatin, yet in the neuronal digests there was little indication of a fast digesting component at the early stages of digestion (Fig. 1). Furthermore, the chromatin repeat lengths that were measured early in neuronal digests (180 base pairs) are substantially shorter than the repeat length (194 base pairs) that was measured for the glial chromatin.

The reduction in repeat length observed in the neuronal chromatin does not arise from trimming during digestion. This can be ascertained by considering the long multiple bands in the 10-min digest point. Here the septamer had a length of 1,044 base pairs which, if one allowed a generous addition of 30 base pairs to compensate for the effects of trimming would be increased to 1,074 base pairs. The repeat lengths of these fragments (obtained by dividing fragment size by band number) would be 149 base pairs without correction for trimming and 153 base pairs after correction. Clearly therefore trimming alone cannot be responsible for the substantial reduction observed in the repeat length of neuronal chromatin during the course of digestion. Although we cannot rule out the possibility of nucleosome sliding during micrococcal nuclease digestion the observations presented below suggests that this activity, under our digestion conditions, does not make a major contribution to the observed repeat length reduction. Taken in this light, our results would indicate that, in the nucleus, neuronal chromatin displays heterogeneity in repeat lengths. Although the bulk chromatin comprises repeat lengths between 147 and 168 base pairs a small but substantial component has repeat lengths ranging from 170 to 180 base pairs.

Polynucleosomes were prepared by mild micrococcal nuclease digestion of neuronal nuclei. The soluble material released by this treatment was fractionated on a sucrose gradient to obtain chromatin fractions of definite size. A typical gradient profile and the corresponding agarose gel size analysis is shown in Fig. 3. Because of the relatively low recovery of chromatin from digested nuclei (an average of ~20%), coupled with the evident heterogeneity in the repeat length of neuronal chromatin (Fig. 2*b*), it was important to determine the repeat lengths of the chromatin fractions to be employed for the subsequent physical studies. The results of one such analysis, carried out on two different sized polynucleosome fractions, is shown in Fig. 2*c*. The shorter chromatin (a weight average 10 mer) had an initial repeat length of 170 base pairs which, during the course of digestion, was reduced to 161 base pairs. For the longer chromatin (a weight average 42 mer) the corresponding repeat lengths were 163 and 156 base pairs. Similar results to those obtained with the 42 mer were produced by digestion of chromatins ranging in size from 20 to 80 nucleosomes long (data not shown).

It should be noticed that the spread in repeat lengths observed during the course of polynucleosomes digests (~9 and 7 base pairs for the 10 and 42 mer, respectively, Fig. 2*c*) were much less than that observed during the nuclear digest (32 base pairs, Fig. 2*b*). This point supports our contention that neuronal nuclear chromatin comprises subpopulations that vary in repeat length. In addition, extended digestion of the polynucleosome fractions did not lead to repeat lengths as short as that measured at the end of nuclei digestion (147 base pairs, Fig. 2*b*), an observation that implies that this repeat length in the nuclear digests did not arise from sliding as both sets of digestions were carried out under similar ionic conditions.

The results described above demonstrate that during the preparation and fractionation of neuronal polynucleosomes the small fraction of long repeat length chromatin (>170 base pairs) was removed from the bulk chromatin and presumably sedimented as small oligomers. Similarly chromatin with a repeat length <155 base pairs was poorly represented even in the largest polynucleosomes tested (80 mer). In view of the low recovery during the preparation of polynucleosomes, this latter material may not have been extracted from the digested nuclei.

Although it is evident from the results above that a single repeat length value cannot be assigned to neuronal chromatin we have assumed for convenience that the polynucleosomes employed in the experiments that follow (mainly 20–40 mers) had a repeat length of 164 base pairs. This value is the figure obtained by extrapolating the repeat length data in Fig. 2*c* (for the 40 mer) to zero digestion time and, as such, constitutes a maximum value. It should be stressed that after isolation from a sucrose gradient each chromatin fraction would be expected to have retained some limited heterogeneity in repeat length. Because longer repeat length chromatin is digested more rapidly than short repeat length material (Fig. 2*b*), extrapolation of the data in Fig. 2*c* to zero digestion time will tend to bias the repeat length in favor of these higher values. Furthermore, as pointed out above, only a small amount of

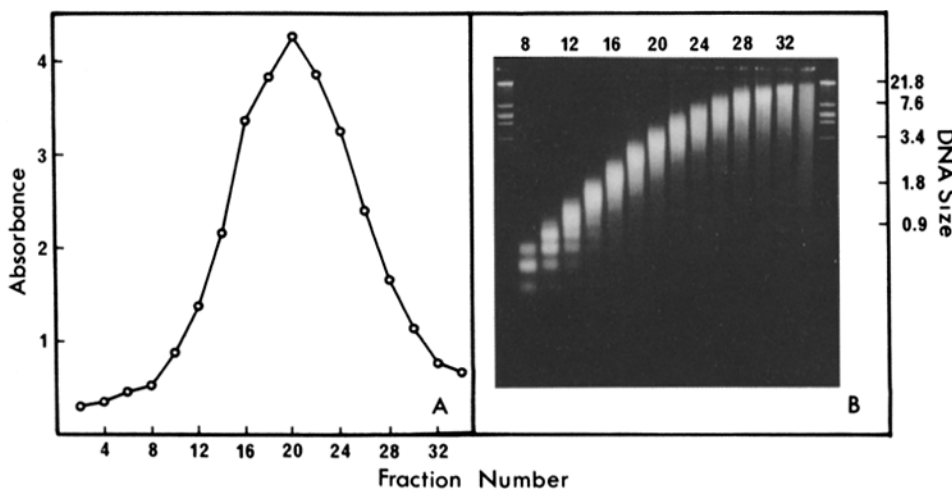

FIGURE 3 (*A*) The sedimentation profile of chromatin isolated from a preparative micrococcal nuclease digest of neuronal nuclei is shown. Sedimentation through the sucrose gradient was from left to right. (*B*) The DNA obtained from chromatin fractions taken from the gradient shown in (*A*) were sized (kilobase) in a 1% agarose gel. The markers were an EcoRI digest of lambda DNA (shown) and an Hae III digest of PM2 DNA (not shown).

digested chromatin is represented in resolvable DNA bands at the earliest stages of digestion (data not shown, but see Fig. 1 for example) and to place equal weight on this data will again bias the result in favor of longer repeat lengths. For these reasons, a more representative, average value for the 40-mer size class of chromatin (Fig. 2*c*) would probably be around 160 base pairs.

Further Nuclease Digests

The DNA products obtained after more extensive micrococcal nuclease digestion of neuronal polynucleosomes were analyzed in 7% polyacrylamide gels. In this study both native and H1-depleted chromatins (DPN) were investigated. The results are shown in Fig. 4. Initially the digestion of depleted neuronal polynucleosomes gave rise to discrete monomer DNA products with sizes of 146, 157, and 168 base pairs, all of which were subsequently trimmed to 146 base pairs. (It should be noted that in this assay the presence of the 168-base pair band need not reflect nucleosomes with a 168-base pair spacing. Chromatin with, for example, a regular 157-base pair repeat, if cleaved randomly at either end of the 10 base pair spacer, would also produce 146-, 157-, and 168-base pair fragments). The products obtained from native neuronal polynucleosomes were very similar to those observed with depleted material and no discrete protection of a band containing 168-base pairs of DNA was noted, although a small fraction did migrate around this position on the gel. When compared with the protection of 168-base pair DNA in a comparable digest of chicken erythrocyte chromatin, the relative protection in the neuronal chromatin was seen to be very limited indeed (Fig. 4). Even depleted neuronal polynucleosomes, when reconstituted with calf thymus H1 and subsequently digested with micrococcal nuclease, failed to provide a protected DNA fragment of 168 base pairs (Fig. 4). Given that the average repeat length of the neuronal polynucleosomes is <168 base pairs, this result may not be surprising. However, the data would appear to eliminate the possibility that neuronal polynucleosomes are comprised of interspersed, irregular length nucleosomes (some at least 168 base pairs long) that have an average repeat length of <168 base pairs.

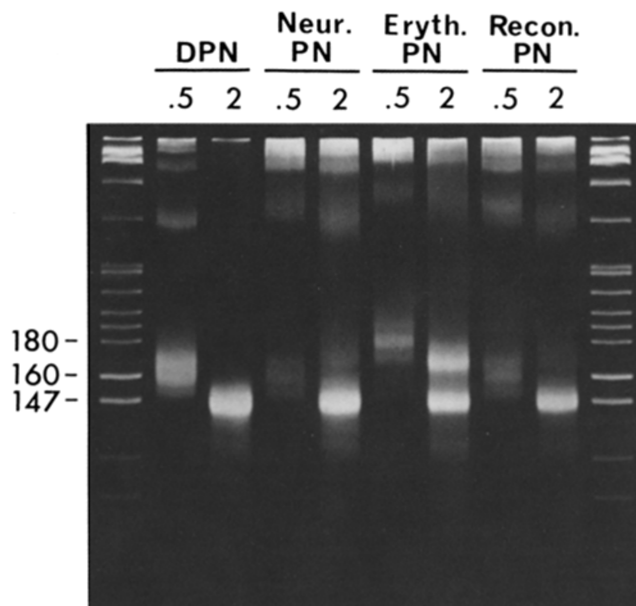

FIGURE 4 An analysis of extensive micrococcal nuclease digestion products in a 7% acrylamide gel is shown. The chromatins digested were depleted (*DPN*) and native neuronal polynucleosomes (*Neur.PN*), native erythrocyte polynucleosomes (*Eryth.PN*), and chromatin formed by the reconstitution of depleted neuronal polynucleosomes with calf thymus H1 (*Recon.PN*), at a linker histone to nucleosome ratio of 1.0. The times of digestion are shown. The restriction marker is an Hpa II digest of PBR322 DNA.

Sedimentation

To provide information concerning the ability of neuronal chromatin to adopt higher order structure the sedimentation properties of this material, both as native and depleted polynucleosomes, were measured. For comparative purposes, a similar analysis of chicken erythrocyte polynucleosomes was also undertaken. Sedimentation velocities were measured as a function of polynucleosome size, taking the repeat lengths of erythrocyte and neuronal chromatins as 212 and 164 base pairs, respectively. Because of the low yield and small average size of the neuronal polynucleosomes prepared for this study, sedimentation coefficients were measured only in the size range of 10 to 40 nucleosomes. Furthermore, it was not possible to investigate the effects of chromatin concentration and sedimentation coefficients have not, therefore, been corrected for this parameter. Analysis was carried out in a buffer containing 80 mM NaCl, which provided the conditions necessary to induce higher order folding of the chromatin (3, 9, 30, 36). A summary of the results of this study are shown in Fig. 5*a*.

Neuronal and erythrocyte polynucleosomes proved to have comparable sedimentation velocities, with respect to polynucleosome size, in either the native or depleted states. In both cases the removal of linker histone from the native material caused a substantial reduction in the sedimentation velocity of the resulting chromatin. For chicken erythrocyte chromatin, it has been demonstrated that in the presence of linker histone the polynucleosome fiber adopts a regular higher order structure in buffers containing 80 mM NaCl, and it is for this reason that native erythrocyte polynucleosomes sediment more rapidly than depleted polynucleosomes (3, 6). The fact that neuronal polynucleosomes behave in a similar manner suggests that this material also adopts a highly folded conformation in the presence of H1. The involvement of H1 in this latter system was confirmed in reconstitution studies where it was demonstrated that the sedimentation velocity of the native polynucleosomes could be recovered by the depleted material on the addition of a suitable amount of calf thymus H1 (Fig. 5*b*).

From the sedimentation data shown in Fig. 5*a*, one can calculate frictional coefficients for the erythrocyte and neuronal chromatins. For this purpose molecular weights of 260, 204, and 293, 181 g/mol were assumed for neuronal and erythrocyte nucleosomes, respectively and the corresponding partial specific volumes taken as 0.66 and 0.64 ml/g, respectively. For fibers 40 nucleosomes long, such calculations provided frictional coefficients for neuronal and erythrocyte native chromatins of 7.14×10^{-7} and 7.38×10^{-7} cm/g/s. These values are essentially the same and as frictional coefficients are largely a reflection of the shape of the molecule in question, the similarity in this property of the polynucleosomes suggests that their folded fibers should be fundamentally comparable in structure.

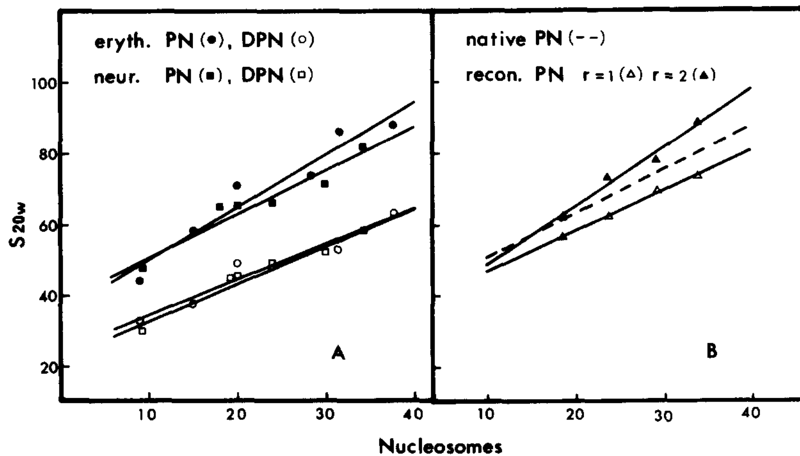

FIGURE 5 The relationship between sedimentation coefficient (S_{20w}) and polynucleosome size are shown for neuronal and erythrocyte, native (PN) and H1-depleted (DPN) chromatins (*A*). A similar analysis for two sets of reconstituted neuronal chromatins (Recon.PN) is compared with the native neuronal data (*B*). The reconstitutes were prepared at calf thymus H1 input ratio (r) of both one and two molecules per nucleosome. Sedimentation was in buffer containing 80 mM NaCl.

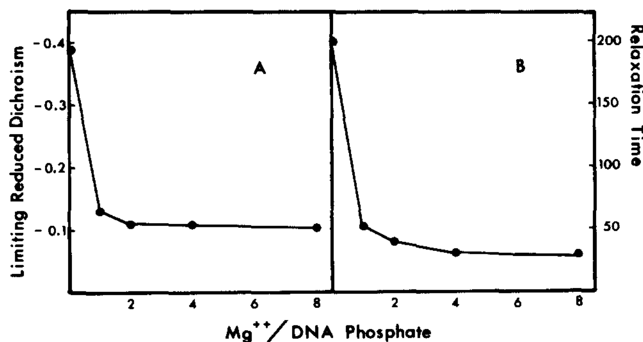

FIGURE 6 The changes observed in the limiting reduced dichroism (*A*) and the relaxation times (microseconds) (*B*) of native neuronal polynucleosomes on the addition of divalent cations are presented. The chromatin fibers were, on average, 25 nucleosomes long (Table I).

Electric Dichroism

A more detailed understanding of both the shape and internal organization of the neuronal higher order fiber was obtained from its dichroism and relaxation properties after orientation in an electric field. This approach has been successfully employed in previous studies on the chromatin fiber (4, 19, 24, 25). A summary of the results obtained in the present study are shown in Fig. 6 and Table I.

In the absence of magnesium, the unfolded native neuronal chromatin fiber provided a limiting reduced dichroism of −0.39. This value is very close to the dichroism predicted for a chromatosome orientated with its flat faces parallel to the applied field (−0.37) (24), and suggests that when the unfolded neuronal polynucleosome chain orientates in the electric field its adjacent nucleosomes are arranged edge to edge rather than face to face. Although at high field strengths the relationship between field strength and reduced dichroism suggested that some DNA was unraveled from the chromatosomes in the native fiber (data not shown), the effect was not nearly so marked as was apparent in depleted neuronal polynucleosomes. With this material, a limiting reduced dichroism of −0.66 was measured in the absence of magnesium, indicative of a substantially unraveled chromatin fiber. It will be noticed from Table I that the stabilizing influence of magnesium was not sufficient to retain the DNA in depleted chromatin within the confines of the nucleosome: the limiting reduced dichroism under these conditions remained very negative (−0.63).

The addition of magnesium to native neuronal polynucleosomes had substantial effects on both the limiting reduced dichroism and relaxation properties of these chromatins. In the presence of one magnesium cation per DNA phosphate the limiting reduced dichroism of native neuronal chromatin dropped to −0.13 (Table I, Fig. 6*a*). Furthermore, the addition of magnesium in excess of the above ratio did not substantially alter this value, which was further reduced, at an eightfold higher magnesium ratio, only to −0.10. The reduced dichroism values reflect the orientation of nucleosomes in the neuronal higher order fiber and suggest that, on average, the radially arranged (24) flat faces of the nucleosomes are tilted, with respect to the fiber axis, by between 27 and 30 degrees. The magnitude of this tilt value is slightly greater than that observed in erythrocyte chromatin (20°; [24]), but falls within the range observed for a variety of different repeat length chromatins (25). In fact, the relatively high tilt value in the short repeat length neuronal chromatin fibers tends to support the tentative relationship between repeat length and nucleosome tilt noted in the study of McGhee et al. (25).

The addition of divalent cations to neuronal native polynucleosomes also produced a substantial reduction in the relaxation times measured (Table I, Fig. 6*b*). In the absence of magnesium the relaxation time for a 25-nucleosome long polymer was 200 μs, which, combined with the nature of the decay curves (data not shown), indicated an extended and flexible fiber. By adding the equivalent of one magnesium cation per DNA phosphate the relaxation time for native neuronal polynucleosomes dropped to 55 μs. However, in contrast to the relationship between reduced dichroism and magnesium ion concentration (Fig. 6*a*), relaxation times for neuronal chromatin continued to fall as the ratio of magnesium to DNA phosphate was increased, reaching a value of 27 μs at the highest cation concentration tested (Table I, Fig. 6*b*). This requirement for higher magnesium to DNA phosphate ratios to induce maximum condensation appears to distinguish the short repeat length neuronal chromatin from other chromatins, of various repeat lengths, that have been studied under similar conditions (24, 25), and may indicate a relatively reduced degree of stabilization in the shorter repeat length folded fiber.

Relaxation times are a function of the frictional properties of the chromatin fiber under the conditions of measurement and can be interpreted in terms of the shape of the complex (24). In Table I the lengths of the cylindrical rods of average

Table I
Dichroism Properties of Neuronal Polynucleosomes

Chromatin	Limiting reduced dichroism	Relaxation time	Tilt	Rod length	Nucleosomes per turn
		μs		*nm*	
Depleted PN (20)	−0.66	280			
+ 200 μM Mg^{++}	−0.63	240			
Native PN (25)	−0.39 (−0.40)	200 (214)			
+ 1 Mg^{++}/DNA PO_4	−0.13 (−0.13)	55 (58)	27.5°	73	3.8
+ 2 Mg^{++}/DNA PO_4	−0.11 (−0.12)	40 (40)	28.5°	61	4.5
+ 4 Mg^{++}/DNA PO_4	−0.11 (−0.11)	29 (31)	28.7°	51	5.4
+ 8 Mg^{++}/DNA PO_4	−0.11 (−0.10)	27 (24)	29.5°	50	5.5

Summary of the data obtained by electric dichroism studies on neuronal polynucleosomes. The lengths of the polynucleosome fibers are shown in parenthesis in column 1. Nucleosome tilt, derived from the limiting reduced dichroism (24), is expressed relative to the fiber axis. The rod lengths were determined from the relaxation times (24), taking the fiber diameter as 30 nm, and the number of nucleosomes per turn of the supposed solenoid were calculated from the rod lengths assuming a pitch of 11 nm. The figures in parenthesis in the second and third columns are the corresponding values obtained with reconstituted neuronal chromatins prepared at a calf thymus H1 ratio of two molecules per nucleosome. Dichroism measurements were made in 0.22 mM cacodylate, 5 μM EDTA, pH 7.0.

diameter 30 nM that would give the observed relaxation times for native neuronal chromatin in the presence of the various ratios of magnesium have been calculated. The reduction in fiber length, with added magnesium, can be interpreted either as an increase in the number of nucleosomes per turn (3.8 up to 5.5) of a solenoid type structure (24) with a constant pitch of 11 nM, or alternatively, as a decrease in the pitch (17.4 down to 12.0 nM) of a solenoid containing six nucleosomes per turn.

Chromatins prepared by the reconstitution of depleted neuronal polynucleosomes with calf thymus H1 were also studied by electric dichroism. As shown in Table I, such material gave reduced dichroism and relaxation data almost indistinguishable from that obtained with native chromatin.

Electron Microscopy

In Fig. 7, micrographs of native, depleted, and reconstituted neuronal polynucleosomes, fixed in the presence of 80 mM NaCl, are presented along with similar specimens prepared from erythrocyte chromatin. Although these two types of chromatin differ substantially in their repeat lengths, they present, in the microscope, structures that share common features in each of the three states studied.

There was, surprisingly, little difference between the structures adopted by each type of depleted polynucleosome. For each material the fibers adopted equally irregular overall structure and in both cases stretches of DNA joining adjacent nucleosomes could be discerned, although this latter feature was more pronounced in the erythrocyte chromatin. The presence of what appears to be substantial stretches of spacer DNA in the short repeat length chromatin could be taken to reflect long repeat length nucleosomes in the neuronal polynucleosomes, an interpretation that conflicts with the digestion results (Fig. 2*c*). This DNA may, however, have been produced by unfolding from the nucleosome (or core particle), a proposal that explains the same phenomenon observed in electron microscope spreads of yeast chromatin (29), which, like neuronal chromatin, also has a very short repeat length (21, 22). This latter interpretation is also consistent with the reduced dichroism measurements of depleted neuronal chromatin, which indicated a substantially unwound fiber, even in the presence of divalent cations (Table I).

Native neuronal chromatin (Fig. 7*f*) appeared in the microscope as 20–30-nM diam fibers. These structures were noticeably irregular with respect to diameter even within individual fibers. In these respects the neuronal chromatin was similar in appearance to erythrocyte higher order fibers (Fig. 7, *b* and *c*), (6, 24), and to the fibers that have been described for other chromatins under similar conditions (36). Chromatin that had been prepared by reconstitution of depleted neuronal polynucleosomes with calf thymus H1 presented similar structures to those seen in the native samples (Fig. 7*f*).

DISCUSSION

The results presented in this study demonstrate that the chromatin of calf cortical neurone nuclei is heterogeneous in repeat length. Although the bulk of this material consists of nucleosomes containing on average between 147 and 170 base pairs of DNA, a small fraction does have a longer repeat length that ranges from 170 to 180 base pairs (Figs. 1 and 2). Our data does not permit us to say whether the heterogeneity exists between subpopulations of the nuclei or whether it is a property of individual nuclei. In contrast, soluble polynucleosomes, isolated by nuclease treatment of neuronal nuclei, had repeat lengths between 155 and 170 base pairs and did not display the heterogeneity in this property which was typical of nuclear digests (Fig. 2).

In spite of its very short repeat length, cortical neurone chromatin can adopt higher order structure. As with most other chromatins (23), this second level of folding is dependent on both ionic strength and the presence of linker histone, H1. The shape of the folded fiber, as reflected by its frictional properties (derived from both sedimentation and electric dichroism studies), is consistent with a cylindrical rod with dimensions matching those determined for other chromatins (25, 36), the best described of which is the chicken erythrocyte fiber (24). Electron microscopy has provided visual evidence to support this conclusion. To fully compact neuronal chromatin relatively high concentrations of divalent cations were required, an observation that may reflect a reduced stability for these short repeat length fibers. Nevertheless, when fully compacted, neuronal chromatin displayed dichroism properties consistent with those expected of a solenoid type structure (11) containing about six nucleosomes per superhelical turn. Furthermore, on average, the nucleosomes within the neuronal fiber were orientated, with respect to the fiber axis, at an angle (30°) which falls within the range (21° to 33°) ob-

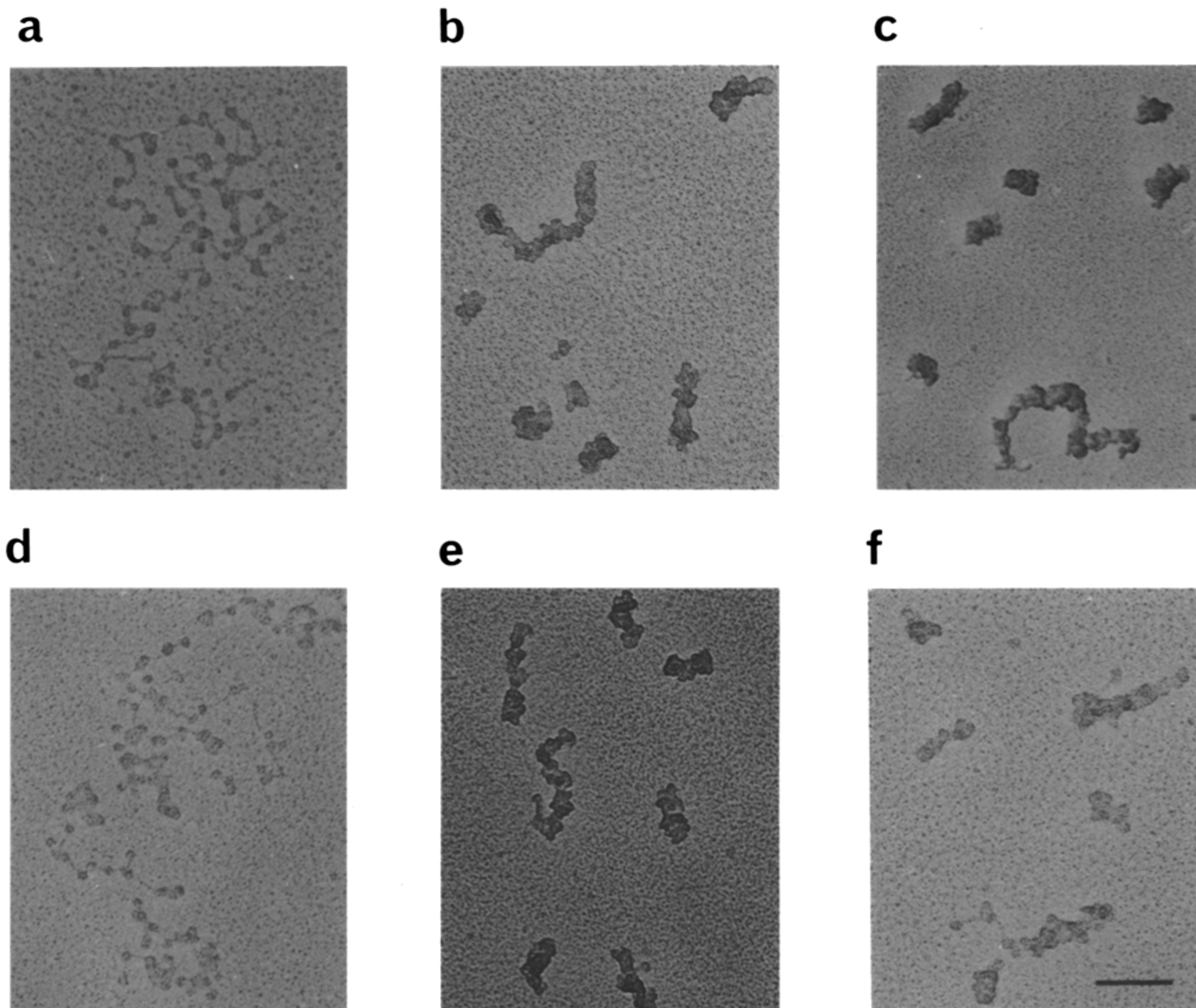

FIGURE 7 Electron micrographs of depleted (*a* and *d*), native (*b* and *e*), and reconstituted (*c* and *f*) erythrocyte (*a*–*c*) and neuronal (*d*–*f*) chromatins, crosslinked with glutaraldehyde in the presence of 80 mM NaCl, are shown. The reconstituted chromatins were prepared at an H1 (neuronal) or H5 (erythrocyte) input ratio of two molecules per nucleosome. The chromatin fibers were, on average, 40 nucleosomes long. Bar, 100 nm. × 115,000.

served for a variety of other chromatins (25). Given the resolution of the techniques employed in this study it would appear, therefore, that the dimensions of the neuronal chromatin higher order fiber and the manner in which nucleosomes are arranged within this structure are fundamentally similar to the same parameters in much longer repeat length chromatins.

In a study based on model building, Worcel (41) proposed that chromatins with repeat lengths as short as 150 base pairs could be accommodated in a solenoid type higher order fiber. However, there is no experimental precedent for H1 induced higher order folding in a chromatin with a repeat length <168 base pairs. In yeast chromatin, the absence of an H1 has led to the suggestion that the 30-nm fiber observed (29) with this very short repeat length chromatin (<168 base pairs, (21, 22)) may be stabilized by an as yet unidentified molecule (29).

Our observations, combined with previous studies (24, 25), show that the ability to form higher order structure extends to cover almost the complete range of documented chromatin repeat lengths. Only chromatins with repeat lengths between 154 and 164 base pairs remain to be tested directly, although it is evident from Fig. 2 that a substantial fraction of the chromatins employed in the present study must have had repeat lengths within this range. It may be, therefore, that the ability to form higher order structure is a universal property of chromatin, and as such may be the major factor responsible for defining repeat length limits. The importance of possessing the ability to form higher order structure presumably derives from its capacity to provide a mechanism for transcriptional repression (and control) and to enable complete chromosome condensation to occur during cell division.

A further implication of our study concerns the location of the linker histone, H1, within the chromatin higher order fiber. Previous work has revealed that H1 is located on a nucleosome, or chromatosome (33), at that point where the DNA both leaves and enters the nucleosome (2, 6, 33). The characteristic protection from nucleases of a DNA fragment 168 base pairs long occurs as a direct result of this interaction (27). A similar location for H1 in unfolded, native, rat polynucleosomes was suggested by the characteristic "zig-zag"

conformation of this material when viewed in the electron microscope (36). In a number of studies concerned with the nature of the higher order fiber, it has been assumed that H1 retains its specific location with respect to the nucleosome as the latter structure adopts a position within the folded fiber (2, 24, 36), and indeed that the specificity of this interaction might be fundamental in determining higher order folding (2).

In neuronal polynucleosomes, H1 cannot adopt a position relative to its associated nucleosome similar to that occupied by H1 in longer repeat length chromatins because not only is the average repeat length of this chromatin too short to be amenable to such an interaction but, in addition, no nuclease resistant DNA fragments consistent with such an interaction can be produced from this chromatin. Nevertheless, this short repeat length chromatin does adopt higher order structure and this property is dependent on H1. These observations imply that in neuronal chromatin alternative H1 binding sites are available on the nucleosome and that these are employed during higher order folding. If such additional sites were also available in longer repeat length chromatins and utilized in the folded fiber, then the location of H1 in this latter fiber need not be so dependent on nucleosome orientation. Therefore, although nucleosomes may be positioned with their DNA entry and exit points within or outside of the chromatin fiber (24), H1 could bind independently of these sites and may, for example, be exclusively located along the central axis of the fiber (36).

As regards the assembly and stability of the higher order chromatin fiber, the placement of H1 along the central axis of this structure is a favored location (11, 36). However, this configuration could also prove advantageous in genetic expression, for the absence of H1 on the outside of the chromatin higher order fiber would leave the DNA on the external surface available for interaction with other protein molecules. Furthermore, this spectrum of exposed DNA sequences could be sensitively controlled by variation of the chromatin repeat length. Such a mechanism could be responsible for the substantial alterations in gene expression which have been shown to accompany very small (<5 base pairs) variations in repeat length (34).

This work was supported in part by a Wellcome Senior Research Fellowship to J. Allan and a Medical Research Council Project Grant (G979/1094/SA) to H. Gould.

Received for publication 25 April 1983, and in revised form 12 December 1983.

Note Added in Proof: Since acceptance of this manuscript, similar results have been reported by Pearson, E. C., P. J. G. Butler, and J. O. Thomas (*Eur. Mol. Biol. Organ. J.*, 8:1367–1372).

REFERENCES

1. Allan, J., D. Z. Staynov, and H. J. Gould. 1980. Reversible dissociation of linker histone with preservation of internucleosomal repeat. *Proc. Natl. Acad. Sci. USA.* 77:885–889.
2. Allan, J., P. G. Hartman, C. Crane-Robinson, and F. X. Aviles. 1980. The structure of histone H1 and its location in chromatin. *Nature (Lond.).* 288:675–679.
3. Allan, J., G. J. Cowling, N. R. Harborne, P. Cattini, R. Craigie, and H. J. Gould. 1981. Regulation of the higher order of chromatin by histones H1 and H5. *J. Cell Biol.* 90:279–288.
4. Allan, J., N. R. Harborne, D. C. Rau, and H. J. Gould. 1982. Participation of the core histone "tails" in the stabilisation of the chromatin solenoid. *J. Cell Biol.* 93:285–297.
5. Arceri, R. J., and P. R. Gross. 1980. Histone variants and histone structure during sea urchin development. *Dev. Biol.* 80:186–209.
6. Bates, D. L., P. J. G. Butler, E. C. Pearson, and J. O. Thomas. 1981. Stability of higher order structure of chicken erythrocyte chromatin in solution. *Eur. J. Biochem.* 119:469–476.
7. Belyavsky, A. V., S. G. Bavykin, E. G. Goguadze, and A. D. Mirzabekov. 1980. Primary organisation of nucleosomes containing all five histones and DNA 175 and 165 base pairs long. *J. Mol. Biol.* 139:519–536.
8. Brown, I. R. 1978. Post natal appearance of a short DNA repeat length in neurones of the cerebral cortex. *Biochem. Biophys. Res. Commun.* 84:285–292.
9. Butler, P. J. G., and J. O. Thomas. 1980. Changes in chromatin folding in solution. *J. Mol. Biol.* 140:505–529.
10. Ermini, M., and C. C. Kuenzle. 1978. The chromatin repeat length of cortical neurones shortens during early postnatal development. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 90:167–172.
11. Finch, J. T., and A. Klug. 1976. Solenoidal model for superstructure in chromatin. *Proc. Natl. Acad. Sci. USA.* 73:1879–1901.
12. Harris, M. R., N. Harborne, B. J. Smith, and J. Allan. 1982. The distribution of the histone H1° in different brain cell types. *Biochem. Biophys. Res. Commun.* 109:78–82.
13. Horgen, P. A., and J. C. Silver. 1978. Chromatin in Eukaryotic microbes. *Annu. Rev. Microbiol.* 32:249–284.
14. Humphries, S. E., D. Young, and D. Carroll. 1979. Chromatin structure of the 5S ribonucleic acid genes of *Xenopus laevis*. *Biochemistry.* 18:3223–3231.
15. Jaeger, A. W., and C. C. Kuenzle. 1982. The chromatin repeat length of brain cortex and cerebellar neurones changes concomitant with terminal differentiation. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:811–816.
16. Klug, A., D. Rhodes, J. Smith, J. T. Finch, and J. O. Thomas. 1980. A low resolution structure for the histone core of the nucleosome. *Nature (Lond.).* 287:509–516.
17. Kornberg, R. D., and J. O. Thomas. 1974. Chromatin structure: oligomers of the histones. *Science (Wash. DC).* 184:865–868.
18. Kornberg, R. D. 1977. Structure of chromatin. *Annu. Rev. Biochem.* 46:931–954.
19. Lee, K. S., M. Mandelkern, and D. M. Crothers. 1981. Solution structural studies of chromatin fibres. *Biochemistry.* 20:1438–1455.
20. Lohr, D., and K. E. Van Holde. 1979. Organisation of spacer DNA in chromatin. *Proc. Natl. Acad. Sci. USA.* 76:6326–6330.
21. Lohr, D., J. Corden, K. Tatchell, R. T. Kovacic, and K. E. Van Holde. 1977. Comparative subunit structure of HeLa, yeast and chicken erythrocyte chromatin. *Proc. Natl. Acad. Sci. USA.* 74:79–83.
22. Lohr, D., R. T. Kovacic, and K. E. Van Holde. 1977. Quantitative analysis of the digestion of yeast chromatin by staphylococcus nuclease. 16:463–471.
23. McGhee, J. D., and G. Felsenfeld. 1980. Nucleosome structure. *Annu. Rev. Biochem.* 49:1115–1156.
24. McGhee, J. D., D. C. Rau, E. Charney, and G. Felsenfeld. 1980. Orientation of the nucleosome within the higher order structure of chromatin. *Cell.* 22:87–96.
25. McGhee, J. D., J. M. Nickol, G. Felsenfeld, and D. C. Rau. 1983. The higher order structure of chromatin: the orientation of nucleosomes within the 30 nm chromatin solenoid is independent of species and spacer length. *Cell.* 33:831–841.
26. Miki, B. L. A., and J. M. Neelin. 1979. DNA repeat lengths of erythrocyte chromatins differing in content of H1 and H5. *Nucleic Acids Research* 8:529–542.
27. Noll, M., and R. D. Kornberg. 1977. Action of micrococcal nuclease on chromatin and the location of histone H1. *J. Mol. Biol.* 109:393–404.
28. Ostima, R., D. Curiel, and E. Linney. 1980. H1 histone and nucleosome repeat length alterations associated with the in vitro differentiation of murine embryonal carcinoma cells to extra-embryonic endoderm. *J. Supramol. Struct.* 14:85–96.
29. Ratner, J. B., C. Saunders, J. R. Davie, and B. A. Hamkalo. 1982. Ultrastructural organisation of yeast chromatin. *J. Cell Biol.* 92:217–222.
30. Renz, M., P. Neils, and J. Hozier. 1977. Involvement of histone H1 in the organisation of the chromatin fibre. *Proc. Natl. Acad. Sci. USA.* 74:1879–1883.
31. Savic, A., P. Richman, P. Williamson, and D. Poccia. 1981. Alterations in chromatin structure during early sea urchin embryogenesis. *Proc. Natl. Acad. Sci. USA.* 78:3706–3710.
32. Shick, V. V., A. G. Belyavsky, S. G. Bavykin, and A. D. Mirzabekov. 1980. Primary organisation of the nucleosome core particles: sequential arrangement of histones along DNA. *J. Mol. Biol.* 139:491–517.
33. Simpson, R. T. 1978. Structure of the chromatosome, a chromatin particle containing 160 base pairs of DNA and all the histones. *Biochemistry.* 17:5524–5531.
34. Sperling, L., and M. C. Weiss. 1980. Chromatin repeat length correlates with phenotype expression in hepatoma cells, their differentiated variants, and somatic hybrids. *Proc. Natl. Acad. Sci. USA.* 77:3412–3416.
35. Strauss, F., and A. Prunell. 1983. Organisation of internucleosomal DNA in rat liver chromatin. *EMBO (Eur. Mol. Biol.) J.* 2: 51–56.
36. Thoma, F., T. Koller, and A. Klug. 1979. Involvement of histone H1 in the organisation of the nucleosome and of the salt dependant superstructure of chromatin. *J. Cell Biol.* 83:403–427.
37. Thomas, J. O., and R. J. Thompson. 1977. Variation in chromatin structure in two cells types from the same tisue: a short DNA repeat length in cerebral cortex neurones. *Cell.* 10:633–640.
38. Thompson, R. J. 1973. Studies in RNA synthesis in two populations of nuclei from the mammalian cerebral cortex. *J. Neurochem.* 21:19–40.
39. Weintraub, H. 1978. The nucleosome repeat length increases during erythropoeisis in the chick. *Nucl. Acids Res.* 5:1179–1188.
40. Whatley, S. A., C. Hall, and L. Lim. 1981. Chromatin organisation in the rat hypothalamus during early development. *Biochem. J.* 196:115–119.
41. Worcel, A. 1977. Molecular architecture of the chromatin fibre. *Cold Spring Harbor Symp. Quant. Biol.* 62:313–324.
42. Zonga, V., and A. P. Matthias. 1979. The variation with age of the structure of chromatin in three cell types of rat liver. *Biochem. J.* 179:291–298.