Presence of nucleosomes within irregularly cleaved fragments of newly replicated chromatin*

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ABSTRACT

In previous reports (Annunziato *et al.*, J. Biol. Chem., 256:11880-11886 [1981]; Annunziato and Seale, Biochemistry 21:5431-5438 [1982]) we have described two classes of newly replicated chromatin which differ in structure, solubility properties, and requirements for maturation. One class is nucleosomal, soluble at low to intermediate ionic strengths, and acquires mature nucleosomal composition and normal repeat length in the absence of concurrent protein synthesis. In contrast, the other class is cleaved irregularly by MNase* (appearing as a smear in DNA gels), is insoluble at moderate ionic strengths, requires protein synthesis to gain normal subunit structure, and comprises ~60% of total new chromatin DNA after mild nuclease digestion. It is now demonstrated that this heterogeneous component (produced by the action of either MNase or Hae III on chromatin replicated in cycloheximide) yields nucleosomes when redigested with MNase. The presence of nucleosomes within heterogeneous chromatin fragments suggests that nucleosomal and non-nucleosomal regions may be juxtaposed during chromatin replication. These findings are discussed with respect to current models of nucleosome segregation.

INTRODUCTION

During chromatin replication, double-stranded progeny DNA becomes complexed with both old (parental) and newly synthesized histones to form nascent nucleosomes (reviewed in ref. 1). Based on the assumption that only pre-fork parental octamers are recycled onto new chromatin, it follows that approximately half of nascent DNA is assembled *de novo* into new nucleosomes, and thus transiently lacks nucleosomal structure following DNA replication. Although non-nucleosomal DNA has been detected in the immediate vicinity of native replication forks (2, 3), its half life is short (2-10 minutes). It has been advantageous for the study of assembly intermediates to analyze the properties of chromatin synthesized in the absence of protein synthesis; when protein synthesis is inhibited, DNA replication continues for a limited time without *de novo* nucleosome assembly, permitting the accumulation of non-nucleosomal replication products (4-7). Chromatin replicated in the presence of cycloheximide (ch-

chromatin) is approximately twice as sensitive to nuclease digestion than control chromatin (4-8), in accord with predicted effects of histone depletion.

In previous experiments, we have shown that the nucleosomal and non-nucleosomal (*i.e.*, unassembled) components of both normal nascent and ch-chromatin can be biochemically separated after digestion with MNase, due to the preferential solubility of newly replicated nucleosomes at low ionic strengths, versus retention of unassembled DNA in the nucleus (perhaps due to association with hydrophobic replication proteins, or with the nuclear matrix) (3, 7). Whereas the soluble components exhibited a clearly defined repeating structure attributable to nucleosomes, the insoluble, unassembled DNA lacked subunit organization, and was distributed in a heterogeneous molecular weight range without evident structure. Furthermore, the unassembled component accumulated in the presence of cycloheximide, while the nucleosomal component regained the mature HeLa repeat length (7).

In this report we have exploited these features of ch-chromatin in order to further study the properties of the non-nucleosomal component, using MNase either alone or in conjunction with the restriction endonuclease, HaeIII. Our results demonstrate that nucleosomes reside with heterodisperse digestion products that apparently lack a defined repeating structure. These findings may have important implications regarding currently accepted models of parental histone segregation at the replication fork.

MATERIALS AND METHODS

Cell Culture and Labeling

HeLa cells were maintained in spinner culture at 37°C in Eagle's minimal essential medium supplemented with 5% calf serum. For pulse labeling, cells ($\sim 4 \times 10^5$ /ml) were harvested by centrifugation (250 xg; 1.5 min), concentrated 5- to 10-fold in prewarmed whole medium, and equilibrated at 37°C for at least 5 min. For 10-30 min labeling, cells were incubated with [methyl-³H]thymidine (70 Ci/mmol, New England Nuclear) at 2-4 μ Ci/ml. Long-term labeling was performed at 0.2 μ Ci/ml for one generation (24 hr). For experiments using HaeIII, labeling was performed at 50 μ Ci/ml. Cycloheximide was added as required to a final concentration of 200 μ g/ml (from a freshly prepared stock solution of 10 mg/ml). At this concentration, protein synthesis is completely and immediately inhibited in HeLa cells, and chromatin DNA replicated under these

conditions is approximately twice as sensitive to nuclease digestion than control chromatin (4, 5, 7). Cells were preincubated for 5-12 min in cycloheximide prior to the addition of radiolabel.

Nuclear Isolation; Digestion With Micrococcal Nuclease and HaeIII; Chromatin Fractionation

For digestion with MNase, nuclei were isolated as described previously (3, 7) in CB buffer (1 mM Tris, 25 mM KCl, 0.9 mM MgCl₂, 0.9 mM CaCl₂, 0.14 mM spermidine, 2 mM sodium butyrate, pH 7.6). Nuclei were resuspended in CB buffer at 40 A₂₆₀/ml (A₂₆₀ measured in 1% SDS) and digested with micrococcal nuclease (Sigma) at 37°C; enzyme concentrations and digestion times are given in the Figure Legends. Reactions were terminated by the addition of EGTA (pH 7.6) to a final concentration of 1 mM, and cooling on ice. Nuclei were pelleted to yield a first supernatant containing acid soluble nucleotides (but no acid precipitable DNA (3)), and then swollen by resuspension in 2 mM EDTA, pH 7.2; insoluble chromatin (P) was removed by centrifugation (12,500 xg; 10 min) yielding a supernatant (S) containing solubilized chromatin. Residual monomers were eluted from the pellet (P) by resuspending nuclei in CB buffer containing 0.2 M NaCl; after 20 min at 0°C, the nuclei were again pelleted (1,500 xg; 10 min), yielding a 0.2 M NaCl eluate (S.2) and a final pellet (P.2). The P.2 was then resuspended in one half the original volume of CB buffer, brought to an additional 0.5 mM CaCl₂, and digested with MNase (2 U/ml) for 2.5 min, to produce redigested chromatin (R). The reaction was terminated with 5mM EDTA.

For digestion with HaeIII, nuclei were isolated in Buffer A (10 mM Tris, 3 mM MgCl₂, 2 mM mercaptoethanol, pH 7.6) as described previously (9). Digestion of nuclei with HaeIII (BRL) was performed as described by Seidman *et al.* (8): nuclei were suspended at 20 A₂₆₀/ml in 20 mM Tris, 60 mM NaCl, 7 mM MgCl₂, 1 mM mercaptoethanol, pH 7.4, and digested with 1,000 U/ml HaeIII for 30 min. Following digestion, nuclei were placed on ice for 1 hr, and then gently pelleted (1,500 xg; 10 min) to yield a first supernatant (SI). The pellet was then resuspended in buffer A containing 0.2 M NaCl; after 20 min the nuclei were again pelleted, to yield a second supernatant (SII). For redigestion with MNase, the SI and SII were brought to 3.5 mM CaCl₂ and incubated at 0.375 U/ml (SI) or 0.75 U/ml (SII) at 37°C. Reactions were halted with 5 mM EGTA. Yields are given in the Figure Legends.

Digestion of isolated DNA with HaeIII was performed at 2.3 U/μgm. To purify DNA, nuclei were brought to 0.5% NaDodSO₄, digested 16 hr with 150

μgm/ml proteinase K (Merck) at 37°C, extracted twice with phenol:chloroform: isoamyl alcohol (50:50:1), twice with chloroform:isoamyl alcohol (24:1), and ethanol precipitated.

Gel Electrophoresis

For DNA size analyses of nuclease digestion products, chromatin samples were adjusted to 10 mM magnesium acetate and ethanol precipitated. NaDodSO₄-polyacrylamide gel electrophoresis was then performed according to the procedure of Loening (10) as described previously (3, 7). Electrophoretic analyses of nucleoprotein particles were performed in composite 0.5% agarose, 2.5% polyacrylamide gels, according to the method of Todd and Garrard (11) as described previously (7). Samples were dialyzed against 2 mM EDTA prior to electrophoresis. Composite gels were prepared for fluorography using EN³HANCE (New England Nuclear); DNA gels were impregnated with 2,5-diphenyloxazole (12).

RESULTS

In our initial attempts to characterize the non-nucleosomal component of nascent chromatin (labeled for 30 sec with [³H]thymidine), we were surprised to observe that redigestion of insoluble, irregularly cleaved chromatin with MNase produced nucleosomes in approximately 50% yield. Because it was possible that these nucleosomes had been assembled de novo with newly synthesized histones during normal replication, the experiments were repeated with ch-chromatin. In this case, only parental histones are present on new DNA.

HeLa cells were preincubated 5-10 min in cycloheximide, in order to interpose an extensive stretch of DNA between normal, assembled chromatin and the region to be labeled. In the presence of cycloheximide, the rate of chain growth in HeLa cells (0.5 - 1 μ/min) is reduced to ~ 20% of controls (5). [³H]thymidine was then added for 30 min in the continuous presence of cycloheximide. Isolated nuclei were digested with MNase, and soluble chromatin was extracted with 2 mM EDTA. Figure 1 shows the products as DNA (panel A) or as nucleoproteins (panel B).

Under the conditions of digestion (~10% acid solubility), 24% of ch-chromatin was released with EDTA, predominantly as monomers (lane S). It has been previously demonstrated by both this and other laboratories that MNase-cleaved ch-chromatin can be resolved as nucleosomal multimers up to 8-mers depending on the extent of digestion (7-9, 13, 14). Such results have generally been interpreted as indicating that parental histone cores

Figure 1. Sequential digestion of ch-chromatin with micrococcal nuclease. (Panel A): HeLa cells were labeled with [3 H]thymidine for 30 min in the presence of cycloheximide. Isolated nuclei were digested with MNase (1.5 U/ml) for 2 min and soluble chromatin released with 2 mM EDTA (lane S). The EDTA-insoluble pellet (lane P) was treated with 0.2 M NaCl to elute residual nucleosomes (lane .2). The remaining pellet (lane P.2) was then redigested with MNase, yielding additional monomers and dimers (lane R) (see Materials and Methods). Chromatin DNA was subjected to electrophoresis and analyzed by fluorography. 24% of total acid precipitable radioactivity was released with EDTA (S), with 76% remaining in the pellet (P); 14% of ch-chromatin was eluted with 0.2 M NaCl (.2), and 30% remained as nucleosomes after redigestion (R). Marker fragments (lane m): HaeIII digest of ϕ X174 [3 H]DNA (New England Nuclear); fragment sizes are 1353, 1078, 872, 603, 310, 278/271 (doublet), 234, 194, and 118 bp. The positions of mono- (M), di- (D) and trinucleosomal DNA (T) are indicated. (Panel B): Ch-chromatin was prepared and fractionated as in Panel A, subjected to electrophoresis as native nucleoproteins, and analyzed by fluorography. Lanes are labeled as in A. The positions of mono- (M) and dinucleosomes (D) are indicated.

are segregated conservatively, or at least in clusters (8, 9, 13, 14).

The insoluble pellet contained ch-chromatin with dual characteristics, displaying some subunit organization in addition to a strong background smear (Figure 1A, lane P). Residual nucleosomes were therefore extracted with 0.2 M NaCl, as described previously (3, 7), releasing 14% of ch-chromatin (Figure 1A, lane .2). Newly replicated mononucleosomes released with 0.2 M NaCl contained atypically long DNA (156 to 282 bp), considerably greater than that of the EDTA-soluble monomers (146-188 bp), or bulk monomers also present in the 0.2 M NaCl eluate (143-214 bp). Similar

extra-long nucleosomes in ch-chromatin have been observed previously (19, 20). The presence of these extra-long linkers suggests that at least some nucleosomal regions of ch-chromatin may be interrupted by stretches of unassembled DNA. This will be further discussed below.

After extraction with 0.2 M NaCl, the pellet contained ch-chromatin ranging in size from 200 bp to several kb (Figure 1A, lane P.2). Ch-DNA extracted from this fraction is heterogeneous in size, exhibiting no nucleosomal periodicity (see also 3, 7), and comprises up to 65% of total ch-chromatin under mild digestion conditions. Significantly, the heterogeneous chromatin possessed a greater molecular weight than the soluble nucleosomal component, irrespective of the extent of digestion; therefore, it must exist as a nucleoprotein complex, not free DNA. Free DNA is rapidly degraded to acid solubility under these conditions (see below; also ref. 3).

In order to investigate the properties of the heterogeneous component, the P.2 fraction was redigested with MNase (Figure 1, lane R). Redigestion produced clearly-resolved nucleosomes in approximately 50% yield, representing 30% of total ch-chromatin DNA. This demonstrates that the regions responsible for the smearing are more susceptible to nuclease digestion than are newly replicated nucleosomes, and suggests that cuts within atypically irregular "spacers" are responsible for the non-nucleosomal appearance of the insoluble fraction.

Summation of the nucleosomal components present in all fractions (including ~50% of the 0.2 M NaCl eluate) revealed that approximately 60% of the total acid insoluble ch-chromatin was nucleosomal. This does not include that portion of ch-DNA rendered acid soluble (~10%), which results from an indeterminate degree of digestion of both linkers and unassembled DNA.

When chromatin was replicated for 10-15 min under control conditions, both the EDTA-soluble and insoluble fractions possessed typical subunit structure and nuclease resistance (3, 7). Also, electrophoretic analysis of the products of MNase digestion as nucleoprotein particles showed that the redigested pellet yielded nucleosomes with typical mobilities (Fig. 1B, lane R).

The use of MNase as the initial step in fractionating nucleosomal and unassembled ch-chromatin is less than ideal for several reasons. As noted above, MNase will begin to digest unassembled chromatin to acid solubility immediately, resulting in its preferential loss; however, when digestion is

limited (such that little acid soluble material is generated) much of the chromatin remains in the insoluble pellet as high molecular weight species, and its structure is difficult to determine (see Figure 1, lane P.2). Thus, it could be argued that nucleosomes obtained by redigestion are derived from undigested regions with typical nucleosomal periodicity. However, attempts to generate heterogeneous chromatin of lower molecular weight (by increasing the extent of digestion) have resulted in extensive loss of the non-nucleosomal component. Finally, the "trimming" action of MNase can quickly remove unassembled DNA from newly replicated monomers and multimers, potentially creating the false impression that they were solely derived from regions of normal spacing. These difficulties can be avoided through the use of a restriction endonuclease as the primary cutting agent. Because HaeIII had been used to analyze the properties of ch-chromatin in an earlier report (8), we have used this enzyme to further study the properties of newly replicated chromatin.

As a control, the action of HaeIII on mature chromatin was examined. Cells were labeled with [³H]thymidine for 30 min, and isolated nuclei digested with HaeIII (see Materials and Methods). After digestion, nuclei were pelleted by low speed centrifugation to yield a first supernatant (SI). The pellet was then suspended in buffer containing 0.2 M NaCl, and again pelleted, producing a second supernatant (SII) and a final pellet.

Consistent with previous findings (15), HaeIII cleaved mature chromatin into a well resolved series of nucleosome-sized fragments (Figure 2A): few, if any, intranucleosomal cuts were introduced. Clearly, HaeIII has a very strong preference for recognition sites exposed in the linkers between nucleosomes (15). Chromatin DNA in the SI fraction had a lower average molecular weight than that in the SII, as well as a somewhat shorter repeat length (SI: 180 bp; SII: 186 bp).

The presence of HaeIII during digestion was absolutely required for the release of nucleosomal fragments (Figure 2B); thus, no endogenous nucleases contributed to the production of the DNA pattern.

Digestion of purified DNA with HaeIII produced a broad smear, upon which several bands were superimposed (Figure 2C). These bands coincided with known human repetitive DNA sequences, including multiple repeats of a 170 bp satellite (16-18). Multiples as large as six to eleven times the basic repeat have been observed, even after exhaustive digestion, indicating sequence heterogeneity (16, 18). In agreement with the data of Manuelidis (16), the 340 bp dimer was the most predominant fragment, and

Figure 2: Digestion of control nuclei and purified DNA with HaeIII.

(Panel A): Cells were labeled with [^3H]thymidine for 30 min in the absence of cycloheximide. Isolated nuclei were digested with HaeIII and gently pelleted, yielding a first supernatant (SI, lane I). Nuclei were then treated with 0.2 M NaCl, and pelleted to yield a second supernatant (SII, lane II) and a final pellet (lane P). Approximately 8% of bulk chromatin DNA was released by these methods (SI: 2.0%; SII: 5.7%) (see Materials and Methods). DNA was subjected to electrophoresis and analyzed by fluorography. (Lane Mn): MNase digest of ch-chromatin. The positions of mono- (M), di- (D), and trinucleosomal DNA (T) are indicated. Arrowhead indicates the position of 170 bp repetitive DNA fragment (see text).

(Panel B): Cells were labeled with [^3H]thymidine for 40 min in the presence of cycloheximide. Isolated nuclei were incubated for 30 min in HaeIII digestion buffer at 37°C without added enzyme. Chromatin was fractionated and analyzed as in panel A. Lanes are labeled as in A. Marker fragments (lane m) are as in Figure 2.

(Panel C): Cells were labeled with [^3H]thymidine for 40 min in the presence of cycloheximide. DNA was purified, digested with HaeIII for 0, 10, 30, 60, 120, and 240 min (lanes a to f, respectively), and subjected to electrophoresis. Marker fragments (lane g) are as in Figure 2. Small arrowhead marks the position of 170 bp repetitive DNA monomer; large arrowhead: 340 bp dimer. For panels A, B, and C, a fluorograph is presented.

bands approximately 1.3 and 2.5 times the 170 bp monomer were also observed (219 and 422 bp, respectively). It should be noted that no contaminating nucleases were present in the HaeIII preparation, since extensive degradation of purified DNA did not occur. Therefore, the nucleosomal repeat observed for control chromatin (Figure 2A) was produced by the action of HaeIII alone.

Digestion of ch-chromatin with HaeIII yielded results strikingly

Figure 3. Digestion of ch-chromatin with HaeIII, and redigestion of the SI and SII with MNase.

Cells were preincubated in cycloheximide for 12 min, and then labeled with [3 H]thymidine for 40 min in the presence of cycloheximide. Isolated nuclei were digested with HaeIII and chromatin fractionated as in Figure 2 (panel A). Less than 2% of total ch-chromatin DNA was rendered acid soluble; 8-11% of ch-chromatin was released into the SI and SII under these conditions; in this case, SI: 3.4%; SII: 4.9%. Chromatin in the SI (lane b) was redigested with MNase (0.375 U/ml) for 1, 2, and 6 min, with 62.9, 51.6, and 45.9 percent of the starting SI chromatin remaining acid precipitable at each time point (lanes c to e, respectively); SII chromatin (lane g) was redigested with MNase (0.75 U/ml) for 1, 2, and 6 min, with 58.4, 50.9, and 41.9 percent of the starting SII chromatin remaining acid precipitable (lanes h to j). Chromatin DNA was subjected to electrophoresis and analyzed by fluorography. (Lane a): MNase digest of ch-chromatin; the positions of mono- (M), di- (D), and trinucleosomal DNA (T) are indicated. Marker fragments (lane f) are as in Figure 2. (Lane k): ch-chromatin DNA from the final HaeIII pellet. Open diamond: 340 bp repetitive DNA dimer; open arrowhead: position of 170 bp repetitive DNA monomer; solid arrowhead: position of 146 bp core nucleosome DNA. Fragments shorter than 70 bp have not been included in the figure.

different from those observed with control chromatin (Figure 3). No extended nucleosomal ladder was found in the SI (lane b). Instead, a broad smear of approximately monomer size extended from 160 to 263 bp, with some smaller fragments also in evidence. The maximum size of the larger fragments was 50 bp greater than the longest monomers released by HaeIII digestion of control chromatin (216 bp). In contrast to a previous report (8), the SI fraction of ch-chromatin was not naked DNA: when redigested

with MNase, 45-60% of the starting SI material was nuclease resistant, and was clearly resolved into nucleosomal monomers (lanes c-e).

Similar results were obtained with ch-chromatin eluted into the SII, which was distributed in a range of 200 to 700 bp, with the majority of the DNA migrating between the positions of normal monomers and trimers (lane g). Unlike control chromatin (Figure 2A, lane II) there was no indication of a nucleosomal ladder, although faint bands, corresponding to the repetitive sequences observed in naked DNA (Figure 2C), were observed. (Note: 0.2 M NaCl effects the release of heterogeneous chromatin after digestion with HaeIII, but not with MNase (cf. Figure 1A, lane .2). This is probably due to the absence of polyamines in the HaeIII digestion). The ch-chromatin pellet (lane k) contained DNA of lower average molecular weight than that of control chromatin (Fig. 2A, lane P). This can be attributed to the relative insolubility of non-nucleosomal DNA, as previously observed after digestion with MNase (Fig. 1; also refs. 3, 7).

The appearance of the SII (Fig. 3) strongly suggests that, with the exception of a few repetitive sequences, HaeIII is introducing cuts at sites in the unassembled regions of ch-chromatin in a nonorderly manner. Since HaeIII has a very strong preference for sites in linker DNA (Figure 2A), the complete absence of a regular nucleosomal repeat suggests that the nucleosomal and unassembled regions may be interspersed. That this is the case was demonstrated by redigestion of the SII fraction with MNase: due to the greater susceptibility of unassembled chromatin to digestion, nuclease-resistant nucleosomes were rapidly unmasked (Figure 3, lanes h-j). The earliest digestion products included extra-long monomers (up to 250 bp), which were trimmed as digestion proceeded. Consistent with intuitive predictions, 40-60% of the starting SII chromatin was found to be nucleosomal, with a final average length of 150 bp (lane j). This was easily distinguished from the 170 bp repetitive fragment (open arrow). Further, the fragment size and nuclease-resistance of the remaining DNA argues strongly against intranucleosomal cutting during the initial HaeIII digestion.

In order to ensure that no histone exchange was occurring in the presence of 0.2 M NaCl, the following control mixing experiment was performed. HeLa cells were incubated for 30 min in the presence of cycloheximide, without [³H]thymidine. Nuclei were isolated, mixed with uniformly labeled free DNA in the presence of 0.2 M NaCl, and then digested with MNase (Figure 4). Because soluble histones can readily leak out of

Figure 4. Histone exchange does not occur in 0.2 M NaCl.

Cells were incubated in cycloheximide for 30 min in the absence of radiolabel. Nuclei were isolated and mixed (in the presence of buffer A containing 0.2 M NaCl) with an equivalent amount of uniformly labeled free DNA. The mixture was maintained at 0.2 M NaCl for 30 min on ice, and then digested at 37°C with MNase (0.75 U/ml) for 0, 0.5, 1, 2, 5, and 10 min (lanes a to f, respectively), as described for ch-chromatin (see Materials and Methods). Chromatin DNA was subjected to electrophoresis, stained with ethidium bromide (panel A) and analyzed by fluorography (panel B). Marker fragments are as in Figure 2. The positions of mono- (M), di- (D) and trinucleosomal DNA (T) are indicated.

intact nuclei (29, 30), we reasoned that histones putatively released by 0.2 M NaCl would traverse the nuclear envelope and bind to the excess DNA. Although the unlabeled chromatin showed typical subunit structure (panel A), the labeled, naked DNA was quickly degraded to non-precipitable nucleotides (panel B). Thus, histone exchange in 0.2 M NaCl does not account for the presence of nucleosomes in the SII fraction of ch-chromatin.

When the SI and SII fractions of HaeIII-digested ch-chromatin were examined as native chromatin in gels (Figure 5), they migrated as heterogeneous nucleoproteins (lanes a and c), not as nucleosomes and naked DNA (lanes b and d). When 0.2 M NaCl-soluble proteins were mixed with free SII DNA, the migration of the DNA was unaffected, again demonstrating the absence of histone exchange (lane e). The migration of the SI and SII as nucleoproteins is fully consistent with the data obtained from DNA gels

Figure 5. Analysis of ch-chromatin as native nucleoproteins.

Cells were labeled with [3 H]thymidine for 40 min in the presence of cycloheximide. Nuclei were digested with HaeIII, and chromatin fractionated as in Figures 2A and 3. Native nucleoproteins were subjected to electrophoresis in a composite agarose-polyacrylamide gel, and analyzed by fluorography. (Lane a), SI; (lane b), free DNA obtained from the SI; (lane c), SII; (lane d), free DNA obtained from the SII. (Lane e): free DNA from the SII was mixed with an equal volume of an extract obtained by incubating unlabeled nuclei in 0.2 M NaCl (20 A₂₆₀/ml); the mixture was then dialyzed against 2 mM EDTA, and subjected to electrophoresis. (Lane f): MNase digest of ch-chromatin; the mononucleosome position is indicated (M).

(Figure 3), and supports the conclusion that MNase unmasks "hidden" nucleosomes by removing adjacent stretches of non-nucleosomal DNA.

DISCUSSION

After digestion with MNase or HaeIII, a significant fraction of ch-chromatin was insoluble at low ionic strengths, and lacked nucleosomal organization. With MNase, ~60% (more or less, depending on the extent of nuclease digestion) possessed the "non-nucleosomal" appearance (this report; also refs. 3, 7). Virtually all digestion products from HaeIII digestion of ch-chromatin appeared as non-nucleosomal DNA, in contrast to the regular nucleosomal ladder obtained from control chromatin. Although HaeIII solubilized less DNA than MNase due to shielding of cleavage sites in nucleosomes, the amounts released from control chromatin and ch-chromatin were essentially equivalent (about 10%).

It must be stressed that these data do not conflict with previous

reports of regular nucleosomal spacing for ch-chromatin (7, 8, 13, 14, 19, 20). Nucleosomal ladders of 6-8 subunits are observed when either soluble chromatin or total DNA is analyzed (7, 8, 13, 14, 19, 20). Analyses of total DNA (soluble plus insoluble) reveal a summation of the two components: a regular nucleosomal ladder superimposed on a high degree of heterogeneous background (discussed in detail below) (8, 19). In addition, nucleosomes with unusually long DNA complements have also been observed (this report; refs. 19, 20), further indicating the juxtaposition of nucleosomes and unassembled DNA. However, by taking advantage of the relative insolubility of unassembled DNA (3, 7), we have been able to isolate the non-nucleosomal component of ch-chromatin, and thereby study its structure.

Surprisingly, the heterogeneous nucleoprotein obtained with either MNase or HaeIII yielded nucleosomes when trimmed with MNase. In order to account for this finding, several features of ch-chromatin must be considered. First, the presence of nucleosomes within irregularly cleaved fragments of ch-chromatin cannot be due to complete loss of regular nucleosomal periodicity. As discussed above, the nucleosomal components of ch-chromatin possess a well-defined repeating structure. After incubation periods of 20 min or longer, the repeat length of soluble ch-chromatin is identical to that found in mature chromatin (7).

Second, it is extremely unlikely that the non-nucleosomal appearance of the insoluble fraction results from gross changes in nucleosomal composition or structure. In previous experiments (7) we have determined that nucleosomes obtained from ch-chromatin display the full spectrum of electrophoretic heterogeneity found in control nucleosomes, and in identical proportions. Also, the observation that ~50% of ch-chromatin is in nucleosomes which possess normal nuclease resistance argues strongly against radical conformational changes (this report; also refs. 7, 8, 13, 14).

Third, control experiments demonstrated that histone exchange did not account for these observations. Because several kb of unlabeled DNA separated the last mature nucleosome from labeled ch-chromatin (due to pre-incubation of cells in cycloheximide), large-scale histone sliding during chromatin preparation is also extremely unlikely to account for these results. It must be emphasized that the nucleosomes of ch-chromatin are not depleted in histone H1 (7), the loss of which has been linked to histone lateral migration (21, 22). Experiments specifically designed to

Figure 6. Possible Modes of Parental Histone Octamer Segregation.

a) Conservative: parental histones are distributed to only one progeny DNA duplex over very long distances. Endonuclease digestion yields two distinct components, nucleosomes (monomers and multimers) and unassembled DNA.

b) Bilateral, Alternating: histone octamers are segregated alternately to both sides of the fork. Endonuclease digestion yields monomers with variably-sized linkers; no nucleosomal multimers are possible.

c) Bilateral in clustered segregation groups: histone octamers are segregated to both sides of the fork in groups of one or more. Endonuclease digestion yields unassembled DNA, along with monomers and multimers containing extra-long linkers.

For (a), redigestion of unassembled DNA (appearing as heterogeneous fragments in gels) produces only smaller, randomly cleaved products. For (b) and (c), heterogeneous chromatin yields nucleosomes upon redigestion. Open circles: parental histone octamers; arrowheads: possible sites of endonucleolytic cleavage.

detect histone rearrangement have observed no histone sliding for native or ch-chromatin at physiological ionic strengths (7, 19, 21, 22). In addition, our conclusions are not restricted to chromatin replicated in cycloheximide: MNase digestion of normal nascent chromatin also produces both nucleosomes and a heterogeneous chromatin fraction (3), the latter of which yields nucleosomes upon redigestion with MNase (unpublished observations).

Given the above considerations, the data are best explained with reference to models of parental histone segregation during replication. Three such models of histone segregation are diagrammed in Figure 6.

In Figure 6a the commonly accepted model, conservative segregation, is presented. Endonuclease digestion will yield two moieties: nucleosomes (monomers and multimers) from one arm and a heterogeneous fraction from the other. Redigestion of the heterogeneous chromatin cannot produce

nucleosomes, but only smaller, randomly cleaved products.

Although the alternating segregation model (Figure 6b) can account for the presence of nucleosomes within apparently unstructured material, it does not allow for the production of polynucleosomes during the initial digestion. Thus, neither of the foregoing can account for the experimental observations.

A third model, bilateral segregation of nucleosome clusters resulting in contiguous regions of nucleosomal and unassembled DNA (Figure 6c), can accommodate our observations on ch-chromatin, since both nucleosomal multimers, and randomly sized fragments containing nucleosomes can be generated in significant yield. This mechanism has been suggested previously (19, 25).

Upon endonucleolytic cleavage of bilaterally segregated nucleosome clusters, fragments would arise from i) cuts situated in unassembled regions, ii) one cut in a nucleosomal domain and one in an unassembled domain, iii) cuts in unassembled domains flanking a nucleosome cluster, and iv) both cuts within a nucleosomal domain. Because HaeIII digestion solely within the nucleosomal component should yield a ladder, but none is observed in ch-chromatin, the abundance of these fragments must be low relative to the three other possible fragment types. This can be attributed to i) the increased exposure of restriction sites in unassembled regions, and ii) the relatively high molecular weight of the HaeIII digestion fragments released from naked DNA (Figure 2c). Therefore, virtually all digestion products (cases ii and iii) should not exhibit nucleosomal structure until trimmed, as observed. Thus we conclude that the heterogeneous molecular weight material arises from endonucleolytic cleavages in unassembled DNA domains interspersed among domains of parental nucleosome clusters. This is supported by the observations of Pospelov et al. (19), who showed that most ch-chromatin solubilized by BspRI (an isoschizomer of HaeIII) migrated with an intermediate bouyant density between that of DNA and nucleosomes. It was therefore suggested that the nucleosomal and unassembled components of ch-chromatin were contiguous (19).

We did not observe the release of free DNA by HaeIII digestion of ch-chromatin in the first supernatant (i.e., the SI) after BspRI or HaeIII digestion as reported by others (8, 19); no data in support of this was presented in either of those reports. In contrast, our results clearly demonstrate that approximately 50% of the ch-DNA present in the SI is in

nucleosome cores, and that the remaining DNA is contiguous with these cores as extra-long linkers. If there were two separate types of fragments of equal abundance, e.g., the 200-300 bp DNA and mononucleosomes, the two components would be readily distinguishable in both DNA and DNP gels. Interestingly, DNA purified from the BspRI supernatant obtained by Pospelov et al. was between 200 and 300 bp in length (19), precisely the size of the SI ch-DNA we observe. It therefore seems probable that the SI DNA observed by those investigators was also complexed in nucleosomes with extra-long linkers.

It should be noted here that nucleosomal ladders obtained from normal nascent or ch-chromatin usually possess an atypical "background smear" in gels, not observed with control chromatin (7, 8, 19, 20). As suggested by Pospelov et al. (19), this background most likely arises from monomers and multimers containing an unusually long DNA complement, due to random cleavages in unassembled DNA between typical nucleosomes. These extra lengths of new DNA appear to contribute to the relative insolubility of newly replicated chromatin whether digested with MNase or HaeIII, perhaps due to association with insoluble replication structures. Therefore, only those monomers and dimers which have been sufficiently trimmed by MNase (or which were derived from within a clustered nucleosomal domain) are soluble at low ionic strengths (this report; also refs. 3, 7).

If parental histone octamers are distributed to both sides of the fork more or less equally, clearly no consistent association of old histones with the leading strand can occur for cellular chromatin, as was concluded for the replication of SV40 minichromosomes (8). Curiously, an examination of SV40 integrated into cellular chromatin indicated that the conservative mode of segregation was maintained, but that parental histones remained with the non-coding strand (23), not with the coding strand as concluded for free viral minichromosomes (8). Our results are also apparently inconsistent with an electron microscopic analysis of cellular chromatin, in which it was observed that the conservative mode of segregation was maintained for up to 100 kb (6). In a recent examination of segregation by electron microscopy, nucleosomes were observed on both sides of the fork of ch-chromatin in CHO cells (C. L. F. Woodcock, personal communication), in accord with the conclusions of an earlier microscopic analysis of SV40 replicated in puromycin (24). Investigations in which the association of parental histones with density-labeled DNA was monitored over several cell generations have failed to support the fully conservative mode of

segregation (25, 26, 27). Similarly, nearest-neighbor analyses have indicated that newly assembled nucleosomes and parental nucleosomes are interspersed (28).

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ABBREVIATIONS (*)

Ch-chromatin, chromatin synthesized during exposure of cells to cycloheximide; ch-DNA, nuclear DNA synthesized during exposure of cells to cycloheximide; MNase, micrococcal nuclease; bp, basepairs; kb, kilobasepairs.

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