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Maturation of Nucleosomal and Nonnucleosomal Components of Nascent Chromatin: Differential Requirements for Concurrent Protein Synthesis†

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ABSTRACT: The DNA of newly replicated chromatin is comprised of two components, distinguishable by their solubility characteristics and requirements for maturation. One of these components possesses core histones, typical nucleosomal structure, a nuclease-resistant core containing 146 base pairs (bp) of new DNA, and all the nucleosomal species found in bulk chromatin (due to bound histone H1 and high mobility group proteins). In addition, this class of nascent chromatin exhibits a shortened repeat length of ~165 bp, as opposed to the 188-bp repeat of bulk chromatin. Within 10 min of DNA synthesis, the spacing of mature chromatin is established; the spacing maturation can occur in the absence of protein synthesis. The second class of nascent DNA is distinguished from the nucleosomal component by its insolubility, lack of discernible nucleosomal organization, and dependence on protein synthesis to attain typical subunit structure. This unassembled component is not free DNA, as demonstrated by its intermediate resistance to nucleolytic degradation. The structural properties and maturation requirements of this material suggest that it is the site of de novo nucleosome assembly.

During the replication of nuclear DNA, the structure of chromatin is transiently altered. Among the effects of such conformational modification is an increased sensitivity of nascent chromatin to nuclease digestion, such that newly replicated chromatin DNA is both cleaved to mononucleosomes and digested to acid solubility more rapidly than bulk chromatin (Seale, 1975, 1976, 1981; Hildebrand & Walters, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Worcel et al., 1978; Klempnauer et al., 1980; Annunziato et al., 1981). Another structural alteration is observed as a shortened repeat length for the nucleosomes on new DNA (Levy & Jakob, 1978; Murphy et al., 1978, 1980; Seale, 1978a, 1981; Galili et al., 1981; Jackson et al., 1981). Lastly, we have recently shown that in the vicinity of the replication fork approximately 40% of the new DNA lacks typical nucleosomal organization (Annunziato et al., 1981). Similar findings have been reported for replicating SV40 minichromosomes (Herman et al., 1979; Cusick et al., 1981).

One useful approach in dissecting the mechanisms of chromatin replication and maturation has been to block concurrent protein synthesis with the inhibitor cycloheximide. DNA replication continues at a reduced rate in the absence of protein synthesis (Weintraub & Holtzer, 1972; Seale & Simpson, 1975), but chromatin DNA so produced is approximately twice as sensitive to digestion to acid solubility with DNases than control chromatin, indicating that approximately half of the new DNA lacks nucleosomes (Weintraub, 1973, 1976; Seale & Simpson, 1975; Seale, 1976, 1978a; Schlaeger & Klempnauer, 1978; Roufa, 1978). In contrast, regularly spaced nucleosomal multimers are observed on the nuclease-resistant fraction of new DNA, as predicted for the segregation of parental histone octamers to the growing daughter DNA molecules (Seale, 1976, 1978a; Weintraub, 1976; Schlaeger & Klempnauer, 1978; Riley & Weintraub, 1979; Seidman et al., 1979). In this paper, we have examined the subunit structure and nucleosomal periodicity of chromatin replicated in either the presence or the absence of protein synthesis, in order to gain an understanding of the processes of chromatin maturation following DNA replication. We provide evidence that the nucleosomal and nonnucleosomal classes of nascent DNA can be distinguished, not only by their structures but also by their differential requirements for protein synthesis for maturation.

Experimental Procedures

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.

Long-term labeling of cells with [^{14}C]thymidine (50 mCi/mmol, Schwarz/Mann) was performed at 0.01 μCi/mL for one generation (24 h). For pulse-labeling experiments, cells were harvested by centrifugation (250*g*, 1.5 min) when at ~4 × 10^5 cells/mL. Pulse labeling with [*methyl*-^{3}H]thymidine (60 Ci/mmol, New England Nuclear) was performed by concentrating cells 20-fold (0.5-min pulse) or 5-fold (5- to 60-min pulse) in prewarmed whole medium followed by equilibration at 37 °C for 5 min. For 30-s labeling, cells were incubated with radioactive thymidine at 50–60 μCi/mL; for longer labeling periods, thymidine was added at 2.5–6.7 μCi/mL.

For measurement of protein synthesis, cells were prelabeled for 24 h with [^{14}C]thymidine and incubated with [^{3}H]lysine (60 Ci/mmol, New England Nuclear) for the times indicated at 25 μCi/mL, in culture medium depleted 80% in lysine.

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Labeling was halted by diluting an aliquot of cells 10-fold with ice-cold buffer A (Seale, 1978a) containing 0.1% sodium azide. Cells were pelleted, resuspended in H_2O, and precipitated with trichloroacetic acid.

Cycloheximide was added as required to a final concentration of 200 μg/mL, from a freshly made stock solution of 10 mg/mL in distilled water. Cells were preincubated for 5–10 min in cycloheximide before the addition of [^{3}H]thymidine. Pulse labeling with thymidine was halted by diluting cells 5-fold in the appropriate ice-cold nuclear isolation buffer (see below) containing 0.1% sodium azide.

Analysis of Nucleosomal Repeat Lengths. For the sizing of newly replicated and bulk DNA, cells were washed twice in PK buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes)[1] and 100 mM KCl, pH 7.0] and resuspended in 10 mM Pipes, 75 mM KCl, and 0.5 mM PMSF. Cells were allowed to swell for 10–15 min and lysed with approximately 20 strokes in a Dounce homogenizer. Nuclei were collected by centrifugation at 1500*g* for 1 min and washed once in PK buffer plus PMSF and once in buffer alone. Nuclei were then resuspended in PK buffer at a concentration of 50 A_{260}/mL (A_{260} determined in 0.3 N NaOH), and $CaCl_2$ was added to a final concentration of 0.5 mM. Digestion with micrococcal nuclease (Sigma) was for the times indicated (text) at 0 °C at 25 units/mL. The reaction was terminated by the addition of EGTA (pH 7.6) to a final concentration of 5 mM. After 15 min on ice, insoluble material was removed by centrifugation (12800*g* for 7 min), and the supernatant, containing the solubilized chromatin, was collected.

For the sizing of nascent core nucleosomal DNA, nuclei were digested in PK buffer plus 0.5 mM $CaCl_2$ at 37 °C for the times indicated (text), with 5 units of micrococcal nuclease/mL of digestion buffer.

Fractionation of Chromatin Using the Procedure of Sanders (1978). Stepwise salt elution of chromatin and analysis of nucleosomes in DNP (deoxyribonucleoprotein) gels were performed as previously described (Annunziato et al., 1981). Briefly, cells were washed twice with CB buffer (1 mM Tris, 25 mM KCl, 0.9 mM $MgCl_2$, 0.9 mM $CaCl_2$, 0.14 mM spermidine, and 2 mM sodium butyrate, pH 7.6), resuspended in CB buffer plus 0.5 mM PMSF, swollen on ice for 15 min, and lysed with 6–10 strokes in a Dounce homogenizer. Nuclei were pelleted (1500*g*, 1 min), washed once in CB buffer plus PMSF and once in buffer alone, and resuspended in CB buffer at a concentration of 46 A_{260}/mL. The nuclei were then equilibrated at 37 °C for 5 min and digested with micrococcal nuclease (1.2 units/mL) for 2 min.

Following digestion, the nuclei were pelleted (1500*g* for 10 min) to yield a supernatant (termed S0) containing acid-soluble nucleotides. Chromatin was then fractionated according to the procedure of Sanders (1978), by resuspending the nuclear pellet, sequentially, in CB buffer containing 0.5 mM PMSF and 1 mM EGTA plus 0.1, 0.2, 0.3, 0.4, and finally 0.6 M NaCl. Each extraction step was for 20 min on ice; nuclei were then pelleted (1500*g*, 10 min) and the fractions collected and denoted S.1–S.6, plus the final pellet (P). Fractions were dialyzed against 2 mM EDTA and, when necessary, concentrated in the B-15 Minicon concentrator (Amicon).

As a control, a total chromatin fraction, T, was obtained by lysis of nuclei with 2 mM EDTA, pH 7.2, and removal of insoluble chromatin (denoted P_t) by centrifugation (12800*g* for 10 min).

Gel Electrophoresis. For DNA size analyses, chromatin fractions were brought to 10 mM magnesium acetate, ethanol precipitated, and solubilized in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, adjusted to pH 7.2 with glacial acetic acid) made 5% in glycerol and 1% in $NaDodSO_4$. The P fraction was resuspended in 2 mM EDTA prior to ethanol precipitation. Polyacrylamide slab gels (3.9%) were prepared according to Loenig (1967) and run at 90–100 V in the presence of 0.1% $NaDodSO_4$, as described previously (Annunziato et al., 1981). Gels were either 12.5 cm or, for greater resolution of multimers, 18.5 cm in length. Band sizes were determined from a calibration curve (log M_r vs. mobility) constructed from labeled restriction fragments (*Hae*III digest of ϕX174 [^{3}H]DNA, New England Nuclear). Measurements were made from the origin to the center of each band in the fluorographic image (Compton et al., 1976; Noll & Kornberg, 1977; Thomas & Thompson, 1977). Several fluorographic exposures were made of each gel in order to clearly resolve the desired oligomer bands.

For analysis of nucleoprotein particles, chromatin fractions were subjected to electrophoresis in composite 0.5% agarose and 3.5% polyacrylamide (DNP) gels by using a modification of the procedure of Todd & Garrard (1977), as described previously (Annunziato et al., 1981). Gels were cast and run in 6.4 mM Tris, 3.2 mM sodium acetate, and 0.28 mM EDTA, pH 8.0; electrophoresis was at 110 V for 4 h at 4 °C, with buffer recirculation.

Gels were stained with ethidium bromide, photographed, impregnated with scintillant, and exposed to Kodak X-OMAT R film. DNP gels were prepared for fluorography with EN^{3}HANCE (New England Nuclear); DNA gels were impregnated with PPO (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Radioactivity Determinations. Aliquots of chromatin fractions were precipitated, together with 50 μg each of DNA and albumin, with 10% trichloroacetic acid, collected on glass fiber filters (Reeve Angel 934-AH, Whatman), and washed twice with trichloroacetic acid and twice with 95% ethanol. Dried filters were incubated with 0.2 mL of NCS (Amersham) containing 5% H_2O and counted in PPO/toluene fluor (4 g/L). The fraction of ^{14}C counts registered in the ^{3}H channel was subtracted by the external standard technique.

Results

Newly Replicated HeLa Chromatin Exhibits a Shortened Nucleosomal Repeat. An unresolved question regarding nascent chromatin structure is whether the shortened repeat of newly replicated chromatin reflects a real intrinsic property or simply a more rapid rate of "trimming" of internucleosomal linker DNA due to its increased nuclease sensitivity. Cells were labeled either for 30 s or for 24 h with thymidine in order to study the nucleosomal periodicity of newly replicated and bulk chromatin; nuclei were isolated and were digested for increasing periods with micrococcal nuclease (MNase). Digestions were performed at 0 °C in order to retard the exonucleolytic trimming of linker DNA (Noll & Kornberg, 1977) and to inhibit histone lateral migration (i.e., sliding) (Beard, 1978; Weischet, 1979; Spadafora et al., 1979; Weischet & Van Holde, 1980). In agreement with the findings of others, newly replicated multimers exhibited a shortened nucleosomal repeat

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid, disodium salt; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), dipotassium salt; PMSF, phenylmethanesulfonyl fluoride; MNase, micrococcal nuclease; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; HMG, high mobility group; ch-chromatin, chromatin synthesized during exposure of cells to cycloheximide; ch-DNA, nuclear DNA synthesized during exposure of cells to cycloheximide.

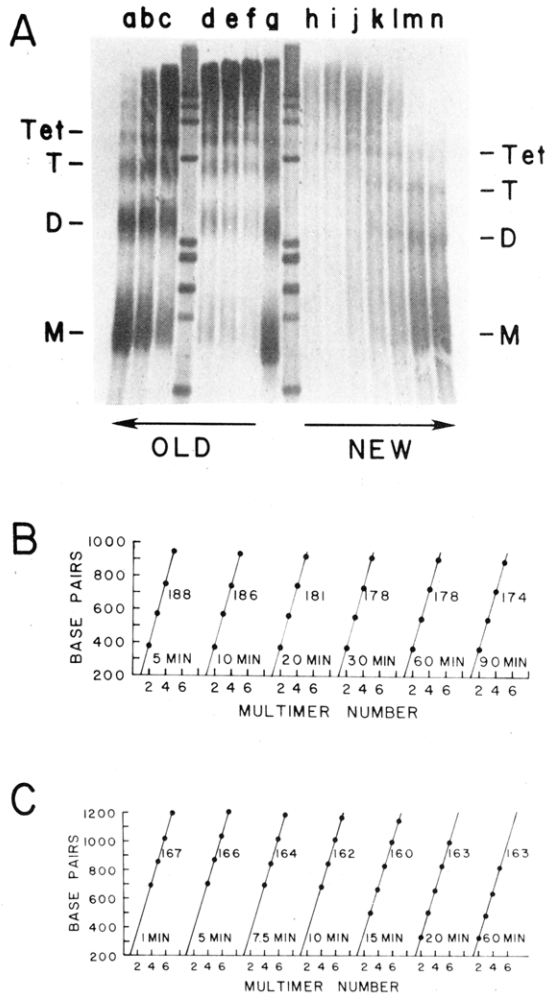

FIGURE 1: Newly replicated HeLa cell chromatin has a shortened nucleosomal repeat. (A) HeLa cells were labeled with thymidine for one generation (lanes a–g) or for 30 s (lanes h–n) and isolated nuclei digested with MNase for increasing periods in PK buffer at 0 °C. Soluble chromatin DNA was subjected to electrophoresis in a 12.5-cm polyacrylamide gel and analyzed by fluorography. (Lanes a–f) Digestion of bulk chromatin for 90, 60, 30, 20, 10, and 5 min, respectively; (lane g) control bulk chromatin, digested at 37 °C for 2 min; (lanes h–n) digestion of nascent chromatin for 5, 7.5, 10, 12.5, 15, 20, and 60 min, respectively. Marker fragments: *Hae*III digest of ϕX174 [^{3}H]DNA. Fragments are 1353, 1078, 872, 603, 310, 278/271 (doublet), 234, 194, and 118 base pairs long. The positions of mono-, di-, tri-, and tetranucleosomal DNA (M, D, T, and Tet, respectively) are indicated. (B) HeLa chromatin DNA was labeled for one generation with [^{14}C]thymidine, digested with MNase for the times indicated, and subjected to electrophoresis in an 18.5-cm gel. The nucleosomal repeat length at each digestion time point was obtained by plotting multimer size (in base pairs) vs. multimer number; the resulting line (obtained by linear regression analysis; correlation coefficients 0.99984 ● 0.00015) is the repeat length, shown for each time point (Noll & Kornberg, 1977; Sperling et al., 1980). (C) HeLa chromatin DNA was labeled for 30 s with [^{3}H]thymidine, digested with NMase for the times indicated, and subjected to electrophoresis. Nucleosomal repeat length was then analyzed as in Figure 1B. Correlation coefficients: 0.99964 ± 0.00035.

relative to the corresponding multimers from bulk chromatin (Figure 1A) (Levy & Jakob, 1978; Murphy et al., 1978, 1980; Seale, 1978a, 1981; Galili et al., 1981; Jackson et al., 1981; Annunziato et al., 1981). Nascent multimers appear distinctly less heterogeneous than bulk multimers, in agreement with earlier findings (Seale, 1978a); however, the tighter multimer bands of new DNA are superimposed upon a high degree of interband background radioactivity not seen in bulk chromatin (Figure 1A). New and old monomers, unlike the multimers, have similar mobilities (compare lanes a and n, Figure 1A), suggesting that the structural alterations that produce the short repeat occur in the internucleosomal linker, not through a reduction in the monomer DNA per se.

For quantitation of the difference in periodicity between newly replicated and bulk chromatin, nucleosomal repeat lengths were determined by plotting the length of each multimeric DNA fragment vs. the number of nucleosomes in each fragment; the slope of the resulting line yields the repeat length (Noll & Kornberg, 1977; Sperling et al., 1980). When this method of analysis was applied to bulk chromatin, it was found that the nucleosomal repeat gradually decreased with increasing digestion time, from 188 to 174 base pair(s) (bp) (Figure 1B) (note: the data presented in Figure 1 were derived from several experiments and fluorographic exposure levels). The decrease in repeat length is most likely due to the heterogeneity of internucleosomal linker lengths in bulk chromatin and the greater resistance of more closely spaced nucleosomes to excision. Multimers with a shorter linker will therefore persist until the later stages of digestion (Noll & Kornberg, 1977; Lohr et al., 1977; Martin et al., 1977; Prunell & Kornberg, 1978, 1982). Also, a limited amount of exonucleolytic trimming at 0 °C cannot be excluded. The 188-bp repeat length for HeLa bulk chromatin at the early stages of digestion (Figure 1B) is in good agreement with previously reported values of 183–188 bp for human cells (Compton et al., 1976; Lohr et al., 1977; Hsiung & Kucherlapati, 1980).

The analysis of newly replicated chromatin (Figure 1C) yielded strikingly different results. Because (1) digestions were performed at 0 °C and (2) the largest discernible multimers were selected for analysis, the influence of exonucleolytic trimming on repeat length was virtually eliminated. Nevertheless, at all stages of digestion, nascent chromatin displayed a repeat length (160–167 bp) significantly shorter than the 188-bp repeat of bulk chromatin. A comparison of panels B and C of Figure 1 shows that the nucleosomal repeat of newly replicated chromatin, after only 1 min of Mnase digestion, is shorter than that of bulk chromatin digested 90 times longer.

The shortened repeat of nascent chromatin might be accompanied by large-scale changes in nucleosomal structure, or by a loss of specific nucleosomal proteins (such as histone H1 and HMG proteins). Alternatively, the reduced spacing might be simply the consequence of a reduced internucleosomal linker, with few major disruptions of nucleosomal organization. In a previous report (Annunziato et al., 1981), it was shown that within 30 s of DNA synthesis nascent DNA was associated with particles possessing the same electrophoretic mobility as H1-containing and HMG protein containing mononucleosomes. This is well within the period in which the shortened repeat of nascent chromatin is observed (Figure 1; Murphy et al., 1978, 1980; Seale, 1981). Thus, it appears that the reduction in repeat length is not accompanied by a loss of histone H1 or HMG proteins.

For elimination of the possibility that the shortened nascent repeat is due to a reduction in the core DNA size, cells were labeled for 30 s with [^{3}H]thymidine and digested with MNase at 37 °C, yielding trimmed core particles (see Experimental Procedures). Analysis of the new DNA by gel electrophoresis and fluorography (Figure 2) revealed that nascent mononucleosomes, like those of bulk chromatin, contained a stable core of 146 bp. It is therefore unlikely that the reduced length of newly replicated multimers arises either from a shortened core DNA size or from scissions within the cores of the terminal nucleosomes. Since the shortened repeat is not contingent upon a loss of nucleosomal integrity, nor, as discussed above, upon the detectable loss of accessory nucleosomal

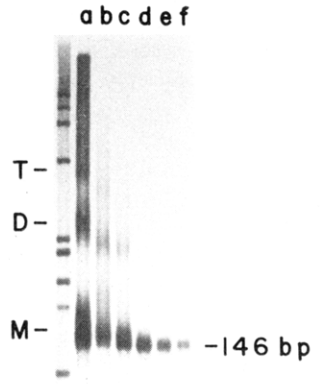

FIGURE 2: Nascent nucleosomal cores contain 146 base pairs of new DNA. Chromatin was labeled for one generation (a) or for 30 s (b–f) with thymidine and digested at 37 °C with MNase. Chromatin DNA was analyzed by gel electrophoresis and fluorography. (Lane a) Control bulk chromatin digested for 2 min; (lanes b–f) digestion of nascent chromatin for 1, 2, 5, 10, and 15 min, respectively. The positions of bulk monomer (M), dimer (D), and trimer (T) DNA are indicated. Marker fragments are as in Figure 1A.

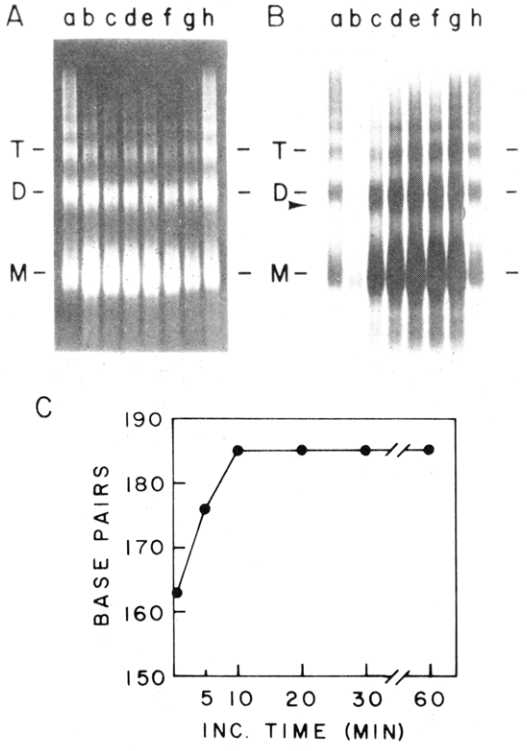

FIGURE 3: Maturation of nascent nucleosomal repeat length. Chromatin was labeled with [^{3}H]thymidine for increasing periods (30 s to 60 min), and isolated nuclei from each time point were digested for 60 min at 0 °C with MNase. Chromatin DNA was subjected to electrophoresis, stained with ethidium bromide (A), and analyzed by fluorography (B). (Lanes a and h) Control chromatin DNA labeled for one generation with thymidine; (lanes b–g) chromatin DNA labeled with thymidine for 0.5, 5, 10, 20, 30, and 60 min, respectively. The positions of bulk monomer (M), dimer (D), and trimer (T) DNA are indicated. The *arrowhead* indicates the position of nascent *dimer* DNA. (C) Nucleosomal repeat length was obtained by linear regression analysis (as in Figure 1) and plotted as a function of label incorporation time.

proteins, we conclude that the most likely explanation is that the internucleosomal linker has been temporarily reduced, and possibly eliminated.

Maturation of Nucleosomal Repeat Length with and without Concurrent Protein Synthesis. To determine the time required for the mature (i.e., bulk) repeat length to be established after DNA synthesis, cells were labeled for increasing periods (0.5–60 min) with [^{3}H]thymidine, and nuclei from each period were digested to the same extent with MNase (Figure 3A). By comparing the electrophoretic mobility of new nucleosomal DNA to that of uniformly labeled bulk chromatin (Figure 3B), and by sizing the DNA (Figure 3C), it was determined that within 10 min of DNA synthesis newly replicated chromatin attained the repeat length characteristic of bulk chromatin.

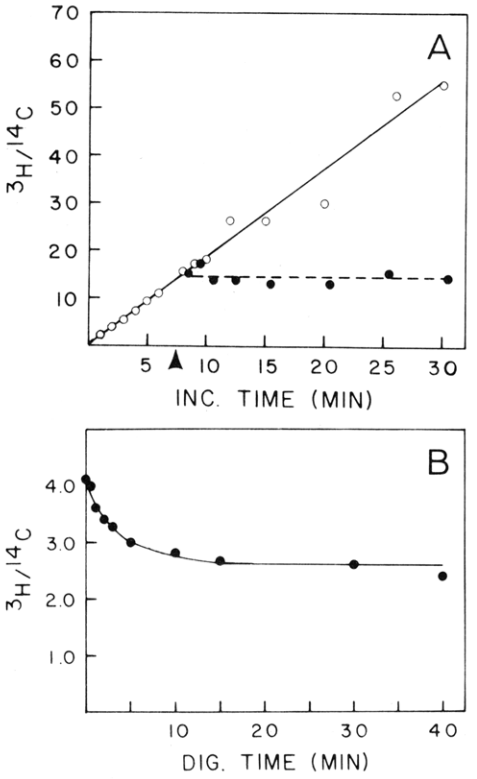

FIGURE 4: Effects of cycloheximide on protein synthesis and nuclease sensitivity of newly replicated chromatin. (A) Cells were prelabeled for one generation with [^{14}C]thymidine and then incubated for the times indicated with [^{3}H]lysine. After 7.5 min, the cells were divided into two portions, and cycloheximide (200 μg/mL) was added to one (*arrowhead*). At each point, an aliquot of cells was removed, washed, and precipitated with trichloroacetic acid. Protein synthesis in the absence (○) and the presence (●) of cycloheximide is expressed as ^{3}H/^{14}C. (B) Cells were prelabeled for one generation with [^{14}C]-thymidine and then incubated for 50 min with [^{3}H]thymidine in the presence of cycloheximide (200 μg/mL). Nuclei (46 A_{260}/mL) were digested at 37 °C with 1.2 units/mL MNase for the times indicated and acid precipitated. The relative nuclease sensitivity of newly replicated chromatin with respect to bulk chromatin is expressed as ^{3}H/^{14}C.

The maturation of chromatin replicated in the presence of cycloheximide was then examined. It has been established by a number of investigators that DNA replicated without concurrent protein synthesis retains many of its immature features. These include a greater sensitivity to nuclease digestion relative to bulk chromatin (Weintraub, 1973, 1976; Seale & Simpson, 1975; Seale, 1976, 1978a; Schlaeger & Klempnauer, 1978; Roufa, 1978) and the accumulation of unassembled, nonnucleosomal "chromatin", arising from the segregation of parental histone octamers (Seale, 1976, 1978a; Weintraub, 1976; Seidman et al., 1979; Riley & Weintraub, 1979). It was therefore of interest to determine if the repeat length of newly replicated chromatin could mature when protein synthesis was blocked.

Cells were labeled with [^{3}H]thymidine for increasing periods (5–45 min) in the presence of 200 μg/mL cycloheximide. Control experiments showed that at this concentration cycloheximide completely and immediately inhibited protein

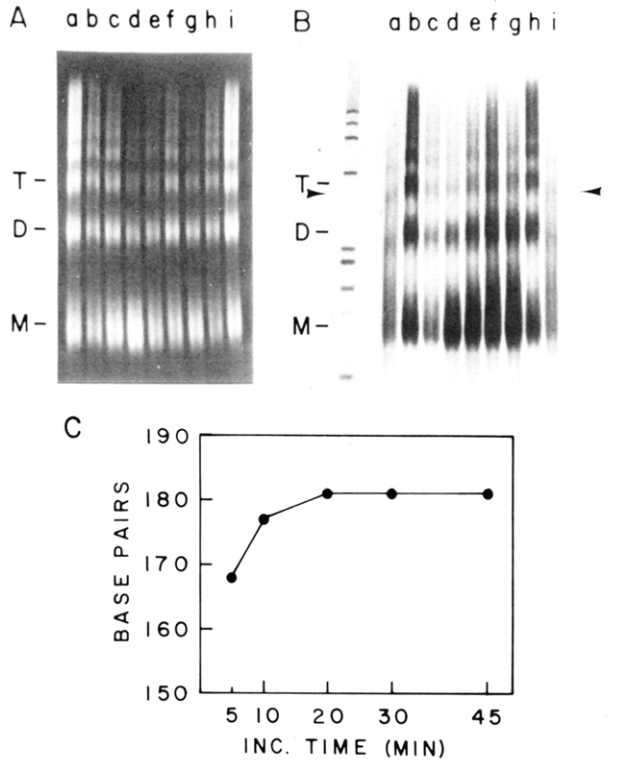

FIGURE 5: Maturation of nascent nucleosomal repeat length in the absence of protein synthesis. Chromatin was labeled with [^{3}H]thymidine for increasing periods (5–45 min) in the presence of cycloheximide, and isolated nuclei from each time point were digested for 60 min at 0 °C with MNase. Chromatin DNA was subjected to electrophoresis, stained with ethidium bromide (A), and analyzed by fluorography (B). (Lanes a and i) Control nascent chromatin DNA labeled for 30 s with thymidine in the absence of cycloheximide; (lanes b and h) control bulk chromatin DNA labeled for one generation with thymidine in the absence of cycloheximide; (lanes c–g) chromatin DNA labeled with thymidine in the presence of cycloheximide for 5, 10, 20, 30, and 45 min, respectively. The positions of bulk monomer (M), dimer (D), and trimer (T) DNA are indicated. Marker fragments are as in Figure 1A. The *arrowhead* indicates the position of nascent *trimer* DNA. (C) Nucleosomal repeat length was obtained by linear regression analysis and plotted as a function of label incorporation time.

synthesis in HeLa cells (Figure 4A), and that, in agreement with other reports (Weintraub, 1973, 1976; Seale & Simpson, 1975), DNA synthesized under these conditions was approximately twice as sensitive to DNase digestion than was bulk chromatin (Figure 4B). Analysis of nucleosomal repeat length as a function of labeling time (Figure 5) revealed that, in the absence of concurrent protein synthesis, histone octamers (presumably of parental origin) are initially spaced on new DNA with a repeat length of approximately 165 bp, but with continued replication, the mature nucleosomal repeat is reestablished.

The observation that ch-chromatin is able to regain the repeat length typical of bulk chromatin, while remaining immature in other respects (as discussed above; see also Figure 4B), raises questions concerning the composition of parentally derived nucleosomes on new DNA. Since the protein composition of nucleosomes has a marked effect on their electrophoretic mobility (Todd & Garrard, 1977; Bakayev et al., 1977, 1978; Albright et al., 1980; Hutcheon et al., 1980; Sandeen et al., 1980; Mardian et al., 1980; Albanese & Weintraub, 1980; Annunziato et al., 1981; Egan & Levy-Wilson, 1981), mononucleosomes that had replicated in the presence or absence of cycloheximide were subjected to electrophoresis and compared.

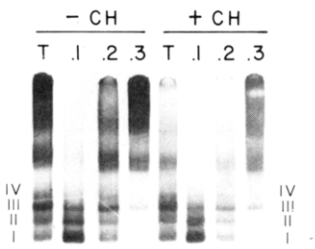

FIGURE 6: Nucleosomal heterogeneity is preserved during replication in the presence of cycloheximide. Cells were labeled with [^{3}H]thymidine for 10 min in the absence (–CH) or for 20 min in the presence (+CH) of cycloheximide, and chromatin was fractionated after MNase digestion by stepwise salt elution (see Experimental Procedures). Chromatin fractions were subjected to electrophoresis in an agarose–polyacrylamide (DNP) gel, and the gel was analyzed by fluorography. Lane designations .1–.3 correspond to the molarity of NaCl used for elution. (Lane T) Total 2 mM EDTA-soluble MNase digest. The positions of mononucleosomes I, II, III, and IV are indicated. HMG protein containing mononucleosomes are eluted with 0.1 M NaCl; H1-containing monomers are extracted in 0.3 M NaCl (Annunziato et al., 1981).

HeLa cells were labeled for 10 min in the absence or for 20 min in the presence of cycloheximide. Nuclei were then digested with MNase, and soluble chromatin was isolated by two different methods (Experimental Procedures). In the first method, nuclei were lysed with EDTA after nuclease digestion, and soluble chromatin (lane T, Figure 6) was collected after insoluble material (denoted P_t) was removed by centrifugation. In the second method, chromatin was fractionated by maintaining the digested nuclei in the presence of magnesium ions and incrementally raising the ionic strength of the medium with NaCl (Sanders, 1978). At each step in the fractionation, chromatin of progressively higher molecular weight is solubilized (Figure 6). Furthermore, as we have previously demonstrated (Annunziato et al., 1981), different species of mononucleosomes are released at each salt concentration. The core mononucleosome and HMG protein containing monomers (containing no H1 histone) are present in the 0.1 M NaCl eluate. H1-containing monomers (lacking HMG 14 and 17) are found in the 0.3 M NaCl fraction; all species are eluted with 0.2 M NaCl, or with EDTA [Figure 6; for details, see Annunziato et al. (1981)].

A comparison of the mononucleosomes synthesized in the presence or absence of cycloheximide revealed no differences in electrophoretic mobility or distribution (Figure 6); H1-containing and HMG protein containing monomers were represented in both cases. Thus, not only are the core histones transferred to new DNA in the absence of protein synthesis (Seale, 1976, 1978a; Weintraub, 1976; Seidman et al., 1979) but also histone H1 and HMG proteins as well.

Identification of a Fraction of Newly Synthesized DNA Which Does Not Mature in the Absence of Protein Synthesis. According to current concepts of histone segregation and nucleosome assembly, approximately half of the new DNA synthesized in the presence of cycloheximide should remain unassembled (i.e., nonnucleosomal) even after prolonged labeling periods [see Seale (1978b), Laskey & Earnshaw (1980), and DePamphilis & Wassarman (1980) for reviews]. From the data of Figure 6, it was clear that most ot the new DNA which eluted in 0.1–0.3 M NaCl was in nucleosomes, for both control and cycloheximide-treated samples. In previous work, it had been shown that nonnucleosomal nascent DNA (labeled for 30–60 s in *normally* replicating cells) was preferentially isolated at NaCl concentrations above 0.3 M and that this relatively insoluble material comprised approximately 40% of

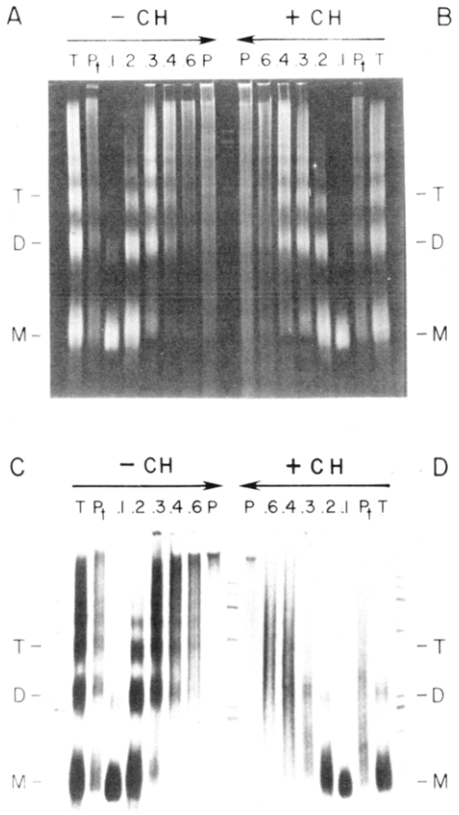

FIGURE 7: Nonnucleosomal DNA does not mature in the presence of cycloheximide. Cells were labeled with [^{3}H]thymidine for 10 min in the absence (−CH; panels A and C) or for 20 min in the presence (+CH, panels B and D) of cycloheximide, and chromatin was fractionated by stepwise salt elution as in Figure 6. Chromatin DNA was subjected to electrophoresis, stained with ethidium bromide (A and B), and analyzed by fluorography (C and D). Lane designations .1–.6 correspond to the molarity of NaCl used for elution. Lane P contains the DNA remaining in the residual nuclear pellet after salt elution. (Lane T) Total 2 mM EDTA-soluble nuclease digest; (lane P_t) 2 mM EDTA-insoluble chromatin DNA (see Experimental Procedures). The positions of monomer (M), dimer (D), and trimer (T) DNA are indicated. Marker fragments are as in Figure 1A. Note: chromatin DNA fractions replicated in the absence and the presence of cycloheximide have been presented in mirror image to facilitate comparison of the higher salt eluates.

the nascent DNA (Annunziato et al., 1981). Therefore, the chromatin fractionation procedure was extended to include elutions with 0.4 and 0.6 M NaCl.

Chromatin was labeled with [^{3}H]thymidine for 10 min in the absence or for 20 min in the presence of cycloheximide, and the old and new DNA in each salt eluate was examined by gel electrophoresis, ethidium bromide staining, and fluorography. The ethidium bromide stain demonstrated that the bulk chromatin DNA showed typical nucleosomal periodicity in both control and ch-chromatin (Figure 7A,B). However, an examination of the position of new DNA in the gel fluorograph (Figure 7C,D) revealed major differences between chromatin DNA from control and cycloheximide-treated cells. After 10 min of replication, all new DNA from control cells showed nucleosomal structure. In contrast, new DNA labeled for 20 min in the presence of cycloheximide showed dual characteristics. Whereas new DNA in fraction T and the 0.1 and 0.2 M NaCl eluates exhibited nucleosomal periodicity, new DNA in fraction P_t and in the 0.4 and 0.6 M NaCl eluates clearly lacked subunit organization, and instead appeared as a smear (Figure 7D). New chromatin DNA extracted with 0.3 M NaCl showed some nucleosomal periodicity, as well as a substantial background smear between the monomer and dimer (Figure 7D, lane 3). The nonnucleosomal component of newly replicated ch-DNA is therefore similar to that labeled for 30–60 s in normal cells (Annunziato et al., 1981).

The lack of nucleosomal structure in the high salt eluates and fraction P_t was not time dependent: increasing the replication period in cycloheximide to 50 min yielded results identical with those shown in Figure 7D.

It also should be noted that the nonnucleosomal DNA of ch-chromatin is of a higher average molecular weight than the *nucleosomal* component of new DNA synthesized under identical conditions (Figure 7D). Thus, the nonnucleosomal material has a greater nuclease resistance than nascent internucleosomal linkers, indicating that this fraction exists as a DNA–protein complex in the nucleus, not as naked DNA. However, it is also clear that the nonnucleosomal fraction is more susceptible to nuclease digestion than mature chromatin (compared lanes P_t and 0.3–0.6 of Figure 7C,D).

Discussion

Newly Replicated Chromatin Has a Shortened Repeat Length. At all stages of MNase digestion, newly replicated HeLa cell nucleosomes exhibit a repeat length shorter than that of bulk chromatin. The shortened repeat is in agreement with the reports of others (Levy & Jakob, 1978; Murphy et al., 1978, 1980; Seale, 1978a, 1981; Galili et al., 1981) and is consistent with correlations between reduced nucleosomal repeat lengths and increased nuclear activity (Compton et al., 1976; Spadafora et al., 1976; Lohr et al., 1977; Thomas & Thompson, 1977; Weintraub, 1978; Savic et al., 1981; Berkowitz & Riggs, 1981). Since the shortened repeat of newly replicated chromatin is not accompanied by changes in mononucleosomal electrophoretic mobility (Annunziato et al., 1981) or by alterations in the size of the mononucleosome core, the simplest explanation for the decrease is that the internucleosomal linker has been reduced.

Although Jackson et al. (1981) observed that at extended times of nuclease digestion nascent chromatin displayed a shortened repeat, they also reported that with more limited cleavage the repeat lengths of newly replicated and bulk chromatin were similar. It was concluded that nuclease digestion at 37 °C induced histone rearrangement (i.e., sliding) on new DNA, thus changing the otherwise normal spacing to a short repeat. We do not observe this. At all stages of digestion at which nascent DNA multimers could be electrophoretically resolved (with particular emphasis on the earliest digestion times), newly replicated chromatin possessed a repeat length significantly shorter than that of the corresponding bulk chromatin.

It is unlikely that the shortened repeat is due to histone redistribution. In isotonic buffers at 0 °C (conditions used in this study), core histone migration is greatly reduced (Germond et al., 1976; Beard, 1978; Weischet, 1979; Spadafora et al., 1979). For example, in analyses of H1-depleted chromatin, which yields a fraction of "compact dimers" after exhaustive cleavage with MNase (presumably due to histone migration), nucleosome sliding did not occur unless 0.35 M NaCl was present throughout the course of the digestion (Weischet & Van Holde, 1980). Exposure of H1-depleted chromatin to ionic strengths up to 600 mM NaCl caused no significant histone sliding for up to 20 h at 4 °C (Spadafora et al., 1979; Weischet, 1979). Furthermore, while H1 depletion was necessary for the generation of compact dinucleosomes in bulk chromatin (Weischet et al., 1979), we have recently shown that histone H1 is present on nascent nucleosomes when the shortened repeat is observed (Annunziato et

al., 1981). Also, whereas the compact dinucleosomes that arise during the digestion of H1-depleted chromatin contain 240–260 bp of DNA (Klevan & Crothers, 1977; Tatchell & Van Holde, 1978), nascent oligomers produced under the conditions of this report reach a plateau of ~320 bp (2 × 160). This is significantly different from the oligomer values which have been interpreted to demonstrate histone sliding and more likely reflects the close spacing of H1-containing and HMG protein containing particles.

The aforementioned observations are subject to certain reservations. The nucleosomal migration studies, cited above, were performed in vitro on reconstituted nucleosomes, and on native and salt-stripped particles. Due to the structural modifications of nascent chromatin (see the introduction), the properties of bulk chromatin may not be applicable in all respects. If new chromatin does possess unique properties that permit histone sliding at physiological ionic strength at 0 °C, then sliding must be extensive in order to produce oligomers as large as heptamers with little or no spacer. If lability of nucleosomal position proves to be the case, rather than an intrinsically shorter repeat length, this property remains unique to nascent nucleosomes for a transient interval (~10 min), after which it is no longer observed. The *observation* of this effect, then, was used as a criterion for further studies on newly replicated chromatin.

Maturation of Newly Replicated Chromatin. Within 10 min of DNA synthesis under normal conditions, the mature nucleosomal repeat is established. When chromatin is replicated in the presence of cycloheximide, nucleosomes initially display the shortened repeat and subsequently gain the mature spacing with continued DNA replication; thus, maturation is independent of concomitant protein synthesis. As in the case of normal replication, all the mononucleosomal species observed in bulk chromatin are represented on DNA replicated in cycloheximide, indicating that HMG proteins and histone H1 are present on ch-chromatin.

Since H1 has been implicated in maintaining chromatin in the condensed state (Varshavsky et al., 1977; Renz et al., 1977; Spadafora et al., 1979; Strätling, 1979; Thoma et al., 1979; Allan et al., 1981), it is possible that changes in the higher order structure during replication, mediated by H1, could modulate nucleosomal periodicity (or vice versa). If the higher order structure of chromatin is lost or altered during replication, as proposed by others (Levy & Jakob, 1978; Worcel et al., 1978), our data suggest that this change is accompanied by a reduced internucleosomal linker. The shift to the mature repeat, then, might be a consequence of (or a prerequisite for) chromatin condensation after DNA synthesis.

The maturation of the nucleosomal repeat of ch-chromatin is in marked contrast to the inability of the nonnucleosomal component of new ch-DNA to acquire subunit organization under identical conditions. The nonnucleosomal component comprises ~40% of new DNA in both pulse labeled (Annunziato et al., 1981) and ch-chromatin; however, unlike the situation in control cells, the nonnucleosomal component of ch-chromatin does not assume typical nucleosomal structure with continued DNA synthesis. Several lines of evidence strongly suggest that cycloheximide prevents the maturation of this fraction through its effect on protein synthesis, and not by inhibiting DNA replication. Although the rate of thymidine incorporation is 20–50% that of untreated cells, replication proceeds linearly from preexisting forks for approximately 1 h in cycloheximide, until replicon-sized DNA is produced (Weintraub & Holtzer, 1972; Seale & Simpson, 1975). In labeling periods in cycloheximide of 50 min (i.e., sufficient time to synthesize the same amount of DNA required for the maturation of control chromatin), the acquisition of subunit structure remained suppressed.

In agreement with previous reports, we find that ch-chromatin is more sensitive to DNase digestion than bulk chromatin (Weintraub, 1973, 1976; Seale & Simpson, 1975; Seale, 1976; Seidman et al., 1979). It has been proposed that part of this increased sensitivity reflects the structure of unassembled (i.e., nonnucleosomal) ch-DNA. Electrophoretic analyses of ch-DNA demonstrate that the nonnucleosomal component possesses an intermediate nuclease sensitivity between that of nascent internucleosomal linkers and mature chromatin. This not only confirms earlier predictions of increased nuclease sensitivity (relative to bulk chromatin) but also demonstrates greater nuclease resistance relative to free DNA [see Seale (1976)], suggesting that the nonnucleosomal component is complexed with protein. Similar conclusions have been reached by buoyant density analyses of chromatin replicated normally, as well as in cycloheximide, in which it was shown that nascent chromatin had a lighter density in CsCl than control chromatin (Fakan et al., 1972; Seale & Simpson, 1975; Jackson & Chalkley, 1981). The relative nuclease resistance (and apparent protein association) of the nonnucleosomal component of somatic cells in contrast to the report that nonnucleosomal DNA in replicating SV40 is protein free. The combined nuclease sensitivities of the nascent linkers and the nonnucleosomal component (both of which are more sensitive than bulk chromatin) may account for the increased overall nuclease sensitivity of ch-chromatin DNA (Weintraub, 1973, 1976; Seale & Simpson, 1975; Hildebrand & Walters, 1976; Seale, 1976, 1981; Levy & Jakob, 1978; Worcel et al., 1978; Schlaeger & Klempnauer, 1978; Seidman et al., 1979; Klempnauer et al., 1980; Annunziato et al., 1981; Jackson & Chalkley, 1981).

In summary, the use of the inhibitor cycloheximide has enabled us to distinguish two classes of newly replicated chromatin that differ greatly in structure and dependence on protein synthesis for maturation. One class is nucleosomal, possesses histone H1 and HMG proteins, and, like normal nascent chromatin, exhibits a shortened repeat length that matures to the bulk value in 10–20 min. The other class lacks typical nucleosomal structure, requires protein synthesis for maturation, and probably represents, at least in part, that portion of nascent DNA that is the site of de novo nucleosome assembly.

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