Dual Nature of Newly Replicated Chromatin

EVIDENCE FOR NUCLEOSOMAL AND NON-NUCLEOSOMAL DNA AT THE SITE OF NATIVE REPLICATION FORKS

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When chromatin is extracted from nuclease-digested nuclei by stepwise salt elution, two different classes of newly replicated chromatin can be distinguished. Nascent DNA eluted from nuclei under conditions of low to moderate ionic strength (0.1-0.3 M NaCl) exhibits nucleosomal periodicity and is found in particles which have the same electrophoretic mobility as bona fide H1-or high mobility group protein-containing mononucleosomes. Thus, factors believed to be involved with both the higher order coiling and transcriptionally active state of chromatin are rapidly complexed with newly synthesized DNA and may be retained on parental nucleosomes throughout replication.

In contrast, approximately 40% of new DNA is resistant to extraction with solutions of moderate ionic strength. Most of this material is eluted from nuclei by 0.4-0.8 M NaCl. While bulk chromatin that is extracted by 0.4-0.8 M NaCl is organized into nucleosomes, most of the newly replicated "chromatin" from the same fractions lacks subunit structure, as determined by DNA size analyses in polyacrylamide gels, whereby distinguishing this nascent material from newly replicated chromatin eluted at lower ionic strength. Within 15 min all newly synthesized chromatin matures and exhibits the solubility and nucleosomal periodicity characteristics of bulk chromatin. The unusual properties of the "nonnucleosomal" fractions may reflect the structure of newly synthesized DNA prior to its assembly into nucleosomes.

Much of our knowledge concerning the nature of newly replicated chromatin is derived from studies using various DNAases as probes of chromatin structure. Such experiments have demonstrated that, within 30 s of DNA synthesis, a major fraction of nascent DNA can be found in nuclease-resistant particles which co-sediment in sucrose gradients with mature nucleosomes and which contain approximately 165 base pairs of DNA (reviewed in Refs. 1 and 2). Furthermore, analyses of replicating chromatin by electron microscopy (3-5) have shown nucleosome-like structures within 300 base pairs of the replication fork. These data suggest that histone-DNA interactions similar to those found in mature chromatin are quickly re-established after, or persist throughout, DNA replication. However, it is also quite clear that newly replicated chromatin differs from mature chromatin in several important respects, as evidenced by the greater extent of conversion of new DNA to acid solubility by nuclease (6,7), and the faster rate at which nascent chromatin is cleaved to mononucleosomes, relative to bulk chromatin (7-11).

Although nuclease digestion studies indicate that a significant fraction of new DNA exhibits nucleosomal periodicity, and is thus rapidly complexed with the core histones (H2A, H2B, H3, and H4), there exists a gap in our knowledge concerning the association of histone H1 and various non-histone chromosomal proteins with newly replicated DNA. The matter is complicated by the presence in interphase chromatin of both transcriptionally active and inactive regions, which must be faithfully replicated or reinitiated to ensure normal cell functions. Of the non-histone proteins, the high mobility group proteins, first identified by Johns and coworkers (12,13), and in particular HMG (14-17) (or equivalent proteins), have been implicated in maintaining the transcriptionally competent state of chromatin (14-19). There now exist several methods for isolating nucleosomes enriched in HMG 14 and 17, based on their differential solubility properties (19-23). We have combined one of these methods, the technique developed by Sanders (20), with high resolution deoxyribonucleoprotein gels (24) in order to examine the composition of nucleosomes immediately after DNA replication. Our results indicate that, within 30 s of DNA synthesis, HMG 14 and 17, and histone H1, are present in the subsets of monomeric nucleosomes found in mature chromatin.

The efficiency of the Sanders procedure in eluting new and old chromatin DNA from nuclei has also been examined. Approximately 40% of DNA labeled for 1 min was refractory to release with solutions of moderate ionic strength. The salt-resistant fraction, much of which could be extracted from nuclei with increased concentrations of NaCl, lacked typical nucleosomal structure. These features of nascent chromatin were transitory; DNA labeled for 15 min possessed the nucleosomal organization and solubility properties of bulk chromatin. The observation of two distinct classes of newly replicated chromatin has important implications with regard to the processes of chromatin maturation after DNA synthesis.

MATERIALS AND METHODS

Cell Culture and Labeling—HeLa cells, the gift of Dr. Andrew Wiseman (Scripps Clinic and Research Foundation, La Jolla, California), were maintained in spinner culture at 37 °C in Eagles minimal essential medium supplemented with 5% calf serum. Long term labeling of cells with [3H]thymidine (50 mCi/mmol, New England Nuclear) was performed at 0.01 μCi/ml of cell culture for one generation (24 h). Cells were prelabeled with [3H]lysine (60 Ci/mmol, New England Nuclear) and [3H]arginine (30 Ci/mmol, New England Nuclear). The abbreviation used is: HMG, high mobility group.
Running buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, graphed, impregnated with scintillant, and exposed to Kodak I11 digest of GX174 DNA) were purchased from New England Nuclear.

0.5% agarose, 2.5-3.5% polyacrylamide (DNP) gels, using a modification yielding a total soluble chromatin fraction (denoted T). A second aliquot of nuclei was removed after digestion, pelleted, and lysed by a modification of residual nuclear material (12,000 g for 10 min) was designated T.2.

Nuclear Isolation, Digestion with Micrococcal Nuclease, and Chromatin Fractionation—Cells were washed twice in CB buffer, resuspended in CB buffer containing 0.5 mM phenylmethylsulfonyl fluoride, and lysed with 6-10 strokes in a dounce homogenizer. Nuclei were collected by centrifugation at 1,000 x g (average) for 1 min, washed once in CB buffer plus phenylmethylsulfonyl fluoride and washed twice in buffer alone. Nuclei were then resuspended in CB buffer at a concentration of 46 A260/ml (A260 measured in 0.3 M NaOH) and digested as described (20). For analysis of replicating chromatin, nuclei were incubated for 2 min at 37 °C with 1.2 units of micrococcal nuclease (Sigma) per mg DNA. When nucleosomal proteins were to be analyzed, nuclei were digested with 0.7 units of micrococcal nuclease/ml. The reaction was terminated by the addition of 100 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EDTA, pH 7.6), to a final concentration of 1 mM, and cooling to 4 °C.

Following nuclease digestion, the nuclei were gently pelleted (1,400 x g for 10 min) to yield a supernatant (termed S0) containing non-histone proteins and acid-soluble nucleotides. Chromatin was then fractionated according to the method of Sanders (20) by resuspending the nuclear pellet, sequentially, in CB buffer containing 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, plus 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M NaCl. Nuclei were incubated in buffer for 20 min at 4 °C; at each step nuclei were pelleted and the supernatant fractions were collected and denoted S1, S2, S3, S4, and S6, respectively. All buffers contained 0.5 mM phenylmethylsulfonyl fluoride. Repeating the extraction after each step yielded no further release of 260-nm absorbing material.

At any stage in the fractionation procedure the nuclei can be lysed with EDTA to release the remaining chromatin. As a control, an aliquot of nuclei was removed after digestion, pelleted, and lysed by resuspension in 2 mM EDTA, pH 7.2. Insoluble chromatin (denoted F1) was then removed by centrifugation (12,000 x g for 10 min), yielding a total soluble chromatin fraction (denoted T). A second aliquot of nuclei was sequestered after the 0.2 M NaCl elution step and was then collected by centrifugation at 1,000 x g for 10 min) was designated T.2.

Gel Electrophoresis and Protein Isolation—The chromatin fractions were dialyzed against 2 mM EDTA, and concentrated in the B-15 “minicon” concentrator (Amicon). The T and T.2 fractions were concentrated without dialysis. For analysis of nucleoprotein particles, chromatin fractions were subjected to electrophoresis in a composite 0.5% agarose, 2.5-3.5% polyacrylamide (DNP) gels, using a modification of the procedure of Todd and Garrard (24). Gels were cross-linked with N,N'-diallyltartartradiamid at an acrylamide:N,N'-diallyltartartradiamid ratio of 20:1. Slab gels were cast and run in 6.4 mM Tris, 3.2 mM sodium acetate, 0.28 mM EDTA, pH 9.3 or 8.0, with no detectable difference at either pH in the migration of newly replicated or bulk chromatin. Electrophoresis was at 110 V for 4 h at 4 °C, with buffer recirculation.

For DNA size analysis, the dialyzed fractions were brought to 10 mM magnesium acetate, ethanol-precipitated, and resuspended in running buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, adjusted to pH 7.2 with glacial acetic acid) made 5% in glycerol and 1% in SDS. Polyacrylamide slab gels (3.5%) were prepared according to Loenig (25), using an acrylamide: bisacrylamide ratio of 21:1. Both the gel and the running buffer contained 0.1% SDS (Bio-Rad). Electrophoresis was at 90 V for 3 h. Labeled restriction fragments (Hae III digest of E174 DNA) were purchased from New England Nuclear.

DNP and DNA gels were stained with ethidium bromide, photographed, and run with scintillant, and exposed to Kodak X-Omat R film. DNP gels were prepared for fluorography using ENHANCE (New England Nuclear); DNA gels were impregnated with 2,5-diphenyloxazole (26, 27).

Electrophoresis of proteins in SDS-polyacrylamide slab gels was according to Thomas and Kernberg (28). For protein analysis, ethidium-stained bands were excised from DNP gels, soaked in SDS-sample buffer for 1 h, and imbedded in 1% agarose (in stacking gel buffer), above the stacking gel. Following electrophoresis, gels were fixed for at least 1 h in 4% ethanol, 5% acetic acid, prior to preparation for fluorography (26, 27).

HMG 14 and 17 were isolated according to the procedure of Weisbrod and Weintraub (14). Nuclei were extracted with 0.35 mM NaCl, and soluble proteins were precipitated successively with 2, 10, and 25% trichloroacetic acid. The 25% trichloroacetic acid precipitate was washed with acetone and separated in polyacrylamide gels in the presence of SDS (Fig. 1).

Radioactivity Determinations—Aliquots of chromatin fractions were precipitated with 10% trichloroacetic acid together with 50 μg each of DNA and albumin, collected on GF/C glass fiber filters (Whatman), and washed twice with trichloroacetic acid and twice with 95% ethanol. Dried filters were incubated with 0.2 ml of NCS (Amer sham) containing 5% H2O and counted in 4 g/l of 2,5-diphenyloxazole/toluene fluor. The fraction of 3H counts registered in the 3H channel was determined by the external standard technique.

RESULTS

Analysis of Nucleosomes Eluted by the Sanders Procedure—Following extraction of micrococcal nuclease digested nuclei with increasing concentrations of NaCl (see “Materials and Methods”), chromatin fractions were subjected to electrophoresis in DNP gels (Fig. 2). In agreement with the data of Sanders (20), the average molecular weight of eluted chromatin increased with the salt concentration. Mononucleosomes were resolved into four distinct components, termed MI, MII, MIII, and MIV in order of decreasing mobility, following the nomenclature of Garrard et al. (24, 29). The various mononucleosomal species have been described by a number of investigators and correlated with differences in both DNA size and protein composition, most notably in content of histone H1 and HMG proteins 14 and 17 (29-32).

In order to verify the protein composition of each electrophoretic species of mononucleosome, cells were labeled for 24 h with [3H]arginine and lysine, and chromatin was fractionated as in Fig. 2. Individual monomer bands were excised and the nucleosomal proteins analyzed in second dimension gels in the presence of SDS (Fig. 3). Only the nucleosomal species pertinent to the study of chromatin replication will be discussed.
were digested with micrococcal nuclease (6.7 units/ml), and chromatin nucleosomal monomers mobility but partition according to salt solubility. The more

As previously noted (29), MIV contains both histone H1 and HMG proteins, suggesting independent binding sites for these proteins on the nucleosome.

In summary, the protein composition of HeLa cell nucleosomes is consistent with previous descriptions of nucleosomal heterogeneity (24, 29-34). Furthermore, in agreement with the data of Sanders (20), the salt elution technique has made it possible to separate HMG-rich nucleosomes (MII and MIIIB in 0.1 M NaCl) from H1-containing nucleosomes (MIIA in 0.3 M NaCl).

Nucleosomal Heterogeneity Following DNA Replication—In order to study nucleosomal composition immediately following DNA replication, HeLa cells were labeled for 30 s in vivo with [3H]thymidine. Nuclei were then isolated and digested, and chromatin was fractionated (see “Materials and Methods”). Fig. 4A shows an ethidium bromide stain of nucleosomes released as the salt concentration was raised sequentially from zero to 0.6 M NaCl. When nuclei were lysed with EDTA after the 0.2 M NaCl elution step (i.e. after most of the monomers had been extracted) the soluble chromatin fraction consisted mainly of high molecular weight chromatin and low amounts of residual monomer (Fig. 4A, lane T.2). Fraction T.2, containing MIIA, is comparable to chromatin eluted with 0.3 M NaCl (Fig. 4A, lane T.3) but has not been exposed to salt concentrations above 0.2 M NaCl.

A fluorograph of the gel (Fig. 4B) shows the distribution of newly replicated chromatin DNA. Within 30 s of DNA synthesis, all the mononucleosomal species contain label. This suggests a rapid, or continuous, association between a major fraction of new chromatin and histone H1 or HMG proteins. As described for a number of systems (7-11), newly replicated chromatin is more rapidly digested than bulk chromatin and, therefore, appears predominantly in the low molecular weight region of gel (Fig. 4B). The fragments that exhibit the greatest separation of new chromatin (in mono- and dinucleosomes) from the high molecular weight bulk chromatin are the T fraction, the 0.3 M NaCl eluate, and the T.2 fraction (Fig. 4).

Assuming a eukaryotic fork migration rate of 0.5-1.0 μm/min (38), sufficient DNA is manufactured in a 30-s period to form 4–8 nucleosomes on either side of the fork. To verify the nascent DNA that was found in the position of HMG-containing nucleosomes was in the immediate vicinity of the replication fork, cells were labeled for 15 min with [3H]thy-
FIG. 4. HMG- and H1-containing nucleosomes are associated with newly replicated DNA. HeLa cells were labeled for 30 s with \[^{3}H\]thymidine, and chromatin was prepared and fractionated as described under "Materials and Methods." Chromatin fractions were subjected to electrophoresis, stained with ethidium bromide, and prepared for fluorography. Lane designations correspond to the NaCl molarity used for elution. Lanes T and T.2 contain chromatin released from nuclei with 2 mM EDTA without salt exposure (T), or after elution with 0.2 M NaCl (T.2). A subnucleosome enriched for new DNA is indicated (s). Nucleosomes are labeled as in Fig. 2. Note: Samples were applied to the gel in amounts designed to yield the best resolution of bulk chromatin. Thus, these data do not reflect the absolute percentage of either new or bulk chromatin present in each fraction. A, ethidium bromide stain; B, fluorograph.

Fig. 5. Maturation of newly replicated chromatin. Cells were labeled for 15 min with \[^{3}H\]thymidine, and chromatin was fractionated and subjected to electrophoresis as in Fig. 4. Lanes and nucleosomes are labeled as in Fig. 4. A, ethidium bromide stain; B, fluorograph.

midine to allow sufficient time for mature chromatin to predominate over nascent chromatin. In agreement with the results of others (7, 8, 11), after 15 min of labeling newly replicated chromatin displayed a nuclease sensitivity equivalent to that of bulk chromatin: the DNA distribution in the ethidium bromide-stained DNP gel and in the fluorograph appeared identical (Fig. 5). Thus, it appears that HMG proteins and histone H1 are present on newly replicated nucleosomes (Fig. 4) well in advance of chromatin maturation.

A Subnucleosome Enriched for Newly Replicated DNA Contains HMG 17—One subnucleosomal fragment, barely detectable by ethidium bromide staining, is enriched in new DNA (Fig. 4B, lanes 1 and 2). After 15 min, this particle was no longer preferentially labeled (Fig. 5B). To determine the protein composition of this subnucleosome, HeLa cells were labeled for 24 h with \[^{3}H\]arginine and lysine. Following electrophoresis, the lane containing the 0.1 M NaCl eluate was excised, and proteins were analyzed by electrophoresis in a second dimension in the presence of SDS (Fig. 6). The fluorograph was over-exposed to reveal minor protein bands. Comparing the position of newly replicated chromatin (Fig. 6, top) with the pattern of labeled proteins (Fig. 6, bottom) revealed one major protein in the position of the nascent subnucleosome. The protein migrated just behind histone H3, with the same mobility as HMG 17. (Note: to facilitate the comparison of the second dimension SDS gel (Fig. 6, top) with the positions of newly replicated chromatin, the first dimension gel (Fig. 6, bottom) shows the distribution of newly replicated chromatin DNA, not labeled proteins.)
Apparently, HMG 17 alone can protect newly synthesized DNA from complete degradation by micrococcal nuclease. The DNA associated with the nascent subnucleosome is approximately 35 base pairs in length. Thus, this particle may be equivalent to subnucleosome(s) SN2 and/or SN3, identified in mouse cells, which contain approximately 35 base pairs of DNA complexed with HMG-G and HMG-E, respectively (15). HMG-G and HMG-E are the mouse non-histone chromatin proteins which are thought to correspond to HMG 17 and HMG 14 (15).

Several high molecular weight proteins were detected in the second dimension gel in the region between the mononucleosomes and the nascent subnucleosome. These proteins evidently originate from subnucleosomal particles that were observed to stain faintly with ethidium bromide. As expected, fluorographs of DNP gels containing chromatin labeled in arginine and lysine display bands between the mononucleosomes and the nascent subnucleosome. However, these additional subnucleosomes are not enriched in newly replicated DNA (Figs. 4 and 6, top).

Elution of Newly Replicated Chromatin—The efficiency of extraction of new chromatin was examined in HeLa cells labeled 24 h with [14C]thymidine and then for 1 min with [3H]thymidine. Most of the [14C]-labeled bulk DNA was eluted with 0.3 M NaCl and little remained in the nucleus after 0.4 M NaCl elution (Fig. 7B). In contrast, a significant level of newly synthesized DNA was insoluble in 0.3 M NaCl and was released only at high salt concentrations, the residual nuclear pellet remaining enriched for newly replicated DNA. This effect is reflected in the "H/I4C ratios of each fraction (Fig. 7A). In separate experiments we have observed a 5- to 10-fold relative enrichment for newly synthesized DNA in the 0.6 M NaCl eluate (and residual pellet), as compared to the 0.3 M NaCl fraction. After 10 min of continuous replication, newly replicated chromatin was no longer resistant to release at moderate ionic strength, and the "H/I4C ratios were more nearly equivalent for each stage in the fractionation (Fig. 7A). When undigested nuclei were treated with salt solutions at concentrations to 2.0 M, no preferential release of either new or bulk chromatin was observed.

Structure of Newly Replicated Chromatin Released by Salt Elution—To examine the size of replicated DNA in each salt eluate, nuclei from cells labeled for 30 s with [3H]thymidine were digested and fractionated; the fractions were subjected directly to electrophoresis in SDS-polyacrylamide gels (24, 39) in order to avoid the loss of nascent DNA fragments during phenol extraction (40). Bulk DNA exhibited nucleosomal organization at each stage in the fractionation (Fig. 8A). A strikingly different pattern emerged when the position of newly replicated DNA was analyzed in the fluorograph of the same gel (Fig. 8B). In agreement with our earlier observations (Fig. 4, B), nascent DNA was digested to smaller fragments relative to bulk chromatin DNA. In addition, new DNA from higher salt eluates apparently lacks typical nucleosomal structure. This appears, in the fluorograph, as a smear of lower molecular weight than the ethidium stain in the same lane (see lanes A and B of Fig. 8). Newly synthesized DNA released in 0.3 M NaCl has dual characteristics, showing both subnucleosome organization plus a background smear between the

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**Fig. 7.** Salt elution profile of newly replicated and bulk chromatin. Cells were prelabeled for 24 h with [14C]thymidine and then incubated for 1 min with [3H]thymidine. After fractionation, samples from each eluate (0-0.6) and the final pellet (P) were acid-precipitated and counted (B). Radioactivity is expressed as per cent of total ([14C]—o, [3H]—o). The [3H]/[14C] ratios for chromatin prelabeled with [14C]thymidine and incubated with [3H]thymidine for 1 min (A—o or 10 min (A—o) are also presented for each eluate (A). The ratios for chromatin labeled 10 min with [3H]thymidine were normalized so that the nuclear ratio before salt elution was equal to that of chromatin labeled for 1 min. Arrowhead denotes the [3H]/[14C] ratios for 1-min and 10-min labeled chromatin before salt elution.

**Fig. 8.** DNA composition of newly replicated chromatin. Cells were labeled for 30 s with [14C]thymidine and chromatin was fractionated ("Materials and Methods"). Electrophoresis for sizing of nucleosomal DNA in 3.9% SDS-polyacrylamide gels was performed as described under "Materials and Methods." Lanes O to P are labeled as in Figs. 4 and 7. Lane P, contains the EDTA-insoluble fraction ("Materials and Methods"). M, D, and T, positions of mono-, di-, and trinucleosomal DNA, respectively. DNA less than 70 base pairs in length has been run off the bottom of the gel. Labeled DNA marker fragments: Hae III digest of φX174 [14C]DNA. Fragments a-j are 1363, 1078, 872, 693, 310, 278/271 (doublet), 234, 194, 118, and 72 base pairs long, respectively. A, ethidium bromide stain; B, fluorograph.
were prepared and subjected to electrophoresis as in Fig. 8 for sizing of fraction exhibited a typical subunit structure over slight back-s.

HMG monomer and dimer. Similar results were obtained when the ground radioactivity (Fig. 8, lane Po). When chromatin was labeled for 15 min, both the extra sensitivity to micrococcal nuclease and the smearing background radioactivity of fraction P, and the high salt eluates were converted to patterns representative of mature chromatin (Fig. 9).

**DISCUSSION**

**Association of Newly Replicated Chromatin with H1 and HMG 14 and 17**—The salt elution technique of Sanders has permitted the separation of nucleosomes possessing HMG 14 or 17 from H1-containing nucleosomes. Identification of the different electrophoretic forms of nucleosomes was confirmed by direct examination of the protein composition. Within 30 s newly replicated DNA was observed not only in the position of HMG-rich nucleosomes (MII and MIIIIB) but also in nucleosomes containing histone H1 (MIIIA), suggesting either rapid, or continuous, association of a major fraction of newly replicated DNA (Fig. 8, lane P). When chromatin was labeled for 15 min, both the extra sensitivity to micrococcal nuclease and the smearing background radioactivity of fraction P, and the high salt eluates were converted to patterns representative of mature chromatin (Fig. 9).

Several lines of evidence indicate that HMG proteins are not artifactually binding to newly replicated DNA. While HMG 17 (or the HMG-like protein H6 from trout) will interact nonspecifically with nucleosomes when added in molar excess (23, 33, 41), at physiological concentrations these proteins are thought to bind specifically to transcriptionally active (or potentially active) regions of chromatin (14-19, 41). This specificity is maintained in isolated nucleosomes (16). When HMG rearrangement was examined under conditions similar to those employed in this report, endogenous HMG 14 or 17 did not undergo significant exchange (29).

Because HMG 14 and 17 are thought to be instrumental in maintaining the transcriptionally active state of chromatin (14-19), the presence of HMG proteins in the vicinity of the replication fork suggests that at least some signals for the potentially active conformation are preserved throughout, or are re-established shortly after, DNA replication. This interpretation is in agreement with electron micrographs of nascent chromatin showing RNA transcripts in the wake of the replication fork in embryonic cells (3, 4, 42).

A subnucleosomal particle enriched in new DNA was apparently complexed with only one major protein, which co-migrated with HMG 17 in SDS-polyacrylamide gels. This particle may correspond to subnucleosome(s) SN2 and/or SN3 from mouse chromatin (15). DNA isolated from SN2 and SN3 is enriched in sequences complementary to nuclear RNA (15), supporting the theory that HMG 14 and 17 are concentrated in transcriptionally active regions of chromatin. However, if the sole source of the HeLa subnucleosome is transcriptionally active chromatin, the absence of newly replicated DNA from this particle after 15 min becomes somewhat perplexing (Fig. 5) since all transcriptionally active chromatin replicated during the 15-min pulse should remain labeled. Thus, the rapid decrease in relative labeling of this particle may indicate a role for HMG proteins in chromatin replication not presently realized. Alternatively, insofar as HMG proteins are specific to transcribed genes, it may be that recently replicated active nucleosomes are exceptionally sensitive to nuclease digestion, relative to mature active chromatin.

**Nonnucleosomal Structure of Nascent Chromatin**—Newly replicated chromatin is relatively resistant to release from nuclei in solutions of low to moderate ionic strength but is extracted by NaCl concentrations above 0.3 M. Similar observations were made previously (43). The insolubility of nascent chromatin is consistent with reports of preferential association of newly replicated DNA with the nuclear matrix (44-48). Nascent material eluted with high salt is not resolved into a typical nucleosomal repeat in DNA gels, although the bulk chromatin in these fractions exhibits a typical subnucleosomal organization. By examining all of the chromatin, we have discovered not only that much of the material of interest is contained in the insoluble fraction but also that this new DNA possesses an altered structure.

What is the origin of the nonnucleosomal nascent chromatin? One possibility is that nucleosomes are present on this fraction of chromatin, but in a highly altered state, resulting in the lack of periodicity after micrococcal nuclease digestion. A similar proposal has been offered to explain the loss of subunit organization during transcription of the Drosophila heat shock genes (49), although the presence of nucleosomes on the active sequences was not unambiguously demonstrated.

A second alternative is suggested by current theories of nucleosomal segregation and assembly. Due to the conservative segregation of parental nucleosomes (5, 9, 50-52), as the replication fork proceeds one daughter DNA duplex will become immediately complexed with pre-fork histone octamers, presumably leaving the other nascent DNA duplex temporar-
ily histone deficient. It is therefore reasonable to propose that the two distinct classes of nascent chromatin reported here arise, in part, because parental histone segregation can proceed more rapidly than de novo nucleosome assembly, as others have suggested (52, 53). The daughter duplex receiving parental chromatin proteins may, within seconds of DNA synthesis, possess histone H1, as well as HMG and other non-histone proteins, and exhibit much the same solubility as bulk chromatin. It is possible that parental histone octamers are transferred with H1 and HMG proteins still bound. In contrast, the DNA not immediately receiving parental proteins may require on the order of minutes to gain mature structure. This interpretation is indirectly supported by experiments performed on MSB cells, in which it was shown that the DNAase I-sensitive state of active genes was acquired after DNA synthesis, but not before (54). For technical reasons that study (54) could only trace the kinetics of reassembly of the newly replicated DNA not receiving parental nucleosomes (52). It was therefore proposed that this class of new DNA lacks mature organization for a measurable period after DNA replication, although the nascent DNA complexed with parental proteins might gain transcriptional competence much more rapidly (54). This is consistent with our observation that H1 and HMG proteins are rapidly complexed with nascent nucleosomes, at a time when a considerable fraction of new DNA lacks mature nucleosomal organization.

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REFERENCES
1. Seale, R. L. (1978) In The Cell Nucleus (Busch, H., ed) Vol. 4, pp. 155–172, Academic Press, New York
2. DePamphilis, M. L., and Wassarman, P. M. (1980) Annu. Rev. Biochem. 49, 627–666
3. McKnight, S. L., Bustin, M., and Miller, O. L., Jr. (1977) Cold Spring Harb. Symp. Quant. Biol. 42, 741–754
4. Basby, S., and Bakken, A. H. (1980) Chromosoma (Berl.) 79, 85–104
5. Riley, D., and Weintraub, H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 328–332
6. Seale, R. L. (1975) Nature 255, 247–249
7. Levy, A., and Jakob, K. M. (1978) Cell 14, 259–267
8. Hildebrand, C. E., and Walther, R. A. (1976) Biochem. Biophys. Res. Commun. 73, 157–163
9. Seale, R. L. (1976) Cell 9, 423–429
10. Schlaeger, E.-J., and Klempnauer, K.-H. (1978) Eur. J. Biochem. 89, 567–574
11. Worcel, A., Han, S., and Wong, M. L. (1978) Cell 15, 969–977
12. Goodwin, G. H., Sanders, C., and Jehns, E. W. (1972) Eur. J. Biochem. 35, 14–19
13. Goodwin, G. H., and Johns, E. W. (1973) Eur. J. Biochem. 40, 215–219
14. Weisbrod, S., and Weintraub, H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 630–634
15. Bakayev, V. V., Schmatchenko, V. V., and Georgiev, G. P. (1979) Nucleic Acids Res. 7, 1525–1540
16. Weisbrod, S., Groudine, M., and Weintraub, H. (1980) Cell 19, 289–301
17. Gazit, B., Panet, A., and Cedar H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1787–1790
18. Albanese, I., and Weintraub, H. (1980) Nucleic Acids Res. 8, 2787–2805
19. Levy, W. B., Connor, W., and Dixon, G. H. (1979) J. Biol. Chem. 254, 609–620
20. Sanders, M. M. (1978) J. Cell Biol. 79, 97–109
21. Goodwin, G. H., Mathew, C. G. P., Wright, C. A., Venkov, C., and Johns, E. W. (1979) Nucleic Acids Res. 7, 1815–1835
22. Kuehl, L., Lyness, T., Dixon, G. H., and Levy-Wilson, B. (1980) J. Biol. Chem. 255, 1090–1095
23. Jackson, J. B., Pollock, J. M., and Rill, R. L. (1979) Biochemistry 18, 3739–3748
24. Todd, R. D., and Garrard, W. T. (1977) J. Biol. Chem. 252, 4729–4738
25. Loenig, U. E. (1967) Biochem. J. 102, 251–257
26. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88
27. Laskey, R. A., and Mills, A. D. (1975) J. Biol. Chem. 250, 335–341
28. Thomas, J. O., and Kornberg, R. D. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2626–2630
29. Albright, S. C., Wiseman, J. M., Lange, R. A., and Garrard, W. T. (1980) J. Biol. Chem. 255, 3673–3684
30. Bakayev, V. V., Bakayeva, T. G., and Varshavyshin, A. J. (1977) Cell 11, 619–629
31. Bakayev, V. V., Bakayeva, T. G., Schmatchenko, V. V., and Georgiev, G. P. (1978) Eur. J. Biochem. 91, 291–301
32. Hutcheon, T., Dixon, G. H., and Levy-Wilson, B. (1980) J. Biol. Chem. 255, 651–655
33. Sandeen, G., Wood, W. I., and Felsenfeld, G. (1980) Nucleic Acids Res. 8, 3757–3778
34. Mardian, J. K. W., Paton, A. E., Bunick, G. J., and Olins, D. E. (1980) Science 209, 1534–1536
35. Walker, J. M., Gooderham, K., Hastings, J. R. B., Mayes, E., and Johns, E. W. (1980) FEBS Lett. 122, 256–260
36. Romani, M., Vidali, G., Tahourdin, C. M., and Bustin, M. (1980) J. Biol. Chem. 255, 468–474
37. Gorden, J. S., Bruno, J., and Lucas, J. A. (1981) J. Cell Biol. 88, 373–379
38. Edenberg, H. J., and Huberma, J. A. (1975) Annu. Rev. Genet. 9, 245–284
39. Levinger, L., and Varshavyshin, A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3244–3248
40. Pakan, S., Turner, G. N., Pagano, J. S., and Hancock, R. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2300–2305
41. Kuehl, L., Barton, D. J., and Dixon, G. H. (1980) J. Biol. Chem. 255, 10671–10675
42. McKeith, S. L., and Miller, O. L., Jr. (1979) Cell 17, 551–563
43. Annunziato, A. T., and Woodcock, C. L. F. (1981) Proc. Nucleic Acid Res. 26, 135–149
44. Berezney, R., and Coffey, D. S. (1975) Science 189, 291–293
45. Dijkwel, P. A., Mullenders, L. H. F., and Wanka, F. (1979) Nucleic Acids Res. 6, 219–230
46. Pardoll, D. M., Vogelestein, B., and Coffey, D. S. (1980) Cell 19, 527–536
47. Vogelestein, B., Pardoll, D. M., and Coffey, D. S. (1980) Cell 22, 79–85
48. Berezney, R. (1979) Exp. Cell Res. 123, 411–414
49. Wu, C., Wong, Y.-C., and Elgin, S. C. R. (1979) Cell 16, 807–814
50. Seale, R. L. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2717–2721
51. Weintraub, H. (1976) Cell 19, 419–422
52. Seidman, M. M., Levine, A. J., and Weintraub, H. (1979) Cell 18, 439–449
53. Herman, T. M., De Pampelitis, M. L., and Wassarman, P. M. (1979) Biochemistry 18, 4683–4671
54. Weintraub, H. (1979) Nucleic Acids Res. 7, 781–792