In Vivo Assembly of Newly Synthesized Histones†

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ABSTRACT: Following a labeling period of 2 min, HeLa histones continue to accumulate in chromatin for 10 min, indicating the presence of a histone pool. During the accumulation period, H2A and H2B enter chromatin immediately, while entry of H3 and H4 is more prolonged. Association of newly synthesized core histones with chromatin does not necessarily indicate assembly. When 2-min [^{3}H]lysine-labeled chromatin is exposed to 0.45 M NaCl, nearly half of the newly synthesized histones are dissociated, while mature core histones are stable. H2A and 70% of H2B are salt stable and remain with newly synthesized polynucleosomes. About 30% of H2B, 50% of H4, and all of H3 are salt labile; thus, both the new nucleosomal core histones and salt-labile new core histones are nonstoichiometric. Pulse-labeled core histones are more trypsin-sensitive than mature histones. When the salt-labile, newly synthesized histones are removed, the remaining proteins have the same trypsin sensitivity as bulk core protein. Examination of the tryptic peptides indicated that the increased trypsin sensitivity was due to complete destruction of the loosely associated core histones which undergo a lag prior to assembly. The altered order of appearance of two peptides in stripped, newly assembled nucleosomes indicates that the conformation in these particles is different from that in mature chromatin.

There are two stages of protein–DNA interactions in chromatin replication; in one, the parental nucleosomal histones are distributed to daughter chromatids, and in the second, the balance of daughter chromatid DNA is assembled with histones into nucleosomes. Parental nucleosomes are inherited by a nonrandom process, passing preferentially to one daughter chromatid (Seale, 1976; Weintraub 1976); the recipient daughter chromatid is the leading, 5′ → 3′, DNA duplex (Seidman et al., 1979). It is not understood how the core histones remain associated with DNA during the DNA denaturation and nucleotide polymerization steps in replication (or whether the core histones remain bound); the experimental observation is that nucleosomes are found on approximately half of daughter chromatid DNA shortly after DNA synthesis in cells (Seale, 1974, 1976), in cells grown in cycloheximide (Seale & Simpson, 1975; Weintraub, 1976; Seidman et al., 1979) and in chromatin DNA synthesized by isolated nuclei in vitro (Seale, 1978a,b).

The relationship of replication to transcription is a most pertinent problem. The transcriptionally active conformation may be temporarily disrupted by passage of the replication fork (Weintraub, 1979); in *Drosophila* embryos transcriptionally active chromatin has been observed on both sides of a replication bubble and very near the replication fork (McKnight & Miller, 1977). The deposition of nucleosomes on the uncomplexed portion of newly synthesized DNA may involve the specification of transcriptional potential at that time; however, the line of reasoning that traces transcriptionally active and inactive chromatin conformations to the replicative assembly of stable, heritable nucleosomes has not been rigorously tested. Equally compatible is the idea that nucleosome assembly is nonspecific and that a given metabolic configuration is specified by a temporal process at some time following histone deposition [for review, see Seale (1978b); De Pamphilis & Wassarman, 1980; Mathis et al., 1980; Laskey & Earnshaw, 1980].

The understanding of nucleosome assembly must include the steps between the release of histone polypeptides from polyribosomes and their integration into nucleosomes. Histone transport into the nucleus is rapid since short exposure to labeled amino acids yields labeled chromosomal histones (Oliver et al., 1974; Ruiz-Carrillo et al., 1975; Jackson et al., 1976); it is not known whether transport is by free diffusion or is mediated. Newly synthesized histones may spontaneously form quaternary complexes prior to assembly with DNA. In embryonic systems, a large histone storage pool exists and provides histones to cleavage nuclei which undertake intense DNA synthesis (Woodland & Adamson, 1977; Laskey et al., 1977; Shih et al., 1980). The storage histones of *Xenopus* oocytes are in 12S quaternary complexes together with an assembly or escort protein pentamer (Earnshaw et al., 1980) which mediates assembly with DNA into nucleosomes.

A vital question is whether a functional histone pool exists in somatic cells or if any such unassociated histone simply represents the transience between release from polyribosomes and nucleosome assembly. Recently, evidence for a storage pool was found that cells in G1 were shown to synthesize histones, most of which were not assembled until the S phase (Groppi & Coffino, 1980).

Assembly factors have not been reported in somatic cells; however, the preferential incorporation of newly synthesized histones into cores (Leffak et al., 1977; G. Tsanev and R. Hancock, unpublished results) may reflect a somatic escort protein. The requirement for an assembly activity in vitro can be fulfilled by eukaryotic DNA nicking–closing enzyme (Germond et al., 1979). Under in vitro conditions, reconstitution (distinguished from biological assembly) has been accomplished at physiological ionic strengths (Ruiz-Carrillo et al., 1979; Stein et al., 1979).

The present study was conducted in an effort to understand facets of in vivo assembly. Although most newly synthesized histones coisolate with chromatin following a pulse label, a substantial fraction does not associate for several minutes. Of the new histones that coisolate with chromatin, a subset undergoes a lag prior to assembly during which it can be preferentially digested by trypsin and can be dissociated by salt concentrations that leave nucleosomes intact. The salt-labile as well as the assembled new core histones are not stoichiometric in the two respective fractions, as predicted from prior studies.

Methods

HeLa cells were maintained in spinner culture in Eagle's

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minimal essential medium supplemented with 5% calf serum (GIBCO).

Prelabeling of cells with [^{14}C]thymidine was performed by adding 1 μCi/100 mL cell culture overnight. [^{14}C]Arginine and [^{14}C]lysine (50 μCi/100 mL cell culture) prelabelings were in medium depleted of those amino acids by 80%.

Cells were pulse labeled by concentrating them 20-fold in the appropriate prewarmed medium. Amino acid pulse labeling was performed in totally arginine- and lysine-deficient medium in which the cells were preincubated 20–30 min prior to addition of the isotope. Controls showed no decrease in incorporation of [^{3}H]leucine for over 1 h in these conditions. For the amino acid chase, arginine and lysine were added to a final concentration 10 times that of the complete medium. Cells were collected by adding directly to 5 volumes of ice-cold buffer A (5 mM Tris, pH 7.6, 3 mM $MgCl_2$, and 2 mM mercaptoethanol) containing 0.1% sodium azide.

Cells were washed twice in buffer A, swollen for 10 min, and lysed in a dounce homogenizer. Nuclei were collected by centrifugation at 1000g and washed twice. In experiments concerning histone acetylation, buffers contained 5 mM sodium butyrate.

Digestion by micrococcal nuclease was in buffer A containing 2.5 × 10^{-4} M $CaCl_2$ with 0.1 unit of enzyme (Sigma) per A_{260} unit of chromatin (measured in 1% $NaDodSO_4$) at 37 °C for 2 min and terminated by addition of EGTA to 2 mM and cooling to 4 °C.

Histones were extracted with 0.2 N HCl, precipitated with 10 volumes of acetone at −20 °C, and dissolved in the appropriate electrophoresis buffer. Aliquots were counted after precipitation together with 50 μg each of DNA and albumin in 10% Cl_3CCOOH and collected on filters.

Salt extraction of chromatin was performed in 0.45 NaCl, 1 mM EDTA, and 5 mM Tris, pH 7.6. The volume was adjusted to 5 mL, and layered over a 2-mL cushion of 50% (w/w) sucrose, 0.45 M NaCl, 1 mM EDTA, and 5 mM Tris, pH 7.6 (and 5 mM butyrate, where applicable). Centrifugation was at 45 000 rpm for 65 h in the Beckman Ti50 rotor at 4 °C. The supernatant was dialyzed against 2 mM EDTA, lyophilized, and dissolved in electrophoresis buffer. The pellet was dissolved in 0.2 mM EDTA, pH 7.6.

Electrophoresis in $NaDodSO_4$ was according to Thomas & Kornberg (1977) and in acid–urea according to Panyim & Chalkley (1969). Individual stained bands were excised, digested with 0.25 mL of NCS solubilizer (Amersham), and counted.

Trypsin digestion was performed on chromatin in 0.2 mM EDTA, pH 7.6, at 37 °C. Controls showed no evidence of autoproteolysis of nuclear proteins for 4 h at 37 °C. Prolonged storage at 4 °C did result in proteolysis of the H1 and H3 histones. Trypsin, prepared fresh and used immediately, was added to levels indicated in the text for 15 min, and digestion was terminated with 1% $NaDodSO_4$.

Results

In order to understand the process of assimilation of newly synthesized histone proteins into nucleosomes, it is imperative to determine whether this occurs with immediacy upon peptide release or whether any lag period is detectable, indicating a pool.

Toward this end, pulse–chase experiments were performed (Figure 1). For an internal reference, cells were incubated with [^{14}C]arginine and lysine for one generation. The cells were then labeled for 2 min with [^{3}H]arginine plus [^{3}H]lysine, and the labeling was stopped by addition of an excess of each unlabeled amino acid. Figure 1 shows the effectiveness of

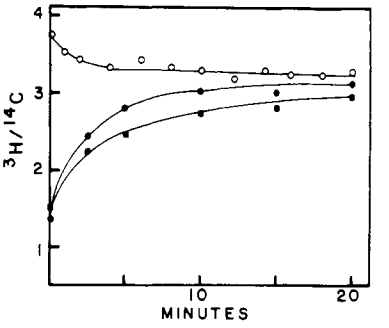

FIGURE 1: Pulse–chase kinetics of arginine plus lysine. Cells prelabeled 18 h with [^{14}C]arginine and [^{14}C]lysine were incubated for 2 min with [^{3}H]arginine plus [^{3}H]lysine. An excess of unlabeled amino acids was added at time zero. (○) Total cellular radioactivity; (●) acid-soluble nuclear proteins; (■) acid-insoluble nuclear proteins.

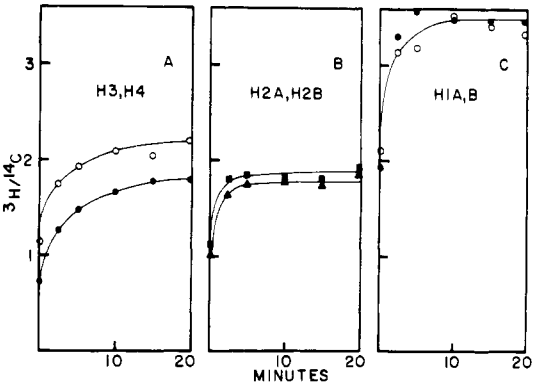

FIGURE 2: Kinetics of incorporation of pulse-labeled radioactivity into individual histones. (Panel A) (●) H4; (○) H3; (Panel B) (▲) H2A; (■) H2B; (Panel C) (●) H1A; (○) H1B.

amino acid excess toward suppression of incorporation of radioactivity. There was a slight decline in cellular acid-precipitable tritium initially, after which there was no increase during the experiment. The reason for the decline in total radioactivity is not understood; it may reflect unstable cellular protein with a high turnover rate. During this time, tritium increased in both the acid-soluble and the acid-insoluble fractions. At the initiation of chase conditions, about 40% of the acid-soluble proteins had already associated with chromatin.

At each point in the chase period, the acid-soluble proteins were separated in $NaDodSO_4$–polyacrylamide gels in order to verify that histone radioactivity followed the kinetics of the total acid-soluble fraction and to test whether individual histones behaved in concert (Figure 2). The H3 and H4 histones increased in specific activity at a rate similar to that of the acid-soluble fraction, while H2A and H2B associated more rapidly. Semihistone A24 increased in radioactivity by a factor of 2.5–3 times that of the other core histones (Seale, 1981). Similarly, histone H1 reached a specific activity about twice that of the core histones, although the kinetics of association with chromatin were similar. Thus, in agreement with Jackson et al. (1976), histones enter chromatin with kinetics that suggest passage through a pool, but such a pool must be of finite size and undergo rapid exchange.

Further experiments involved the use of salt concentrations that can cause aggregation of whole nuclei, so nuclease cleavage of chromatin was employed in order to enhance solubility.

Digestion of nuclei with micrococcal nuclease to 5–10% acid solubility yields three fractions of chromatin by the procedure of Todd & Garrard (1979). The nuclease-soluble fraction, S2, and the remaining insoluble chromatin pellet, P, were both investigated whenever practicable; since these fractions contain

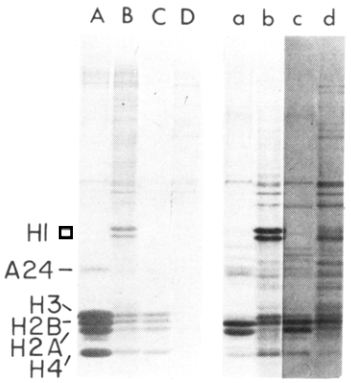

FIGURE 3: Salt sensitivity of newly synthesized histones. Nuclei were isolated from cells incubated for 2 min with [^{3}H]arginine and [^{3}H]lysine and digested with micrococcal nuclease. The nuclease-soluble chromatin (S2) and the insoluble chromatin (P) were stripped with 0.45 M NaCl. Proteins from polynucleosomes and the salt washes were separated in $NaDodSO_4$ gels. (Lane A) S2 polynucleosomal protein; (lane B) S2 salt wash; (lane C) pellet polynucleosomes; (lane D) P salt wash. (Lanes A–D) Coomassie blue stain; (lanes a–d) autoradiogram.

about 80% and 15% of total DNA, respectively, we are examining essentially all chromatin. When the S2 and P were extracted with 0.45 M NaCl, the newly synthesized core histones showed strikingly enhanced salt sensitivities (Figure 3). This salt concentration extracts nonhistone proteins, H1, and the HMG proteins, leaving the core histones and DNA in stable complexes. Both the S2 and P fractions lost a considerable portion of pulse-labeled core histone radioactivity to the salt eluates. The salt sensitivity of new core histone protein decreased with time; by 10 min of continuous labeling, the distribution of core histone label between the stripped nucleosomes and the salt eluate reflected the protein mass distribution.

The partitioning of individual core histones between the stripped nucleosomes and the salt eluate was different for each core histone. H2A and H2B coisolated primarily with the stripped nucleosomes of the S2, but about 30% of radiolabeled H2B was salt labile (Figure 3). H4 was approximately equally distributed between stripped S2 nuclesomes and the salt eluate in the shortest pulse-labeling periods (1 and 2 min), but H3 was almost entirely removed by 0.45 M salt.

Ruiz-Carrillo et al. (1975) and Jackson et al. (1976) showed that newly synthesized H4 is hypermodified with two acetyl and one phosphoryl groups, later removed upon association with chromatin. The possibility that the salt-labile histones are more extensively modified than those that are integrated into mature nuclesomes was examined by electrophoresis in acid–urea gels. The extensively modified H4 histones were not selectively removed by salt washing; to the contrary, the most modified forms of H4 were present in the same relative abundance in both fractions (data not shown).

The property of 0.45 M salt sensitivity of newly synthesized histones indicated that these proteins were not assembled into nucleosomes, despite their coisolation with chromatin. Since nucleosomes have been reported to have a characteristic resistance to trypsin digestion (Weintraub & Van Lente, 1974), trypsin digestion was chosen as a probe for altered structure.

Nuclease-solubilized (S2) chromatin was prepared from cells prelabeled 18 h with [^{14}C]lysine and then pulse-labeled for 2 min with [^{3}H]lysine. The chromatin was digested with increasing amounts of trypsin for 15 min, and digestion to acid solubility is expressed as $^3H/^{14}C$ (Figure 4). The newly synthesized proteins were distinctly more trypsin sensitive than those uniformly labeled, by an increment of 50%. The increment in digestibility could be primarily from the nonassembled histones. So that this could be tested, nucleosomes were stripped in 0.45 M NaCl and then digested with trypsin (Figure 5). During the course of digestion of the stripped nucleosomes, a constant $^3H/^{14}C$ ratio was maintained; thus, no increment in digestibility was evident when the salt-sensitive proteins were removed.

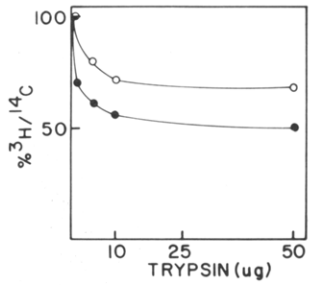

FIGURE 4: Trypsin digestion of whole chromatin to acid solubility. Cells were ^{14}C prelabeled and ^{3}H pulse labeled for 2 min as in Figure 1. S2 chromatin (●) and P chromatin (○) were digested with the indicated amounts of trypsin. Data are expressed as the ratio of percent acid-soluble ^{3}H/acid-soluble ^{14}C.

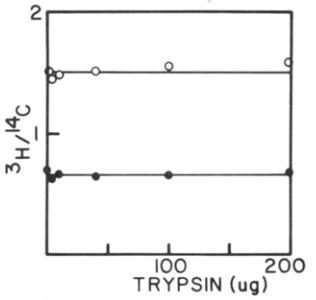

FIGURE 5: Trypsin digestion of stripped chromatin. Chromatin was prepared as in Figure 4 and stripped in 0.45 M NaCl prior to trypsin digestion. (●) S2; (○) P.

The tryptic peptides were separated in $NaDodSO_4$–polyacrylamide gels and examined by fluorography in order to understand the nature of the increased trypsin sensitivity of the newly synthesized, chromatin-associated histones. Comparison of the staining patterns with the autoradiographic patterns revealed a most interesting potential for artifact (Figure 6). The digestion of chromatin by an incremental addition of trypsin generates a set of discrete peptide products which agree well with the limit digest products reported by Weintraub & Van Lente (1974). Figure 6A (lanes L and M) show trypsin alone; contaminants in commercial trypsin preparations coelectrophorese precisely with those apparently derived from chromatin. This set of stained bands was present in both Sigma and Worthington TPCK trypsin preparations and was identical in samples dissolved directly in $NaDodSO_4$ buffer and in 30-min autodigested trypsin solutions. Fluorography of the gel revealed the true histone tryptic peptides which comigrated with the peptide contaminants in the commercial enzyme. The radioactive histone tryptic peptides were discrete in size, but not stable. Each peptide was progressively degraded, such that the soluble chromatin fraction was digested to the extent of 85% acid solubility at the final point in Figure 6B (lane K). The tryptic histone peptides are labeled P1–P6, in accord with the nomenclature of Weintraub & Van Lente; however, we observed more bands than have been reported previously (Figure 6B). A limit digest was *not* observed, either in terms of acid-soluble products or in terms of stable peptide intermediates.

The digestion of pulse-labeled ([^{3}H]lysine for 2 min) chromatin yielded a set of tryptic peptides similar to those

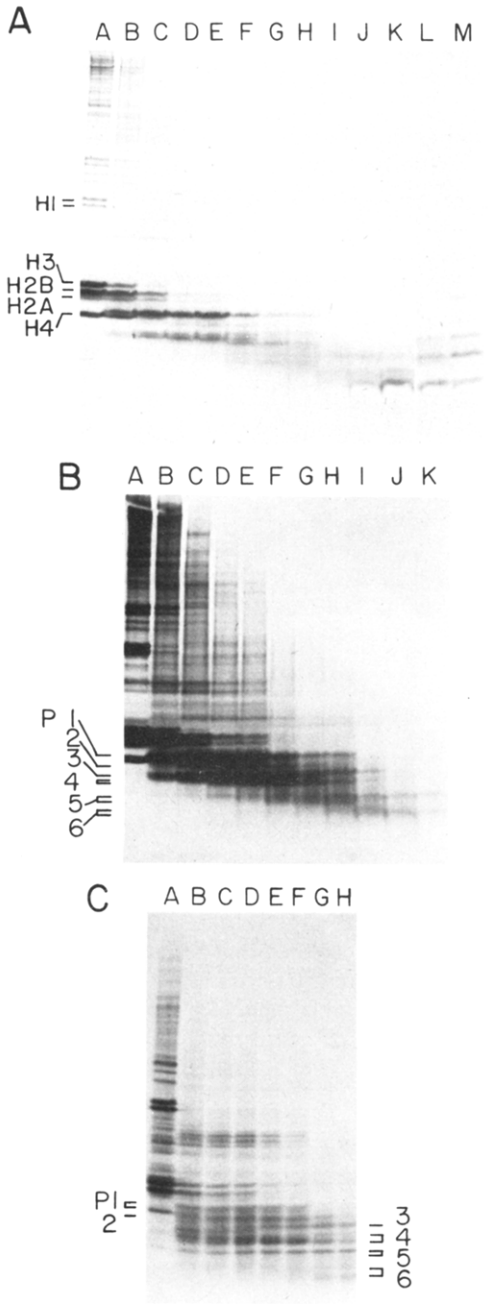

FIGURE 6: Tryptic peptides from S2 chromatin. (Panel A) Coomassie blue stain, 18-h [^{3}H]lysine label; (panel B) autoradiogram of panel A; (panel C) fluorograph of 2-min [^{3}H]lysine-labeled S2 chromatin. In panels A and B, lanes A–K contained 0.2 A_{260} units of chromatin plus trypsin (μg). (Lane A) 0; (lane B) 0.025; (lane C) 0.125; (lane D) 0.25; (lane E) 0.5; (lane F) 1.25; (lane G) 2.5; (lane H) 5; (lane I) 12.5; (lane J) 25; (lane K) 50 μg; (lane L) trypsin (Sigma) used for digestion; (lane M) Worthington TPKC trypsin. (Panel C) Fluorograph of S2 chromatin labeled 2 min with [^{3}H]lysine. (Lane A) 0 trypsin; (lane B) 0.025; (lane C) 0.125; (lane D) 0.25; (lane E) 0.5; (lane F) 1.25; (lane G) 2.5; (lane H) 5 μg.

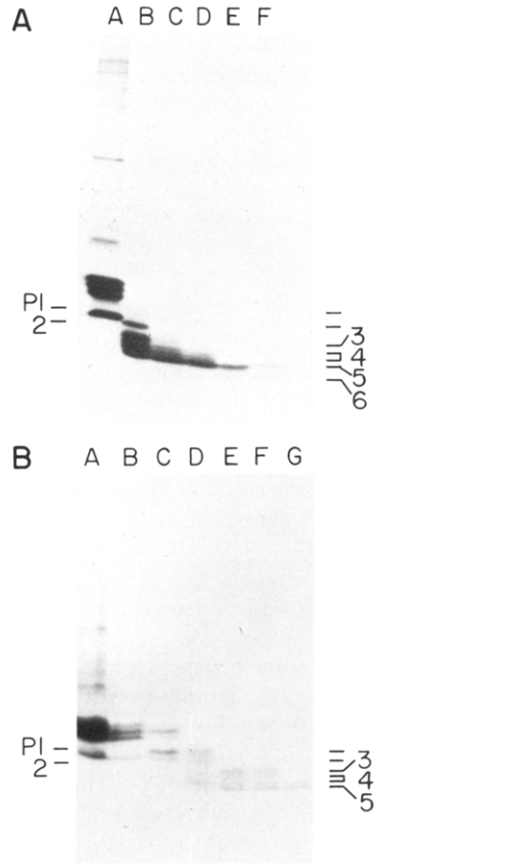

FIGURE 7: Tryptic peptides from stripped chromatin. (Panel A) Fluorograph of [^{3}H]lysine and [^{3}H]arginine uniformly labeled, stripped S2. (Lane A) No trypsin; (lane B) 0.025; (lane C) 0.125; (lane D) 0.25; (lane E) 0.5; (lane F) 1.25 μg. (Panel B) Fluorograph of 2-min [^{3}H]lysine-labeled, stripped S2 chromatin. (Lane A) No trypsin; (lane B) 0.03; (lane C) 0.125; (lane D) 0.25; (lane E) 0.5; (lane F) 1.25; (lane G) 2.5.

generated from uniformly labeled chromatin (Figure 6C). The relative intensities of the tryptic peptides differ from those in uniformly labeled chromatin, presumably because the H3 and H4 histones are more weakly labeled.

When chromatin stripped in 0.45 M NaCl was digested, a more simple pattern of intermediates was produced. Figure 7A shows a fluorogram of the tryptic peptides of uniformly labeled histones. The stripped nucleosomes were markedly less resistant than whole chromatin, and digestion proceeded rapidly to the complete destruction of core histones; this enhanced sensitivity may be due, in part, to the lack of competition of other nuclear proteins for trypsin. Once the salt-dissociable proteins are removed, the stripped cores show much less diversity in trypsin digest patterns. Recent experiments involving nuclease digestion of nucleosomes following salt washing and restoration of the sensitivity of active genes after reconstruction of HMG proteins (Weisbrod & Weintraub, 1979; Albright et al., 1980) may be related to this observation.

When pulse-labeled, salt-stripped chromatin was digested, the pattern of tryptic peptides was similar to that of stripped bulk chromatin (Figure 7B). Here, also, the relative intensities of the bands are different; at least part of this effect may be due to different specific activities in the starting material. However, we note that P3 survives to a later stage of digestion in the pulse-labeled material, and P4 is degraded more rapidly, as compared to the order of digestion in stripped, mature chromatin. This indicates a conformational difference in the newly assembled nucleosome, and we are investigating the nature of this difference. There is the distinct absence of the P6 peptides in both sets of stripped nucleosomes. The difference between new and mature nucleosomes appears to be kinetic rather than resulting from exposure or blockage of sites, and in general, the enhanced protease sensitivity of pulse-labeled chromatin proteins can be attributed to the destruction of a subset of core histones that cosiolate with chromatin but are partially assembled, if at all.

Discussion

When histones are released from the soluble cytoplasmic polyribosomes, they either diffuse or are transported into the

nucleus for chromatin assembly. This takes as long as 10 min following polypeptide release. The lag between termination of protein synthesis and nucleosome assembly is characterized by two stages. Nonassembled new histones exist in both the soluble and the chromatin-associated states. Since the time for soluble histone association and for integration of chromatin-associated histones into salt-resistant structures (presumably mature nucleosomes) is similar, it is not evident that there is a pathway of association prior to assembly or whether both stages occur simultaneously.

The demonstration that the coisolation of new histone with chromatin is not necessarily synonymous with their functional integration into nucleosomes was made in two ways. First, a salt concentration that does not dissociate native nucleosomes (0.45 M) removed about 40% of core histones labeled for 1 or 2.5 min. Second, trypsin digestion preferentially destroyed pulse-labeled histones by an increment of 50% relative to uniformly labeled chromatin. When the weakly bound fraction was removed, no preferential degradation of pulse-labeled histone was evident.

Several possibilities may account for the state of nonassembled, newly synthesized histones. The delayed kinetics of assembly strongly indicate the presence of a histone pool. Recently, Groppi & Coffino (1980) reported the synthesis of histones in the G1 period and their later migration to the nucleus, also indicative of a histone pool. This is the first direct evidence for a somatic cell histone pool like that established for embryonic systems (Woodland & Adamson, 1977; Laskey et al., 1977). The function of the somatic cell pool is not yet evident; it may simply be transitory storage prior to assembly, or it may result from nucleosome exchange. It may prove significant in regard to the possibility of histone exchange that 18% of core histones made in G1 were nuclear, despite the absence of DNA synthesis (Groppi & Coffino, 1980), and that histones made in hydroxyurea-blocked cells enter chromatin (Tsanev et al., 1980). If histones do not exchange, it is not evident how either of these two processes could occur. However, the coisolation of new core histones with chromatin in these reports could represent nonassembled protein, as shown in the present study.

A second possibility is that new, chromatin-associated histones represent immature nucleosomes. The correlation of new H3 and H4 with new DNA has been noted by others (Worcel et al., 1978; Senshu et al., 1978; Cremisi & Yaniv, 1980; Jackson & Chalkley, 1981a,b; A. T. Annunizato and R. L. Seale, unpublished results). One caveat to this observation is that the new H3 and H4 histones have not been demonstrated to exist in stable complexes with new DNA. The new DNA which is most enriched for the new histones has no subunit structure, as assessed by micococcal nuclease digestion (Annunziato et al., 1981).

The third possibility for chromatin-associated, new histones is that they occupy low-affinity sites on chromatin and later come into contact with a region of DNA suitable for nucleosome assembly. In vitro studies have demonstrated that histone octamers may migrate along chromatin to a DNA region suitable for core protein–DNA assembly (Ruiz-Carrillo et al., 1979; Stein, 1979; Stein et al., 1979). However, the separate behavior of H3 and H4 from H2A and H2B during assembly (discussed below) argues against this model in replication.

One particularly interesting feature of the new core histones in either state is their lack of stoichiometry. Both in the salt-resistant fraction and in the salt eluate, the new histones were not in uniform abundance. Newly synthesized histones H2A and H2B were the primary salt-resistant species, although about 30% of H2B was salt labile. The most weakly bound proteins were H3 and H4, H3 being more salt labile than H4.

Examination of the data in other reports has revealed similar phenomena. In the gradient fractionations of nuclease-solubilized chromatin pulse labeled with amino acids, Worcel et al. (1978) showed that the H3–H4 pair migrated independently of the H2A–B2B pair. Significantly, the relative proportions between the proteins of each pair were not constant across the gradient, as expected for stoichiometric assembly. Leffak et al. (1977) reported that nucleosome cores were assembled exclusively from new proteins. One notes that [^{3}H]lysine was employed for labeling. About 30% of the lysine label enters H1, which banded as a cross-linked H1 polymer. Together with the contribution of radioactivity from nonhistone proteins, one estimates that about 50% of the radioactivity was in core histones; i.e., each of the individual core histones contained 10–15% of total radioactivity in the gradient. Thus, in bouyant density gradients, substantial deviation from assembly exclusively by new histones could occur without detection.

In a study of new histone (8-min label) association with SV40 viral chromatin (Cremisi & Yaniv, 1980), the abundance of new H3 on replication intermediates was approximately twice that of new H4, after correction for the methionine content of the ^{35}S-labeled proteins. H2B was preferentially localized on nonreplicating minichromatin which is a physically distinct species. On the basis of the lack of stoichiometry of newly synthesized core histones, the histone octamer of the nucleosome core may not be comprised exclusively from new histones. Whether this proves to be borne out, it is consistently observed that H3 and H4 are integrated into chromatin differently from H2A and H2B.

The stepwise assembly of newly synthesized histones in somatic cells is distinctly different from the apparent single-step transfer of the histone octamer to DNA observed in embryonic systems. The lack of detection of somatic assembly factors to date possibly results from assays which score complete nucleosomes. Assembly intermediates may not give positive results for the introduction of supercoils, protection against micrococcal nuclease, and an 11S sedimentation coefficient, and they may be unstable when subjected to manipulations used for mature chromatin.

References

Albright, S. C., Wiseman, J. M., Lange, R. A., & Garrard, W. T. (1980) *J. Biol. Chem. 255*, 3673–3684.

Annunziato, A. T., Schindler, R. K., Thomas, C. A., Jr., & Seale, R. L. (1981) *J. Biol. Chem.* (in press).

Cremisi, C., & Yaniv, M. (1980) *Biochem. Biophys. Res. Commun. 92*, 1117–1123.

De Pamphilis, M. L., & Wassarman, P. M. (1980) *Annu. Rev. Biochem. 49*, 627–666.

Earnshaw, W. C., Honda, B. M., Laskey, R. A., & Thomas, J. O. (1980) *Cell (Cambridge, Mass.) 21*, 373–383.

Germond, J. E., Yaniv, J. R., Yaniv, M., & Brutlag, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3779–3783.

Groppi, V. E., Jr., & Coffino, P. (1980) *Cell (Cambridge, Mass.) 21*, 195–204.

Jackson, V., & Chalkley, R. (1981a) *Cell (Cambridge, Mass.) 23*, 121–134.

Jackson, V., & Chalkley, R. (1981b) *J. Biol. Chem. 256*, 5095–5103.

Jackson, V., Shires, A., Tanphaichitr, N., & Chalkley, R. (1976) *J. Mol. Biol. 104*, 471–483.

Laskey, R. A., & Earnshaw, W. C. (1980) *Nature (London) 286*, 763–767.
Laskey, R. A., Mills, A. D., & Morris, N. R. (1977) *Cell (Cambridge, Mass.) 10*, 237–243.
Leffak, M., Grainger, R., & Weintraub, H. (1977) *Cell (Cambridge, Mass.) 12*, 837–845.
Mathis, D., Oudet, P., & Chambon, P. (1980) *Prog. Nucleic Acid Res. Mol. Biol. 24*, 1–55.
McKnight, S., & Miller, O. (1977) *Cell (Cambridge, Mass.) 12*, 795–804.
Oliver, D., Granner, D., & Chalkley, R. (1974) *Biochemistry 13*, 746–749.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1975) *Science (Washington, D.C.) 190*, 117–128.
Ruiz-Carrillo, A., Jorcano, J. L., Eder, G., & Lurtz, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3284–3288.
Seale, R. L. (1974) *Nature (London) 255*, 247–249.
Seale, R. L. (1976) *Cell (Cambridge, Mass.) 9*, 423–429.
Seale, R. L. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2717–2721.
Seale, R. L. (1978b) *Cell Nucl. 4*, 155–172.
Seale, R. L. (1981) *Nucleic Acids Res. 9*, 3151–3158.
Seale, R. L., & Simpson, R. T. (1975) *J. Mol. Biol. 94*, 479–501.
Seidman, M. M., Levine, A. J., & Weintraub, H. (1979) *Cell (Cambridge, Mass.) 18*, 439–449.
Senshu, T., Fukada, M., & Ohashi, M. (1978) *J. Biochem. (Tokyo) 84*, 985–988.
Shih, R. J., Smith, L. D., & Keem, K. (1980) *Dev. Biol. 75*, 329–342.
Stein, A. (1979) *J. Mol. Biol. 130*, 103–134.
Stein, A., Whitlock, J. P., Jr., & Bina, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5000–5004.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Todd, R. D., & Garrard, W. T. (1979) *J. Biol. Chem. 254*, 3074–3083.
Tsanev, G., Vassilev, L., & Russev, R. (1980) *Nature (London) 285*, 584–486.
Weintraub, H. (1976) *Cell (Cambridge, Mass.) 9*, 419–422.
Weintraub, H. (1979) *Nucleic Acids Res. 7*, 781–792.
Weintraub, H., & Van Lente, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4249–4253.
Weisbrod, S., & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 630–634.
Woodland, H. R., & Adamson, E. D. (1977) *Dev. Biol. 57*, 118–135.
Worcel, A., Han, S., & Wong, M. L. (1978) *Cell (Cambridge, Mass.) 15*, 969–977.

Complete Nucleotide Sequence of the Chicken Chromosomal Ovalbumin Gene and Its Biological Significance†

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ABSTRACT: The nucleotide sequence of the entire chicken chromosomal ovalbumin gene has been determined. The gene is 7564 nucleotides in length to code for a mature messenger RNA of 1872 nucleotides. Comparison of the sequence at the 5′-terminal region of the gene with that reported by others has revealed multiple polymorphic nucleotides in the structural, intervening, and flanking DNA sequences. Some of the polymorphic sites occur at positions very close to splice junctions or the eucaryotic promoter sequence, yet apparently have little or no effect on the expression of this gene. The heptanucleotide promoter sequence TATATAT present in the 5′-flanking region of the ovalbumin gene does not occur within the confines of the gene. Nevertheless, multiple Hogness box sequences similar to those found in other eucaryotic genes were delineated within the boundaries of the gene. These internal Hogness box sequences are not used for transcription initiation. Similarly, the hexanucleotide sequence AATAAA common to all eucaryotic messenger RNAs at the 3′-untranslated region occurs seven additional times within the ovalbumin gene. These sites are not used for transcription termination or polyadenylation. Thus, although these sequences may play important roles in the initiation or termination of gene transcripts as well as polyadenylation of the transcripts, the specificity for such biological functions must not reside within these sequences alone. Furthermore, sequences complementary to the highly conserved rat U1 small nuclear RNA have been found throughout the gene. Many of these regions of complementarity occur in the structural sequences. If the small nuclear RNA does play a role in splicing, the specificity must be provided also by other as yet undefined components.

Expression of the ovalbumin gene in the chicken oviduct is regulated by steroid hormones (O'Malley & Means, 1974; Woo & O'Malley, 1975; Harris et al. 1973; Sullivan et al., 1973; Palmiter, 1975). Further insight into the molecular mechanism by which steroids regulate ovalbumin gene expression requires detailed knowledge with regard to the molecular structure and nucleotide sequence of the natural gene. We and others have previously reported the cloning and characterization of various overlapping genomic chick DNA fragments containing the ovalbumin gene, which had led to the discovery that the structural ovalbumin gene sequences are separated into eight segments by seven intervening DNA sequences (Woo et al., 1978; Dugaiczyk et al., 1978, 1979;

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