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Replicative Conformation of Parental Nucleosomes: Salt Sensitivity of Deoxyribonucleic Acid–Histone Interaction and Alteration of Histone H1 Binding†

Ernst-Jürgen Schlaeger

ABSTRACT: The transiently altered DNA–histone interaction of parental chromatin during replication was studied by micrococcal nuclease digestion. A large amount of nuclease-resistant pulse-labeled DNA and a small fraction of nonreplicating DNA are released from chromatin fragments by treatment with 0.5 M NaCl and appear as protein-free DNA. As shown by reconstitution experiments, the salt sensitivity of digested nascent chromatin is most probably a consequence of the shorter DNA fragment size (55 ± 15 base pairs) in these complexes. This new DNA is associated with parental chromatin fragments which are structurally changed in such a way that parts of nucleosomal DNA were more susceptible to nuclease attack. The core histones of these particles are probably not distinct from those of salt-stable nucleosomes. However, histone H1 and probably high-mobility group proteins appear to be more weakly bound during replication as shown by electrophoresis under nondenaturating conditions. The results agree with the assumption that the transient alteration of nucleosomal conformation describes a state in which DNA could be replicated without leaving the associated core histone complexes. A possible attachment of pulse-labeled chromatin with nuclear matrix is discussed.

Newly replicated chromatin is transiently changed in chromatin structure [for a review, see DePhamphilis & Wassarman (1980)]. The higher nuclease susceptibility (Seale, 1975, 1976, 1978; Hildebrand & Walters, 1976; Weintraub, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Seidman et al., 1979; Klempnauer et al., 1980) and the altered nuclease-resistant cleavage products of newly replicated chromatin (Hildebrand & Walters, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Schlaeger & Knippers, 1979; Klempnauer et al., 1980) are two important features of the structural differences from mature chromatin and have been observed in several systems (DePhamphilis & Wassarman, 1980).

A portion of the nascent chromatin is cleaved by micrococcal nuclease to DNA fragments which are shorter in size than those produced under the same conditions from nonreplicating chromatin, indicating that the pulse-labeled DNA is differently organized (Seale, 1978; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Klempnauer et al., 1980). The amount of nascent chromatin which differs in the DNA–protein interaction could be measured by the release of protein-free short DNA fragments (4–5 S) in 0.5 M salt as previously described (Schlaeger & Knippers, 1979).

From the salt-sensitive behavior of nascent chromatin fragments, it is suggested that almost all parental chromatin regions associated with new DNA are subjected to a conformational change during replication (E.-J. Schlaeger and R. L. Seale, unpublished experiments).

In an effort to understand the altered DNA–protein interaction of newly replicated chromatin, the structural properties of pulse-labeled DNA have been studied in more detail by using micrococcal nuclease as a probe of chromatin structure. In the first section of this work, pulse-labeled chromatin was nuclease digested in intact nuclei and analyzed, whereas in the second part, the structural properties of nascent DNA on isolated nucleosomes were investigated.

From the data obtained, it is suggested that during DNA replication the parental nuleosome structures are subjected to a conformational change in such a way that parts of nucleosomal DNA become more susceptible to nuclease cleavage cuts. This altered DNA–histone interaction was also observed for a minor class of nonreplicating chromatin subunits. The unusual DNA fragments are released from salt-sensitive nucleosomes as a consequence of the shorter size. Evidence will be presented showing that the histone H1 is transiently more

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weakly bound on the parental chromatin during replication.

Materials and Methods

Cell Culture and Preparation of Nuclei. Preparation and cultivation of lymphocytes from bovine retropharyngeal lymph nodes have been described (Peters, 1975). Long-term labeling of cells with 0.5 μCi of [^{3}H]thymidine per cell suspension was performed for about 15–18 h. For pulse labeling, the cells were concentrated 10-fold to (3–5) $\times$ 10^7 cells/mL in medium and incubated further for 3–5 min. These cells were labeled with 100 μCi of [^{3}H]thymidine/mL for 30 s. Labeling was terminated by addition of ice-cold Hanks balanced salt solution followed by rapid centrifugation (360g, 1 min). In a pulse–chase experiment, the cell sediment was diluted in prewarmed culture medium to 5 $\times$ 10^6 cells/mL and incubated 15 min more. Nuclei were prepared as previously described (Schlaeger, 1978).

Pulse labeling with [^{3}H]- or [^{32}P]dTTP in isolated S-phase nuclei was performed as previously described (Schlaeger, 1978). A pulse–chase experiment was performed by dilution of the sample with 1 volume of unlabeled reaction buffer in which the concentration of unlabeled dTTP was increased 50-fold with an additional incubation of 30 min.

Nuclease Digestion with Micrococcal Nuclease. Nuclei were transferred into nuclease buffer (10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 40 mM NaCl, and 10 mM 2-mercaptoethanol) and then digested at 37 °C with micrococcal nuclease (Boehringer-Mannheim, Worthington). The reaction was terminated by addition of ice-cold nuclease buffer, and the treated nuclei were rapidly transferred after centrifugation into lysis buffer (5 mM Tris-HCl, pH 7.5, and 2.5 mM EDTA) for 30–60 min on ice. Salt-treated nuclear lysates were prepared by adding NaCl to 0.5 M to the digested chromatin at 0 °C. Digestion kinetics were performed as previously described (Schlaeger & Klempnauer, 1978).

Reconstitution experiments with DNA fragments were performed in a nuclear lysate of nuclease-treated nuclei. Unlabeled nuclei (2 $\times$ 10^7) were digested with micrococcal nuclease (30 units) in 0.5 mL of nuclease buffer for 8 min. The treated nuclei were transferred into lysis-reconstitution buffer (5 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 25 mM KCl, and 10 mM 2-mercaptoethanol) and maintained 30–60 min on ice. Purified DNA fragments were added after this period, and the assembly products were analyzed by sucrose gradient centrifugation as previously described (Schlaeger, 1981).

Centrifugation. The nuclear lysates were layered onto linear 5–25% sucrose gradients containing 2 mM EDTA, pH 7.5, and 10 mM 2-mercaptoethanol, centrifuged in an SW 40 rotor, fractionated, and counted (Schlaeger & Knippers, 1979). The gradient contained 0.5 M NaCl when salt-treated nuclear lysates were investigated.

Gel Electrophoresis. For DNA size analysis, the nuclease-resistant DNA was purified as previously described (Schlaeger & Klempnauer, 1978) and analyzed on a 5% polyacrylamide (Tris–borate–$MgCl_2$) slab gel according to Maniatis et al. (1975). Gels were stained with ethidium bromide, photographed, and analyzed further by fluorography (Laskey & Mills, 1975) on Kodak XR-5 film.

The heterogeneity of nucleoprotein particles was analyzed on a 5% polyacrylamide gel under nondenatured conditions (Pospelov et al., 1977). This DNA slab gel was run in 10 mM Tris–borate, pH 8.3, and 1 mM EDTA. Electrophoresis was carried out at 50 V for 2.5 h at 18–20 °C. Gels were stained with ethidium bromide, photographed, and prepared for autoradiography on Kodak XR-5 film.

Protein analysis was performed by using a 15% polyacrylamide gel according to Laemmli (1970).

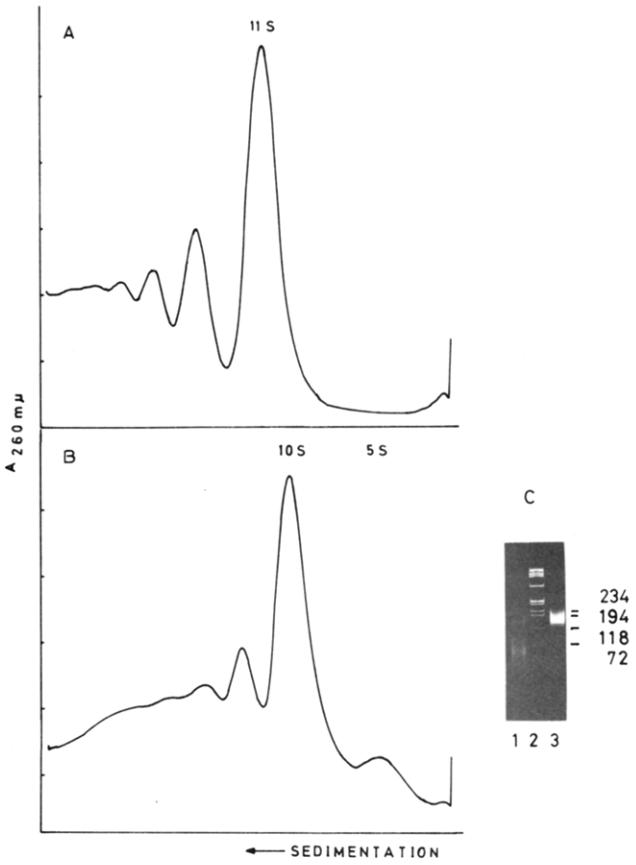

FIGURE 1: Sucrose gradient analysis of nuclease-digested chromatin in low salt and in 0.5 M salt. Unlabeled nuclei (2 $\times$ 10^7) were digested with micrococcal nuclease (30 units) to 30% acid solubility, and the nuclear lysates were centrifuged through a sucrose gradient without salt (A) and containing 0.5 M NaCl (B). (C) The isolated 4–5S and 10S fractions from the gradient shown in panel B were analyzed by gel electrophoresis and stained with ethidium bromide. Lane 1, DNA from the 4–5S peak; lane 2, ϕX174 *Hin*dIII DNA fragments; lane 3, DNA from the 10S peak.

Results

Occurrence of Salt-Sensitive Chromatin Fragments in Nonreplicating and Newly Replicated Chromatin. When unlabeled nuclei from resting or concanavalin A activated bovine lymphocytes were digested with micrococcal nuclease and chromatin fractionated by sucrose gradient sedimentation without salt or in the presence of 0.5 M salt (Figure 1A,B), an additional 4–5S peak of UV-absorbing material in the gradient containing 0.5 M salt could be observed. Gel electrophoretic analysis of the nuclease-resistant DNA fragments from the 10S mononucleosomes and the 4–5S fraction showed that the slow sedimenting peak contained a heterogeneous mixture of short DNA fragments with an average length of 55 $\pm$ 15 base pairs (bp), whereas the monomer appeared as a band of 165 $\pm$ 3 bp in length. The amount of monomeric DNA in the 4–5S fraction was probably a contamination from the predominant 10S peak in the gradient as indicated from recentrifuged 4–5S material.

The possibility that these unusual structures occur as a simple intermediate of an advanced process of chromatin degradation was excluded by the fact that the amount of 4–5S DNA does not increase after nuclease digestion of nuclear lysates as well as of isolated nucleosomes. Furthermore, a very short digestion of naked DNA does not pass through such an intermediate (not shown). These results indicate that the small

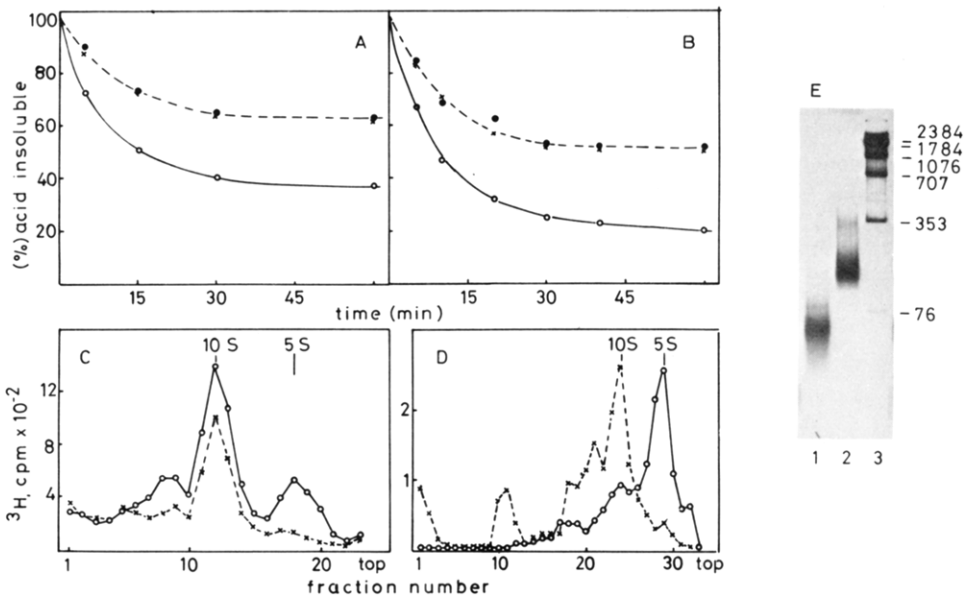

FIGURE 2: Nuclease digestion kinetics and sedimentation analysis of nascent chromatin pulse labeled in vivo and in vitro. [^{14}C]Thymidine prelabeled cells were incubated for 30 s with [^{3}H]thymidine. In the case of in vitro synthesis, isolated nuclei were pulse labeled with [^{3}H]dTTP for 1 min. Chase incubations were performed as described under Materials and Methods. Both labeled nuclei were transferred into nuclease buffer and then digested with micrococcal nuclease. After several time points, acid solubility was determined as described earlier (Schlaeger & Klempnauer, 1978). (A) In vivo pulse (2×10^7 nuclei/30 units of nuclease); (B) in vitro pulse (6×10^7 nuclei/60 units of nuclease); (○) ^{3}H-pulse-labeled DNA; (×) ^{3}H pulse–chase label; (●) ^{14}C-prelabeled DNA. The 8-min digestion products were treated with 0.5 M NaCl and centrifuged through sucrose gradients containing 0.5 M salt: (C) in vivo pulse; (D) in vitro pulse; (○) pulse-labeled DNA; (×) pulse–chase label. (E) DNA size analysis by fluorography of the in vivo pulse-labeled 10S and 4–5S fractions (C) on a polyacrylamide gel. Lane 1, 4–5S DNA; lane 2, 10S DNA; lane 3, ^{3}H-labeled Col E_1 *Hae*II fragments.

fraction of salt-sensitive fragments obviously originates from some part of the native chromatin structure.

The amount of salt-sensitive chromatin fragments was significantly increased in newly replicated chromatin. This is shown in Figure 2, where the amounts of nascent 4–5S DNA fragments of lymphocyte chromatin pulse labeled in vivo, as well as in vitro, were analyzed and the nuclease degradation kinetics were investigated.

As expected, newly synthesized DNA was more susceptible to nuclease cleavage than nonreplicating chromatin (Figure 2A,B). A significant portion of in vivo pulse-labeled DNA was associated with salt-sensitive chromatin fragments (Figure 2C). The amount of pulse-labeled 4–5S DNA fragments, however, was much higher when the pulse label was performed in isolated nuclei (Figure 2D), indicating that a large portion of parental chromatin regions at the replication points are subjected to a transient change in structure during replication. The low efficiency of the chromatin maturation process coupled with a reduced DNA replication velocity is probably responsible for the increased amounts of pulse-labeled DNA which are cut to 4–5S fragments in the cell-free DNA synthesis system.

The observed changes in chromatin structure disappeared completely after a short chase period in vivo, as well as in the isolated nuclei (Figure 2A–D).

It should be mentioned that often during a short incubation period in vitro a slower sedimenting peak of nuclease-resistant new DNA was observed in the absence of salt. However, the ratio of these fragments to nascent 11S mononucleosomes is much lower. With increasing incubation time in vitro, the amount of the 4–5S DNA in the absence of salt decreases. After 2.5–5 min of incubation, almost no unusual DNA fragments could be detected in low salt when the ratio of 4–5S DNA to 10S monomers was about 1 in 0.5 M salt (not shown).

DNA fragments obtained from the 4–5S and the 10S fraction were analyzed by polyacrylamide gel electrophoresis followed by fluorography. The calculated length of these

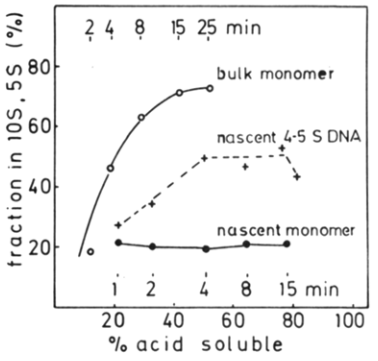

FIGURE 3: Relative accumulation rate of pulse-labeled DNA in the monomer and 4–5S fractions. Nuclei from [^{3}H]thymidine-prelabeled cells were incubated with [^{32}P]dTTP for 1 min and then digested with micrococcal nuclease for 1, 2, 4, 8, 12, and 25 min. Aliquots were treated with EDTA (10 mM final concentration), and the whole nuclear lysates were centrifuged as in Figure 2. The constructed plots were obtained by measuring the amount of radioactivity in the 10S and 4–5S fractions relative to the total radioactivity in each gradient as a function of the also measured acid solubility.

fragments was 55 ± 15 and 145 ± 3 bp, respectively. Mild digestion at 0 °C or nuclease treatment in the presence of 0.35 M salt did not change either the higher ratio of pulse-labeled material in the 4–5S peak to the monomeric DNA or the size of the shorter DNA fragments, excluding the possibility that the shorter DNA fragments arose mainly from exonucleolytic degradation (Weischet et al., 1979).

The released 4–5S DNA fragments appear to be largely free of associated proteins as shown by their gel electrophoretic mobility and their binding capacity to nitrocellulose filters (<10%) as recently described (Schlaeger & Knippers, 1979).

The kinetics of the relative accumulation of pulse-labeled DNA in the 4–5S and 10S fractions have also been measured. Cells were grown for 16 h with [^{3}H]thymidine; nuclei were prepared and incubated in vitro with [^{32}P]dTTP for 1 min and exposed to micrococcal nuclease for various times. Aliquots

of the digested samples were analyzed in a sucrose gradient containing 0.5 M NaCl. The amount of radioactivity measured in the 4–5S and 10S fractions relative to the total radioactivity in each gradient was determined as a function of the concomitant acid solubility (Figure 3). The ^{3}H radioactivity in bulk monomers increased linearly until 60–70% of the total radioactivity was accumulated in the 10S fraction. The production rate of 4–5S bulk DNA fragments was not determined in this experiment. A strikingly different result was observed with the resistant pulse-labeled fractions. Within 1 min of digestion, about 20% of the total ^{32}P radioactivity accumulated in the mononucleosome peak, and this value remained constant during a 25-min digestion when more than 77% of the newly replicated DNA became acid soluble. The quick release of nascent monomers indicates that these nucleosomes were probably differently spaced by more exposed linker regions. This confirms an earlier observation that about 10% of the labeled monomeric DNA fragments of a 60-min in vitro incubation were produced at a much higher rate than the other 90% which were produced with a similar rate than the corresponding fragments from bulk chromatin (Schlaeger & Klempnauer, 1978). The release of pulse-labeled 4–5S DNA fragments from replicating chromatin occurred at a slower rate than that of nonreplicating monomers and increased until 50% of the total ^{32}P label was accumulated in this fraction. This indicates a more heterogeneous structure of the parental chromatin regions close to the replication points.

In summary, the results of the sedimentation studies of nuclease-digested chromatin indicate that the parental chromatin regions associated with pulse-labeled DNA are altered in structure compared to the maturated chromatin. The appearance of the unusual cleavage pattern of nascent DNA which is associated with salt-sensitive chromatin fragments not only is coupled with the DNA replication process but also was observed (in small amounts) in nonreplicating chromatin.

Reconstitution Analysis with the Nascent 4–5S DNA. There exist at least two possibilities to explain the peculiar salt-sensitive behavior of the nascent chromatin fragments. First, the short size of the 4–5S DNA fragment causes a DNA–protein interaction in a salt-sensitive manner, or the involved chromatin proteins are modified in a manner unknown. However, in the latter case, a salt-stable reassociation of the shorter DNA fragments with unmodified proteins could be possible.

During the course of the nuclease cleavage as more and more DNA becomes acid soluble, an increasing amount of core histones is released from the disintegrated chromatin (Schlaeger, 1981). The free histones are in a native state which allows exogenous DNA to be converted into nucleoprotein complexes in the presence of 0.5 M salt which appear to exhibit a typical nucleosomal structure as tested by several criteria (Schlaeger, 1981). Therefore, the nuclear lysates of extensive nuclease-digested chromatin were used to study the salt stability of the assembly products with the 4–5S DNA fragments. Unlabeled nuclei were extensively digested with micrococcal nuclease and transferred into a lysis-reconstitution buffer. Purified ^{32}P-labeled 4–5S DNA was incubated with the nuclear lysate in low salt, as well as in 0.5 M NaCl. After 30–60 min on ice, the whole reaction mixture was centrifuged in a sucrose gradient with and without 0.5 M NaCl. Most of the 4–5S DNA fragments formed nucleoprotein structures which sedimented at 11 S in low salt concentration, like bulk chromatin subunits (Figure 4A). However, in the presence of 0.5 M NaCl, the shorter DNA fragments appear to be unable to form stable nucleoprotein complexes, since most of the radioactivity

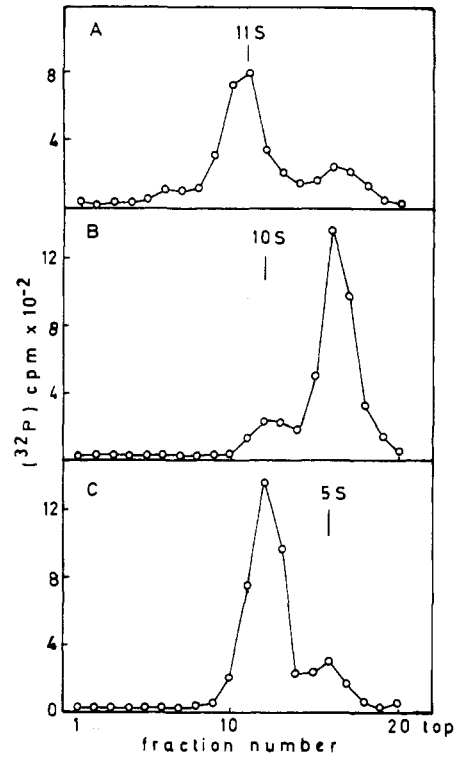

FIGURE 4: Reconstitution of nucleoprotein complexes using 4–5S DNA and 10S nucleosomal DNA. Unlabeled nuclei (2×10^{7}) were digested with micrococcal nuclease to 30% acid solubility and the nuclei lysed in appropriate buffer. Purified ^{32}P-labeled 4–5S DNA was added to the nuclear lysates with low salt and in the presence of 0.5 M NaCl. After 30-min incubation on ice, the samples were centrifuged without salt (A) and in 0.5 M NaCl (B). (C) ^{32}P-labeled monomeric DNA (165 bp) reconstituted and centrifuged in 0.5 M NaCl.

was found unbound in the 4–5S fraction of the gradient (Figure 4B). A control experiment with ^{32}P-labeled monomeric DNA yielded reconstitution products of 10S particles which were stable in high salt concentration (Figure 4C). It is apparent that the salt-stable electrostatic interaction between DNA and the chromatin protein complexes depends on the length of the DNA fragment used, assuming that the salt-sensitive behavior of degraded chromatin may be a consequence of the shorter DNA fragment size in these complexes.

Recentrifugation of 11S Mononucleosomes. In the following experiment, evidence will be presented that the pulse-labeled 4–5S DNA fragments arose from 11S chromatin fragments by dissociation of the nucleoprotein complexes after treatment with 0.5 M salt. This is shown with the help of the reconstitution approach as described above. The experiment was based on the following assumption: when salt-sensitive chromatin particles are present in the 11S mononucleosome peak, the shorter DNA fragments should be released after treatment with salt. Furthermore, exogenously added monomeric DNA fragments could be complexed in part by the released proteins to form salt-stable nucleoprotein complexes sedimenting at 10 S (see Figure 4C).

Nuclei were incubated with [^{32}P]dTTP for 1.5 min and subjected to micrococcal nuclease digestion until about 40–45% of the nascent DNA became acid soluble. After centrifugation through a low salt sucrose gradient, the 11S particles were isolated, divided into two equal samples, and mixed with purified mononucleosomal [^{3}H]DNA (165 bp). After a 30-min incubation on ice, without salt and in the presence of 0.5 M salt, the reaction mixtures were analyzed in a sucrose gradient with and without salt. A large fraction of ^{32}P-labeled 4–5S DNA fragments was released from the 11S particles in 0.5

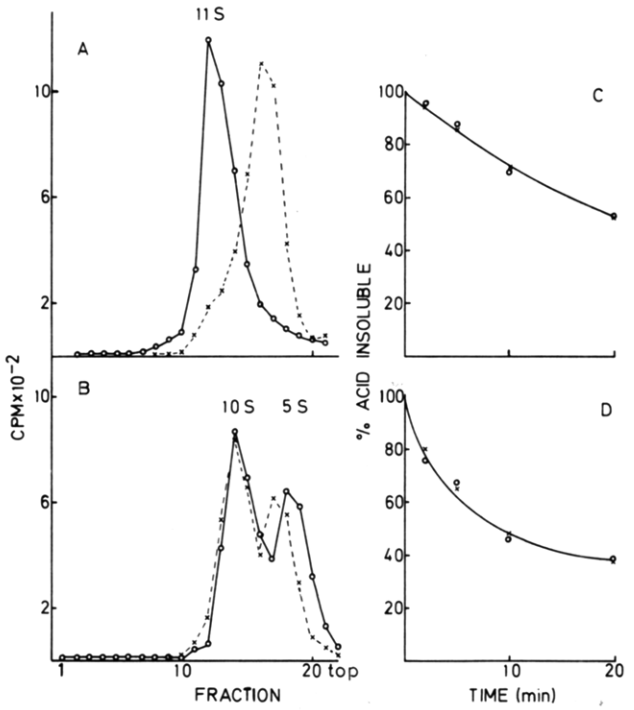

FIGURE 5: Recentrifugation of 11S mononucleosomes associated with nascent DNA. Nuclei (6×10^7) were pulse labeled in vitro with [^{32}P]dTTP for 1.5 min and then digested with micrococcal nuclease (75 units) to 48% acid solubility. After centrifugation through a sucrose gradient containing low salt, the 11S monomer fraction was isolated and then incubated with ^{3}H-labeled nucleosomal DNA fragments (165 bp) for 30 min at 0 °C without salt and in 0.5 M NaCl followed by recentrifugation without salt (A) and in 0.5 M NaCl (B). (○) ^{32}P-Labeled DNA; (×) ^{3}H-labeled monomeric DNA. Nuclease digestion kinetics of the recentrifuged mononucleosomes, shown in panels A and B, were performed in a parallel experiment with ^{3}H-labeled monomers from prelabeled cells. (C) Degradation of 11S nucleosomes; (D) degradation of 10S nucleosomes; (○) ^{32}P-pulse-labeled DNA; (×) ^{3}H-prelabeled DNA.

M salt (Figure 5B), but this was not observed in the absence of salt (Figure 5A). This result correlates with the sedimentation behavior of the added [^{3}H]DNA. At low ionic strength, the [^{3}H]DNA sedimented mainly in the position of free DNA with a tail in the 11S region, whereas in 0.5 M salt a larger portion of [^{3}H]DNA cosedimented with the 10S nucleosomes as nucleoprotein complexes. This observation clearly demonstrates that salt-sensitive chromatin fragments from the 11S peak dissociate in 0.5 M NaCl into the unusual pulse-labeled 4–5S DNA fragments and chromatin proteins (probably histones) which could be used to assemble monomeric DNA to 10S nucleoprotein complexes.

Degradation kinetics of the 11S and 10S nucleosomes after recentrifugation (Figure 5C,D) revealed that the nuclease susceptibility of pulse-labeled DNA is indistingusihable from that of the bulk DNA, indicating further that the shorter DNA fragments in the 11S fraction are not unspecifically associated with monomers.

So that the protein composition of the salt-sensitive chromatin fragments could be analyzed, a larger DNA was used. When the phage SPP1 DNA (M_r 25×10^6) was substituted for the short nucleosomal DNA fragments into the salt-treated 11S fraction, containing unlabeled mononucleosomes, it was possible to separate the assembly products by sucrose gradient centrifugation as shown in Figure 6.

The SPP1–nucleoprotein peak and the 10S mononucleosomes were isolated and analyzed by gel electrophoresis. The protein content of SPP1–chromatin, which reflects mainly the salt-sensitive nonreplicating chromatin fragments, was

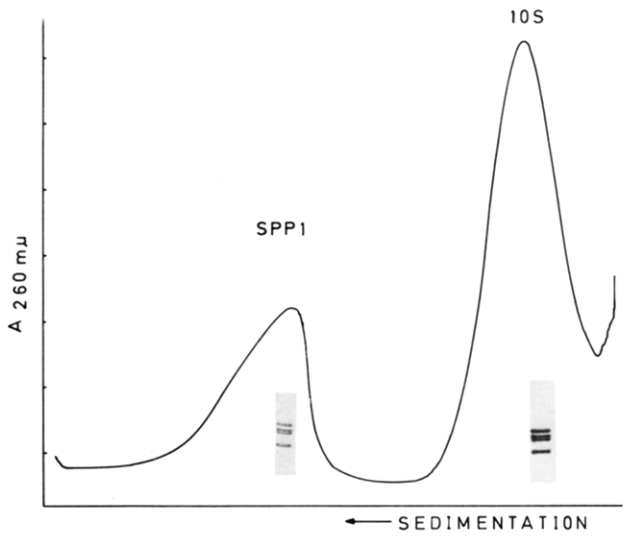

FIGURE 6: Protein analysis of salt-sensitive nucleosomes. Unlabeled nuclei were digested, and the 11S nucleosomes were prepared as described in Figure 5. The concentrated fraction was adjusted to 0.5 M NaCl and then incubated with SPP1 DNA (80 μg) on ice, followed by centrifugation in the presence of 0.5 M salt. The separated SPP1–nucleoprotein complexes and the 10S fraction were dialyzed, lyophilized, and subjected to 15% sodium dodecyl sulfate gel electrophoresis (insert).

identical with that of salt-stable 10S nucleosomes of the same gradient. All four core histones were detectable in equimolar amounts (Figure 6). The same result was obtained by using acid–urea gels according to Panyim & Chalkley (1969) (not shown).

The result of these experiments supported the earlier assumption that the salt-sensitive structure arose from altered DNA–histone interactions of nucleosomal structure (Schlaeger & Knippers, 1979).

Heterogeneity of Pulse-Labeled 11S Nucleosomes. Mononucleosomes produced after micrococcal nuclease digestion are heterogeneous in DNA size and protein composition, most notably in content of histone and high-mobility group (HMG) proteins 14 and 17 (Albright et al., 1980). The structural heterogeneity of various species is limited to five to six distinct components at low ionic strength [for a review, see McGhee & Felsenfeld (1980)].

In the following investigation, the electrophoretic mobility of the 11S monomers with pulse-labeled DNA was analyzed. First, the heterogeneity of lymphocyte chromatin subunits was determined after varying digestion times with micrococcal nuclease. Mononucleosomes from a low salt sucrose gradient were subjected to gel electrophoresis under nondenaturing conditions, followed by staining with ethidium bromide. Figure 7A shows that the degree of heterogeneity depends on the extent of chromatin degradation.

The different nucleosome bands were termed M0, MI, MII, MIII, MIV, and MV in order of decreasing mobility following the nomenclature of Todd & Garrad (1979). When less than 10% of the DNA was acid soluble, monomers were produced which migrated in the gel as a broad band around the position of band MIII. After more extensive digestion, when more than 20% of the DNA was rendered acid soluble, the homogeneous peak of mononucleosomes was resolved into five to six discrete bands. All nucleosomal fractions were represented when more than 60% of the DNA was degraded.

A short characterization of nucleosome bands is given in Figure 7B. The DNA size and the histone components obtained from lymphocyte mononucleosomes confirm results described by others (Albright et al., 1980).

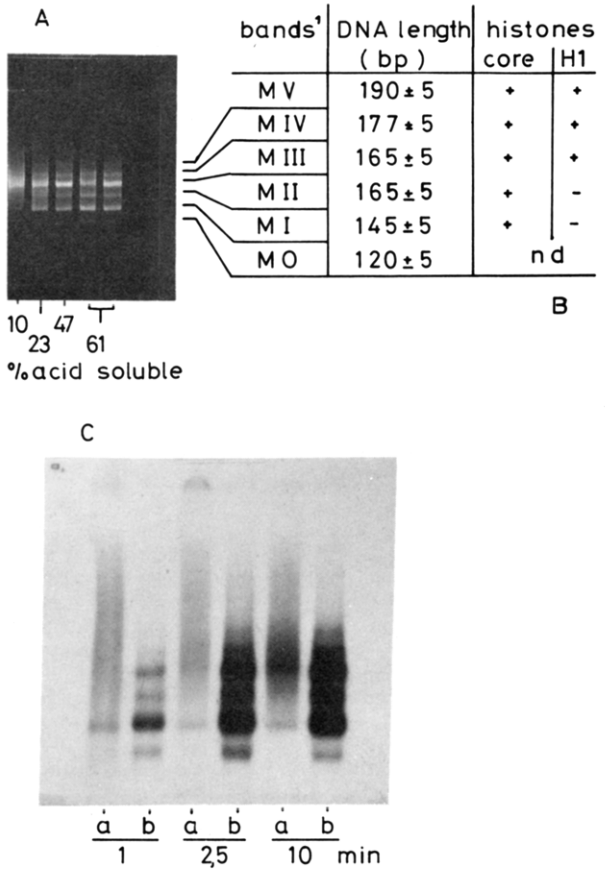

FIGURE 7: Separation of 11S mononucleosomes by polyacrylamide gel electrophoresis. Unlabeled nuclei were digested with micrococcal nuclease for 2, 6, 12, and 20 min, and mononucleosomes were prepared as in Figure 5. The 11S monomers were concentrated and analyzed on a 5% polyacrylamide gel under nondenaturating conditions followed by ethidium bromide staining (A). Slices of the monomer bands from nonstained parallel lanes were incubated with 5% sodium dodecyl sulfate for 15 min at 37 °C and analyzed on a 5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate for DNA size determination and on a 15% sodium dodecyl sulfate–polyacrylamide gel, respectively, to analyze the protein composition (B). Nuclei were pulse labeled with [^{32}P]dTTP for 1, 2.5, and 10 min. After nuclease digestion, 11S mononucleosomes were prepared and subjected to gel electrophoresis. The gel was stained with ethidium bromide followed by autoradiography (C): (a) digestion for 1 min at 37 °C and 30 min at 0 °C; (b) digestion for 8 min at 37 °C. The nomenclature of the mononucleosomes (seen in panel B) is after Todd & Garrad (1979).

Next, the mobility of mononucleosomes associated with pulse-labeled DNA was analyzed. Nuclei were incubated with [^{32}P]dTTP for 1, 2, 5, and 10 min and subjected to nuclease treatment until 6–40% of the labeled DNA became acid soluble. The digested samples were centrifuged in a sucrose gradient, and the isolated 11S mononucleosome fractions were analyzed by gel electrophoresis. The gel was subjected to autoradiography. As shown, newly replicated DNA comigrates with the nucleosomal bands (Figure 7B). No changes in the bands were observed. However, pulse-labeled DNA after a 1-min synthesis differed somewhat from bulk nucleosomes independent of the extent of degradation. The 11S chromatin particle carrying nascent DNA migrated somewhat slower than maturated fragments after short nuclease incubation, indicating a higher protein:DNA ratio. This may be due to the observation that nascent chromatin fragments have a slightly lower buoyant density than bulk chromatin (Seale & Simpson, 1975; Murphy et al., 1980; Jackson & Chalkley, 1981a,b). After extensive digestion, nascent DNA was found predominantly in the monomer band MI. This fraction is characterized by a DNA piece of 145 bp in length and the absence of the histone H1 and HMG proteins (Albright et al., 1980). The fact that only small amounts of the pulse label were found in bands with histone H1 suggests that the parental chromatin regions close to the replication points are not associated with H1 in a state similar to that of bulk chromatin. Furthermore, the HMG proteins, if present, appear to be more weakly bound during replication. ^{32}P-Labeled mononucleosomes prepared after a 10-min incubation exhibited no significant differences compared to nonreplicating chromatin subunits.

Discussion

In this work, experiments are described in which altered DNA–protein interactions close to the replication points were investigated. During micrococcal nuclease digestion of pulse-labeled nuclei, a large fraction of chromatin fragments are released from nascent chromatin which are associated with shorter (55 ± 15 bp) than usual (165 bp) DNA fragments. The unusual DNA fragments are released from the nucleoprotein complexes in 0.5 M salt probably as a consequence of their smaller size. The histone content of the salt-sensitive nucleosome-like structures did not appear to differ from that of the salt-stable particles. It is therefore suggested that during the transfer of parental chromatin to the daughter duplex DNA, the nucleosomes are transiently changed in structure possibly by exposure of parts of the protected nucleosomal DNA in a manner unknown. It should be mentioned, however, that the conformational transition of nucleosomes was also observed for a small fraction of nonreplicating chromatin fragments, indicating that this altered structure is not confined to the replicating chromatin at the growing points alone. This makes it unlikely that proteins which are involved specifically in DNA replication are responsible for the observed unusual cleavage pattern. This assumption is supported by experimental results showing that nuclei extracted with 0.5 M salt before nuclease digestion to remove a large amount of nonhistone protein from the chromatin exhibited the same amount of altered DNA–protein interaction as the control (E.-J. Schlaeger and R. L. Seale, unpublished experiments).

Additionally, the conclusion that the altered structure of newly replicated chromatin was indeed a transient state during replication arose from experiments which showed that the maturation process to bulk chromatin structure depends strongly on continuous DNA synthesis (E.-J. Schlaeger and W. Pülm, unpublished results).

At this point, it is not known why newly replicated chromatin acquires an altered structure leading to an increased level of nuclease cleavage cuts relative to bulk chromatin during digestion. However, an interesting relationship to the changed DNA–protein interaction of nascent chromatin arose from the observation that a large portion of pulse-labeled DNA (if not all) is attached to the nuclear matrix in several eukaryotic cell systems (Berezney & Coffey, 1975; Dijkwel et al., 1979; Pardoll et al., 1980; Vogelstein et al., 1980; Hunt & Vogelstein, 1981). With increasing times of synthesis, the pulse-labeled DNA was released from the attachement sites. It is suggested that the involvement of the nuclear matrix with eukaryotic DNA replication is a general one (Hunt & Vogelstein, 1981). A model was proposed in which the replication complexes or the replicating DNA, probably at the growing points, is attached at fixed sites on the nuclear matrix, and the DNA is reeled through these association points as it is duplicated (Pardoll et al., 1980).

Concerning the change of chromatin structure during replication, it is possible that during the association of nascent

chromatin to nuclear matrix the pulse-labeled DNA could lose, in part, the nucleosomal protection, i.e., by fewer turns around the histone octamer complexes or by more exposed sites on DNA in this structure to nuclease cleavage. Clearly, other models could be advanced.

Polyacrylamide gel electrophoresis resolved the homogeneous 11S peak of mononucleosomes into five to six distinct bands dependent on the state of the chromatin degradation (see Figure 7). Within 1 min, nascent monomers including the altered chromatin fragments comigrated with these bands. No different bands or smears have been observed. Based on the migration behavior, which is determined by the protein composition and length of the DNA fragments (Todd & Garrad, 1979), more than one 4–5S fragment has to be associated with these nucleoprotein particles. A large portion of pulse-labeled DNA was accumulated in the monomer band MI which possesses no histone H1 and no HMG proteins (Albright et al., 1980), indicating that this fraction arose from nascent chromatin on which these proteins are more weakly bound. Small amounts of radioactivity, however, migrated with these components due to MII, MIII, and MIV.

The alteration of the histone H1 binding on nascent chromatin agrees with results of sedimentation studies with pulse-labeled DNA (Worcel et al., 1978; E.-J. Schlaeger and W. Pülm, unpublished results), showing that chromatin subunits associated with new DNA were released at a significantly faster rate from the native chromatin than bulk nucleosomes. Noll & Kornberg (1977) and Weischet et al. (1979) have shown that in histone H1 depleted nuclei the monomer production was significantly faster. Recently, Renz and coworkers (Renz et al., 1977) have shown that the monomer fraction produced under limited digest conditions contains only small amounts of H1, but the level of associated histone H1 increased with increasing amounts of multimeric nucleosomes. Due to the possible role of the outer histone H1 for the maintenance of the chromatin superstructure (Thoma et al., 1979), it is reasonable to suggest that the altered form of histone H1 binding on nascent chromatin could induce a less compact structure of replicating chromatin. Possibly a more unfolded structure of parental chromatin could be an important requirement for the regular DNA replication, i.e., for the attachment to the nuclear matrix site.

The observation that a small amount of HMG proteins is also associated with nascent chromatin fragments (bands MII, MIII, and MIV) indicates further that very early after the replication fork has passed a transcriptionally active chromatin region these essential proteins are rebound (if they have really left the chromatin) to establish again the active structure (Bakayev et al., 1979; Weisbrod & Weintraub, 1979; Albanese & Weintraub, 1980; Weisbrod et al., 1980). The rapid reactivation of the structural properties of the transcriptional chromatin region after replicating has also been proposed by others (Weintraub, 1979; Annunziato et al., 1981). However, direct evidence was obtained from electron microscopic analysis showing nascent transcripts very close to the replication points (McKnight & Miller, 1979).

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Characterization of in Vitro Deoxyribonucleic Acid Breakage and Cross-Linking Induced by Bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV)†

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ABSTRACT: Bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV) (CHIP or JM-9), a derivative of Cisplatin, was found to have DNA breakage and interstrand cross-linking activities in vitro. DNA breakage was detected by alkaline and neutral sucrose gradient analysis, agarose gel electrophoresis, and alkaline ethidium bromide fluorescence assay employing covalently closed circular PM2 DNA. DNA cross-linking activity was detected by alkaline sucrose gradient analysis and by the "snap-back" assay employing PM2 DNAs. Non-sulfhydryl-containing reducing agents, e.g., $NaBH_4$ and NADPH, stimulated both cross-linking and breakage activities. Alkaline buffers, cyanide, or sulfhydryl group containing agents inhibited both types of activities. The hydroxyl free radical scavenger sodium benzoate (100 mM) was found to inhibit 99% and 25% of DNA breakage and cross-linking activities, respectively, suggesting DNA breakage and cross-linking may be independently mediated.

Platinum coordination complexes have been shown to be active antitumor agents in many in vitro and in vivo systems (Rosenberg et al., 1969; Prestayko et al., 1979; Cleare et al., 1978). The mechanism of cytotoxicity of platinum complexes has been attributed to their interaction with tumor cell DNA, since it was found that the compound *cis*-diamminedichloroplatinum(II) (CDDP)[1] can bind to cellular DNA and inhibit cellular DNA biosynthesis (Rosenberg et al., 1969; Roberts & Thomson, 1979). Using isolated purified covalently closed circular (CCC) DNA, Cohen et al. (1979) and this laboratory reported that CDDP induced tertiary conformational changes in CCC DNA (Mong et al., 1980a). CDDP apparently binds to the CCC DNA in vitro causing intrastrand cross-linking and results in an unwinding and then subsequent rewinding-like phenomenon as evidenced by the reversal of the DNA viscosity and the gel electrophoretic mobility (Macquet & Butour, 1978; Mong et al., 1980a,b, 1981). DNA interstrand cross-linking induced by CDDP was also detected (Horaček & Drobnik, 1971; Macquet & Theophonides, 1975; Pascoe & Roberts, 1974a,b). It was concluded, however, that CDDP does not induce DNA breakage and that DNA intrastrand cross-linking is probably the predominant mode of DNA binding in vitro (Mong et al., 1980a; Kleinwachter, 1978; Roberts & Thomson, 1979).

When the so-called "second generation platinum analogues" were examined, several octahedral platinum(IV) compounds were shown to induce DNA breakage (Mong et al., 1980b) and DNA interstrand cross-linking in vitro (Mong et al., 1981) instead of intrastrand cross-linking, DNA shortening, unwinding, and rewinding as did CDDP and other platinum(II) analogues. Since DNA degradation has been considered an important mechanism of action of many antineoplastic agents and CDDP has *not* been shown to induce DNA breakage in vitro or in vivo, platinum(IV) compounds may represent a distinct class of platinum drugs potentially having a different mechanism of action from CDDP. The purposes of this study were to characterize the mechanism(s) of DNA breakage and cross-linking induced by CHIP and to determine whether the primary types of damage induced by CHIP are different from those induced by CDDP, i.e., DNA shortening, unwinding, and rewinding. Using several highly sensitive assay systems tailored for each type of DNA interaction, we have also investigated the conditions and cofactors that either stimulated or inhibited the DNA breakage and cross-linking effects. The results suggest that the mechanism(s) of action of CHIP is

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[1] Abbreviations: EB, ethidium bromide; CDDP, *cis*-diamminedichloroplatinum(II) or Cisplatin; CHIP or JM-9, bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV); JM-28, bis(isopropylamine)-*trans*-(dihydroxymalonato)platinum(IV); Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; 2ME, 2-mercaptoethanol; CCC PM2 or form I PM2 DNA, covalently closed circular supercoiled PM2 DNA; form II, single-strand broken circular form PM2 DNA; form III, double-strand broken linear duplex form PM2 DNA; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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