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Structure of Chromatin at Deoxyribonucleic Acid Replication Forks: Nuclease Hypersensitivity Results from both Prenucleosomal Deoxyribonucleic Acid and an Immature Chromatin Structure†

Michael E. Cusick,‡ Keun-Su Lee,§ Melvin L. DePamphilis, and Paul M. Wassarman*

ABSTRACT: Relative to nonreplicating DNA in mature simian virus 40 (SV40) chromosomes, newly synthesized DNA in replicating SV40 chromosomes was found to be hypersensitive to the nonspecific endonucleases, micrococcal nuclease (MNase), DNase I, and DNase II. Nascent DNA, pulse labeled in either intact cells or nuclear extracts supplemented with cytosol, was digested about 5-fold faster and about 25% more extensively than uniformly labeled DNA in mature viral chromosomes. Pulse–chase experiments in vitro revealed a time-dependent chromatin maturation process that involved two distinct steps: (i) conversion of prenucleosomal DNA (PN-DNA) into immature nucleosomal oligomers and (ii) maturation of newly assembled chromatin into a structure with increased nuclease resistance. PN-DNA was hypersensitive to MNase, releasing short DNA fragments which were subsequently solubilized by the nuclease. However, when the nascent PN-DNA was specifically removed by digestion of replicating viral chromosomes with *Escherichia coli* exonuclease III (3′–5′) and phage T7 exonuclease (5′–3′), subsequent digestion of the remaining chromatin with MNase revealed the same degree of hypersensitivity observed prior to exonuclease treatment. Furthermore, newly assembled nucleosomal oligomers, isolated after a brief MNase digestion of replicating viral chromosomes, were also hypersensitive to MNase relative to oligomers isolated from mature chromosomes. Hybridization analysis of the DNA in these immature oligomers revealed that it originated from both sides of replication forks. Inhibition of DNA polymerase α by aphidicolin inhibited conversion of PN-DNA into nucleosomes but did not inhibit loss of nucleosomal hypersensitivity to MNase. In contrast, components in the soluble fraction of the subcellular system ("cytosol") were required for both DNA replication and chromatin maturation. Analysis of the nucleoprotein products from a MNase digestion of replicating and mature SV40 chromosomes failed to detect a change in nucleosome structure that corresponded to the loss of nuclease hypersensitivity. However, the results presented demonstrate that *both* PN-DNA and newly assembled immature chromatin, present on both arms of SV40 replication forks, contribute to the commonly observed hypersensitivity of newly replicated chromatin to endonucleases.

Since eukaryotic chromatin structure varies as a function of gene activity (Weisbrod, 1982) and DNA sequence (Wu et al., 1979; Wigmore et al., 1980; Bryan et al., 1981; Cremisi, 1981; Herbomel et al., 1981), it seems likely that chromatin structure greatly influences where and when transcription, replication, and, perhaps, recombination occur. Therefore, to preserve these structural landmarks, chromosome replication presumably requires accurate duplication of protein organization, as well as DNA sequence (Weintraub, 1979). To understand the mechanisms involved in this duplication process requires detailed knowledge of the assembly pathway at DNA replication forks.

Chromatin assembly primarily involves the organization of nucleosomes containing a minimum of 145 base pairs (bp)[1]

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‡ Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.
§ Present address: Department of Chemistry, University of Wisconsin, Madison, WI 53706.

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of DNA coiled around an octamer of histones consisting of two each of histones H2A, H2B, H3, and H4; mature chromatin can also contain histone H1 and nonhistone proteins, and protein modification such as phosphorylation and acetylation can occur (Cremisi, 1979; DePamphilis & Wassarman, 1980, 1982; McGhee & Felsenfeld, 1980). In this connection, it has been found that newly replicated mammalian and papovavirus DNA is rapidly assembled into chromatin with nucleosome-like structures on both arms of replication forks within an average of 125 bp from the sites of DNA synthesis (McKnight & Miller, 1977; Shelton et al., 1978a; Cremisi et al., 1978; Seidman et al., 1978; Schlaeger & Knippers, 1979; Herman et al., 1979, 1981; Cusick et al., 1981; Tack et al., 1981). However, despite the rapid appearance of nucleosome-like structures, newly assembled chromatin is hypersensitive to various endonucleases, suggesting that a maturation process follows nucleosome assembly (Hildebrand & Walters, 1976; Hewish, 1977; Levy & Jakob, 1978; Seale, 1975, 1978; Schlaeger & Knippers, 1979; Klempnauer et al., 1980; Yakura & Tanifuji, 1980). Replicating chromosomal DNA is digested more rapidly and to a greater extent than DNA in nonreplicating chromatin, releasing small (3–7S) nascent DNA fragments that are subsequently digested completely. However, the interpretation that chromatin maturation is attributable to the presence of immature nucleosomes is complicated by the fact that replicating chromosomes contain a significant amount of nucleosome-free DNA at the sites of DNA synthesis (prenucleosomal DNA, PN-DNA) that is extremely sensitive to nucleases (Herman et al., 1979, 1981; Cusick et al., 1981). It is conceivable that PN-DNA alone, and not the nascent nucleosomes, could account for the behavior of replicating chromatin in the presence of endonucleases. PN-DNA, which is unique to replicating chromatin, is rapidly released by MNase as small DNA fragments that are then digested further. Therefore, nascent nucleosomes may be released more rapidly by MNase simply because they are close to the fork (i.e., PN-DNA), and the smaller DNA digestion products may represent PN-DNA rather than degraded immature nucleosomes. When the radiolabeling period is increased or a pulse–chase protocol is used, the fraction of radiolabel in PN-DNA would diminish and the newly synthesized chromatin would mature in terms of its nuclease sensitivity.

In this report, we have used replicating and nonreplicating mature SV40 chromosomes to demonstrate that the process of chromatin maturation involves *both* assembly of PN-DNA into nucleosomes and the subsequent conversion of immature chromatin into mature chromatin. These two components of chromatin maturation were distinguished from one another by (i) excision of nascent DNA from the PN-DNA region with exonucleases leaving nascent, nucleosomal DNA behind and (ii) isolation of nucleosomal dimers and trimers from replicating and mature viral chromosomes. In each case, nascent nucleosomes were found to be hypersensitive to the nonspecific endonucleases, MNase, DNase I, and DNase II, relative to nucleosomes associated with nonreplicating chromatin. Immature nucleosomes were present on both arms of replication forks, but chromatin maturation could not be readily correlated with a change in the nucleoprotein MNase digestion products.

Experimental Procedures

Cells and Virus. Simian virus 40 wild-type 800 (Mertz & Berg, 1974) was grown at a low multiplicity of infection and used to infect a CV-1 African Green monkey kidney cell line at a multiplicity of infection sufficient to give the maximum rate of viral DNA replication at 36-h postinfection. The conditions for growth of both cells and virus have been previously described (Wilson et al., 1976; Anderson et al., 1977).

Radiolabeling of Replicating and Mature SV40 Chromosomes. (*a*) *In Intact Cells.* Before SV40 chromosomes were isolated from infected cells, mature SV40 chromosomes containing SV40(I, II) DNA were radiolabeled by incubating infected cells with [^{14}C]thymidine (0.5 μCi/mL, 50 mCi/mmol; Schwarz/Mann) from 24–36-h postinfection (Herman et al., 1979, 1981). Replicating SV40 chromosomes were briefly labeled with [^{3}H]thymidine at 36–38-h postinfection, when the rate of viral DNA synthesis was maximal, by first incubating cells with [^{3}H]thymidine (167 μCi/mL, 85 Ci/mmol; New England Nuclear) for 10 min at 0 °C to maximize the specific radioactivity of dTTP pools in the absence of DNA synthesis (Perlman & Huberman, 1977; Herman et al., 1979). The plates of cells were then shifted to 30 °C for 1–2 min to allow DNA replication to continue. Replication was stopped by floating the dishes in ice–water and washing the cells with two 5-mL aliquots of ice-cold 15 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and 25 mM NaCl. Chromosomes were then prepared as described under In Nuclear Extracts.

(*b*) *In Nuclear Extracts.* Nuclear extracts containing both mature and replicating SV40 chromosomes were pepared from SV40-infected cells at 36–38-h postinfection by using the hypotonic extraction procedure of Su & DePamphilis (1976, 1978), but with only half the volume of hypotonic solution [10 mM Hepes (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol). Extracts were routinely prepared from cells radiolabeled with [^{3}H]thymidine (2.5 μCi/mL, 85 Ci/mmol; New England Nuclear) from 24–36-h postinfection. Replicating SV40 chromosomes were then labeled with [α-^{32}P]dCTP (200–400 Ci/mmol; New England Nuclear) for 1–5 min at 30 °C in the presence of added cytosol (Su & DePamphilis, 1976, 1978; Shelton et al., 1980; Cusick et al., 1981). To stop further incorporation of radiolabel, a 100-fold excess of unlabeled dCTP was added, and the reactions were continued at 30 °C for up to 1 h. In some cases, [α-^{32}P]dNTPs were omitted, and only the fate of SV40(RI) [^{3}H]DNA, prelabeled in intact cells, was followed; no incorporation of residual [^{3}H]thymidine was observed in vitro.

To stop replication, samples were placed on ice, and EDTA was added to a final concentration of 10 mM. SV40 replicating and mature chromosomes were routinely fractionated by sedimentation through a linear 5–30% sucrose gradient containing either 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 200 mM NaCl or hypotonic extraction buffer with 200 mM NaCl (Herman et al., 1979). Gradients were fractionated from the top to avoid contamination with virions in the pellet. Fractions containing mature or replicating SV40 chromosomes were pooled and then concentrated by pressure dialysis (Spectrapor membrane) under N_2 gas against 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA in preparation for digestion by various nucleases. DNA concentrations of chromosomal samples were routinely determined by a modification (Shelton et al., 1978b) of a standard fluorometric assay (Kissane & Robbins, 1958).

[1] Abbreviations: SV40, simian virus 40; SV40(I) DNA, covalently closed, superhelical mature form of viral DNA; SV40(II) DNA, duplex circular viral DNA containing at least one single-strand interruption; SV40(RI) DNA, normal replicating intermediates of SV40 DNA; bp, base pairs; EDTA, sodium ethylenediaminetetraacetate; Exo III, *Escherichia coli* exonuclease III; T7 Exo, *E. coli* bacteriophage T7 gene 6 exonuclease; MNase, micrococcal nuclease; DNase I, pancreatic deoxyribonuclease; DNase II, spleen deoxyribonuclease; PN-DNA, prenucleosomal DNA; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

(*c*) *In Nucleoprotein Complexes.* Experiments in which the contribution of cytosol was evaluated were carried out with SV40 nucleoprotein complexes prepared by centrifuging a nuclear extract at 50000 rpm for 50 min in a Beckman SW60 rotor. The supernatant (nucleosol) was discarded, and the pellet (nucleoprotein complexes) was resuspended in 0.02 mL of hypotonic solution per original 100-mm diameter dish of cells (Su & DePamphilis, 1978). The nucleoprotein complexes were then mixed either with cytosol or with 20 mM Hepes (pH 7.8), 50 mM potassium acetate, 0.5 mM $MgCl_2$, 0.25 mM dithiothreitol, and all chemical components essential for in vitro replication (Su & DePamphilis, 1978). Potassium acetate was substituted for KCl because Cl^- inhibits DNA replication in vitro (Richter et al., 1980). DNA replication and radiolabeling procedures were then identical with those described for nuclear extracts. Replication was stopped, and SV40 chromosomes were purified and concentrated as described above.

Preparation of Cytosol. Cytoplasm left over from the preparation of nuclei for the nuclear extract was centrifuged at 100000*g* for 60 min, and potassium acetate was added to the supernatant (cytosol) to a final concentration of 50 mM. Cytosol was used either immediately or stored at −70 °C for up to 6 months with no apparent loss of activity.

Endonuclease Digestion of SV40 Chromosomes. (*a*) *Micrococcal Nuclease* (*MNase*). SV40 chromosomes (5–10 μg of DNA/mL) were incubated with MNase (Worthington; 0.1 unit/μg of DNA) at 37 °C in a 0.65 mL reaction volume containing 10 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, and 1 mM $MgCl_2$. When the viral DNA concentration was less than 1–2 μg/mL (e.g., with isolated nucleosomal oligomers), unlabeled CV-1 cell chromatin (Shelton et al., 1980) was added to the digestion at 5 μg/mL. Acid-insoluble radioactivity was determined at various times during the digestion by adding an aliquot of the reaction mixture to 3 mL of 1 N HCl and 0.5% sodium pyrophosphate. After 10 min on ice, precipitates were collected on Whatman GF/C filters, washed with cold 1 N HCl, 0.5% sodium pyrophosphate, and then ethanol. The filters were dried, and their radioactivity was measured in a standard toluene-based liquid scintillation cocktail. When nucleoprotein products were to be analyzed by velocity sedimentation, the digestion was stopped with 10 mM EDTA and then the sample layered directly onto a neutral sucrose gradient. Sedimentation conditions are described below.

(*b*) *Deoxyribonuclease I* (*DNase I*). SV40 chromosomes (5–10 μg of DNA/mL) were incubated at 37 °C with 0.2 unit/μg of DNA of DNase I (Sigma) in a 0.65 mL reaction volume containing 10 mM Tris-HCl (pH 7.5) and 3 mM $MgCl_2$ (Shelton et al., 1980). Acid-insoluble radioactivity was determined at various times during the digestion as described above for MNase.

(*c*) *Deoxyribonuclease II* (*DNase II*). SV40 chromatin (5–10 μg/mL) was incubated at 37 °C in a 0.65 mL reaction volume containing 10 mM Tris-HCl (pH 7.5) and either 100 units of DNase II (Sigma DN-II-HP) per μg of DNA and 0.01 mM EDTA or 1000 units/μg of DNA and 1 mM $CaCl_2$ (Horz et al., 1980).

Exonuclease Digestion of SV40 Chromosomes. (*a*) *E. coli Exonuclease III* (*Exo III*). SV40 chromosomes (5–10 μg of DNA/mL) were incubated at 20 °C with 40 units of Exo III (New England Biolabs) per μg of DNA in a 0.30 mL reaction volume containing 50 mM Tris-HCl (pH 8.0), 0.26 mM $MgCl_2$, 0.06 mM EDTA, and 1 mM dithiothreitol (Herman et al., 1981). Acid-insoluble radioactivity was determined at various times during the digestion as described above for MNase. When chromosomes were digested first with Exo III and then MNase, the first reaction was stopped with 5 mM EDTA and the mixture filtered through Sephadex G-50 (Pharmacia) in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. SV40 chromosomes recovered in the void volume were then digested with MNase.

(*b*) *Bacteriophage T7 Exonuclease* (*T7 Exo*). The T7 Exo used in these experiments was prepared by the procedure of M. Engler and C. C. Richardson (unpublished results) as previously described (Cusick et al., 1981). SV40 chromosomes (5–10 μg of DNA/mL) were incubated at 20 °C with T7 Exo (16 units/μg of DNA) in a 0.30 mL reaction volume containing 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 0.15 mM $MnCl_2$, 0.05 mM EDTA, and 1 mM dithiothreitol (Herman et al., 1981). Digestions were stopped and analyzed as described above for Exo III. When SV40 chromosomes were digested concurrently with Exo III and T7 Exo, the conditions for T7 Exo were used; Exo III retains full activity under T7 Exo conditions.

Velocity Sedimentation Analysis. Nucleoprotein products of MNase digestions of SV40 chromosomes were routinely analyzed by velocity sedimentation in linear 5–20% neutral sucrose gradients containing 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 50 mM NaCl. Sedimentation was for 14 h at 35000 rpm in a Beckman SW41 rotor at 4 °C. Fractions were collected from the bottom of the tube, and their contents were precipitated by the addition of 3 mL of cold 1 N HCl and then collected on Whatman GF/C filters. Filters were washed with ethanol and dried, and radioactivity was measured in a standard toluene-based liquid scintillation cocktail.

Results

Maturation of Nascent Chromatin in Nuclear Extracts. Nascent DNA in replicating SV40 chromosomes (isolated from virus-infected cells) is digested at a faster rate and to a greater extent by micrococcal nuclease (MNase) than is DNA in mature (nonreplicating) SV40 chromosomes (Klempnauer et al., 1980; Cusick et al., 1981). Since nucleosomes appear rapidly on both arms of eukaryotic replication forks (Herman et al., 1981; Cusick et al., 1981), the MNase hypersensitivity of newly replicated chromatin indicates that completion of chromatin assembly (chromatin "maturation") is a slower process than DNA replication. To examine further the relationship between chromatin maturation and DNA replication, experiments have been carried out in nuclear extracts under conditions shown to support both faithful continuation of DNA replication (Su & DePamphilis, 1978) and assembly of nascent DNA into nucleosome-like structures (Shelton et al., 1978a, 1980; Cusick et al., 1981). As previously reported, with uninfected CV-1 cells no DNA synthesis was observed in nuclear extracts and no radiolabeled DNA sedimented in the position of viral chromosomes. Nuclear extracts allow incorporation of radiolabeled precursors at higher specific activities, permit rapid and extensive reduction of specific radioactivities by unlabeled precursors during a "chase" period, and remove permeability barriers to drugs and other molecules.

In the experiments described here, SV40(RI) DNA in viral chromatin was radiolabeled by two different methods: (i) incubating intact cells (in vivo) for 1 min in the presence of [^{3}H]thymidine (pulse), and then continuing viral DNA replication in a nuclear extract (chase), or (ii) incubating a nuclear extract (in vitro) for 1 min in the presence of [α-^{32}P]dCTP (pulse), and then continuing viral DNA replication in the presence of enough unlabeled dCTP to prevent further incorporation of radiolabel (chase). In neither case was further incorporation of radiolabel observed while SV40(RI) [^{3}H]-

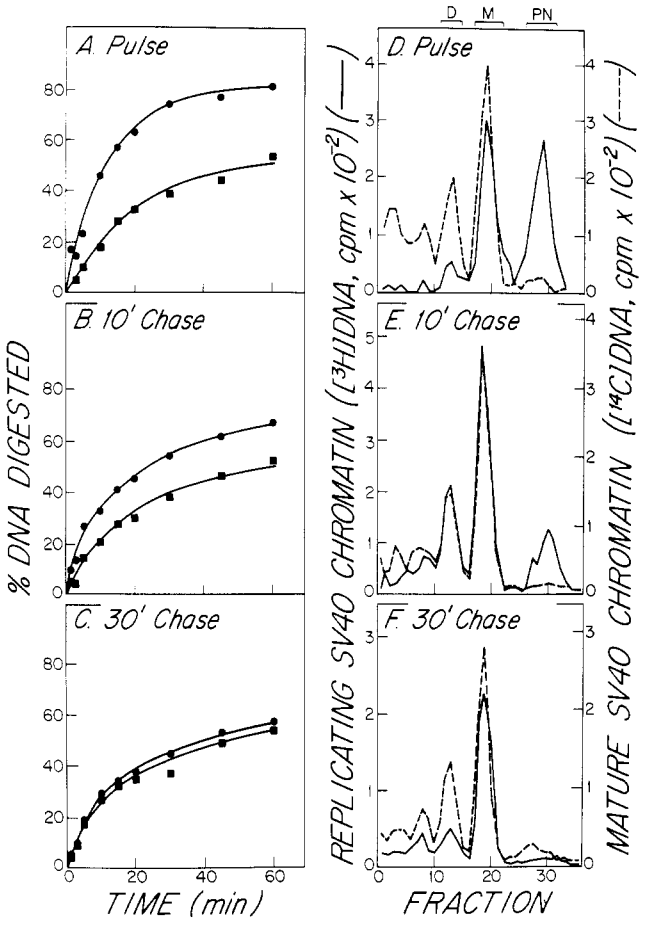

FIGURE 1: MNase digestion of newly replicated and mature SV40 chromatin. Replicating SV40 chromosomes, radiolabeled for 1 min with [^{3}H]thymidine in intact cells, were allowed to continue replication (chase) in a hypotonic nuclear extract supplemented with cytosol for 0 (A, D), 10 (B, E), or 30 min (C, F). The replicating SV40 ^{3}H chromosomes (●) were mixed with mature SV40 ^{14}C chromosomes (■) that had also been labeled in intact cells and then digested with MNase. The fraction of acid-insoluble radioactivity remaining was measured as a function of the time of digestion (A–C). Samples were taken from these digestions for sedimentation analysis of the nucleoprotein products when the fraction of replicating [^{3}H]chromatin (—) that was solubilized was 30% in the 0-min chase experiment (D), 30% in the 10-min chase (E), and 26% in the 30-min chase (F). The fraction of mature [^{14}C]chromatin (---) solubilized in the same reactions was 12%, 16%, and 24%, respectively. Fractions were collected from the bottom of the tube. Nucleosomal monomers are in fractions 19–20, and PN-DNA is in fractions 26–30.

or [^{32}P]DNA completed replication in vitro.

At various times during viral DNA replication, replicating SV40 chromosomes were isolated by sedimentation through neutral sucrose gradients, mixed with mature SV40 chromosomes containing uniformly labeled SV40(I, II) [^{3}H]- or [^{14}C]DNA, and then digested with MNase. Replicating chromosomes, pulse labeled either in vivo (Figure 1A) or in vitro (data not shown), were digested faster (≃2–6-fold) and to a greater extent (≃25%) than mature chromosomes. However, when viral DNA replication was allowed to continue in a nuclear extract (chase), the sensitivity of newly replicated chromatin to MNase diminished until it was comparable to that of mature viral chromatin (Figure 1B,C). The time course of nuclease digestion of replicating chromatin never became coincident with that of mature chromatin (Figure 1C), even when the chase period was extended, suggesting that a small fraction of the chromatin did not mature in vitro. The results shown in Figure 1 were essentially the same by using SV40 chromosomes purified on gradients in either hypotonic solution

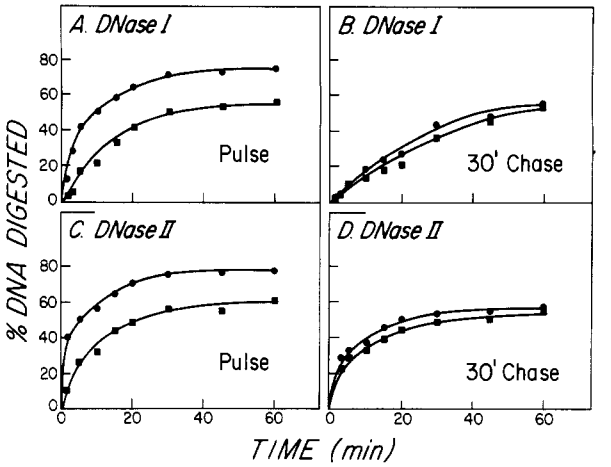

FIGURE 2: DNase I and DNase II digestion of newly replicated and mature SV40 chromatin. Replicating SV40 ^{32}P chromosomes (●), radiolabeled for 1 min with [α-^{32}P]dCTP in a hypotonic nuclear extract supplemented with cytosol [pulse (A, C)], were allowed to continue replication for up to 30 min in the presence of excess unlabeled dCTP [chase (B, D)]. These viral chromosomes were mixed with mature SV40 ^{3}H chromosomes (■) prelabeled in intact cells and then digested with either DNase I (A, B) or DNase II (C, D). The fraction of acid-insoluble radioactivity remaining was measured as a function of time of digestion.

[10 mM Hepes (pH 7.8), 5 mM KCl, and 0.5 mM $MgCl_2$] or 10 mM Tris-HCl (pH 7.8), 2 mM EDTA, and 600 mM NaCl, instead of the usual 200 mM NaCl. MNase digestions carried out at 0–4 °C also revealed the same differences between replicating and mature chromatin that were observed at 37 °C but exhibited a more distinct biphasic time course. Furthermore, the MNase results were typical of those obtained with two other DNA endonucleases, DNase I (McGhee & Felsenfeld, 1980) and DNase II (Horz et al., 1980); these two endonucleases are distinctly different from MNase and from each other in their mode of digestion of chromosomal DNA. However, these endonucleases each digested nascent SV40 [^{32}P]chromatin, pulse labeled in vitro, faster (≃2–5-fold) and more extensively (≃20–25%) than mature SV40 chromatin (Figure 2A,C). As in the case of MNase, the sensitivity of replicating SV40 chromosomes to either DNase I or DNase II became comparable to that of mature chromatin after a 30-min incubation in a nuclear extract (Figure 2B,D). Therefore, hypersensitivity to DNA endonucleases in general is a characteristic of newly assembled chromatin, whether radiolabeled in intact cells or in a subcellular system, and apparently reflects nucleosomal organization rather than the presence or absence of salt-labile proteins.

Relationship of Prenucleosomal DNA and Chromatin Maturation. Sedimentation analyses of nucleoprotein products from MNase digestion of SV40 chromosomes revealed a substantial fraction of radiolabeled DNA that was specifically released from replicating chromatin and was significantly smaller (3–7S) than nucleosomal monomers (Figure 1D). This material was previously shown to be nonnucleosomal DNA, originating from the prenucleosomal DNA region (PN-DNA) at replication forks, that was subsequently converted into acid-soluble material as MNase digestion continued (Cusick et al., 1981). PN-DNA clearly contributed to the rapid and extensive release of acid-soluble ^{3}H label from replicating chromosomes (Figure 1A). Furthermore, as DNA replication continued, not only did the rates and extents of DNA digestion diminish to those characteristic of mature chromatin but so did the fraction of PN-DNA released by MNase (Figure 1D–F). Therefore, the rate of disappearance of pulse-labeled

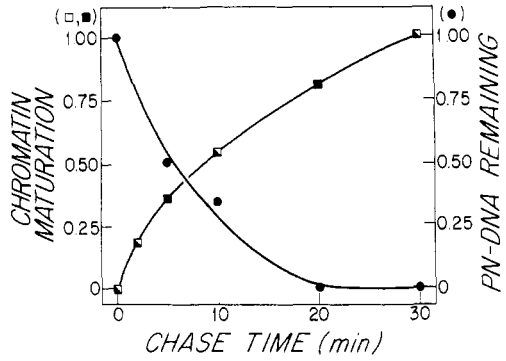

FIGURE 3: Time course for the maturation of SV40 chromatin during viral chromosome replication. Pulse-labeled, replicating SV40 chromosomes were allowed to continue replication in hypotonic nuclear extracts supplemented with cytosol (chase) as described in Figures 1 and 2. The chromosomes were isolated after varying periods of chase time, mixed with mature SV40 chromosomes, and digested with either MNase (■) or DNase I (□). The time courses for each digestion (e.g., Figures 1 and 2) were compared by measuring the *area* between the curve describing replicating chromatin and that describing mature chromatin. These areas were then normalized so that the difference between replicating and mature chromatins observed at 0 min of chase was defined as 0 chromatin maturation and that observed after 30 min of chase was defined as 1 (data points at longer chase times were identical with those at 30 min). The fraction of pulse-labeled, replicating SV40 DNA that was released by MNase as PN-DNA was calculated from sedimentation profiles of the type shown in Figure 1. Since the fraction of PN-DNA observed depended on both the extent of MNase digestion and the chase time, all of these data points were collected when the fraction of acid-soluble, pulse label was 30 ± 5%. The fraction of radiolabel remaining in PN-DNA (●) was normalized so that the amount observed at 0-min chase was defined as 1, and the amount observed at 30 min of chase was defined as 0.

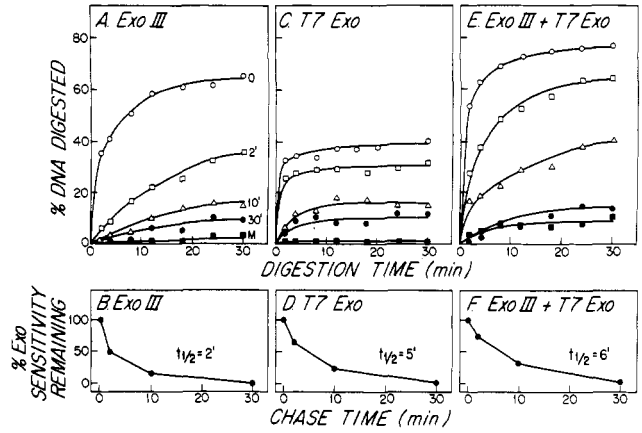

FIGURE 4: Sensitivity of newly replicated SV40 chromatin to *E. coli* exonuclease III (Exo III) and phage T7 exonuclease (T7 Exo). Replicating SV40 chromosomes were pulse labeled for 1 min with [α-^{32}P]dCTP in a hypotonic nuclear extract supplemented with cytosol, and then replication was continued in the presence of excess unlabeled dCTP (chase) for 0 (○), 2 (□), 10 (△), or 30 min (●). These chromosomes were then mixed with mature SV40 ^{3}H chromosomes (■), prelabeled in intact cells, and digested with Exo III (A), T7 Exo (C), or Exo III plus T7 Exo (E). The fraction of acid-insoluble radiolabel remaining was measured as a function of the time of digestion. From the digestion time courses (A, C, E), the time required during viral chromatin replication to protect the pulse-labeled region against exonuclease attack (i.e., maturation) was calculated (B, D, F). The distance between the plateau of the digestion curve for replicating chromatin and that for the 30-min chase time was plotted as a function of the chase period.

PN-DNA during an in vitro chase was compared with the rate of chromatin maturation (i.e., loss of MNase hypersensitivity) during the chase period. The result of such a comparison (Figure 3) was consistent with PN-DNA accounting for a significant proportion of the acid-soluble radioactivity released by MNase digestion of newly replicated chromatin. However, it was noted that radiolabeled PN-DNA disappeared somewhat faster ($t_{1/2} \simeq 6$ min) than chromatin maturation occurred ($t_{1/2} \simeq 9$ min).

Results of previous experiments (Herman et al., 1981; Cusick et al., 1981) demonstrated that *E. coli* exonuclease III (Exo III; degrades one strand of duplex DNA from the 3′ to 5′ end) and phage T7 exonuclease (T7 Exo; degrades one strand of duplex DNA from the 5′ to 3′ end) would together excise at least 95% of nascent DNA in the PN-DNA region of replication forks, without degrading DNA in nucleosomes. Exo III and T7 Exo each excise Okazaki fragment DNA on replicating SV40 chromosomes (90% on retrograde arms), while Exo III digests the remaining nascent PN-DNA from forward arms and T7 Exo digests PN-DNA from retrograde arms (Herman et al., 1981). Therefore, these nucleases can be used to measure the fraction of nascent DNA present as PN-DNA during replication. In the experiments described here, replicating SV40 ^{32}P chromosomes, pulse labeled for 2 min in vitro and isolated after various chase times, were mixed with mature SV40 ^{3}H chromosomes and digested with Exo III and/or T7 Exo (Figure 4). The results indicate that the nascent DNA at replication forks which is accessible to exonucleases (PN-DNA) disappears with a half-life of about 6 min or less, consistent with the previous assessment of MNase sensitive material at replication forks (Figure 3). While these results may also reflect a difference in rates of nucleosome assembly on forward (Figure 4B) and retrograde (Figure 4D) sides of replication forks (2 vs. 5 min, respectively), a more detailed analysis would be required to confirm this possibility.

Organization of Immature Nucleosomes. In order to determine whether or not newly assembled nucleosomes were hypersensitive to MNase, a mixture of replicating SV40 [^{32}P]chromatin and mature SV40 [^{3}H]chromatin were digested: first with an exonuclease to remove [^{32}P]DNA from the PN-DNA region and then with MNase to compare the rates and extents of digestion of nascent and mature chromatin. As above, Exo III excised about 60% (Figure 4A) and T7 Exo about 40% (Figure 4C) of the radiolabeled DNA from replicating SV40 chromosomes. However, even after digestion with either Exo III or T7 Exo, the *remaining* [^{32}P]DNA was found to be just as hypersensitive to MNase digestion (Figure 5B,C) as the original, untreated chromatin sample (Figure 5A). Even the excision of all PN-[^{32}P]DNA by both Exo III and T7 Exo failed to convert immature nascent chromatin (endonuclease hypersensitive) into mature, nonreplicating chromatin (Figure 5D). It was noted that pulse-labeled chromosomes that had completed maturation during a 30-min chase in nuclear extracts did not exhibit MNase hypersensitivity after treatment with Exo III and T7 Exo (data not shown); therefore, exonuclease digestion of chromatin did not in itself induce MNase hypersensitivity. Comparison of the areas between the two digestion curves in each panel of Figure 5, with the corresponding areas under the curves for mature SV40 [^{3}H]chromatin (as in Figure 3), revealed that replicating chromatin was initially more sensitive to MNase. Even after treatment with Exo III, T7 Exo, or both enzymes, replicating chromatin remained significantly more sensitive to MNase than mature chromatin. Apparently, some feature of nucleosomes containing nascent DNA at replication forks (immature nucleosomes) makes the nucleosomal DNA nearly as sensitive to MNase digestion as PN-DNA.

To demonstrate directly that newly assembled nucleosomes containing nascent DNA were hypersensitive to MNase, nucleosomal dimers and trimers were isolated from MNase digests of replicating and mature chromosomes and then reex-

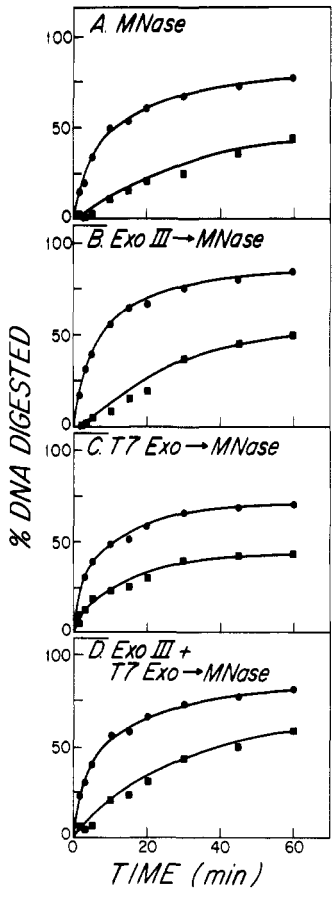

FIGURE 5: Sensitivity of replicating SV40 chromosomes to MNase after predigestion with Exo III and T7 Exo. Replicating SV40 ^{32}P chromosomes (●), labeled for 1 min in a nuclear extract plus cytosol, and mature SV40 ^{3}H chromosomes (■), labeled in intact cells, were digested together with either MNase (A), Exo III followed by MNase (B), T7 Exo followed by MNase (C), or both Exo III and T7 Exo followed by MNase (D). Exonuclease digestions were taken to completion as in Figure 4, and the fraction of acid-insoluble radiolabel remaining was then measured as a function of MNase digestion time.

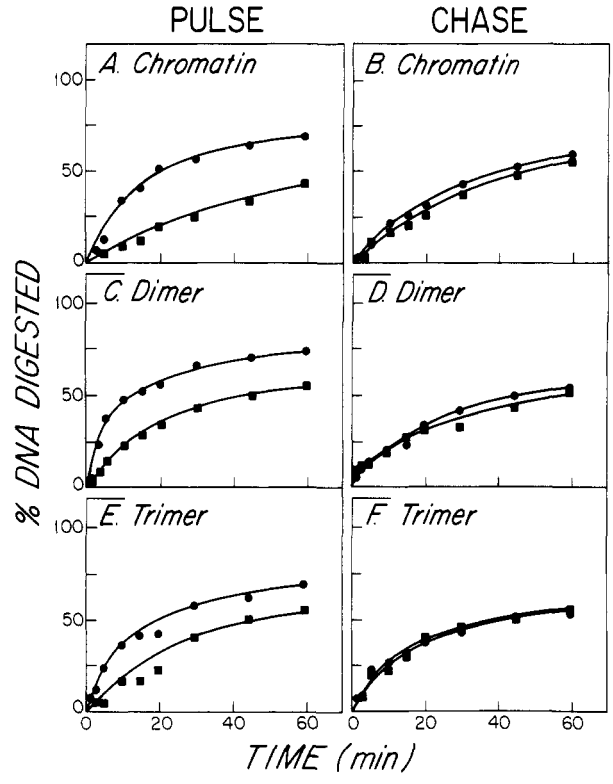

FIGURE 6: MNase digestion of purified nucleosomal oligomers. Replicating SV40 ^{32}P chromosomes (●), pulse labeled for 5 min in a nuclear extract plus cytosol (pulse) and then allowed to continue replication for an additional 30 min in the presence of excess unlabeled nucleotides (chase), were digested with MNase and the nucleoprotein products separated by sedimentation in a sucrose gradient (e.g., Figure 1). Fractions containing the maximum amount of nucleosomal dimers and trimers were pooled and then concentrated by pressure dialysis. Nucleosomal dimers and trimers were also prepared from mature SV40 ^{3}H chromosomes (■) radiolabeled in intact cells. Samples of replicating viral chromosomes (A, B), dimers (C, D), and trimers (E, F) were mixed with the corresponding samples from mature viral chromatin and then digested with MNase. The fraction of acid-insoluble radiolabel remaining was measured as a function of digestion time.

posed to MNase. In these experiments, replicating SV40 chromosomes were pulse labeled in vitro for 5 min, rather than 1 min, to position more radiolabel further away from replication forks and then chased for 30 min without additional [α-^{32}P]dCTP incorporation. A separate nuclear extract was prepared containing mature SV40 ^{3}H chromosomes that were prelabeled in intact cells. MNase digestion of [^{32}P]chromatin isolated from the 5-min pulse–30-min chase, and mixed with mature [^{3}H]chromatin, revealed the maturation process seen previously in experiments by using a 1-min pulse (Figure 6A,B). To obtain nucleosomal oligomers, radiolabeled chromosomes were mixed with a 100-fold excess of unlabeled cellular chromatin, digested with MNase until 25–35% of the radiolabel was acid soluble, and fractionated by sucrose gradient sedimentation as in Figure 1. The addition of cellular chromatin was necessary in order to stabilize the isolated viral nucleosomes; at low concentrations, nucleosomes "unravel", releasing their DNA (Cotton & Hamkalo, 1981; T. M. Herman, M. L. DePamphilis, and P. M. Wassarman, unpublished observations). ^{32}P-Labeled nucleosomal dimers and trimers from replicating chromosomes were combined with the corresponding ^{3}H-labeled material from mature chromosomes and digested with MNase. In both cases, newly assembled nucleosomal oligomers (pulse) were hypersensitive to MNase (Figure 6C,E) but lost the MNase hypersensitivity (i.e., "matured") as DNA replication continued in vitro (chase) (Figure 6D,F). When the same experiment was carried out with monomers isolated from mature and replicating SV40 chromosomes, they were degraded at the same rate and to the same extent as bare DNA, indicating that isolated viral monomers were unstable even under these conditions. Comparison of the areas between the two digestion curves in each panel of Figure 6, with the area defined by mature chromatin, revealed that nascent chromatin, dimers, and trimers were all significantly more sensitive to MNase before maturation than after.

Immature Chromatin Exists on Both Arms of Replication Forks. Immature chromatin was shown to exist on both sides of replication forks by the ability of nascent DNA from rapidly released monomers to anneal equally well with either strand of an SV40 DNA restriction fragment, as previously described by Cusick et al. (1981). Replicating SV40 ^{32}P chromosomes were radiolabeled for 2 min in a nuclear extract plus cytosol, and one aliquot was allowed to continue replication in the presence of unlabeled nucleotides for an additional 2 min. Viral chromosomes were then purified by sedimentation in a sucrose gradient and digested with MNase until 10% and 17%, respectively, of the [^{32}P]DNA was solubilized. In each case, monomer [^{32}P]DNA annealed equally well to both strands of each restriction fragment. The results were virtually identical with those shown in Figure 6 of Cusick et al. (1981). Symmetrical hybridization was not the result of excessive ratios of probe to template, since excess template was present (0.5–1

μg of DNA/band) and the results were the same when the specific radioactivity of the [^{32}P]DNA was varied over a 100-fold range. In addition, when labeled Okazaki fragments were annealed to the same DNA blots, at least 90% of the label annealed only to the strand representing the retrograde side of replication forks. Since digestion of newly replicated chromatin (Figures 1 and 2; Cusick et al., 1981), even without PN-DNA present (Figures 5 and 6), released nucleosomal monomers faster than digestion of mature chromatin (Figure 1; Cusick et al., 1981, Figure 3), these data strongly suggest that immature chromatin exists on both arms of replication forks.

Relationship of Chromatin Maturation and DNA Replication. Aphidicolin, a specific inhibitor of DNA polymerase α (Sugino & Nakayama, 1980; DePamphilis & Wassarman, 1980), retards all DNA synthesis at replication forks (Krokan et al., 1979), including joining of Okazaki fragments (Wist & Prydz, 1979; Weaver et al., 1980). To determine whether or not chromatin maturation required concomitant DNA synthesis, a nuclear extract, containing replicating SV40 chromosomes with pulse-labeled [^{32}P]DNA and supplemented with cytosol, was incubated further in the presence or absence of aphidicolin and an excess of unlabeled deoxyribonucleotide substrates (chase) (Figure 7). Under these conditions, aphidicolin inhibited further DNA synthesis 80–90%, as shown by a 5–10-fold decrease in [α-^{32}P]dCTP incorporation (data not shown) and a 5–10-fold decrease in the rate of joining Okazaki fragments to longer DNA chains (Figure 7A). Aphidicolin also decreased by 5–10-fold the rate that pulse-labeled DNA became resistant to either Exo III or T7 Exo (Figure 7B). Therefore, inhibition of DNA synthesis produced a corresponding inhibition in the conversion of nascent PN-DNA into nucleosomes. This would be expected if nucleosome assembly required a minimum amount of duplex DNA which could only be provided by advancing the replication forks. In contrast to the exonuclease analysis of chromatin maturation, MNase analysis represents the combined result of digesting both the PN-DNA region and newly assembled nucleosomes. The rate at which pulse-labeled DNA became resistant to MNase was that expected if maturation of newly assembled nucleosomes was not dependent on concomitant DNA synthesis (Figure 7C). From 40% to 60% of the pulse label was in PN-DNA, as defined by its sensitivity to exonucleases (Figure 4). During the chase period the rate at which the fraction of label in PN-DNA decreased and appeared in immature nucleosomes depended on whether or not aphidicolin was present (Figure 7B). The difference between the rate that MNase hypersensitivity disappeared in the presence of this drug and the rate observed in its absence (Figure 7C) was that expected if the fraction of label in PN-DNA was chased, as shown in Figure 7B, but the fraction of label in nucleosomes matured in the presence of aphidicolin at the same rate as it did in its absence. Therefore, inhibition of DNA synthesis appeared to distinguish between the two components of chromatin maturation by inhibiting the transition of nascent PN-DNA into immature nucleosomes, while not inhibiting the conversion of immature into mature nucleosomes.

Chromatin maturation requires a soluble component found in the cytosol fraction. Replicating SV40 chromosomes were sedimented out of a nuclear extract, and then the total nucleoprotein complexes were resuspended in the DNA synthesis assay solution, either with or without cytosol present as described by Su & DePamphilis (1978). In the absence of cytosol, DNA synthesis (Su & DePamphilis, 1978) and joining of Okazaki fragments were blocked (Su & DePamphilis, 1978; Figure 7A). Omission of cytosol also blocked conversion of PN-DNA into nucleosomes (Figure 7B) and the loss of MNase hypersensitivity (Figure 7C). In the presence of cytosol, the rates of SV40 DNA replication (Su & DePamphilis, 1978) and chromatin maturation were the same as observed in nuclear extracts supplemented with cytosol (data not shown). Thus, cytosol may contribute one or more factors required for nucleosome assembly and maturation.

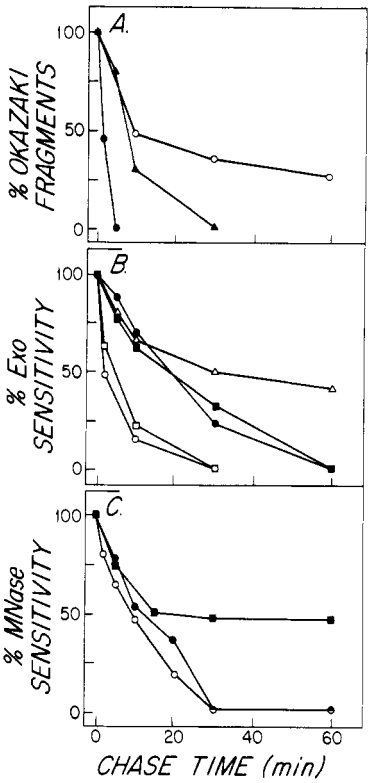

FIGURE 7: Effect of DNA synthesis inhibitors or absence of cytosol on DNA synthesis and chromatin maturation. (A) Normalized percentage of Okazaki fragments remaining as a function of chase time. DNA was extracted from replicating SV40 chromosomes at each chase time and analyzed on a 5–20% alkaline sucrose gradient. The amount of DNA in Okazaki fragments (the peak of DNA sedimenting at 4 S) was calculated as a percentage of the total DNA on the gradient. Data from each set of experiments were then normalized to produce the curves shown. Control chromosomes (●), chromosomes chased in the presence of aphidicolin (▲), and chromosomes chased in the absence of cytosol (○) are shown. (B) Normalized percentage of exonuclease sensitivity remaining as a function of chase time. Replicating SV40 chromosomes from each chase time were mixed with mature SV40 chromosomes and digested together with Exo III or T7 Exo. The percentage of acid-insoluble radiolabel remaining was measured as a function of digestion time to produce time courses such as those in Figure 4A,C,E. The distance between the digestion curve of replicating SV40 chromosomes and the digestion curve of mature chromosomes was computed for each chase time, and then these values were normalized to produce the data shown. Control chromosomes digested with Exo III (○) or with T7 Exo (□), chromosomes chased in the presence of aphidicolin and digested with Exo III (●) or with T7 Exo (■), and chromosomes chased in the absence of cytosol and digested with Exo III (▲) are shown. (C) Normalized percentage of MNase hypersensitivity remaining as a function of chase time. Replicating SV40 chromosomes from each chase time were mixed with mature SV40 chromosomes and digested together with MNase. The percentage of acid-insoluble radiolabel remaining was measured as a function of digestion time to produce time courses like those depicted in Figure 1A–C. The area between the digestion curve of replicating SV40 chromosomes and the digestion curve of mature SV40 chromosomes was computed for each chase time, and then these values were normalized to produce the curves shown. Control chromosomes (○), chromosomes chased in the presence of aphidicolin (●), and chromosomes chased in the absence of cytosol (■) are shown.

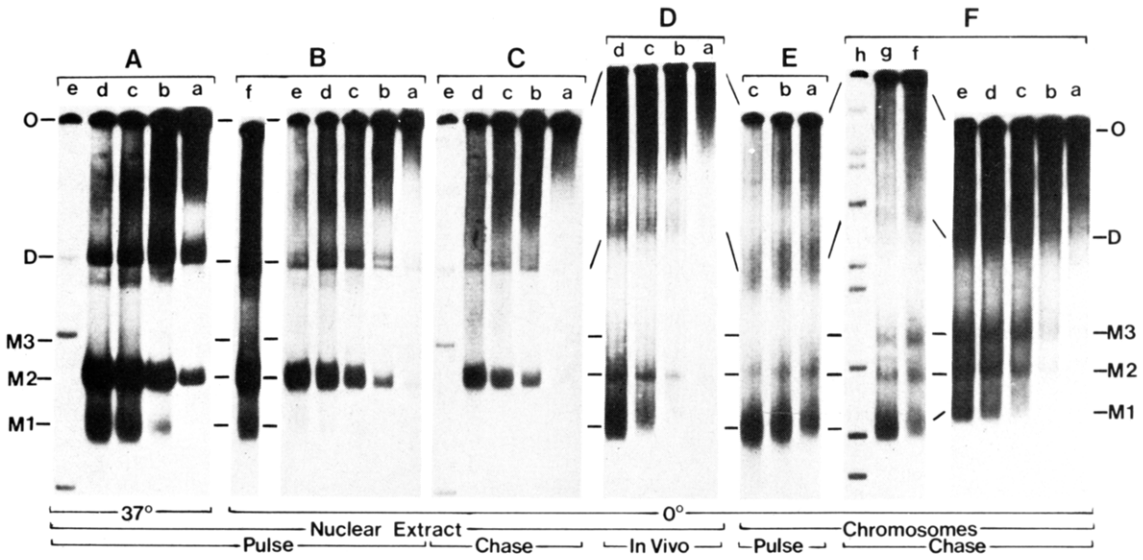

FIGURE 8: Gel electrophoresis of the nucleoprotein products from MNase digestion of SV40 chromatin. Chromatin samples containing 10–20 μg of DNA/mL were adjusted to 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and then incubated either on ice or at 37 °C with 0.5 unit of MNase/μL for up to 4 h. Digestion was stopped with 10 mM EDTA plus 5 mM EGTA. Samples were analyzed directly by electrophoresis in 5% acrylamide and 0.18% bis(acrylamide) at 4 °C with a circulating buffer consisting of 10 mM Hepes (pH 7.5), 1 mM EDTA, and 0.5 mM EGTA at a constant voltage of 150 V. XCFF was included as a useful dye marker that migrated just ahead of the mononucleosomal band designated M3. Gels containing ^{32}P were dried, and the radioactivity was detected by autoradiography. Gels containing ^{3}H were prepared for and the radioactivity was detected by fluorography. (A) Replicating SV40 [^{32}P]chromatin in a nuclear extract digested at 37 °C for increasing times (a–d). Lane e shows SV40 DNA restriction fragments that acted as reference points to compare different gels. (B) Same as (A), but digestion was on ice. Increasing digestion times are shown in lanes a–e; lane f is a longer exposure of lane e that clearly reveals M3. (C) Mature SV40 [^{32}P]chromatin in a nuclear extract digested on ice with increasing times of digestion from lane a to lane d. Lane e contains SV40 DNA restriction fragment markers. (D) Mature SV40 [^{3}H]chromatin in a nuclear extract digested on ice with increasing digestion times from lane a to lane d. (E) Replicating SV40 ^{32}P chromosomes isolated by sedimentation in a hypotonic sucrose gradient and digested on ice with increasing digestion times from lane a to lane c. (F) Mature SV40 ^{32}P chromosomes prepared as in (E) and digested on ice with increasing digestion times from lane a to lane e. Lanes f and g are a separate sample in which M1, M2, and M3 are particularly well resolved. Lane h contains SV40 DNA restriction fragments for comparison. The positions of the gel origin (O), nucleosomal dimers (D), and nucleosomal monomers (M1–3) are indicated on the vertical axis.

Nucleoprotein Organization during Chromatin Maturation. Nucleosomal monomers can be fractionated by gel electrophoresis as a function of their protein composition and DNA length (Levinger & Varshavsky, 1980; Albright et al., 1980). Therefore, we used this technique to analyze the nucleoprotein products from MNase digestion of replicating SV40 [^{32}P]-chromatin that was pulse labeled in vitro (Figure 8A,B,E), mature SV40 [^{32}P]chromatin that was formed in vitro during a chase period (Figure 8C,F), and mature SV40 [^{3}H]-chromatin that was formed in intact cells (Figure 8D). In each case, hypotonic nuclear extracts were prepared from virus-infected cells. Mature viral [^{3}H]chromatin was taken from infected cells that had been incubated for 12 h with [^{3}H]Thd. Replicating SV40 chromatin and mature SV40 [^{32}P]chromatin were taken from nuclear extracts supplemented with cytosol and incubated first with [α-^{32}P]dCTP for 6 min at 30 °C (pulse) and then with an excess of dCTP for 35 min at 30 °C (chase) as described by Cusick et al. (1981). A 6-min pulse was equivalent to a 1-min pulse in distinguishing immature from mature chromatin (compare Figures 1 and 6). DNA synthesis was arrested at 0 °C, and the nuclear extract was either filtered through Sephadex G-50 in 10 mM Hepes (pH 7.8), 0.5 mM $MgCl_2$, and 5 mM KCl at 4 °C to remove unincorporated nucleotides (Figure 8A–D) or sedimented through a sucrose gradient containing the same hypotonic buffer used to isolate viral chromosomes (Figure 8E,F) (Su & DePamphilis, 1978). Appropriate fractions were pooled, dialyzed under N_2 pressure against 10 mM Tris (pH 7.8) and 0.1 mM EDTA, and then digested with MNase either on ice (Figure 8B–F) or at 37 °C (Figure 8A) for various periods of time. The nucleoprotein digestion products were then analyzed directly by gel electrophoresis.

The most easily recognized nucleoproteins released by MNase were similar to multiple forms of nucleosomal monomers previously described for mammalian chromosomes (Albright et al., 1980; Levinger & Varshavsky, 1980; Annunziato et al., 1981; Schlaeger, 1982). We refer to these as M1, M2, and M3. In addition, material equivalent to nucleosomal dimers was observed which we designated as D. The relative amounts of each of these nucleoprotein products, as well as the breadth of individual bands observed following gel electrophoresis, depended on the method of chromatin preparation, as well as the extent and conditions of MNase digestion. However, the loss of MNase sensitivity observed during chromatin maturation could not be readily correlated with a change in the nucleoprotein digestion products.

MNase digestion of replicating SV40 [^{32}P]chromatin in nuclear extracts rapidly released M2 monomers, a distinctive doublet as dimers, and unresolved oligomeric products (Figure 8B). Neither omission of the gel filtration step nor changing the gel filtration buffer to 10 mM Tris (pH 7.8) and 0.1 mM EDTA altered the time course of MNase digestion or the nucleoprotein products observed. However, MNase digestion at 37 °C instead of on ice increased the amount of M1 that was released, from about 1% or less (Figure 8B) to about 30% (Figure 8A). Maturation of SV40 [^{32}P]chromatin in vitro did not change the result (compare part C with B of Figure 8), suggesting that loss of MNase hypersensitivity did not involve a significant alteration in nucleosome structure or composition. Digestion of mature SV40 [^{3}H]chromatin, which represents the total intracellular pool, revealed that although M2 was the initial product released, the final product was predominantly M1 (Figure 8D). Therefore, two steps are indicated in chromatin maturation; loss of MNase hypersensitivity occurs

first, followed by a change in nucleosome structure or composition that results in the conversion of M2 to M1.

When SV40 chromosomes were isolated from nuclear extracts by sedimentation through sucrose in hypotonic buffer, two changes in the nucleoprotein digestion products were apparent. First, M1, M2, and M3 were now readily identified in MNase digests of mature SV40 ^{32}P chromosomes (Figure 8F), and second, the presence of M3 was not often observed in similar digestions of replicating SV40 ^{32}P chromosomes, although M1 and M2 were present (Figure 8E). However, since isolated replicating chromosomes were so rapidly degraded by MNase that early time points were difficult to obtain, the absence of M3 may simply reflect a difference in the rate of digestion, rather than a difference in chromatin organization between replicating (Figure 8E) and mature (Figure 8F) chromosomes. Mature SV40 ^{3}H chromosomes gave a result similar to mature SV40 ^{32}P chromosomes; in both cases M2 and M3 were released before M1 appeared (data not shown). When this experiment was repeated with sucrose gradients containing 200 mM NaCl, 0.1 mM EDTA, and 10 mM Tris (pH 7.8) as described by Shelton et al. (1980), the only monomeric digestion product observed was M1 (data not shown). Presumably, the proteins responsible for generating M2 and M3 were removed by this treatment. Although we do not yet know the protein composition of M1, M2, and M3, SV40 chromosomes isolated under these hypotonic conditions do contain histones H1, H2A, H2B, H3, and H4 in the same relative amounts as found in the CV-1 viral host cell (Shelton et al., 1980). However, the relative amounts of M1, M2, and M3 clearly depend on the conditions of MNase digestion (0 vs. 37 °C), chromosome isolation (crude nuclear extract vs. isolation from sucrose gradients containing low or high salt concentrations), and time after replication (12 h in vivo label vs. 0.5 h in vitro chase). Therefore, the release of these three forms of monomer may depend on chromatin conformation as well as protein composition.

Despite the changes in the number and homogeneity of nucleoprotein products of MNase digestion observed under different conditions, the DNA extracted from these samples gave patterns indistinguishable from each other after electrophoresis (data not shown); in each case, they were the same broad band patterns previously reported for SV40 chromatin (Shelton et al., 1978b, 1980; Cusick et al., 1981), in contrast to the relatively sharp bands observed with nucleoprotein particles. When two-dimensional gel electrophoresis was used, the size of the DNA in the M1, M2, and M3 regions was found to be an average of 145, 165, and 200 bp, respectively. When the extent of digestion was sufficient to convert most of the radiolabeled material into M1, analysis of the total DNA products revealed six bands of varying intensity that contained 145 (monomeric core DNA), 122, 100, 97, 59, and 48 bp (±5 bp), respectively. These represent the normal DNA products from a limit MNase digest of chromatin (Shelton et al., 1980; McGhee & Felselfeld, 1980).

Discussion

It has often been observed that newly replicated cellular (Hildebrand & Walters, 1976; Hewish, 1977; Levy & Jakob, 1978; Seale, 1975, 1978; Schlaeger & Knippers, 1979; Klempnauer et al., 1980; Yakura & Tanifuji, 1980; Schlaeger, 1982) and SV40 (Klempnauer et al., 1980; Cusick et al., 1981) chromatin is more rapidly and extensively degraded by MNase and DNase I than nonreplicating chromatin. To account for these observations, it has been suggested that nascent DNA is first incorporated into "immature" nucleosomes (hypersensitive to endonucleases) relatively close to the actual sites of DNA synthesis and that as the replication forks move away from these nucleosomes they "mature", becoming indistinguishable from nucleosomes of nonreplicating chromatin. However, SV40, and presumably mammalian, replicating chromosomes contain an average of 125 bases of nascent prenucleosomal DNA (PN-DNA) on each arm of a replication fork, in addition to an average of one Okazaki fragment (135 bases) per fork [reviewed in DePamphilis & Wassarman (1980, 1982)]. Since the fraction of radiolabel associated with this PN-DNA region is greatest when the labeling period is shortest (Klempnauer et al., 1980; Cusick et al., 1981; Herman et al., 1981), the process of chromatin maturation could be explained by the presence of PN-DNA alone. In contradistinction, the results presented here demonstrate that chromatin maturation involves the conversion of immature nucleosomal oligomers into mature chromatin, consistent with the model previously proposed for replicating SV40 chromosomes [see Figure 11 in Cusick et al. (1981)].

Replicating SV40 chromosomes were radiolabeled either in a subcellular system or in intact cells for periods in which the average fork advanced only 200–300 bp and, therefore, contained just one or two nucleosomes with labeled DNA on each side of the replication fork (Tapper et al., 1979; Herman et al., 1981). These chromosomes were hypersensitive to three different nonspecific endonucleases (MNase, DNase I, and DNase II), allowing in each case the region of labeled nascent DNA to be degraded 2–5-fold faster and about 25% more extensively than uniformly labeled, nonreplicating, mature SV40 chromosomes present in the same reaction. Furthermore, 40–60% of the labeled nascent DNA was accessible to digestion by Exo III and T7 Exo and could be released as 3–7S pieces of DNA by MNase; this material represented the PN-DNA region. When replication was allowed to continue in nuclear extracts supplemented with cytosol, but in the absence of radiolabeled nucleotides, nascent viral DNA disappeared from the PN-DNA region with a half-life of 6 min or less, and nascent viral chromatin lost its hypersensitivity to endonucleases with a half-life of about 9 min (the time required to replicate about 40% of the SV40 genome). When replicating chromosomes were predigested with Exo III and T7 Exo prior to digestion with MNase, the rapidly released 3–7S DNA fragments were no longer observed, but the nascent DNA that remained associated with the chromosomes was still more rapidly and extensively digested by MNase than mature chromatin.

Although the properties of PN-DNA fragments released by MNase were strikingly similar to those of bare DNA (Cusick et al., 1981), these data, together with previous studies using S1 nuclease (Herman et al., 1979), suggest the accessibility of this region is limited either by proteins still associated after treatment in 200 mM NaCl or by chromosome conformation. Isolated nucleosomal oligomers containing nascent DNA that annealed to both strands of unique DNA restriction fragments were also more rapidly and extensively digested by MNase than oligomers isolated from mature chromatin. Therefore, *both* PN-DNA and newly assembled immature chromatin are present on both arms of replication forks and *both* contribute to the commonly observed hypersensitivity of newly replicated chromatin to endonucleases.

The molecular basis for the conversion of immature chromatin into mature chromatin is not yet understood. Although it is clear that multiple forms of nucleosomal monomers can be identified, the disappearance of nuclease hypersensitivity could not be readily correlated with the appearance of a particular nucleosomal monomer (Figure 8). Similarly, newly

assembled nucleosomes closest to the sites of DNA synthesis in SV40 or cellular chromatin cannot be distinguished from mature nucleosomes by sedimentation (Figure 1; Hildebrand & Walters 1976; Schlaeger & Klempnauer, 1978; Schlaeger & Knippers, 1979; Herman et al., 1981; Cusick et al., 1981; Schlaeger, 1982), by gel electrophoresis of nucleoprotein particles (Figure 8; Annunziato et al., 1981; Galili et al., 1981; Jackson et al., 1981; Schlaeger, 1982), by gel electrophoresis of nucleosomal DNA (Seale, 1978; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Klempnauer et al., 1980; Yakura & Tanifuji, 1980; Annunziato et al., 1981; Cusick et al., 1981; Jackson et al., 1981), or by extensive endonuclease digestion (Seale, 1975; Jackson & Chalkley, 1981a; Galili et al., 1981).

The absence of H1 has been reported to increase the accessibility of linker DNA to endonucleases (Noll & Kornberg, 1977; Weischet et al., 1979) which could account for the extreme rapidity of digestion, while an altered nucleosome structure could account for the increased extent of digestion. Histone H1 appears to be either absent or loosely bound to nascent chromatin. Newly synthesized H1 is not associated with newly replicated SV40 or cellular chromatin (Cremisi et al., 1978; Jackson & Chalkley, 1981a,b; Jackson et al., 1981). Nascent DNA appears preferentially associated with nucleosomal monomers lacking H1 (Jackson et al., 1981; Galili et al., 1981; Schlaeger, 1982), although in one case (Annunziato et al., 1981) histone H1 was found associated with newly replicated DNA. These results are complicated by the fact that exchange of H1 occurs during isolation of chromatin at moderate salt concentrations (Cremisi & Yaniv, 1980; Caron & Thomas, 1981). The absence of H1 on nascent chromatin could also explain the observation that spacing between newly assembled nucleosomes sometimes appears closer than spacing of nucleosomes on mature chromosomes (Seale, 1978; Murphy et al., 1978; Levy & Jakob, 1978; Yakura & Tanifuji, 1980; Galili et al., 1981). In contrast, with mature nucleosomes, the spacing of nascent nucleosomes can decrease with the extent of MNase digestion (Jackson et al., 1981). This may result from nucleosome sliding to form contiguous core particles in the absence of H1 (Klevan & Crothers, 1977; Tatchell & van Holde, 1978; Liggins et al., 1979; Glotov et al., 1982). Therefore, the shorter repeat distances observed between nucleosomes at replication forks is apparently an artifact of MNase digestion of H1-depleted chromatin. Otherwise, such a high density of nucleosomes on nascent DNA would mean that nucleosomes are ejected as chromatin matures and nucleosomes achieve their normal spacing.

The notion that nascent nucleosomes have an altered structure is suggested by the fact that newly synthesized H3 and H4, which preferentially associate with newly replicated SV40 and cellular DNA (Cremisi & Yaniv, 1980; Worcel et al., 1978; Senshu et al., 1978; Jackson & Chalkley, 1981a,b; Jackson et al., 1981), are bound unusually weakly when they first associate with chromatin (Jackson et al., 1981). Furthermore, newly assembled nucleosomal monomers radiolabeled in washed nuclei (Seale, 1978) or sea urchin embryos (Levy & Jakob, 1978) appear more sensitive to internal cleavage by MNase than do mature nucleosomes. Alternatively, modification of histones associated with nascent DNA could result in an altered nucleosome structure and, consequently, in unusual susceptibility to endonucleases. For example, hyperacetylated chromatin is very sensitive to DNase I (Sealy & Chalkley, 1978; Vidali et al., 1978), but not to MNase (Mathis et al., 1978); however, since nascent SV40 chromatin does not appear to be hyperacetylated (Chestier & Yaniv, 1979) and is extremely sensitive to both DNase I and MNase, it is unlikely that this particular type of histone modification is involved in chromatin maturation.

The results presented here provide further evidence that replicating SV40 chromosomes are composed of at least four different regions: (i) prereplicative chromatin that consists of mature nucleosomes in front of replication forks, (ii) prenucleosomal DNA (PN-DNA) that encompasses the actual sites of DNA synthesis, including Okazaki fragments, at replication forks, (iii) postreplicative chromatin containing nascent DNA assembled into immature nucleosomes, and (iv) postreplicative chromatin containing nascent DNA incorporated into mature nucleosomes as described in Cusick et al. (1981). In most of these experiments, viral chromatin was washed in 200 mM NaCl to remove replication proteins without disturbing nucleosomes, although similar results were also obtained with chromosomes washed in 50 mM NaCl (Cusick et al., 1981). PN-DNA appears to be free of nucleosome-like structures because the DNA fragments released by either S1 nuclease or MNase had a buoyant density and nuclease sensitivity equivalent to bare DNA and a sedimentation rate that was unaffected by treatment with salt, detergent, and protease. In addition, nascent PN-DNA was easily excised by exonucleases, whereas nucleosomal DNA was not (Herman et al., 1979, 1981; Cusick et al., 1981). Reconstruction experiments with purified DNA demonstrated that a nucleoprotein complex was not necessary to explain the release of 3–7S DNA by MNase (Cusick et al., 1981). However, in experiments with replicating lymphocyte chromatin, the small nascent DNA fragments rapidly released during MNase digestion were shown to sediment with nucleosomal monomers in 2 mM EDTA (pH 7.5) and 10 mM 2-mercaptoethanol, but not in 0.5 M NaCl (Schlaeger, 1982). These results were interpreted to suggest the presence of structurally altered nucleosomes at replication forks that were hypersensitive to MNase, releasing subnucleosomal lengths of DNA in high salt. An alternative interpretation is that, at this exceptionally low ionic strength, short DNA fragments adventitiously associate with either nucleosomes or core histones generated during MNase digestion. For example, we have noted that addition of SV40(I) DNA to hypotonic nuclear extracts can generate nucleoprotein complexes that sediment like viral chromosomes, but which do not have nucleosomes (R. Su, unpublished results); in this connection, Cotton & Hamkalo (1981) have shown that low concentrations of isolated nucleosomal monomers reversibly dissociate at physiological ionic strengths.

Whatever the basis for the altered structure of newly assembled chromatin, it is related both to DNA replication and transcription. The dramatic difference observed between the nucleoprotein digestion products containing nascent DNA from viral chromosomes digested in nuclear extracts with those digested after isolation from sucrose gradients in hypotonic medium (Figure 8) is, to our knowledge, the first time a change in chromatin structure has been correlated with a change in the ability of chromosomes to continue DNA replication. Replicating SV40 DNA intermediates in nuclear extracts supplemented with cytosol are able to faithfully continue viral DNA replication, while isolated replicating chromosomes only complete the synthesis of Okazaki fragments and, in the presence of cytosol, join them to the long nascent DNA strands (Su & DePamphilis, 1978). Thus, the structural characteristics that generate nucleosomal monomers, designated as M2, appear to be required for replication forks to advance. The fact that both immature chromatin and actively transcribed

chromatin are hypersensitive to nonspecific endonucleases suggests similarities in their structures. Weintraub (1979) has proposed that immature chromatin is more accessible to those gene regulation proteins that eventually determine whether or not the chromatin structure remains in an active or inactive form.

Registry No. MNase, 9013-53-0; DNase I, 9003-98-9; DNase II, 9025-64-3.

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Enthalpy–Entropy Compensation and Heat Capacity Changes for Protein–Ligand Interactions: General Thermodynamic Models and Data for the Binding of Nucleotides to Ribonuclease A†

Maurice R. Eftink,* A. C. Anusiem, and Rodney L. Biltonen

ABSTRACT: General thermodynamic models are presented that can account for the existence of heat capacity changes and compensation between the enthalpy and entropy changes in protein–ligand interactions. The models involve the coupling between some type of transition in the state of the protein (or ligand) and the binding process. The coupled transition may be a proton dissociation, the binding of a second ligand, a change in the degree of aggregation, or a conformational change in either the protein or ligand. Both mandatory coupling and nonmandatory coupling between the binding process and the transition are considered. The model is also extended to include a multistate transition of the protein. Computer simulations show that apparently linear compensation plots (plots of $\Delta H°$ vs. $\Delta S°$) with a slope approximately equal to the experimental temperature are to be expected for the binding of a ligand to a protein when such coupled reactions exist. Also heat capacity changes, which may be either positive or negative, are to be expected to accompany the reaction. Experimental thermodynamic data for the binding of cytidine 3′-phosphate to ribonuclease A are presented. These data demonstrate apparent enthalpy–entropy compensation when pH and ionic strength are varied. A negative heat capacity change, ranging from −145 (at μ = 1.0 M) to −225 cal/(mol·deg) (at μ = 0.05 M), is also observed for this protein–ligand interaction. The apparent compensation and heat capacity change data are interpreted according to the models presented.

The compensation between the enthalpy change, $\Delta H°$, and the entropy change, $\Delta S°$, for the binding of ligands to proteins has been observed for many systems, and the molecular basis for such compensating behavior has been a matter of much interest. An extensive discussion of enthalpy–entropy compensation data and possible interpretations of this effect have been provided by Lumry & Rajender (1970).

Also a number of studies in recent years have demonstrated that the binding of a ligand to a protein is accompanied by a change in the heat capacity, ΔC_p°, for the system (Hinz et al., 1971; Suurkuusk & Wadsö, 1972; Schmid et al., 1976; Niekamp et al., 1977; Fisher et al., 1981). The basis for the heat capacity changes has been proposed to be related to changes in the solvation of the ligand and protein upon binding (Sturtevant, 1977; Eftink & Biltonen, 1980a).

In this report, we discuss some simple models that provide a possible explanation of both enthalpy–entropy compensation and heat capacity changes in terms of ligand-induced changes in the state of the protein. We will use these models in considering thermodynamic data for the interaction of cytidine 3′-phosphate (3′-CMP)[1] with ribonuclease A (RNase A).

Theory

Studies suggest that the globular structure of proteins in solution is quite fragile, being marginally stabilized by a large number of individually weak intramolecular interactions (hydrogen bonds, van der Waals contacts, etc.) (Lumry & Biltonen, 1969; Lumry & Rosenberg, 1976; Ikegami, 1977). Rapid fluctuations in the structure of proteins have been sensed by a number of techniques. These fluctuations may involve the rotation of side chains, vibration of bonds, and the making/breaking of hydrogen bonds and van der Waals contacts. As a result of these structural fluctuations, a protein can be considered a macroscopic ensemble of a large number of closely related microstates.

Proteins may also exist in different macroscopic structural states, each macrostate having a distinctly different folding pattern (in at least a part of the structure). The interconversion of macrostates [Lumry & Biltonen's (1969) "refolding transition"] can be considered to be a two-state process and may occur slowly [an example is the alkaline transition of α-chymotrypsin (Stoesz & Lumry, 1978)]. Different states of aggregation of a protein (i.e., monomer ↔ dimer equilibrium) may also exist. In addition to these different structural states, a protein may also exist in various states of protonation. All of the different states (structural, aggregation, protonation)

† From the Department of Chemistry, University of Mississippi, University, Mississippi 38677 (M.R.E.), School of Chemical Science, University of Harcourt, Port Harcourt, Nigeria P.M.B. 5323 (A.C.A.), and the Departments of Biochemistry and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22903 (R.L.B.).
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[1] Abbreviations: 3′-CMP, cytidine 3′-phosphate; 2′-CMP, cytidine 2′-phosphate; RNase A, bovine pancreatic ribonuclease A; μ, ionic strength.

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