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Chromatin assembly in isolated mammalian nuclei

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ABSTRACT

Cellular DNA replication was stimulated in confluent monolayers of CV-1 monkey kidney cells following infection with SV40. Nuclei were isolated from CV-1 cells labeled with [^{3}H]thymidine and then incubated in the presence of [α-^{32}P]deoxyribonucleoside triphosphates under conditions that support DNA replication. To determine whether or not the cellular DNA synthesized *in vitro* was assembled into nucleosomes the DNA was digested *in situ* with either micrococcal nuclease or pancreatic DNase I, and the products were examined by electrophoretic and sedimentation analysis. The distribution of DNA fragment lengths on agarose gels following micrococcal nuclease digestion was more heterogeneous for newly replicated than for the bulk of the DNA. Nonetheless, the state of cellular DNA synthesized *in vitro* (^{32}P-labeled) was found to be identical with that of the DNA in the bulk of the chromatin (^{3}H-labeled) by the following criteria: (i) The extent of protection against digestion by micrococcal nuclease or DNase I. (ii) The size of the nucleosomes (180 base pairs) and core particles (145 base pairs). (iii) The number and sizes of DNA fragments produced by micrococcal nuclease in a limit digest. (iv) The sedimentation behavior on neutral sucrose gradients of nucleoprotein particles released by micrococcal nuclease. (v) The number and sizes of DNA fragments produced by DNase I digestion. These results demonstrate that cellular DNA replicated in isolated nuclei is organized into typical nucleosomes. Consequently, subcellular systems can be used to study the relationship between DNA replication and the assembly of chromatin under physiological conditions.

INTRODUCTION

Digestion of plant, animal, or yeast chromatin with micrococcal nuclease results in the production of DNA fragments which are multiples of the smallest fragment that accumulates during the initial phase of the digestion. These fragments reflect the organization of DNA and histones into discrete particles (containing about 200 base pairs) termed nucleosomes (1). Further digestion of chromatin with micrococcal nuclease results in the production of a "core particle" containing about 140 base pairs of DNA and two each of the histones H2A, H2B, H3 and H4. Due to the interaction of DNA and histones, more extensive digestion ("limit digest") of the core particle gives rise to a charac-

teristic array of DNA fragments (2, 3-5). The size of the core particle appears to be conserved in all species examined thus far, whereas the length of the DNA between particles ("linker") varies (1). Despite this variation, digestion of chromatin with pancreatic DNase I always gives rise to a characteristic pattern of DNA fragments on denaturing gels (5, 6-9).

While considerable effort has been focused on the molecular mechanism of DNA replication in eukaryotes, the closely related problem of nucleosome assembly has been largely ignored. We have used isolated mammalian nuclei to determine whether or not DNA replicated *in vitro* is also assembled into nucleosomes. Electrophoretic analysis of DNA fragments extracted from nuclei following *in situ* digestion with either micrococcal nuclease or pancreatic DNase I has been used to compare the state of DNA newly synthesized *in vitro* with that of the bulk of the DNA. In addition, nucleoprotein particles released by micrococcal nuclease have been characterized by sedimentation analysis. The results demonstrate that DNA replicated in isolated nuclei is organized into typical nucleosomes. This subcellular system provides a new avenue for investigating the assembly of mammalian chromosomes under physiological conditions.

MATERIALS AND METHODS

Materials. [^{3}H]thymidine was purchased from New England Nuclear and [α-^{32}P]dATP and [α-^{32}P]dCTP were prepared by the method of Symons (10, 11). *Hae* III restriction endonuclease was purchased from New England Biolabs and *Hind* II and III were prepared according to the procedure of Smith and Wilcox (12). Pancreatic DNase I, micrococcal nuclease, and pancreatic RNase A were purchased from either Sigma or Worthington and Proteinase K from Boehringer-Mannheim. Aliquots of micrococcal nuclease containing BSA (1 mg/ml) and aliquots of DNase I dissolved in 50 mM Tris-HCl, pH 7.6, were stored frozen until just prior to use.

Isolation of Nuclei. The experiments reported here used a CV-1 monkey kidney cell line grown to 90% confluency and infected with SV40 as previously described (13). Twenty-four hours before infection, while the cells were actively growing, 2.5-5.0 μCi of [^{3}H]thymidine (50 Ci/mmole) was added to 10 ml of medium in each culture dish to label the DNA. Following virus infection the medium was replaced, but [^{3}H]thymidine was omitted to prevent further labeling of DNA prior to isolation of nuclei. Fifteen hours after infection, when cellular DNA synthesis was maximal, cells were harvested and nuclei isolated (13).

DNA Synthesis in Isolated Nuclei. Isolated nuclei (prelabeled with [^{3}H]thymidine) were incubated in the presence of cytosol (14) and DNA synthesis was followed by measuring the incorporation of [α-^{32}P]dATP and [α-^{32}P]dCTP (100 Ci/mmol, each) as previously described (13). After incubation at 30 ^{o}C for 60 min the assays were terminated by chilling tubes in ice water. To prevent further incorporation of [α-^{32}P]deoxyribonucleotides during subsequent incubations, the DNA synthesis inhibitor (13) Ara-CTP (200 µM) was added to each assay.

Digestion of Cellular Chromatin with Nucleases. Digestion of cellular chromatin *in situ* was carried out by the addition of 5 µl of micrococcal nuclease (50 units) or 45 µl of pancreatic DNase I (150 µg) directly to ^{3}H/^{32}P-labeled nuclei (105 µl). The reaction mixture contained either 10 mM $CaCl_2$ (micrococcal nuclease) or 10 mM $MgCl_2$ (DNase I). Incubations were carried out at 37 ^{o}C for various times and terminated by the addition of 40 µl of 200 mM EDTA and cooling to 0 ^{o}C.

To determine acid-insoluble DNA remaining after nuclease digestion, each reaction mixture was made 1 M in NaCl in a final volume of 0.5 ml to lyse the nuclei. Ice-cold 5% TCA containing 1% sodium pyrophosphate was added and after 15 min at 0 ^{o}C precipitates were collected on Whatman GF/C filters. The filters were wetted with 0.15 ml water, digested for 1 hr at 50 ^{o}C with 0.75 ml NCS (Amersham Searle Corp.), and analyzed by liquid scintillation counting.

Extraction of DNA. DNA was extracted from nuclease digests in the following manner: Samples were brought to a final volume of 200 µl containing 100 mM NaCl and 0.25% sodium dodecyl sulfate and were then digested with 20 µg Proteinase K for 1 hr at 37 ^{o}C. Samples were deproteinized by mixing first with one-volume of cold phenol (saturated with 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 100 mM NaCl) and then with one volume of $CHCl_3$:isoamyl alcohol (24:1, v/v). After centrifugation the upper aqueous phase, containing DNA, was incubated with 16 µg of pancreatic RNase A (preincubated at 100 ^{o}C for 5 min to inactivate any DNase) for 1 hr at 37 ^{o}C. Samples were then extracted twice with $CHCl_3$:isoamyl alcohol, precipitated overnight with two volumes of ethanol at -35 ^{o}C, washed once with 70% ethanol, and dried *in vacuo*. To remove contaminating [α-^{32}P]deoxyribonucleotides, the pellet was first resuspended in 0.5 ml of buffer (10 mM Tris, 125 mM potassium acetate, 400 mM K_2HPO_4, 2 mM EDTA, titrated to pH 8 with H_3PO_4) and then incubated with 0.2% hexadecyltrimethylammonium chloride for 30 min at 4 ^{o}C. Following centrifugation at 12,000 x g for 5 min, the precipitate was washed with 70% ethanol

containing 0.1 M sodium acetate, then with absolute ethanol, and dried in vacuo. These extraction procedures yielded approximately 50% of the DNA.

Electrophoretic Analysis of Isolated DNA. Native gels. Extracted DNA was reprecipitated by the hexadecyltrimethylammonium chloride procedure described above and the precipitate was dissolved in 25 µl of sterile water. DNA from a single assay was mixed with one volume of 9 mM Tris-borate, pH 8.3, 0.25 mM EDTA, and 50% sucrose containing 0.05% bromphenol blue and 0.05% xylene cyanol FF as tracking dyes. The sample was then subjected to slab gel electrophoresis (1.5 mm x 10 cm x 15 cm) either in 2% agarose (40 mM Tris-acetate, pH 7.9, 20 mM sodium acetate, 2 mM EDTA) overnight at 6 volts/cm and 4 ^{o}C, or in 8% polyacrylamide (15) for 3 hr at 10 volts/cm and room temperature. DNA was stained with ethidium bromide (0.5 µg/ml) for 0.5 hr and the gels were photographed under UV illumination using Polaroid Type 107 film and a Kodak 23A filter. Autoradiography using Kodak XR-5 X-ray film was carried out on gels dried down in vacuo onto Whatman 3 MM filter paper.

Denaturing gels. The procedure of Maxam and Gilbert (16) was followed with certain modifications. The precipitated DNA was dissolved in 10 mM Tris-borate, pH 8.3, 0.2 mM EDTA, then brought to 0.1 M in NaOH and diluted with an equal volume of 10 M urea containing 0.01% bromphenol blue and 0.01% xylene cyanol FF. The samples were heated at 95 ^{o}C for 20 sec, cooled in ice-water, loaded onto 12% polyacrylamide slab gels (1.5 mm x 10 cm x 15 cm) containing 7 M urea (pre-electrophoresed for 1 hr), and run at 360 volts for 2.5 hr. Autoradiography was carried out at -70 ^{o}C using Kodak XR-5 film sandwiched between the gel and a Kodak X-Omatic regular intensifying screen. Gels were stained with ethidium bromide (5 µg/ml) for 30 min, destained for 30 min, and photographed as described above.

RESULTS

DNA Replication in Isolated Nuclei. SV40 infection stimulates quiescent monkey cells to initiate DNA replication and to synthesize associated enzymes and other proteins, such as histones (17-19). This observation was used to maximize the number of cells synthesizing DNA when nuclei were isolated. Since at 15 hr post-infection the rate of DNA replication was maximal in the host (20, data not shown), nuclei were isolated at this time. It is important to note that SV40 DNA replication was not detected until 5 hr later (20, data not shown). Prelabeled nuclei, containing [^{3}H]DNA that had completed replication in vivo, were incubated with [α-^{32}P]deoxyribonucleoside triphosphates under conditions previously found to be optimal for the continuation of either SV40

(13) or cellular (21) DNA replication. The addition of cytosol stimulated cellular DNA synthesis 3 to 5-fold. Incorporation of [^{32}P]dCTP and [^{32}P]dATP stopped after 1 hr at 30 ^{o}C. Sedimentation analysis in alkaline sucrose gradients showed that 80% of the [^{32}P]DNA synthesized in the first 30 sec was 4 to 6S. Continued incubation in the presence of a 100-fold excess of unlabeled substrates showed that these "Okazaki pieces" were completely converted into higher molecular weight DNA (the ratio [^{32}P]DNA/[^{3}H]DNA remained constant). These results were typical of viral and cellular DNA replication systems previously developed and characterized in this (13, 14, 22-24) and other laboratories (21).

Analysis of Chromatin Structure Using Micrococcal Nuclease. Micrococcal nuclease digestion of chromatin from a variety of cell types has been shown to render about 50% of the DNA acid-soluble (2, 25-29). Electrophoretic analysis of the DNA remaining at various times during the digestion has demonstrated a repeating subunit structure for chromatin (1). We have examined the time-course and products of micrococcal nuclease digestion of DNA replicated in isolated nuclei to determine whether or not it is assembled into nucleosomes. Nuclei isolated from cells labeled with [^{3}H]thymidine were incubated with [α-^{32}P]deoxyribonucleotides to label newly synthesized DNA and then treated with micrococcal nuclease. At early times of digestion [^{32}P]DNA was solubilized approximately 1.6 times faster than [^{3}H]DNA (Fig. 1). Despite the initial difference in rates of digestion, 45% of both [^{3}H]DNA and [^{32}P]DNA remained acid insoluble even after extensive incubation. The time course of digestion of [^{3}H]DNA was similar to that reported by others for nonreplicating chromatin and was the same whether or not the nuclei were incubated under the *in vitro* DNA synthesis conditions.

The DNA extracted during the initial rapid phase of the digestion was analyzed by electrophoresis in 2% agarose gels. Ethidium bromide staining revealed at least 12 bands, each being an integral multiple of 180 $\pm$ 18 base pairs of DNA (Fig. 2A). These bands clearly represented DNA from the bulk of the chromatin, since we have calculated from the amount and specific activity of the [α-^{32}P]deoxyribonucleotides incorporated that less than 0.1% of the stained DNA was [^{32}P]-labeled *in vitro*. Autoradiography of [^{32}P]DNA present in the same gel also revealed a series of bands that were multiples of 179 $\pm$ 14 base pairs (Fig. 2B). However, unlike the stained gels, only 5 or 6 bands could be detected on the autoradiograms owing to larger amounts of interband material (Figs. 2C and 2D). Since the [^{32}P]DNA is initially digested nearly twice as fast as the [^{3}H]DNA, we compared a densitometer scan of an

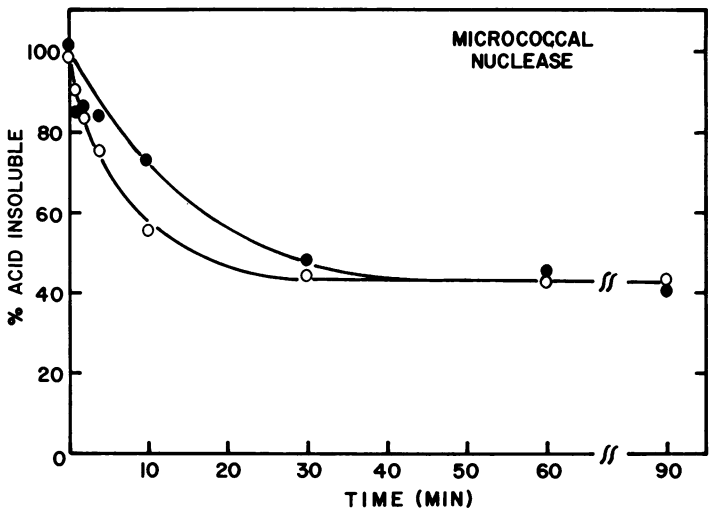

Figure 1. Production of acid soluble DNA by micrococcal nuclease. Cellular DNA was labeled with 2.5 μCi of [^{3}H]thymidine per 100 mm plate of cells for 24 hr prior to viral infection. Nuclei were isolated from these cells and DNA synthesized *in vitro* was labeled using 5 μCi of [α-^{32}P]dATP and [α-^{32}P]-dCTP (133 Ci/mmol) per assay. Digestion *in situ* was carried out by the addition of micrococcal nuclease directly to the reaction tubes as described in Materials and Methods. ● - ●, [^{3}H]DNA labeled *in vivo*; O - O, [^{32}P]-DNA labeled *in vitro*.

autoradiogram of [^{32}P]DNA after 1 min of digestion (Fig. 2D) with a scan of stained DNA after 2 min of digestion (Fig. 2E). Although the overall shape of these two scans was similar, the higher multiples seen on the stained gel were still not discernible on the autoradiogram. This difference in the number of DNA bands resolved was not an artifact of the techniques employed because autoradiograms of cellular [^{32}P]DNA were indistinguishable from the same gels stained with ethidium bromide.

The DNA extracted at late stages of the digestion was analyzed by electrophoresis in 8% polyacrylamide gels. Ethidium bromide staining and autoradiography of the same gel each revealed a series of 6 DNA bands ranging in size from 55 to 145 base pairs (Figs. 3A and 3B). This type of electrophoretic pattern has been shown by others to be a characteristic of the "core particle" of chromatin (2-5).

To obtain additional evidence for the assembly of newly synthesized DNA into nucleosomal particles, products of micrococcal nuclease digestion at a stage when nearly all the DNA contained 180 base pairs or less were sedimented in neutral sucrose gradients. The radioactivity profile showed a major peak

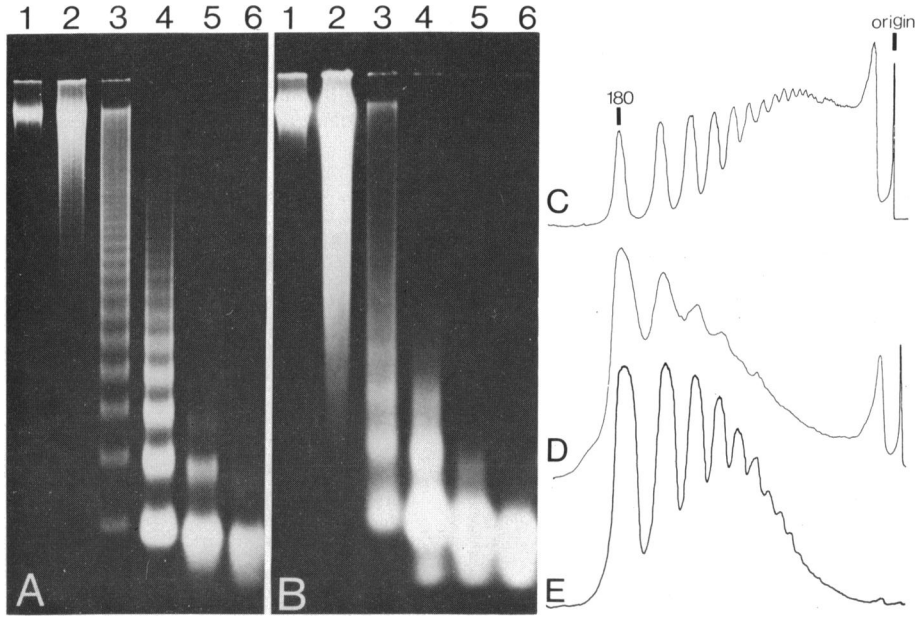

Figure 2. Agarose gel electrophoresis of DNA extracted from a micrococcal nuclease digest of nuclei. Nuclei were isolated, labeled with 20 μCi of [α-^{32}P]deoxyribonucleotides (91 Ci/mmole) in vitro, digested with micrococcal nuclease, and DNA extracted for analysis on 2% agarose slab gels as described in Materials and Methods. All native gels were calibrated using Hae III and Hind II and III restriction fragments of SV40 DNA. The SV40 genome was taken to be 5000 base pairs and previously determined values for the relative sizes of the fragments (36) were used. A plot of log of SV40 fragment size (number of base pairs) against distance migrated was used to determine the sizes of the cellular DNA bands. The lengths of neighboring multiples were subtracted from one another and the mean difference calculated to obtain repeat values of 180 ± 18 (A) and 179 ± 14 (B). (A). Ethidium bromide stained gel. Note that at early times of digestion (1 min) DNA bands up to 12-times the repeat size are visible. Lanes 1-6 represent incubation times of 0, 0.5, 1, 2, 11, and 60 min, respectively. (B). [^{32}P]-autoradiogram of the gel shown in (A). The autoradiogram was exposed for 3 days. To facilitate comparison of panels (A) and (B), note that the autoradiogram has been printed such that [^{32}P]DNA bands are white and the background is dark. (C). Tracing from a densitometer scan of a photographic negative of lane 3 as shown in (A). (D). Tracing from a densitometer scan of a photographic negative of lane 3 as shown in (B). (E). Tracing from a densitometer scan of a photographic negative of lane 4 as shown in (A).

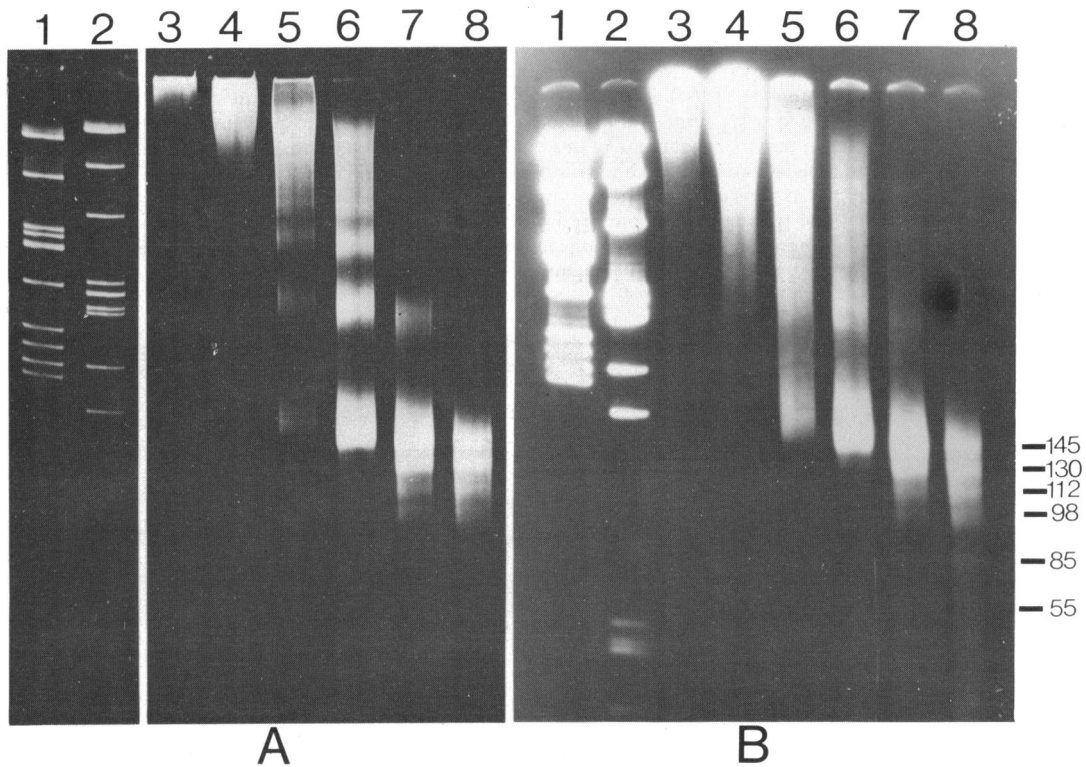

Figure 3. Polyacrylamide gel electrophoresis of DNA extracted from a micrococcal nuclease digest of nuclei. Samples were prepared for analysis on 8% polyacrylamide slab gels as described in the legend to Fig. 2. (A). Ethidium bromide stained gel; lane 1, Hind II and III digest of SV40 [^{32}P]DNA; lane 2, Hae III digest of SV40 [^{32}P]DNA; and lanes 3-8 represent incubation with micrococcal nuclease for 0, 0.5, 1, 2, 11, and 60 min, respectively. It should be noted that the 55 base pair fragment is seen more clearly in an over-exposed photograph of the gel. (B). [^{32}P]-autoradiogram of the gel shown in (A). The autoradiogram was exposed for 3 days and is reproduced here as described in the legend to Fig. 2.

at either 14S (Mg^{++} present) or 11S (EDTA present) for both [^{3}H]DNA synthesized in vivo and [^{32}P]DNA synthesized in vitro (Fig. 4). Nearly 80% of the [^{32}P]-labeled nucleoprotein complex co-sedimented with the in vivo labeled complex, while the remainder sedimented more slowly. The sedimentation properties described here are typical of nucleosomes released by micrococcal nuclease digestion of chromatin (30, 21).

Analysis of Chromatin Structure Using Pancreatic DNase I. When the DNA extracted from DNase I digests of chromatin is subjected to electrophoresis on denaturing gels, a series of single stranded fragments which vary from 10

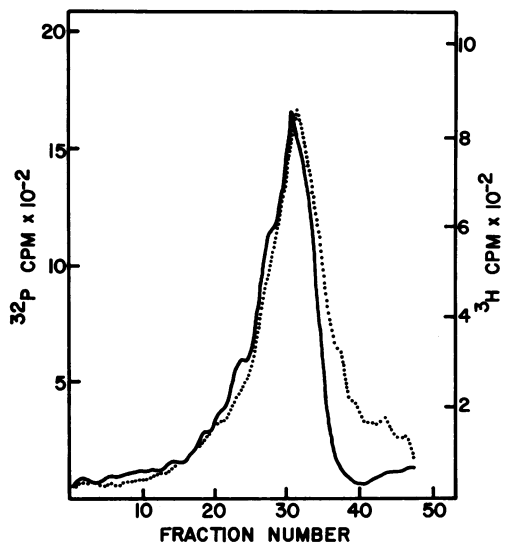

Figure 4. Neutral sucrose gradient analysis of micrococcal nuclease digestion product. Nuclei isolated from cells labeled with [^{3}H]thymidine were incubated with 3 µCi of [α-^{32}P]deoxyribonucleotides (91 Ci/mmol) and then digested for 2 min with micrococcal nuclease as described in Materials and Methods. At this time 35% of the [^{3}H]DNA and 47% of the [^{32}P]DNA was acid soluble. The digestion was terminated with 35 µl of 200 mM EDTA and samples were centrifuged at 12,000 x g. The entire supernatant was layered on a 5-10% linear sucrose gradient containing 10 mM Tris-HCl, pH 7.4, 25 mM KCl, 2 mM $MgCl_2$ and was centrifuged in a Beckman SW50 rotor at 4 °C for 3 hr at 50,000 rpm. Fractions collected from the bottom of the tube were acid precipitated on GF/C filters and subjected to liquid scintillation counting. In a separate experiment, the gradient contained 10 mM Tris HCl, pH 7.4, 50 mM NaCl and 1 mM EDTA. The appearance of the [^{3}H] and [^{32}P]-labels were similar under both sets of conditions. A calibration curve for sedimentation coefficient was constructed from linear DNA of known sizes centrifuged in a separate tube (37). The standards used were full length linear SV40 DNA and the fragments produced by Eco R1 and Hpa 1 digestion of SV40 DNA (11). [^{3}H]DNA (——); [^{32}P]DNA, (- - - -).

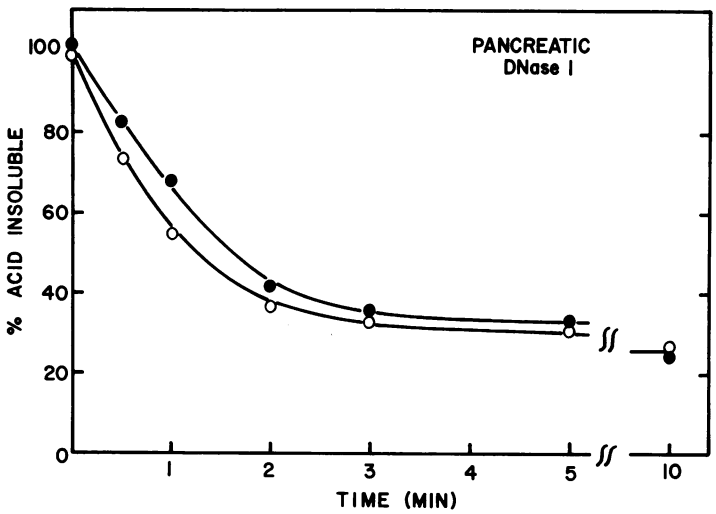

Figure 5. Production of acid-soluble DNA by pancreatic DNase I. These experiments were carried out as described in the legend to Fig. 1, except that 3 μCi of [α-^{32}P]dATP and [α-^{32}P]dCTP (50 Ci/mmol) was used. ● - ●, [^{3}H]DNA labeled *in vivo*; ○ - ○, [^{32}P]DNA labeled *in vitro*.

to 140 nucleotides in length and are integral multiples of 10 nucleotides has been observed (5-7). This pattern is believed to reflect the organization of the DNA within the nucleosome. We found that at very early times of digestion in isolated nuclei, DNase I solubilized [^{32}P]DNA labeled *in vitro* slightly faster than [^{3}H]DNA labeled *in vivo*; nevertheless, the final extent of digestion was the same in both cases (Fig. 5). Ethidium bromide staining of DNA fragments extracted from a DNase I digest and subjected to gel electrophoresis under denaturing conditions revealed a series of bands 10 to 140 nucleotides in length, spaced at intervals of 10 nucleotides (Figs. 6A and 6B). Autoradiograms of [^{32}P]DNA in the same gel also showed a pattern of bands indistinghishable in spacing from the stained material (Fig. 6C).

DISCUSSION

The results of experiments reported in this paper using micrococcal nuclease and pancreatic DNase I support the conclusion that DNA synthesized in isolated nuclei is assembled into nucleosomes. In all experiments the properties of DNA synthesized *in vitro* ([^{32}P]DNA) were compared with those of DNA in the bulk of the chromatin ([^{3}H]-labeled or ethidium bromide stained DNA) within the same nuclei. We found that both types of DNA were digested to the same extent by either micrococcal nuclease or pancreatic DNase I.

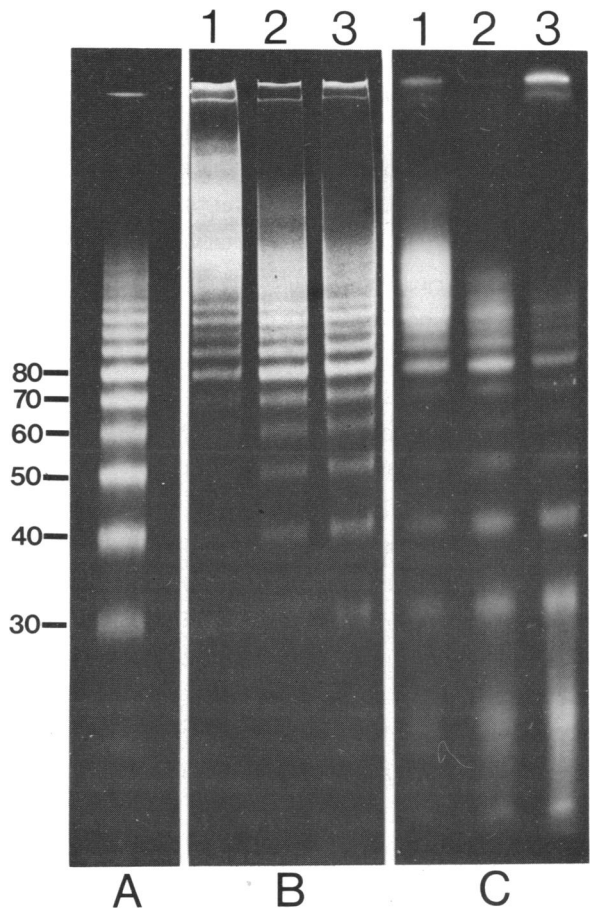

Figure 6. Polyacrylamide gel electrophoresis of DNase I digestion products under denaturing conditions. These digestions were carried out as described in the legend to Fig. 5. Samples were prepared and analyzed on 12% polyacrylamide gels containing 7 M urea as described in Materials and Methods (A). Ethidium bromide stained gel of DNA extracted from a DNase I digest of rat liver chromatin. (B). Ethidium bromide stained gel of DNA extracted from isolated monkey cell nuclei after DNase I digestion for 1, 2, and 3 min (lanes 1-3, respectively). (C). [^{32}P]-autoradiogram of the gel shown in (B). The autoradiogram was exposed for 29 hr and is reproduced here as described in the legend to Fig. 2. Differences in the relative intensities among DNA bands in (B) compared with DNA bands in (C) are inherent in the methods of DNA detection. The amount of [^{32}P] is directly proportional to the amount of DNA present whereas the amount of ethidium bromide that binds to single stranded DNA decreases with decreasing chain length (38).

Electrophoretic analysis of the micrococcal nuclease digestion products demonstrated that DNA synthesized *in vitro* was assembled into nucleosomes containing 180 base pairs, with core particles containing 145 base pairs. These results were identical to those obtained for the DNA in bulk chromatin. Furthermore, the [^{3}H] and [^{32}P]-labeled nucleosomes released by micrococcal nuclease co-sedimented on neutral sucrose gradients. Finally, electrophoretic analysis on denaturing gels of DNA extracted from DNase I digests produced an array of single-stranded DNA fragments characteristic of nucleosomes.

Although nucleosomes associated with DNA synthesized *in vitro* were typical of those present in the bulk of the chromatin by several criteria, the state of the linker regions appears to differ. We found that both the bulk of the DNA and [^{32}P]DNA had a 180 base pair repeat and were digested to the same extent by micrococcal nuclease. These results indicate that all of the DNA synthesized *in vitro* is assembled into nucleosomes and that the number of core particles per given length of DNA must be the same as in the bulk of the chromatin. However, micrococcal nuclease initially digested [^{32}P]DNA more rapidly than the bulk of the DNA and the distribution of DNA fragment lengths on agarose gels following digestion was significantly more heterogeneous for [^{32}P]DNA than for the bulk of the DNA. These data suggest that the linker regions in the newly assembled nucleosomes are more exposed than those in the bulk of the chromatin, permitting a more rapid attack by nuclease at a wider range of sites.

The chromatin assembly observed in isolated mammalian nuclei must occur either by reassortment of preexisting nucleosomes over newly synthesized DNA, or by *de novo* assembly of nucleosomes from histones and DNA (32-34). The cytosol added to nuclei during *in vitro* replication could serve as a source of histones or other factors required for chromatin assembly. In the absence of cytosol apparently normal core particles were formed, but the extent of digestion of newly synthesized DNA by micrococcal nuclease was greater than in the presence of cytosol (data not shown). Laskey *et al.* (35) have recently shown that in a supernatant fraction from *Xenopus laevis* eggs, SV40 DNA is converted into a nucleoprotein complex resembling naturally occurring SV40 chromatin. This *in vitro* assembly requires a thermolabile factor found in the supernatant fraction. Whether or not a comparable factor is present in the cytosol in our experiments is not yet clear.

Isolated nuclei have now been shown to provide physiological conditions that maintain a close relationship between DNA replication and chromatin assembly. This subcellular system permits direct manipulation of the environ-

ment, isolation and characterization of intermediate structures and identification of factors that may be involved in chromatin assembly.

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