Chromatin Replication in Vitro. Properties of a HeLa Nuclear System†

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ABSTRACT: An in vitro HeLa chromatin replication system was developed and characterized. Purified nuclei synthesized DNA from replication forks which were undergoing chain elongation at the time of cell fractionation. DNA replication in isolated nuclei was deficient in DNA maturation functions; the Okazaki fragments synthesized in vitro accumulated and were ligated to high-molecular-weight DNA with low efficiency. The chromatin protein components of nuclei incubated in the DNA replication assay conditions were investigated for displacement, degradation, and exchange. Displacement of total nuclear protein during in vitro incubation occurred to the extent of 3%, and involved only the nonhistone nuclear proteins. Degradation was not detectable, assayed both by loss of acid-soluble radioactivity and by protein electrophoretic patterns in polyacrylamide gels. No detectable protein was exchanged from chromatin to exogenous DNA.

DNA synthesis in vitro has been demonstrated in a wide variety of eukaryotic systems, but has been characterized most extensively in HeLa cell nuclei (Kidwell and Mueller, 1969; Friedman and Mueller, 1968, 1969; Hershey et al., 1973a,b; Krokan et al. 1975a,b; Fraser and Huberman, 1977). When nuclei are isolated and incubated with ATP, magnesium, and deoxynucleoside triphosphates, they synthesize a limited amount of DNA, reaching a maximum of 1–5% of total DNA. In most cases in which the mode of DNA synthesis was characterized sufficiently, chain elongation in vitro represented extension from replication forks which existed at the time of cell fractionation (Hallick and Namba, 1974; Hershey et al., 1973a; Krokan et al., 1975a; Fraser and Huberman, 1977). However, severe fractionation procedures which fragment nuclei have been demonstrated to cause incorporation at sites other than replication forks (Grisham et al., 1972; Kaufman et al., 1972).

The cytoplasmic fraction of cell lysates contains soluble factors which stimulate nuclei to synthesize DNA at a greater rate and extent (Friedman and Mueller, 1968; Kumar and Friedman, 1972; Hershey et al., 1973a,b; Tseng and Goulian, 1975; Fraser and Huberman, 1977). Although the characterization and fractionation of these cytosol components is an important and necessary step toward elucidation of the molecular nature of eukaryotic replication, little progress in this vital area has been reported.

The behavior of the protein component of chromatin during replication in vitro has received little attention. It was the purpose of this investigation to establish an in vitro DNA replication system derived from HeLa cells, with purified nuclei alone (no crude cytosol extract added), and to characterize the effect of in vitro incubation conditions on chromosomal proteins. The chromatin proteins were surveyed for degradation, displacement, and exchange. The results from this study establish that the HeLa system is well suited for the study of chromatin replication in vitro, for none of the protein artifacts examined were found to be significant.

Materials and Methods

HeLa cells were grown to a density of 2–5 × 10^5 cells/mL as previously described (Seale, 1976) and harvested in exponential growth for all experiments.

When prelabeled cells were to be utilized, the radioisotope was added 16–20 h prior to cell harvest. The concentrations of the radioactivities utilized for prelabeling were 0.01 μCi of [^{14}C]thymidine/mL, 0.2 μCi of [^{3}H]thymidine/mL, 0.25 μCi of [^{3}H]lysine/mL, or 0.02 μCi of [^{14}C]lysine/mL. For lysine labeling, medium depleted of lysine by 50% was utilized.

The procedures for cell fractionation and in vitro incubation were essentially those described by Krokan et al. (1975a). Cells were washed and swelled in buffer A (10 mM Tris,[1] pH 7.8, 3 mM $MgCl_2$, 2 mM EGTA, and 2 mM mercaptoethanol). Lysis was accomplished by 15 gentle strokes in a tight-fitting glass homogenizer. One-third volume buffer B (340 mM Tris, pH 8.1, 3 mM $MgCl_2$, 2 mM EGTA, 150 mM glucose) was added, and nuclei were pelleted at 800*g* for 60 s. Nuclei were suspended in the 2:1 buffer A and B mixture and the nuclear density was determined in a hemacytometer. The volume containing 5 × 10^6 nuclei was dispensed to tubes, 2 mL of buffers A–B was added, and the tubes were centrifuged at 800*g* for 60 s. This procedure yields a nuclear population containing less than 2% whole cells as monitored by light microscopy and contains minimal cytoplasmic contaminants (Krokan et al., 1975a,b). Omission of buffer B had no effect. The supernatant was then decanted and residual buffer was removed by aspiration. One hundred microliters of assay mixture (4 °C) was added, nuclei were dispersed by gentle agitation, and the reaction was initiated by placing the tubes in a 37 °C bath. The assay mixture contained 50 mM glucose, 12 mM $MgCl_2$, 10 mM ATP, 1 mM EDTA, 0.1 mM each of dATP, dGTP, and dCTP, 2 mM dithiothreitol, 60 mM Hepes, pH 8.0, and 4 μCi of [^{3}H]TTP (40 Ci/mmol), unless indicated otherwise. Reactions were terminated by placing the tubes on ice and adding 4 mL of buffer A. The suspension was centrifuged, the supernatant was decanted, and nuclei were precipitated with 10% Cl_3AcOH, 10 mM sodium pyrophosphate. Nuclei were col-

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; BUdR, 5-bromo-2′-deoxyuridine; FUdR, 5-fluoro-2′-deoxyuridine; Tris, tris(hydroxymethyl)aminomethane; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DEAE, diethylaminoethyl.

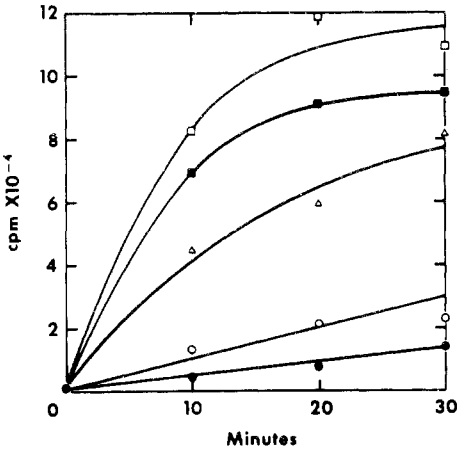

FIGURE 1: Extent of [^{3}H]TTP incorporation as a function of number of nuclei. The nuclear densities were 1×10^6/100 μL (●), 2×10^6/100 μL (○), 4×10^6/100 μL (△), 8×10^6/100 μL (□), and 1×10^7/100 μL (■).

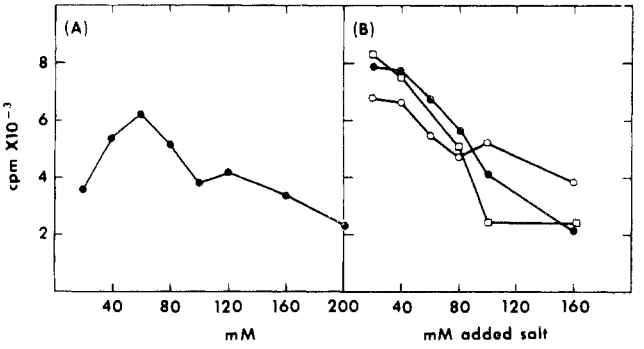

FIGURE 2: Effect of ionic strength on in vitro [^{3}H]TTP incorporation. (A) Incorporation as a function of buffer concentration (Tris or Hepes). (B) Incorporation as a function of monovalent cations added to a reaction mixture containing 60 mM buffer.

lected by filtration on Reeve Angel 1034 AH glass-fiber filters, and digested with 0.2 mL of NCS solubilizer (Amersham Searle) containing 5% water at 50 °C for 2 h. Scintillation cocktail was added, vials were incubated at 37 °C for 12 h, and the radioactivity was determined. ^{14}C spill was determined and subtracted from tritium counts by the automatic external standard method.

Samples to be centrifuged in alkaline sucrose gradients were first digested overnight with 100 μg/mL proteinase K (EM Laboratories) in 10 mM EDTA, 0.05% sodium dodecyl sulfate. One volume of 0.4 N NaOH, 2% Sarkosyl (K and K Laboratories), and 20 mM EDTA were added, and the tubes were agitated on a vortex mixer for 30 s. This mixing step was included to shear high-molecular-weight DNA to about 30–40 S and thus reduce the viscosity of the sample without affecting the molecular weight of the smaller DNA fragments to be analyzed (Figure 3). Samples were layered on 5–28.1% isokinetic sucrose gradients (McCarty et al., 1974) containing 0.8 M NaCl, 0.2 N NaOH, and 0.1% Sarkosyl. Centrifugation was for 16 h at 25 000 rpm in a Beckman SW27 rotor.

Nuclei to be analyzed in CsCl gradients were lysed by dissolving in 5.5 mL of CsCl, density 1.70, in 2 mM EDTA, 10 mM Tris, pH 7.6. The sample was sheared by four passages through a 22-gauge needle and centrifuged at 30 000 rpm for 48 h in a Beckman SW 50.1 rotor. Ten-drop fractions were collected from the bottom of the tube.

Density gradient fractions were precipitated with 10% Cl_3AcOH, 50 μg each of DNA and albumin were added as carrier, and precipitates were collected on filters, digested with NCS, and counted, as described above.

TABLE I: Effect of Deletion of Reaction Mixture Components.

	cpm	% of control
Complete	87 500	100
−EDTA	79 100	90
−Glucose	79 700	91
−ATP	15 750	18
−Mg	400	0.5
−dATP	19 500	22.2
−dCTP	8 800	10
−dGTP	9 000	10.3

TABLE II: Properties of the in Vitro [^{3}H]TTP Incorporation Product.

Treatment	cpm	% of control
None (control)	24 700	100
DNase I, 100 μg/mL, 30 min	90	0.4
Proteinase K, 100 μg/mL, 12 h	24 400	98.8
0.3 N KOH, 37 °C, 12 h	23 500	95.1
10% Perchloric acid, 90 °C, 15 min	345	1.4

Histones were extracted from nuclei with 0.4 N H_2SO_4. The proteins were precipitated with 10 volumes of acetone at −20 °C, collected by centrifugation, and dissolved in 10 M urea, 0.9 N acetic acid. Electrophoresis was performed at 2 mA/tube at 4 °C as described by Panyim and Chalkley (1969), with cytochrome *c* as marker in a separate tube.

Nonhistone proteins in the residue remaining after histone extraction were dissolved directly by boiling in sample buffer and subjected to electrophoresis as described by Weber and Osborn (1969).

Proteins were stained with Coomassie blue in 40% ethanol, 7% acetic acid, and the gels were destained in 7% ethanol, 7% acetic acid containing DEAE resin.

Gels were cut into 1-mm fractions with a Joyce-Loebl gel slicer, digested 2 h with 0.2 mL of NCS solubilizer, and counted.

Results

DNA Replication. The in vitro replication assay utilized here was based on the procedures of Krokan et al. (1975a), a modification of the system first described by Friedman and Mueller (1968). When nuclei were isolated and incubated in the reaction mixture as described under Methods, the nuclei incorporated [^{3}H]TTP into acid-insoluble material for at least 30 min. The degree of incorporation was dependent on the nuclear density (Figure 1); a density of $4–6 \times 10^6$ nuclei per 100 μL of reaction mixture was found to be optimal.

Each component of the reaction mixture was titrated for its ability to stimulate maximal [^{3}H]TTP incorporation. Magnesium and ATP were combined at a ratio of 6:5, respectively, and this mixture was optimal at 12 mM:10 mM. Using a reaction mixture containing the optimal Mg-ATP level, the addition of magnesium chloride (1–20 mM) depressed in vitro DNA synthesis with increasing concentration (not shown). Likewise, ATP added to this reaction mixture, at concentrations of 1 to 20 mM, progressively inhibited incorporation. Incorporation showed a sharp optimum at pH 8.0.

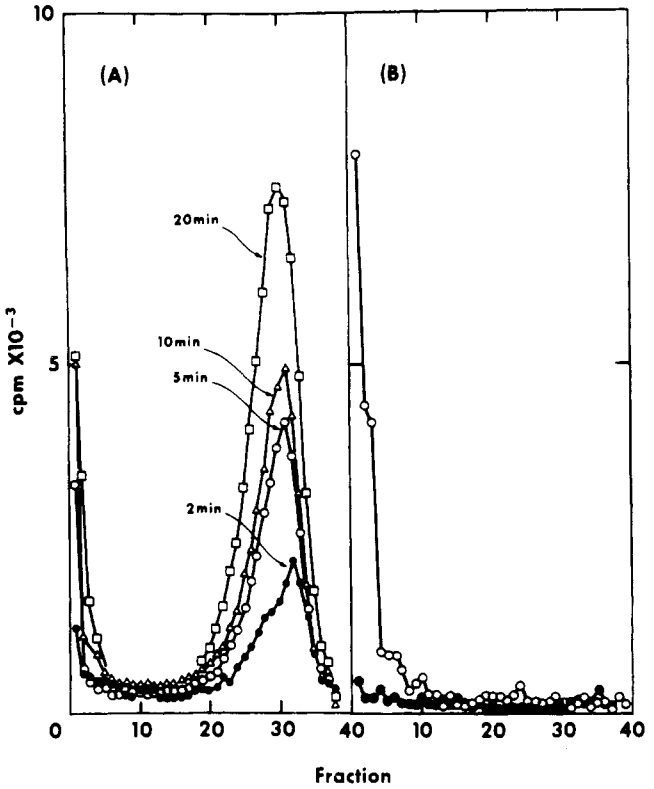

FIGURE 3: Alkaline isokinetic sucrose gradient sedimentation of DNA synthesized in vitro. (A) Samples were withdrawn from the reaction at 2 (●), 5 (○), 10 (△) and 20 min (□) and prepared for centrifugation as described under Methods. (B) Controls: (○) ^{14}C-labeled nuclear DNA from cells prelabeled for 18 h prior to incubation in vitro for 30 min, and (●) nuclei in complete reaction mixture at 0 °C for 30 min prior to preparation for sedimentation analysis. Sedimentation is from right to left.

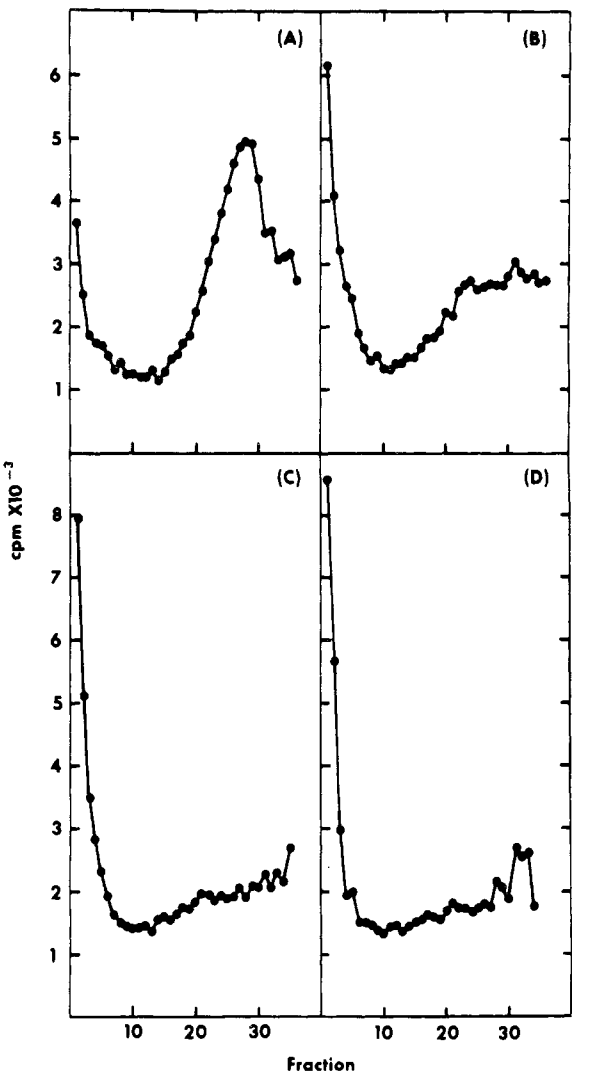

FIGURE 4: Conversion of precursor DNA fragments to high-molecular-weight DNA, following their synthesis. Nuclei were incubated in complete reaction mixture for 5 min, at which time nonradioactive TTP was added to 2 mM. Samples were withdrawn at this time (A) and following 5 (B), 15 (C), and 25 min (D) of further incubation. Further procedures were as in Figure 3.

Because this study was especially concerned with protein displacement and exchange during incubation in vitro, the effect of ionic strength was investigated (Figure 2). Both Tris-HCl and Hepes buffers were found to be optimal at 60 mM. Addition of other ions (NaCl, KCl, NH_4Cl) to increase the ionic strength only served to decrease the amount of [^3H]TTP incorporated (Figure 2B). This result is at variance with the reports of Hershey et al. (1973a,b) and Krokan et al. (1975a) which claimed an ionic strength optimum of 130 mM; the reason for this difference is unknown.

Incorporation was strongly dependent on the presence of all four deoxynucleoside triphosphates (Table I). The low level of synthesis attained upon deletion of one dNTP was not investigated further, but was likely due to residual dNTP pools, for this synthesis was true replication (see Figures 3-5).

Incorporation was strongly dependent on ATP (Table I) and absolutely dependent on magnesium (other divalent cations were not tested). Other components of the system were not obligate for activity, but exclusion caused varying degrees of inhibition.

In order to survey for nonspecific binding of unincorporated tritium, several procedures were tested. Nuclei which had been incubated with [^3H]TTP in vitro at 4 or 37 °C were washed with 5 mL of buffer A and the pellets were (1) precipitated with 10% trichloroacetic acid containing 10 mM sodium pyrophosphate directly, (2) incubated with 0.05% sodium dodecyl sulfate and 100 μg/mL proteinase K at 37 °C for 16 h before acid precipitation, or (3) incubated at 90 °C for 15 min in 10% perchloric acid, and the supernatant was analyzed for radioactivity. The background levels (4 °C incubation) of isotope were subtracted from all values routinely. The radioactivity incorporated was found to be the same for all procedures. Therefore, the simplest procedure, i.e., direct acid precipitation of washed nuclei, was utilized.

The product of in vitro incubation was DNA (Table II). The product was stable to alkali and proteinase K, and was quantitatively degraded to acid solubility by incubation at 90 °C with 10% trichloroacetic acid or by incubation with deoxyribonuclease I.

The sedimentation properties of the DNA product were next investigated. Nuclei were incubated in vitro and samples were withdrawn at 2, 5, 10, and 20 min. Nuclei were digested with proteinase K, and the solution was then made alkaline (pH 12.3) and centrifuged in alkaline isokinetic sucrose gradients (Methods). The product of incorporation by purified nuclei in 2 min was 4.9S DNA fragments which did not mature to high-molecular-weight DNA to a significant degree (Figure 3A). These DNA precursor fragments increased steadily in size during incubation to a size of 5.1 S in 20 min, in accord with results of Krokan et al. (1975b) and Fraser and Huberman (1977). The amount of radioactivity recovered from the

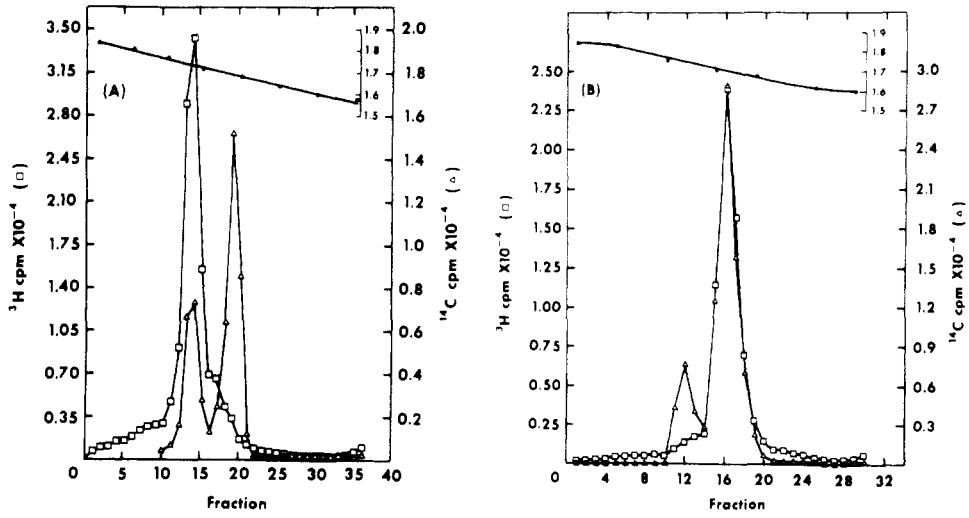

FIGURE 5: Replication fork chain elongation in vitro. The labeling protocol is given in the text. (A) Nuclei incubated in vitro immediately following BUdR incorporation. (B) Nuclei incubated in vitro following 2 h of growth in fresh medium after the BUdR incorporation period. (△) ^{14}C-prelabeled DNA; (□) ^{3}H in vitro synthesized DNA.

TABLE III: Measurement of Nuclear Protein Degradation during Incubation in Vitro.[a]

Minutes at 37 °C	cpm acid insoluble	cpm acid soluble	% acid soluble
0	278 500	2400	0.86
10	276 700	1820	0.65
20	282 000	3450	1.22
30	268 600	3375	1.26

[a] Nuclei were prepared and incubated as described in Table IV. At each time indicated the nuclei were lysed by the addition of 1 mL of 0.1% sodium dodecyl sulfate. Tubes were agitated vigorously on a vortex mixer for 60 s to reduce the viscosity. One aliquot (0.2 mL) was withdrawn to determine total counts and an identical aliquot was precipitated with 10% Cl_3AcOH to determine acid-insoluble radioactivity.

gradients was 85–95% of the material applied. ^{14}C-prelabeled total cellular DNA sedimented to the bottom of the tube, and nuclei exposed to isotope at 0–4 °C and processed as above yielded no acid-precipitable radioactivity in the gradients (Figure 3B).

As shown by the conversion of radioactivity from the precursor DNA peak to more rapidly sedimenting fractions in Figure 3, some ligation did occur. This was better demonstrated by pulse-chase experiments (Figure 4). Nuclei were labeled in vitro for 5 min, unlabeled TTP was added to 2 mM, and samples were withdrawn at the end of the pulse period and at 5, 15, and 25 min of TTP chase. Control experiments showed that there was no additional incorporation following the addition of TTP. The precursor fragments slowly increased in size over the 25-min period examined. Maturation was still incomplete at the termination of the experiment, and 30% of the newly synthesized DNA attained a size of 26.5 S or larger (fractions 1–6). The amount of radioactivity present in the gradient profile in the 5-min sample was totally conserved after 25 min of nonradioactive TTP excess. That ligation could be observed in pulse-chase experiments, but was barely discernible in continuous incorporation experiments (Figure 3), means that chain elongation proceeded at a much faster rate than maturation and ligation, and thus obscured the latter. We have unpublished evidence, in accord with that of Tseng and Goulian (1975) and of Fraser and Huberman (1977), that the functions necessary for DNA precursor maturation and ligation are largely extracted into the cytoplasmic fraction during isolation and washing of nuclei.

In order to demonstrate that the DNA synthesis thus far described was true replicative synthesis and not repair or terminal addition, we demonstrated replication fork elongation in vitro (Figure 5). Cells were prelabeled for one generation with [^{14}C]thymidine, and then washed and grown in fresh medium for 6 h prior to administration of a single 2 mM hydroxyurea block for 12 h. The cells were washed free of hydroxyurea and grown in 10^{-5} M BUdR and 10^{-6} M FUdR for 4 h. Cells were then divided into two aliquots. One was harvested immediately, and one was grown in fresh medium for an additional 2 h prior to harvest. Nuclei were prepared and incubated in vitro with [^{3}H]TTP for 20 min and then digested with proteinase K. DNA was sheared by four passages through a 22-gauge needle and banded in neutral CsCl gradients. [^{3}H]TTP incorporated in vitro immediately after growth in BUdR-containing medium was found exclusively associated with the hybrid density DNA peak (ρ = 1.750), with slight tailing toward normal density DNA (ρ = 1.700), as predicted for random shear of interspersed hybrid density and normal density regions in linear DNA molecules (Painter and Schaefer, 1969). When cells were allowed to grow an additional 2 h prior to in vitro DNA synthesis, the in vitro ^{3}H label banded with DNA of native density (Figure 5B). These results indicate that DNA synthesis in vitro proceeded from replication forks active at the time of cell harvest, and did not reflect either random repair synthesis or repair synthesis in the BUdR-substituted regions of DNA.

Chromatin Proteins. Proteins comprise 65% of the mass of HeLa cell chromatin; experiments were performed in order to investigate the nature and degree of alterations suffered by chromatin proteins during in vitro incubation.

Protein degradation was monitored by release of acid-soluble radioactivity from prelabeled proteins during in vitro incubation, and by electrophoresis of the proteins in polyacrylamide gels. Cells prelabeled for 18 h with [^{3}H]lysine were fractionated, nuclei were incubated in vitro with unlabeled dNTPs, and samples were removed for the determination of whole and acid-precipitable radioactive material (Table III). The loss of

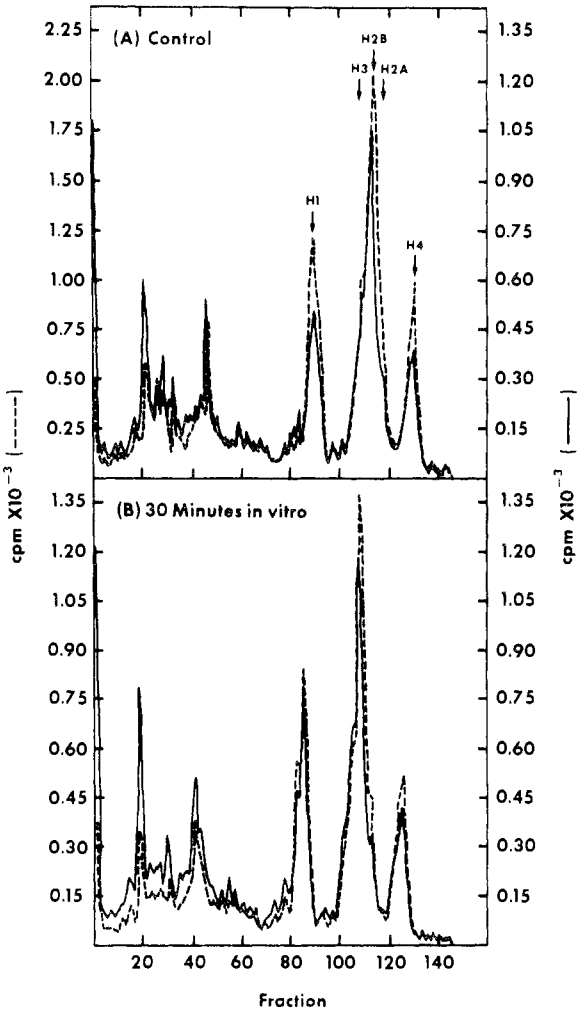

FIGURE 6: Electrophoresis of acid-extractable nuclear proteins prepared before (panel A) and after (panel B) incubation of nuclei in vitro at 37 °C for 30 min. (- - -) ^{3}H-prelabeled acid extract; (—) ^{14}C control (not incubated) acid-extractable nuclear protein.

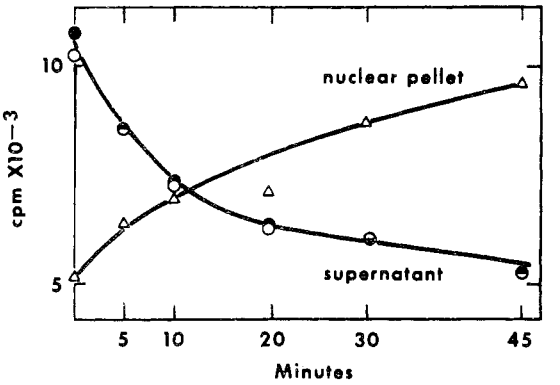

FIGURE 7: Effect of coincubation of exogenous DNA with nuclei in vitro. 5×10^6 nuclei were mixed with 20 μg of HeLa [^{3}H]DNA (1.45×10^4 cpm) in reaction mixture without isotope. At the indicated times, 2 mL of isotonic (to the reaction) mixture (60 mM Tris, pH 8.0, 2 mM mercaptoethanol, 1 mM $MgCl_2$) was added and the suspension was centrifuged at 17 000g for 10 min. Duplicate aliquots of the supernatant were collected for determination of whole (○) and acid-precipitable radioactivity (●) and the pellet (△) was acid precipitated and collected.

TABLE IV: Measurement of Protein Displacement during Incubation in Vitro.[a]

Minutes at 37 °C	cpm Supernatant	Pellet	Total	% Supernatant
0	8 350	249 900	258 250	3.3
5	14 000	210 200	224 200	6.2
10	15 300	247 600	262 900	5.8
20	16 500	254 500	271 000	6.1
30	17 200	246 700	263 900	6.5

[a] Nuclei were isolated from cells incubated for 16 h with [^{3}H]lysine. Nuclei were then incubated in complete reaction mixture lacking radioactive dNTPs for the indicated times. Tubes were removed and put in ice and 1 mL of isotonic mixture was added (legend to Figure 7). Tubes were centrifuged at 5000 rpm for 10 min and the supernatant was separated from the pellet for acid-precipitable radioactivity determination.

acid-insoluble lysine during incubation in vitro was found to be negligible.

[^{3}H]Lysine-labeled (20 h) nuclei were extracted with 0.4 N H_2SO_4 to separate the histone and nonhistone chromatin proteins for gel electrophoretic analysis of samples collected both before and after incubation. In addition, ^{14}C-labeled control proteins were mixed with ^{3}H-labeled proteins extracted from unincubated and incubated nuclei. The histone profiles of samples taken before and after incubation for 30 min at 37 °C are shown in Figure 6. The proteolysis-susceptible H1 proteins were intact after 30-min incubation in vitro, and no change of electrophoretic mobility was detected for any acid-soluble protein. The same lack of degradation was found in the nonhistone nuclear protein population.

Displacement of proteins during incubation of nuclei in vitro was tested by centrifuging nuclei after incubation and measuring radioactive protein remaining in the supernatant. Three percent of total nuclear radioactivity was released from non-incubated nuclei held on ice during the experiment (30 min). During incubation at 37 °C, an additional 3% of total radioactive protein was released from nuclei (Table IV). These proteins were shown to be exclusively nonhistone proteins by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels.

Chromatin protein exchange was to be measured by the transfer of protein to exogeneous DNA during incubation in vitro. However, the exogenous DNA progressively adsorbed to the nuclei during incubation (Figure 7). All the radioactive exogeneous DNA was present after 30 min of incubation and the size of the exogenous DNA was slightly reduced, as measured by sedimentation. Because added DNA adhered to nuclei, the analysis of soluble exogenous DNA-protein complexes was not possible. It was necessary to rely on the nuclease sensitivity of exogenous DNA following incubation with nuclei in vitro, in order to detect exchanged protein, on the premise that such exchanged protein would shield DNA from nucleolytic attack (Clark and Felsenfeld, 1971). When DNA, mixed with nuclei, was digested following 0, 10, 20, and 30 min of incubation in vitro, it was found to be completely nuclease sensitive in all cases (Figure 8). Thus, protein exchange was not detectable. The slower kinetics of DNA degradation with increasing time of in vitro incubation was probably due to the adsorption of the DNA to the nuclei (Figure 7), creating hindrance to the accessibility of the enzyme. As an internal control, the degradation of the nuclear DNA in these experiments was not altered by incubation in vitro with exogenous DNA (Figure 8B).

Discussion

The product of TTP incorporation by isolated HeLa nuclei

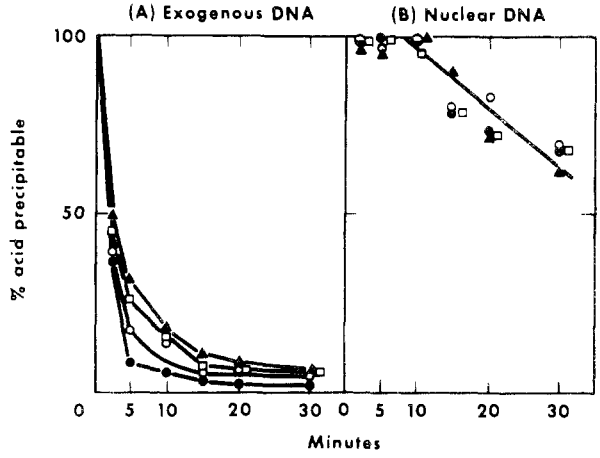

FIGURE 8: Micrococcal nuclease digestibility of exogenous DNA following incubation of nuclei in vitro. Samples were collected at 0 (●), 10 (○), 20 (▲), and 30 min (□) of incubation in vitro. Nuclei were washed and suspended in RSB (10 mM Tris-HCl, pH 7.6, 1.5 mM $MgCl_2$, 10 mM NaCl) and 0.3 volume of 10^{-4} M $CaCl_2$ was added. Digestion was performed at 37 °C by addition of 6 units of enzyme and points were collected at the indicated times. Panel A, exogenous [^{3}H]DNA, panel B, ^{14}C-prelabeled nuclei in the same test mixtures.

is DNA. The product was sensitive to pancreatic deoxyribonuclease and to acid hydrolysis. In accord with other properties of DNA, the product was resistant to alkaline hydrolysis and to proteolysis.

By titrating each component of the reaction mixture, the various optima were found to be in good agreement with previously reported results (Hershey et al., 1973a,b; Krokan et al., 1975a) with the sole exception of ionic strength. Our results indicated an ionic strength optimum at 60 mM rather than 130 mM. This lower ionic optimum is actually more favorable for the study of chromatin protein behavior in vitro, for nonspecific exchange of proteins is minimized (Germond et al., 1976) while optimizing the system for maximal DNA replication.

The DNA which is synthesized in vitro accumulates as DNA precursor, or Okazaki fragments. While some ligation did occur, as was shown in pulse-chase experiments, (1) little of the DNA which was processed to higher molecular-weight species attained a size of 30 S during the course of the experiment, and (2) the rate of Okazaki fragment initiation and chain elongation greatly exceeded the rate of maturation of these precursors. The ligation-defective nature of isolated nuclei is attributable to the extraction of maturation functions during nuclear isolation (Krokan et al., 1975a,b; Tseng and Goulian, 1975; Fraser and Huberman, 1977).

Greater than 80% of the DNA synthesized in vitro was recovered in alkaline gradients in the form of single-stranded Okazaki fragments. On chasing with an excess of unlabeled TTP, the peak of radioactivity was lost from the 5S region, and appeared in the higher molecular-weight regions of the gradient. All the initial radioactivity was accounted for at the termination of the experiment. Thus, the DNA precursor fragments did not undergo turnover. Fraser and Huberman (1977) demonstrated that, upon addition of the cytoplasmic fraction to nuclei during the chase period, the DNA precursor fragments are converted to high-molecular-weight DNA. These results suggest that eukaryotic DNA replication proceeds by a two-strand discontinuous mode of synthesis.

The DNA synthesized in vitro was an extension of growing forks which existed at the time of cell fractionation. When cells were fractionated immediately following a period of BUdR incorporation in vivo, the DNA synthesized in vitro was associated with the density-substituted DNA. If cells were allowed to grow for 2 h after the density labeling period, so as to allow chain elongation to proceed away from the density-substituted regions, the DNA synthesized in vitro was then of native density. Thus, in vitro DNA synthesis by HeLa nuclei is true replicative synthesis, and the association of [^{3}H]TTP incorporated in vitro with the hybrid density DNA does not reflect repair synthesis in the density-substituted regions.

The chromatin proteins were investigated for several in vitro artifacts. Proteolysis during incubation was not detectable. No acid-soluble peptides were produced during a 30-min incubation. Electrophoretic patterns of proteins extracted from incubated nuclei showed a normal profile for both histone and for nonhistone chromatin proteins. Mixing experiments of ^{14}C-labeled control proteins with ^{3}H-labeled proteins extracted from nuclei incubated in vitro provided additional proof that proteolysis during in vitro incubation does not generate artifacts, in this system. Indeed, the extremely proteolysis-susceptible histone H1 (Panyim et al., 1968, 1971) was totally intact following 30 min of incubation in vitro.

Displacement was measured by the loss of nuclear radiolabeled protein to the supernatant. There was a progressive loss of radioactivity from nuclei to the supernatant during the course of incubation in vitro. The amount of radioactive protein lost during a 30-min incubation period was 3% above the 3% displaced from nuclei held at 0 °C for the same period. In polyacrylamide gels these proteins were shown to be nonhistone proteins. Since whole nuclei were utilized for these experiments, it is quite likely that this protein displacement represents leakage of soluble, nonchromatin nuclear proteins, and not the displacement of true chromatin-bound proteins. Displacement of histones was not observed.

Measurement of protein displacement detects only proteins that do not bind chromatin or that have not found a suitable binding site after dissociation. In order to detect exchangeable protein, free DNA was added to nuclei to provide excess binding sites, and the mixture was incubated in vitro. Unfortunately, the exogenous DNA progressively adhered to the nuclei during incubation. This precluded experiments designed to detect proteins bound to free DNA by the assay of exogenous DNA–protein complexes after their separation from nuclei. The alternative approach was to measure the nuclease accessibility of the exogenous DNA. The DNA added to nuclei in vitro was found to be totally digestible even after 30 min of incubation with nuclei in vitro. The slightly slower kinetics of digestion of the exogenous DNA after incubation probably reflect the aggregation of the DNA with nuclei, rather than exchange of protein. In the ionic environment utilized, there is little possibility that nucleosomes could exchange to DNA (Camerini-Otero et al., 1976; Germond et al., 1976). The pattern and degree of protection of DNA from micrococcal nuclease digestion, due to nucleosome association with the exogenous DNA, was not evident. During incubation, the exogenous DNA was quite stable: no loss of acid-soluble radioactivity occurred, but the DNA was slightly reduced in size in neutral isokinetic sucrose gradients.

In summary, HeLa nuclei in vitro incorporate TTP into DNA by the replicative mode. The observed behavior of chromosomal proteins seems to exclude the artifact-inducing problems of degradation and exchange, and displacement of proteins is not only on a very low scale, but does not involve the histone proteins. This system is thus not only exceedingly useful for the detection and characterization of factors required for DNA synthesis, but has also proven most suited for the study

of the interrelationship of parental nucleosomes and DNA during DNA replication (Seale, manuscript in preparation).

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Structural Investigations of Chromatin Core Protein by Nuclear Magnetic Resonance†

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ABSTRACT: A complex derived from chromatin containing one molecule of each of histones H2A, H2B, H3, and H4, termed core protein, was studied by ^{13}C and ^{1}H nuclear magnetic resonance. ^{13}C line widths, when analyzed and compared with those of native and thermally unfolded representative globular proteins, showed that regions of the core protein possess considerable mobility. Studies of C_α and C_β line widths, and C_α spin–spin relaxation times, show that this mobility arises from sections of random-coil polypeptide. It is argued that these regions are N-terminal "tails", attached to C-terminal globular polypeptides. The 270-MHz ^{1}H nuclear magnetic resonance spectrum shows numerous ring current shifted resonances, indicating that the C-terminal globular domain has a precise tertiary structure. The globular domain most likely forms the histone "core" of the chromatin monomer particle, whilst the basic tails probably wind around the grooves of the double helix, enabling the basic side chains to interact with the DNA phosphate groups. Some biological implications of this model are considered.

Whilst the subunit structure of chromatin is now well established (Woodcock, 1973; Van Holde et al., 1974; Olins and Olins, 1974; Kornberg, 1974; Burgoyne et al., 1974; Noll, 1974a,b; Richards et al., 1976), many details of the substructure of the repeat unit await elucidation. Nuclease resistant monomer core particles from chromatin are composed of two molecules each of histones H2A, H2B, H3, and H4 (Thomas and Kornberg, 1975a), which are enclosed by a segment of DNA double helix comprising 140 base pairs (Pardon et al., 1975). Further structural description requires analysis of both histone–histone and histone–DNA interactions.

The isolation of a complex containing all four histones (Thomas and Kornberg, 1975b; Weintraub et al., 1975), which

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