DNA Synthesis in Isolated HeLa Cell Nuclei. Optimalization of the System and Characterization of the Product[†]

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ABSTRACT: DNA replication in isolated nuclei from synchronized HeLa cells has been studied in an effort to optimalize the system and characterize the product. The synthesis was highly dependent on the four deoxyribonucleoside triphosphates, ATP, and Mg^{2+}. Optimum pH was about 7.8. The system was further stimulated by monovalent ions with NH_4Cl and Tris-HCl (each 65 m*M*) being the most effective. The four ribonucleoside triphosphates and glycerol gave a slight but very reproducible and additive stimulation. Low concentrations of spermine and spermidine (0.2–1.5 × 10^{-4} *M*) were also slightly stimulatory (10–15%) whereas higher concentrations were inhibitory. The reaction product was DNase sensitive, and banded at 1.699 g/ml in neutral CsCl together with bulk HeLa nuclear DNA. When studied by neutral CsCl and alkaline Cs_2SO_4 gradients, the incorporation of [^{3}H]TTP was mainly (more than 85%) due to further elongation of strands initiated in vivo as evidenced by BrdUrd labeling.

The replication of DNA in eucaryotic cells is a very complex process. As in procaryotes, studies with more or less purified DNA polymerases from eucaryotic cells have not given sufficient information, and more intact systems have been looked for.

Several studies have therefore appeared on DNA synthesis in vitro in isolated nuclei from different cells [rat liver (Morioka et al., 1973; Lynch et al., 1970, 1972; Hyodo and Ono, 1970; Kaufman et al., 1972; Probst et al., 1972; Cook, 1972), rat thymus (Lagunoff, 1969), rat brain (Shimada and Terayama, 1972), Ehrlich ascites cells (Teng et al., 1970), *Physarum polycephalum* (Brewer and Rusch, 1965, 1966), BHK cells (Lazarus, 1973), L cells (Kidwell, 1972), HeLa cells (Friedman and Mueller, 1968; Kidwell and Mueller, 1969; Kumar and Friedman, 1972; Bernard and Brent, 1973; Radsak, 1973; Hershey et al., 1973a,b), and polyoma-infected mouse fibroblasts (Magnusson et al., 1972; Winnacker et al., 1972)]. These systems generally give a very low synthesis of DNA compared to the input of template and only a few reports (Lynch et al., 1970; Kidwell and Mueller, 1969; Hershey et al., 1973a,b; Winnacker et al., 1972) give evidence for a true replication of DNA, i.e. in vitro synthesis as a continuation of the in vivo process. In some systems the observed synthesis is mainly a repair process (Hyodo and Ono, 1970; Kaufman et al., 1972) for which inactive nuclei may be activated by centrifugation in hypertonic sucrose. Initiation of new replication sites has not been clearly demonstrated in any system using the endogenous template in isolated nuclei from animal cells.

In an effort to improve the incorporation into DNA in a truly replicating system, we have investigated the optimal conditions for isolation and incubation of HeLa cell nuclei in aqueous media. To allow a subsequent study of stimulatory cytoplasmic factors a nuclear system was chosen. The final system incorporates about 5 nmol of TMP per mg of DNA. More than 85% is incorporated into strands labeled with a pulse of BrdUrd[1] in vivo before isolation of nuclei.

Materials and Methods

HeLa S_3 cell suspension cultures were maintained and synchronized according to Mueller and Kajiwara (1966) and reversed with thymidine (3 μg per 10^6 cells) or BrdUrd (6 μg per 10^6 cells). The cells were harvested 3 hr after reversal of the block.

Deoxynucleoside 5′-triphosphate (dNTP) and nucleoside 5′-triphosphates (NTP) were from Sigma, St. Louis, Mo., and Schwarz/Mann, Orangeburg, N.Y. [^{3}H]TTP (Radiochemical Centre, Amersham, U.K.) was dried from the ethanolic solution by evaporation and dissolved in doubly distilled water. Suitable aliquots were frozen until use.

Actinomycin D, RNase A, spermine, spermidine, and putrescine were obtained from Sigma. Rifampicin AF/012 and AF/013 were kindly donated by Dr. G. Lancini, Lepetit, Milano, Italy; α-amanitin was obtained from Calbiochem, San Diego, Calif. Eagle's minimum essential medium (MEM) (Gibco, Grand Island, N.Y.) was supplemented with 0.1 m*M* glycine, 0.1 m*M* serine, and 5% calf serum (Gibco).

Isolation of Nuclei. A modification of the method of Friedman and Mueller (1968) was used. Buffer A contained 2 m*M* EGTA,[1] 3 m*M* $MgCl_2$, and 2 m*M* 2-mercaptoethanol in 10 m*M* Tris-HCl (pH 7.5) and buffer B contained 2 m*M* EGTA, 3 m*M* $MgCl_2$, 150 m*M* glucose, and 0.15% Triton X-100 or Brij 58 in 340 m*M* Tris-HCl (pH 8.1). Buffer C is a mixture of buffers A and B (2:1).

The cells were pelleted at 2000*g* min at 37°, washed once, and resuspended in ice-cold buffer A at 70 × 10^6 cells/ml.

All solutions and containers subsequently used were kept ice cold. After swelling for 10 min the cells were broken in a Dounce homogenizer. Twenty to thirty strokes were usually

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[1] Abbreviations used are: SSC, standard saline citrate is 150 m*M* NaCl in 15 m*M* sodium citrate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BrdUrd, bromodeoxyuridine.

necessary. The preparations were controlled by phase contrast microscopy. Isotonicity was restored by adding 0.5 vol of buffer B. Buffer C (10 ml) was then added to increase the washing fluid volume. The diluted homogenate was centrifuged (2400*g* min) and the pellet resuspended in buffer C without Triton X-100. This suspension was added in 1-ml aliquots to the number of centrifuge tubes necessary for the experiment. The nuclei were pelleted (810*g* min) and immediately resuspended for use in incubation experiments.

Incubation Mixture. Unless otherwise stated, the test mixture contained 50 m*M* glucose, 10.7 m*M* $MgCl_2$, 1 m*M* EDTA, 0.1 m*M* of each of dATP, dCTP, dGTP, and [^{3}H]TTP (50 μCi/μmol), 10 m*M* ATP, and 2 m*M* 2-mercaptoethanol in 130 m*M* Tris-HCl (pH 8.1) (20°). The pellet was resuspended in a 0.5-ml test mixture and incubated at 37° for the desired length of time. Usually about 10^7 nuclei corresponding to about 100 μg of DNA were used for each sample. The reaction was stopped by cooling on ice. One milliliter of bovine serum albumin (Sigma) (2.5 mg/ml) and 1 ml of 0.8 *M* perchloric acid, both in ice-cold 10 m*M* sodium pyrophosphate, were added. The mixture was left for 15 min in an ice bath.

Assay of [^{3}H]TTP Incorporation. The perchloric acid precipitates were washed three times by resuspension in 0.4 *M* perchloric acid containing 5 m*M* sodium pyrophosphate and centrifugation (5000*g* for 15 min at 2°), dissolved in cold 0.3 *M* KOH, and reprecipitated with 2 vol of 0.8 *M* perchloric acid containing 10 m*M* sodium pyrophosphate. The precipitates were washed once as above. This last washing fluid was checked for radioactivity and the washing repeated if any activity was present. The precipitates were then extracted with 2 ml of 0.6 *M* perchloric acid at 90° for 20 min and two aliquots of 0.5 ml were withdrawn for counting in 10 ml of Instagel (Packard Instrument International, Zürich, Switzerland) in a Packard Tri-Carb liquid scintillation counter. The counting efficiency was 31-32%. The remainder of the extract was used for DNA determination by the method of Burton (1956).

DNase Treatment. The nuclear pellet after incubation was washed free of radioactivity with 0.4 *M* perchloric acid in 10 m*M* sodium pyrophosphate and incubated for 1 hr at 37° with 100 μg of pancreatic deoxyribonuclease I (Sigma) in 120 m*M* Tris-HCl (pH 8.0) and 10 m*M* $MgCl_2$. Acid-soluble and insoluble radioactivity was determined as well as the remaining acid-insoluble DNA.

Electron Microscopy. The nuclei were submitted to fixation according to Paracchia and Mittler (1972) and to postfixation with uranyl acetate (Silva et al., 1971). After dehydration in ethanol and propylene oxide the specimens were embedded in Epon-araldite and sections were contrasted with lead citrate (Reynolds, 1963). A Hitachi HU-12 electron microscope was used.

Marker Enzyme Assays. Four marker enzymes were assayed to allow estimation of the contaminating cytoplasmic organelles in the nuclear preparations. Assays were carried out on nuclei isolated in the absence of 2-mercaptoethanol.

NAD(P)H Dehydrogenase (DT-Diaphorase) (EC 1.6.99.2). The assay was carried out according to Dallner (1963) using NADH and dichlorophenolindophenol as substrates. The reaction was started by adding 20-100 μl of whole homogenate or purified nuclei and the decrease in absorbance at 600 nm followed.

NADH dehydrogenase (cytochrome *c* reductase) (EC 1.6.99.3) and NADH-cytochrome reductase (EC 1.6.2.4) were determined as described by Dallner (1963) with horse heart cytochrome *c* (Sigma) as an acceptor.

Cytochrome c Oxidase (EC 1.9.3.1). The enzyme activity was determined according to de Duve et al. (1955).

Extraction of DNA. DNA was extracted as described by Hershey et al. (1973a,b) and further deproteinized by alternate phenol and chloroform-isoamyl alcohol (5:2) extractions.

Density-Gradient Centrifugation. When normal DNA was examined 4 g of CsCl was mixed with 3 ml of 0.1 × standard saline citrate (0.1 × SSC[1]) containing 10-20 μg of DNA and the density adjusted to about 1.72. The sample was centrifuged at 35,000 rpm and 25° for 48 hr in a Spinco SW 65 rotor. Fractions of three drops were collected and the density was determined in every five-eight fractions. The fractions were diluted with 0.5 ml of water. The absorbance at 260 nm and the radioactivity were determined, the latter by adding 0.5 to 10 ml of Instagel and counting as described. The density was determined with a refractometer.

BrdUrd-labeled DNA was examined in neutral CsCl (mean density adjusted to 1.73-1.74 before centrifugation) and alkaline Cs_2SO_4. In the latter case 2.5 g of Cs_2SO_4 was mixed with 3.65 ml of 0.1 × SSC containing about 80 μg of DNA and the pH was adjusted to about 12.5 with 0.2 ml of 2 *M* NaOH. DNA was sheared by five passages through a 25 gauge needle before centrifugation.

The calculation of radioactivity in fractions of higher than normal density was carried out according to Painter and Schaefer (1969). The light peak was taken to be normal DNA. This may give a slight underestimate of DNA with higher than normal density.

Other Methods. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as standard. Osmolarity was measured by an Advanced DigiMatic Osmometer, Model 3D (Advanced Instruments, Needham Heights, Mass.). Mg^{2+} was determined by atomic absorption.

Results

Influence of Detergents on Marker Enzyme. The influence of Triton X-100 and Brij 58 on the marker enzyme assays was tested (Table I). At a concentration of 0.05%, Triton X-100 did not essentially influence cytochrome *c* oxidase activity. NADH dehydrogenase activity was reduced about 40%. Brij 58 from 0.05 to 0.5% did not decrease cytochrome *c* oxidase activity but did reduce the activity of NADH dehydrogenase. NADPH-cytochrome reductase was not influenced by the detergents. NAD(P)H dehydrogenase was stimulated (two- to threefold) but this was not observed in the assays when the incubation mixture contained Tween-20.

Purity of Nuclei. In phase contrast microscopy of nuclei not treated with Triton X-100, a small amount of adherent cytoplasmic material was seen, but regularly fewer than 3% whole cells. Nuclei isolated from a detergent-containing homogenate looked markedly cleaner and no whole cells were seen. After 20 min of incubation the morphology of the nuclei was essentially unaltered. Electron microscopy (Figure 1) showed that 0.05 and 0.1% Triton X-100 largely but not completely stripped off the outer nuclear membrane. Brij 58 in the same concentration effectively removed the outer membrane. Higher concentrations of Triton X-100 (0.2-0.4%) also removed the membrane completely. Nuclei isolated without Triton X-100 had esentially complete double nuclear membranes and several cytoplasmic tags. Marker

Table I: Effect of Detergents on Activity[a] of Marker Enzymes.[b]

		Triton X-100				Brij 58			
Enzyme	No Detergent	0.05%	0.1%	0.2%	0.5%	0.05%	0.1%	0.2%	0.5%
NAD(P)H dehydrogenase	7.71	7.38		6.93	6.51	9.49		10.3	8.73
NADH dehydrogenase	1.91	1.18	0.42	0.24	0.24	0.40		0.11	0.07
NADPH–cytochrome reductase[c]	0.28	0.28		0.30		0.25	0.32		
Cytochrome *c* oxidase	1.04	1.16	0.90	0.65	0.31	1.17		1.09	1.10

[a] Nanokatals/milligram of protein (average of three or more experiments). [b] Synchronized cells in buffer A (without 2-mercaptoethanol) were homogenized. Buffer B and buffer C (with varying detergent concentrations) were added to a final concentration of 13–15 × 10^6 nuclei/ml. Effect of detergent on enzyme activity varied with concentration of cell homogenate; more dilute homogenates were more inhibited. [c] Average of two experiments.

Table II: Activity[a] of Negative Marker Enzymes in Preparations of Nuclei.[b]

		Triton X-100				Brij 58			
Enzyme	No Detergent	0.05%	0.1%	0.2%	0.5%	0.05%	0.1%	0.2%	0.5%
NAD(P)H dehydrogenase	0.89	0.67		0.18	0.23	0.45		0.30	0.20
NADH dehydrogenase	18.2	9.9	5.1			7.0	7.3	8.5	
NADPH–cytochrome reductase	20.6	8.7	6.4						
Cytochrome *c* oxidase	14.5	2.5	2.8			2.3	2.4	3.4	2.6

[a] Percent of total activity in whole homogenate and corrected for influence of detergent (see text). [b] Nuclei were isolated as described except that the concentration of detergent varied.

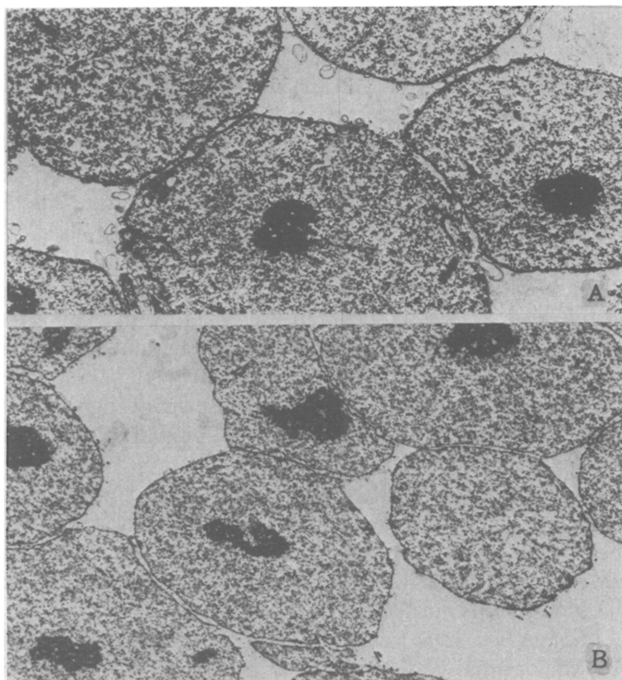

FIGURE 1: Electron micrographs of HeLa cell nuclei isolated from homogenates containing: (A, top) 0.05% Triton X-100, primary magnification 3000; (B, bottom) 0.05% Brij 58, primary magnification 2400.

enzyme analyses (Table II) revealed that nuclei isolated in the absence of detergents were contaminated with a significant amount of cytoplasmic organelles. Treatment with detergents at concentrations down to 0.05% reduced this contamination by 50–82%, giving a final cytoplasmic contamination of 1–8% (Table II). This is an upper limit since it is based on the assumptions that pure nuclei are absolutely devoid of these marker enzymes and that the inactivation caused by the detergent is not reversed by washing without

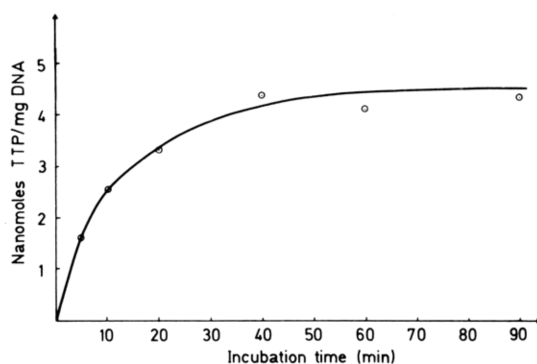

FIGURE 2: Incorporation of [^{3}H]TTP into DNA of isolated nuclei. The test mixture contained NH_4Cl (65 m*M*), Tris-HCl (pH 8.1) (20°) (65 m*M*), glycerol (10%), and CTP, UTP, and GTP (each 0.1 m*M*). The other components of the test mixture were as stated under Materials and Methods.

detergent. Control experiments showed that cytochrome oxidase activity was not restored and cytochrome *c* reductase activity only marginally increased after removal of detergent by centrifugation or dialysis.

DNA Synthesis. When nuclei were incubated with test mixture, [^{3}H]TTP was incorporated into the acid precipitable fraction for about 40 min (Figure 2). This incorporation required the presence of ATP, dGTP, dCTP, dATP, and Mg^{2+} (Table III). Nuclei isolated from synchronized cells harvested in the middle of the S phase were about 5 times more active in DNA synthesis than nuclei from unsynchronized cells in exponential growth. The time course in incorporation was, however, the same. Under optimal conditions S-phase nuclei incorporated about 5 nmol of [^{3}H]TTP per mg of DNA added. Nuclei isolated from early (10 min after reversal) or late (6 hr after reversal) S phase were less active in DNA synthesis (32 and 36% activity, respectively) than nuclei from the middle of S phase (100%). This corresponds to the pattern of rate of DNA synthesis

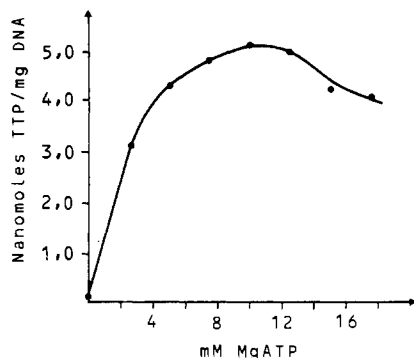

FIGURE 3: Effect of varying MgATP concentration on incorporation of [^{3}H]TTP. Nuclei were incubated for 20 min. The Mg^{2+} concentration was always 0.7 mM higher than the ATP concentration.

Table III: Incorporation of [^{3}H]TTP in the Absence of Various Components of the Incubation Mixture.

	Act. (%)
Complete system	100
− dGTP	13.8
− dATP, dCTP	5.4
− dATP, dGTP, dCTP	3.8
− MgATP (Mg^{2+} reduced to 0.7 mM)	3.5
− Mg^{2+} (reduced to about 0.3 mM)	29

found in intact cells pulse labeled at various times through S phase after synchronization by the same method (Johnsen and Prydz, unpublished). The presence of Triton X-100 in the homogenate reduced the incorporation of [^{3}H]TTP in the nuclei even after washing to remove the detergent. Triton X-100 (0.05 and 0.2%) reduced the incorporation about 10 and 30%, respectively. Nonidet P-40 and Brij 58 had similar effects in equal concentrations.

Deoxyribonucleotides. A final concentration of each deoxyribonucleotide above 25–30 μM saturated the system for about 20–30 min when 8×10^6 nuclei were used. Higher concentrations (up to 300 μM) or repeated additions of dNTP did not influence the incorporation rate. The apparent K_m for the mixture of dNTP was 3.2 μM and V_{max} was about 0.46 nmol of TTP/min per mg of DNA. Removal of one or more of the dNTPs resulted in a marked reduction in the incorporation (Table III).

Mg^{2+} and Ribonucleotides. At an ATP concentration of 5.3 mM the optimum Mg^{2+} concentration was 5–7 mM. Without Mg^{2+} in the test mixture the incorporation was 29% of that in the complete system (Table III). There was, however, a small carry-over of Mg^{2+} with the nuclear pellet (estimated to give a final concentration of 0.2–0.4 mM Mg^{2+}).

Replacement of $MgCl_2$ with $MnCl_2$ (10.7 mM) reduced incorporation to the level seen in the absence of Mg^{2+}. Addition of 2 mM $MnCl_2$ to standard test mixture gave about 10% inhibition. The optimal concentration of ATP was determined to be 10 mM by varying the amount of ATP in conjunction with Mg^{2+} (Figure 3). The Mg^{2+} concentration was kept at 0.7 mM above the ATP concentration. In the absence of MgATP the incorporation was 2–3% of that in the complete system (Table III). Neither oligomycin (final concentration 2.5–40 μg/ml) nor dinitrophenol (final concentration 0.01–0.1 mM) inhibited the effect of ATP,

Table IV: Incorporation of [^{3}H]TTP in the Presence of Various Inhibitors of RNA Synthesis.

	Final Concn (μg/ml)	Act. (%)
Standard system		100
+ α-amanitin	5.3	98
	10.5	102
	21	92.5
	42	100
	200	75
+ rifampicin AF/012	31.5	93.5
	125	93
	500	60
+ rifampicin AF/013	31.5	91
	125	110
	500	81
+ actinomycin D	6.3	71
	12.5	66
	25	39
	50	13
+ RNase A	31.5	66
	125	51
	250	62

suggesting that the ATP effect was not due to energization of nuclear membranes.

EGTA (1 mM) was found to give about 30% better incorporation than EDTA (1 mM).

In repeated experiments we found a slight stimulatory effect (7–26%) when CTP, UTP, and GTP were added at a final concentration of 0.1 mM in the presence of optimal concentrations of ATP and the dNTPs. The Mg^{2+} concentration was increased correspondingly. The effect appeared only after more than 15–20 min incubation and was not abolished by repeated additions of new test mixture providing optimal dNTP for the whole incubation period. The effect was additive with that of glycerol and NH_4Cl–Tris.

We therefore studied the effect of various inhibitors of RNA synthesis on the DNA synthesizing activity of isolated nuclei (Table IV). Actinomycin D had a marked effect as reported earlier by others (Goldberg and Friedman, 1971) whereas the rifampicin derivative AF/013 and α-amanitin had no or only a slight effect even at high concentrations. Rifampicin AF/012 at 0.5 mg/ml reduced the DNA synthesizing activity by 40%.

RNase A had a significant inhibitory effect on DNA synthesis when added at the start of the incubation. The dTMP incorporated did not become acid soluble when RNase was added 50 min after the start of incubation, indicating that the product was not RNase degradable.

Monovalent Ions and pH. With 50 mM Tris-HCl (pH 7.8) the optimal NaCl concentration was 90 mM. This was so also at 2 mM ATP–10.7 mM $MgCl_2$. The corresponding osmolarity of the test mixture was 290 mosM. KCl did not fully replace NaCl, but Tris (130 mM) replaced NaCl and gave the same incorporation. NH_4Cl (65 mM) together with Tris (65 mM) increased the incorporation 30–35% above that given by the optimal NaCl concentration. The effect of ions was not due to osmolarity alone, since the same optima were found when the osmolarity was held constant by glucose. Glycerol (final concentration 5–15%), rendering the test mixture highly hyperosmotic, increased the incorporation by about 20%. The effects of NH_4Cl–Tris and glycerol were additive.

The pH optimum for the incorporation of [^{3}H]TTP was 7.7–7.9 measured at 37° (Figure 4). Lowering the ATP

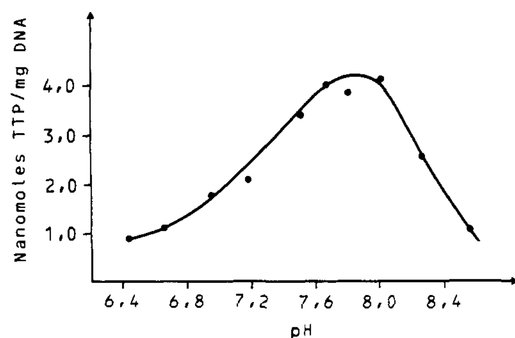

FIGURE 4: Effect of varying pH on incorporation of [^{3}H]TTP. Tris-HCl buffer was used. Nuclei were incubated for 20 min.

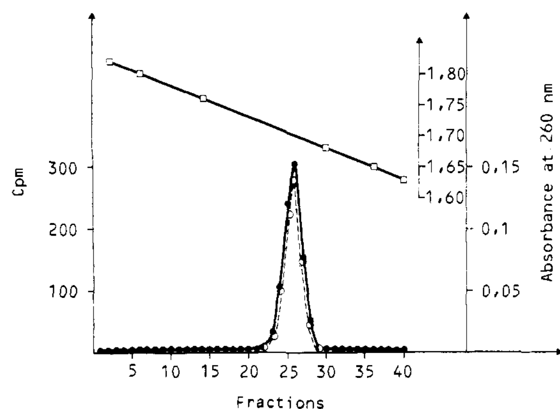

FIGURE 5: Isopycnic centrifugation of DNA in neutral CsCl. Nuclei prepared from synchronized cells 3 hr after reversal were allowed to synthesize DNA in vitro for 20 min. DNA was isolated and 12 μg centrifuged in neutral CsCl (see Materials and Methods): (●—●) radioactivity; (O- - -O) absorbance at 260 nm; (□—□) density.

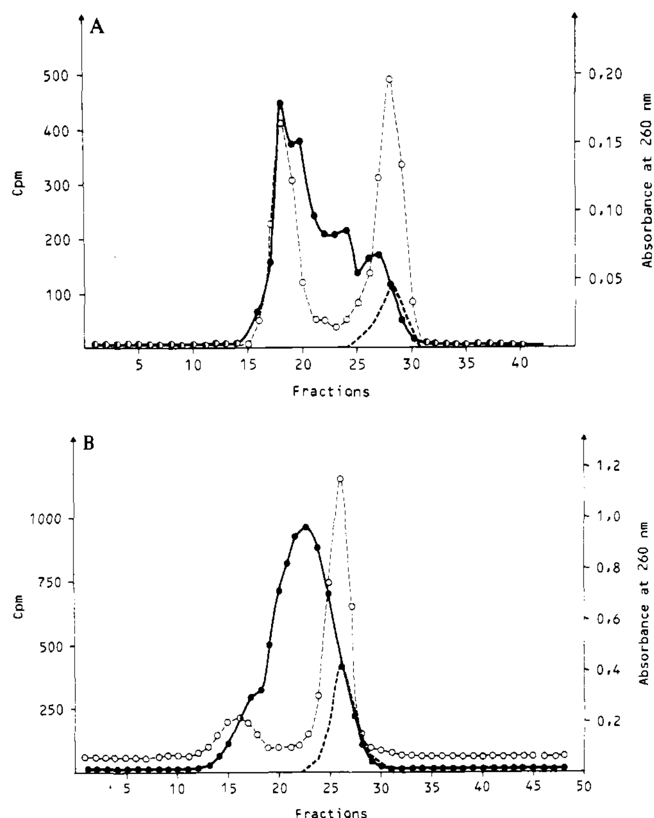

FIGURE 6: Isopycnic centrifugation of DNA labeled with BrdUrd and [^{3}H]TTP in neutral CsCl (A) and alkaline Cs_2SO_4 (B). DNA isolated from [^{3}H]TTP-labeled nuclei prepared from synchronized BrdUrd-labeled cells (see Results section) was sheared (5 passages through a 25 gauge needle) and centrifuged (40 μg) in neutral CsCl (A) and alkaline Cs_2SO_4 (80 μg) (B) (see Materials and Methods): (●—●) radioactivity; (O- - -O) absorbance at 260 nm; (- - -) adjusted DNA peak of normal density.

concentration to 2 mM and thereby increasing free Mg^{2+} concentration did not change the pH optimum.

Polyamines. Spermine (up to 40 μM) and spermidine (up to 150 μM) had a very slight stimulatory effect (about 10%), whereas at higher concentrations both substances were inhibitory. Putrescine was less inhibitory and spermine more inhibitory than spermidine. The order of inhibition spermine > spermidine > putrescine corresponds to the number of amino groups per molecule.

Characterization of the DNA Product. The acid-insoluble radioactivity was not released by treatment with 0.5 N KOH for 12 hr at 37°. By DNase treatment of nuclei, 80–85% of the counts were made acid soluble.

Isolated DNA gave a sharp peak in CsCl gradient centrifugation at a density of 1.699 g/ml corresponding to a G + C content of 40%. Neither material absorbing at 260 nm nor radioactivity was found outside this peak (Figure 5).

To see if the incorporation of [^{3}H]TTP was due to unscheduled (repair) DNA synthesis or was a continuation of the in vivo replication, synchronized cells were labeled with BrdUrd by reversing the amethopterin block with this nucleoside (6 μg/10^6 cells). After 3 hr the nuclei were isolated and incubated in test mixture for 7 min. The cells and nuclei were protected against light. In neutral CsCl and alkaline Cs_2SO_4, 85–87% of the radioactivity banded at densities above normal (Figure 6).

Discussion

The nuclear DNA replicating system presented here is based on that described by Friedman and Mueller (1968). We have introduced certain changes to reduce the cytoplasmic contamination of nuclei with minimal reduction of the ability of the system to incorporate [^{3}H]TTP. We have also optimalized the incubation conditions so that the initial incorporation rate and the amount of DNA synthesized are approximately doubled compared with the data of Hershey et al. (1973a,b) before stimulatory cytosol factors are added. The system requires all four dNTPs, ATP, and Mg^{2+} and has an optimum pH of 7.8. The content and osmotic activity of ions in nuclei are not well understood, but the present system has maximum activity with equal concentrations (65 mM) of NH_4^+ and Tris-HCl buffer. Na^+ gave higher activity than K^+ under isoosmotic conditions. However, glucose with the same osmolarity did not replace the monovalent ions as an activating agent and glycerol in amounts rendering the test mixture strongly hyperosmotic stimulated incorporation 20%.

The product of the reaction was resistant to alkali and RNase and sensitive to DNase. The radioactivity incorporated banded with bulk DNA at 1.699 g/ml in agreement with data for other strains of HeLa cells (Volpe and Eremenko, 1973; Koch and Stokstad, 1967).

Incorporation due to a random repair process might be expected to be randomly located in both heavy and light strands of DNA in BrdUrd-labeling experiments in proportion to the amount of DNA present. A selective repair of BrdUrd-containing DNA would give [^{3}H]TTP incorporation in proportion to the amount of heavy label present, i.e.

the heavy peak would appear symmetrically labeled. Continuation of in vivo synthesis would give [^{3}H]TTP incorporation into the heavy peak and especially into intermediate fractions (Painter and Schaefer, 1969). Counts in the light peak may be due either to initiation in vitro, shearing of DNA imposing breaks between heavy labeled and isotope-labeled parts of continuous strands, or the random repair mentioned above.

Studies with BrdUrd indicated that [^{3}H]TTP was incorporated to a large extent into material of intermediate density (Figure 6), suggesting that the synthesis in vitro elongated the strands already initiated in vivo. Less than 15% of the radioactivity was found in light DNA. The incorporation into light DNA may represent repair synthesis, in vitro initiation, or [^{3}H]TMP-labeled DNA fragments broken off from BrdUrd-labeled strands by shearing. Kumar and Friedman (1972) suggested that initiation of new replicons in vitro may take place to a small extent in isolated nuclei.

In the accompanying paper (Krokan et al., 1975), we report direct evidence for the initiation of primary DNA pieces in isolated nuclei in vitro. The role of RNA as a primer for the synthesis of primary DNA pieces in eucaryotic cells is not established (Gautschi and Clarkson, 1975). If such a role is assumed, the stimulatory effect upon DNA synthesis of CTP, GTP, and UTP (each at 0.1 m*M*) may be explained. The stimulation was observed in the presence of an optimal concentration of ATP and Mg^{2+} and was therefore not due to an effect via the level of these substances.

Furthermore, if synthesis of RNA is involved in the initiation of primary DNA pieces, the absence of the effect of α-amanitin on the incorporation of TTP suggests that RNA polymerase II and probably also polymerase III are not involved in this process, since both are strongly inhibited at the concentration of α-amanitin used (Price and Penman, 1972; Weinman and Roeder, 1974). The effect of the rifampicin derivatives was also very slight. The inhibitory effect of polyamines and of actinomycin D was probably caused by their interaction with the template.

Isolated nuclei are well suited for a study of the mechanism of DNA replication and of the effect of various cytoplasmic factors and other substances on DNA replication with an endogenous template, provided the system is really optimalized so that unspecific stimulation may be reduced. Furthermore, a reasonably high efficiency is desirable. The present system may fulfill both of these requirements. The initial rate of [^{3}H]TTP incorporation corresponded to a DNA synthesis of about 1.8 nmol/min per mg of DNA added (in nuclei). This is about 30% of the in vivo rate if a total S phase length of 6 hr and a constant replication rate during this phase are assumed. The system synthesized about 1% of the HeLa cell genome during 30-40 min.

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