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Deoxyribonucleic Acid Synthesis in Isolated Nuclei from Chicken Embryo Fibroblast Cell Cultures†

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ABSTRACT: A procedure for the isolation of high yields of nuclei from cultures of chicken embryo fibroblast cells is described and a detailed characterization of the DNA synthesis activity of these nuclei is presented. DNA synthesis by the isolated nuclei requires Mg^{2+}, ATP, and all four dNTPs. The activity is stimulated markedly by the addition of exogenous DNA template. If nuclei are isolated from various stages of the cell cycle, both the endogenous and exogenous activities *in vitro* are proportional to the rate of DNA synthesis *in vivo*. Further evidence that the DNA synthesis activity is replication rather than repair is given by the results from experiments with density markers. DNA synthesis is semiconservative and extends for at least 500 nucleotides. The DNA replicated *in vitro* is an extension of growing points previously initiated *in vivo*. Such a cell-free system capable of valid replication should offer a convenient way to study the mechanisms of the elongation and initiation of DNA snythesis.

The molecular mechanism of DNA replication and its initiation are poorly understood at the present time. In the last few years, the development of crude *in vitro* systems has provided a convenient way to dissect some of the components of the replication system in bacteria (Moses and Richardson, 1970; Vosberg and Hoffmann-Berling, 1971; Schaller *et al.*, 1972; Wickner *et al.*, 1972). These systems are capable of elongating bacterial chains on preexisting replication forks (Burger, 1971; Geider and Hoffmann-Berling, 1971; Matsushita *et al.*, 1971).

Nuclei isolated from eucaryotic cells have been shown to be capable of incorporating deoxyribonucleotides into DNA with activities proportional to the *in vivo* rates of DNA synthesis (Friedman and Mueller, 1968; Lynch *et al.*, 1970). Two principle approaches are being used in eucaryotic systems to characterize the DNA made *in vitro* and to attempt to distinguish between repair and replicative synthesis. The first is to study the replication of viral DNA in isolated nuclei so that the product can be characterized in detail by sucrose gradient sedimentation and hybridization (Winnacker *et al.*, 1972; Magnusson *et al.*, 1972; Hunter and Francke, 1974a; DePamphilis and Berg, personal communication). The second approach is to show that the DNA made in isolated nuclei is a continuation at the growing points of chains initiated *in vivo* (Kidwell and Mueller, 1969; Lynch *et al.*, 1970; Hershey *et al.*, 1973).

The purpose of the following experiments is to establish a cell-free system in which to study the regulation of the initiation and elongation of DNA replication in eucaryotic cells. With such a system, combined with knowledge of the events prior to initiation, it would be possible to study the mechanisms by which a tissue limits its final size in the differentiated state and yet retains the capacity to proliferate following injury. We feel that it is necessary to understand the control mechanisms of normal cells before it will be possible to understand the nature of cellular defects which result in failure to divide or in uncontrolled proliferation.

In this report we describe an isolation procedure for obtaining high yields of nuclei from cultures of partially synchronized chicken embryo fibroblasts, and the detailed characterization of the DNA synthesis activity of these nuclei is presented. In addition, we present several lines of evidence that the activity is likely to be replication rather than repair synthesis.

Experimental Section

Cell Culture Techniques. Primary cell cultures were prepared from 10-day-old chicken embryos as described previously (Rein and Rubin, 1968; Rubin, 1973). All experiments reported here were done with secondary chick fibroblast cultures. They were prepared by trypsinizing 4–5-day-old primary cultures (Rubin, 1973) and seeding the cells on 100-mm Falcon plastic petri dishes in growth medium 199 (Grand Island Biological) supplemented with 2% tryptose phosphate broth (TPB,[1] Difco) and 1% each of calf and chicken serum (Microbiological Associates) (designated 199 (2.1.1)). Unless other-

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[1] Abbreviations used are: MalNEt *N*-ethylmaleimide; araCTP, cytosine β-D-arabinofuranonucleoside 5′-triphosphate; BrdUrd, bromodeoxyuridine; BrdUTP, bromodeoxyuridine 5′-triphosphate; TPB, tryptose phosphate broth.

wise noted, the cells were seeded at an initial density of 2–4 × 10^6/dish; 1–2 days later the medium was removed and replaced with 199 plus 2% TPB and no serum (2.0.0) for 12–16 hr ("turn down"); and then serum was added back or the medium was changed to 199 plus 2% TPB, 1% calf serum, and 1–4% chicken serum (2.1.1, 2.1.4, etc). "Active" nuclei were isolated from these cells 10–12 hr later. "Inactive" nuclei were isolated from a control set of cultures grown in parallel in 199 (2.1.1) until 12–16 hr before the isolation, at which time the medium was changed to 199 (2.0.0).

[^{3}H]Thymidine Incorporation and Autoradiography. *In vivo* DNA synthesis rates were determined by incubating the cells in 199 (0.0.0) containing 0.2 μCi/ml of [^{3}H]thymidine (New England Nuclear, specific activity 20 Ci/mmol) for 1 hr. The medium was then removed and the amount of radioactivity/μg of protein per dish was determined as previously described (Martin *et al.*, 1971). To determine the fraction of cells synthesizing DNA, parallel cultures were incubated in 199 (0.0.0) containing 1.0 μCi/ml of [^{3}H]thymidine for 1 hr, the cells were then treated with 5% CCl_3COOH for 10 min, washed with 5% CCl_3COOH, fixed with Bouins for 30 min, washed with 80% ethanol, and prepared for autoradiography by a modification of the technique described by Gurney (1969). Before treating with emulsion, the fixed cells were incubated for 30 min at 37° with 100 μg/ml of preboiled ribonuclease in standard saline–citrate (SSC) buffer, washed with distilled water, and air-dried. The dishes were stored with Ilford emulsion at 4° for 3–5 days, developed, and stained with Harris' hematoxylin, and the fraction of cells with silver grains was determined.

Preparation of Isolated Nuclei. The entire procedure was carried out on ice. The medium was removed from the cells by aspiration, and the cells were washed quickly with cold P buffer (10 mM potassium phosphate, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$) and allowed to swell 10 min in 2 ml of P buffer/dish. The cells were then scraped into a Dounce homogenizer with a rubber policeman, ruptured with 5–10 even strokes of the pestle and concentrated sucrose was immediately added to a final concentration of 0.3 M (PS buffer). The nuclei were washed twice by low-speed centrifugation in a table top Sorvall centrifuge, resuspended in PS buffer at a final concentration of 50 × 10^6 nuclei (100 μg of DNA)/ml (determined by counting in a hemacytometer) and stored at −70°. The yield was typically 50–75% with less than 1% whole cells. If the cell culture was rather dense, the nuclei were lightly "tagged" with cytoplasmic debris. Cleaner preparations could be obtained by treatment with 0.5% Triton N101 or X100, but this decreased the initial rates of *in vitro* DNA synthesis in "active" nuclei and occasionally increased the rate in "inactive" nuclei. Detergent treatment also allowed *in vitro* incorporation to continue for a longer time, perhaps due to repair synthesis stimulated by damage done during the isolation. Storage at −4° for several days markedly increased the endogenous activity (3–10-fold), perhaps due to activation by nucleases. For this reason, nuclei were always stored at −70°, where the activity remained constant for several weeks.

DNA Synthesis Assay. Unless otherwise noted, nuclei were incubated in a reaction volume of 200 μl containing 50 mM Tris (pH 7.4), 5.75 mM $MgCl_2$, 2.5 mM NaCl, 75 mM sucrose, 2.5 mM K-PO_4, 5 mM ATP, 0.1 mM each of dTTP, dGTP, and dCTP (Schwarz/Mann or Sigma), 5 μCi of [^{3}H]dATP (Schwarz/Mann, specific activity 15–18 Ci/mmol), 2.5 × 10^6 nuclei, and 25 μg of calf thymus DNA where indicated. The K-PO_4, NaCl, sucrose, and 0.75 mM $MgCl_2$ were carried over from the nuclei buffer. Denatured calf thymus DNA was boiled for 10 min. Activated calf thymus DNA was prepared by a modification of a procedure by Wickner and Kornberg (personal communication). This consisted of hydrolysis of 0.5 mg/ml of DNA for 10–30 min at 37° with 0.5 μg/ml of pancreatic DNase I (bovine, Sigma, 2200 Kunitz units/mg) in 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 20 mM KCl, and 0.2 mg/ml of bovine serum albumin followed by incubation of the mixture 10 min at 68° to inactivate the enzyme. The inhibitors MalNEt and araCTP were purchased from Schwarz/Mann and Terra-Marina Biological, respectively.

The DNA synthesis reactions were incubated at 37° and stopped by the addition of 5 ml of cold 10% CCl_3COOH + 1% $Na_4P_2O_7$. The precipitates were collected on Whatman GF/C glass fiber filters, washed with *ca.* 75 ml of 5% CCl_3COOH and 15 ml of 95% ethanol, dried, and counted in toluene Omnifluor (New England Nuclear) in a liquid scintillation counter.

CsCl Density Equilibrium Gradients. The DNA synthesis assays were carried out as described above with the following modifications. The reaction volume was increased by a factor of 3–5, 0.1 mM of all four deoxyribotriphosphates were used, and 25–75 μCi each of [^{32}P]dATP and [^{32}P]dGTP (New England Nuclear, specific activity 10–18 Ci/mmol) were added in place of [^{3}H]dATP. Where indicated, BrdUTP (Terra Marina Biological) was used in place of dTTP. Following incubation the nuclei were treated for 2 hr at 37° in lysis buffer (SSC, 10 mM EDTA, 1 mg/ml of predigested pronase, and 0.3% sarkosyl). Solid CsCl was then added to give a final density of about 1.73 g/ml (6.0 g plus 4.8 ml of buffer).

Samples to be sheared were either forced through a no. 27 needle of a 5-ml syringe two times (double-strand mol wt *ca.* 2 × 10^6) or sonicated for 10 min at 0° in a Raytheon sonic oscillator, Model DF-101, at full power (double-strand mol wt *ca.* 3 × 10^5). The samples were centrifuged for 70–90 hr at 30,000 rpm and 20° in the SW 50.1 rotor of the Spinco Model L centrifuge. The tubes were punctured, 5-drop fractions were collected from the bottom, 10 μg of native calf thymus DNA was added to each fraction, and the fractions were precipitated with CCl_3COOH, filtered, washed, and counted as described above.

Results

Characterization of the DNA Synthesis Reaction. The kinetics of DNA synthesis by isolated nuclei under standard conditions are shown in Figure 1a. The incorporation of deoxyribonucleotides into DNA proceeds at a constant rate for at least 10 min, continues to increase rapidly for 30–60 min, and then increases very slowly for several hours (not shown). Incubation at 30° rather than 37° did not appreciably prolong the linear period. Incorporation of deoxyribonucleotides is stimulated markedly by the addition of calf thymus DNA (Figure 1b; note change of scale). This stimulation is greatest when the added DNA has been "activated" by partial DNase digestion.

The reaction is linear with respect to the number of nuclei added over a fivefold range (Figure 2). The kinetics of the reaction are similar over the same range of nuclei concentrations (data not shown). These two observations imply that the decrease in incorporation rate by 30 min is not due to the depletion of any of the components of the reaction mixture.

Some of the factors affecting the rate of DNA synthesis in isolated nuclei are given in Tables I and II. The reaction requires all four deoxyribotriphosphates and is inhibited by the deoxycytidine triphosphate analog araCTP and by the sulfhydryl reagent MalNEt (Table I). Both Mg^{2+} and ATP are required for maximum activity. Since ATP can act as a chelator for Mg^{2+}, there is a complex relationship between the Mg^{2+}

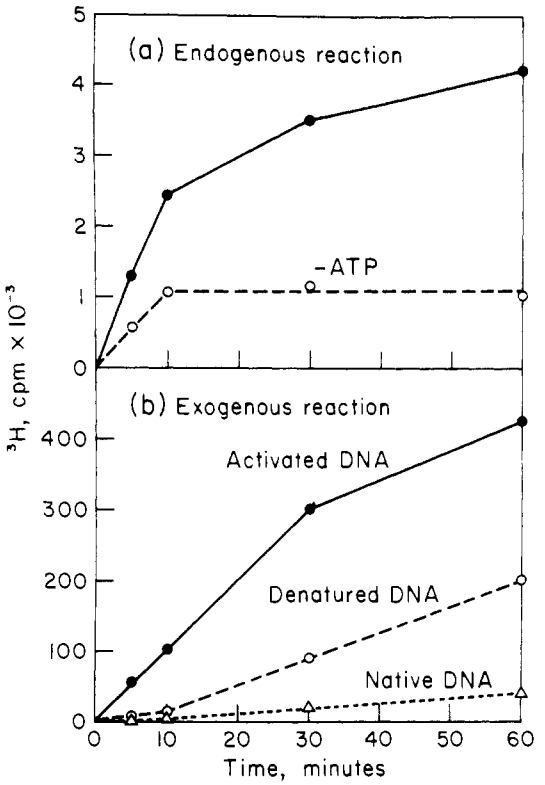

FIGURE 1: Kinetics of DNA synthesis in isolated nuclei. The reactions were carried out for the time indicated as described in the Experimental Section. (a) No exogenous DNA: complete reaction mixture (●); ATP omitted from the cocktail (○). (b) 25μg of calf thymus DNA added to reaction: activated DNA (●); denatured DNA (○); native DNA (△).

requirement (Table I), the inhibition by high concentrations of Mg^{2+} (see Figure 3), and the stimulation by ATP. As illustrated in Figure 3, the optimum Mg^{2+} concentration is higher in the presence than in the absence of ATP. In addition, the extent of stimulation by ATP is dependent upon the length of the reaction; in the absence of ATP, incorporation ceases after approximately 10 min (Figure 1a).

Mn^{2+} can substitute for Mg^{2+} in the endogenous (but not the exogenous) reaction, with an optimum concentration well below that of Mg^{2+} (Table II). The endogenous reaction is stimulated by low concentrations of EDTA or EGTA, perhaps because these chelators overcome an inhibition due to divalent cations such as Ca^{2+} or Zn^{2+}, which may remain bound to the nuclei throughout the isolation procedure (see Table II).

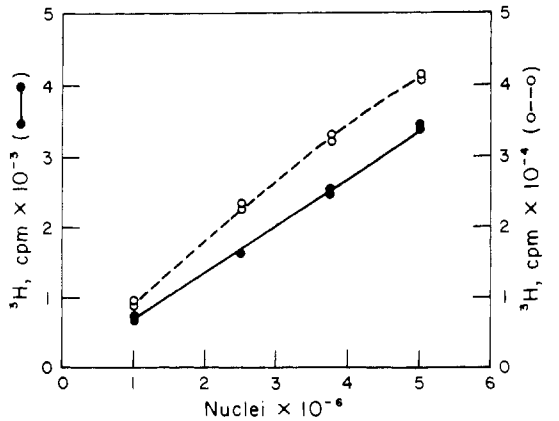

FIGURE 2: Incorporation *vs.* the concentration of nuclei added. The reaction mixtures were incubated for 30 min under standard assay conditions with the amount of nuclei indicated: no exogenous DNA (●); 25 μg of denatured calf thymus DNA (○).

TABLE I: Properties of the DNA Synthesis Reaction in Isolated Nuclei.[a]

Condition	Endogenous[b] (%)	Exogenous[c] (%)
Complete	100	100
− Mg^{2-} [d]	10	3
− ATP	25–60	30–60
− dNTPs	6	7
− dTTP	8	20
− dGTP	13	17
− dCTP	8	22
+ 1 mM araCTP	15	5
+ 1 mM MalNEt	10	5

[a] The reactions were incubated for 10 min under the standard assay conditions described in the Experimental Section with the deletions or additions listed. Because not all of the experiments were done at the same time, the numbers given are the percentages of the control, *i.e.*, the cpm of [^{3}H]-dAMP incorporated by 2.5 × 10^6 nuclei in the complete reaction mixture during a 10-min incubation. Each value represents the average of three or more determinations. [b] The reactions contained no exogenous DNA. [c] The reactions contained 25 μg of denatured calf thymus DNA. [d] The reactions contained 0.75 mM $MgCl_2$ (from the nuclei buffer).

The exogenous reaction is severely inhibited by the addition of NaCl (Table II) or KCl (data not shown), whereas the endogenous reaction is not appreciably affected even by salt concentrations as high as 0.25 M.

Correlation of the Activity of Nuclei in Vitro with DNA Synthesis in Vivo. One of the most interesting aspects of the *in vitro* system with isolated nuclei is its capacity to mirror the regulatory features of the cell cycle *in vivo*. Cell cultures of chicken embryo fibroblasts can be partially synchronized in a gentle and reversible manner by removing serum from the medium for 12–16 hr and then adding the serum back to the medium (Rubin, 1971; Rubin and Koide, 1973). Following the addition of serum, there is a lag in the rate of DNA synthesis

TABLE II: Effect of Cations on the DNA Synthesis Reaction in Isolated Nuclei.[a]

Condition	Endogenous[b] (%)	Exogenous[c] (%)
Complete	100	100
+ 1 mM $MnCl_2$	100	120
+ 5 mM $MnCl_2$	41	7
+ 1 mM $MnCl_2$, − $MgCl_2$[d]	120	20
+ 5 mM $MnCl_2$, − $MgCl_2$[d]	29	7
+ 0.1 mM EDTA	150	
+ 0.1 mM EGTA	145	
+ 50 μM $ZnCl_2$	59	
+ 2.0 mM $CaCl_2$	60	
+ 100 mM NaCl	120	61
+ 250 mM NaCl	85	1

[a] The relative rates of DNA synthesis were determined as in Table I with the additions or deletions noted. [b] The reactions contained no exogenous DNA. [c] The reactions contained 25 μg of denatured calf thymus DNA. [d] The reactions contained 0.75 mM $MgCl_2$ (from the nuclei buffer).

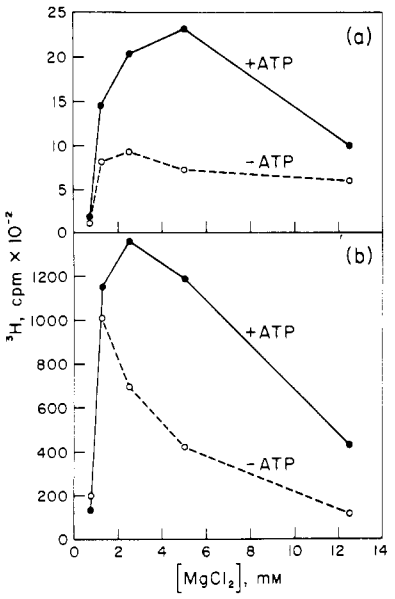

FIGURE 3: Effect of ATP and Mg^{2+} concentrations on the DNA synthesis activity of isolated nuclei. The reactions were incubated for 30 min under standard assay conditions with the modifications detailed below. The ATP concentration was either 5 mM (●) or 0 (○) and the $MgCl_2$ was varied as indicated. (a) No exogenous DNA, (b) 25 μg of activated DNA/reaction.

followed by an increase which reaches a maximum at 9–13 hr and then declines (Figure 4). The extent of synchrony can be estimated by labeling cells with [^{3}H]thymidine for a brief period followed by autoradiography to determine the fraction of labeled nuclei. Typically, 40–70% of the nuclei were labeled at the peak of DNA synthesis. The ratio of the fraction of labeled nuclei at the peak to the fraction before serum addition was nearly identical to the corresponding ratio of thymidine incorporation in all experiments where the two were compared (see Figure 4). Therefore, the amount of [^{3}H]thymidine incorporated in a 1-hr pulse-labeling period was generally used as a measure of the number of cells synthesizing DNA at the time of the pulse, and as an indication of the relative rate of DNA synthesis *in vivo*.

The results of one such cell cycle experiment are given in Figure 5 and two others are summarized in Table III. In all of the experiments a close correspondence between the *in vitro* and the *in vivo* ratios of the active to inactive nuclei was observed. A more detailed analysis of the cell cycle is presented in Table IV. It can be seen that the rate of DNA synthesis *in vitro* in both the endogenous and the exogenous reactions correlates with the corresponding rate of DNA synthesis *in vivo*.

Physical Characterization of the Product Made in Vitro. In order to distinguish between repair and replicative synthesis, two types of experiments were done. The first was designed to determine whether the *in vitro* DNA synthesis was semiconservative and to give an approximate measure of the size of the DNA product made *in vitro*. To do this, nuclei isolated from [^{3}H]thymidine-labeled cells were incubated in reaction mixtures containing dCTP, dATP, dGTP (at least one of which carried a ^{32}P label), and *either* dTTP *or* BrdUTP. Following incubation, the nuclei were lysed and treated with Pronase and the DNA was sheared and analyzed by sedimentation to equilibrium on a CsCl density gradient. Figure 6a illustrates the results of the control experiment in which nuclei were incubated in the normal reaction mixture (containing dTTP). The ^{32}P- and ^{3}H-labeled DNA have nearly identical densities. In the experiment shown in panels b and c of Figure 6, the nuclei were incubated in the presence of BrdUTP. The reaction mixture

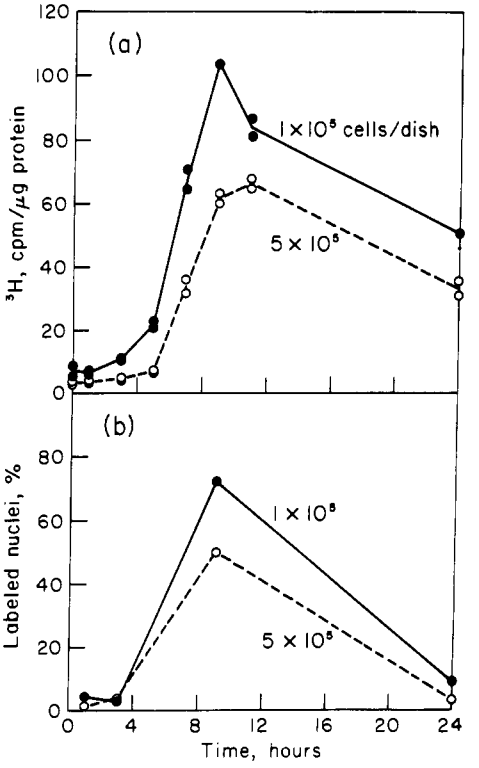

FIGURE 4: *In vivo* DNA synthesis in partially synchronized cultures of chick embryo fibroblast cells. Secondary cultures were seeded as described in the Experimental Section at a density of 1 × 10^5 (●), or 5 × 10^5 (○) cells per 60-mm dish. Serum was removed for 16 hr by changing the medium to 199 (2.0.0) 48 hr after seeding. The medium was then changed to 199 (2.1.4) and DNA synthesis was followed as a function of time after serum addition by: (a) [^{3}H]thymidine incorporation, or (b) determination of the fraction of labeled nuclei by autoradiography (as described in the Experimental Section). The time indicated for each point is the beginning time of an hour pulse label.

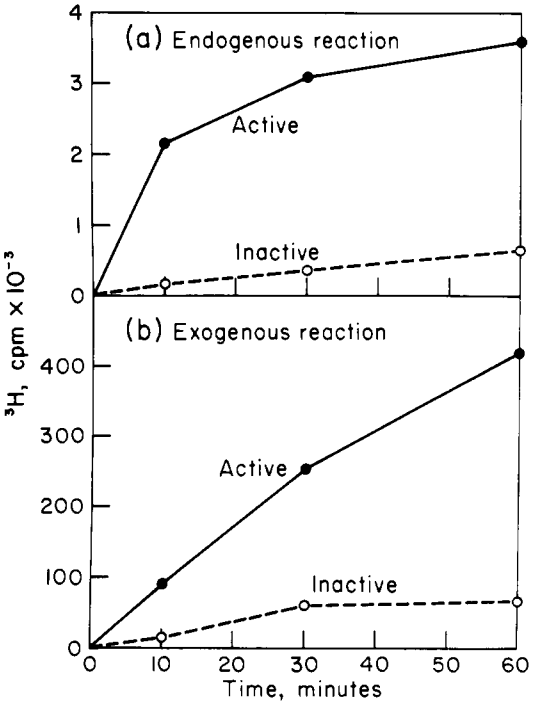

FIGURE 5: Comparison of DNA synthesis in active and inactive nuclei. Nuclei were isolated from cultures which had been partially synchronized by serum deprivation (○) followed by addition of serum for 12 hr (●) as described in the Experimental Section. Incubations were carried out under standard conditions for the time indicated either (a) with no exogenous DNA, or (b) with 25 μg of activated calf thymus DNA.

TABLE III: Comparison of the Rates of DNA Synthesis *in Vivo* and *in Vitro*.

	Active	Inactive	Ratio[e]
	Expt I		
In vivo[a]	18.0	2.8	6.4
In vitro[b]			
Endogenous[c]	8,390	1,410	6.0
Exogenous[d]	182,000	36,100	5.1
	Expt II		
In vivo	38.0	2.5	15.2
In vitro			
Endogenous	7,820	640	12.2
Exogenous	70,900	8,700	8.2

[a] Rates of DNA synthesis *in vivo* were determined as described in the Experimental Section. The value given is the cpm of [^{3}H]thymidine incorporated per μg of protein per dish. A typical dish contained 2–3 mg of protein. Each value is the average of duplicate determinations. [b] DNA synthesis assays were carried out under the standard assay conditions described in the Experimental Section. The value given is the cpm of [^{3}H]dAMP incorporated by 2.5×10^6 nuclei in 30 min. [c] The reactions contained no exogenous DNA. [d] The reactions contained 25 μg of denatured calf thymus DNA. [e] Ratio of active to inactive values.

was then divided into two parts; one was exposed to mild shear conditions by forcing the DNA through a needle (Figure 6b) and the other was sheared extensively by sonication (Figure 6c). The double-strand molecular weights obtained by these two techniques were determined by sedimentation velocity to be approximately 2×10^6 and 3×10^5, respectively (Stavnezer and Gurney, unpublished results).

It can be seen from Figure 6c that if the DNA has been sheared extensively, most of the newly synthesized (^{32}P labeled) DNA sediments at the hybrid density position. This indicates that the DNA is replicated semiconservatively and that replication continues for at least 500 nucleotides (1.5×10^5). Since little density shift is seen when the DNA is sheared less extensively (Figure 6b), the length of DNA replicated at any one site is less than 3000 bases. A small fraction of the DNA synthesized *in vitro* is found at a density greater than that of the hybrid-density marker (Figure 6b, c). This may be due to the fact that the marker was synthesized *in vivo*, where the extent of substitution is limited by endogenous thymidine pools, whereas the DNA synthesized *in vitro* is presumably fully substituted. Alternatively, some of the heavy DNA may be the product of repair synthesis on newly replicated (*i.e.*, hybrid density) templates. This experiment argues against the possibility that the bulk of *in vitro* synthesis is due to numerous small "patching" or repair sites; however, it does not eliminate the possibility that repair synthesis, once initiated, continues for a considerable length.

To eliminate the latter possibility and to demonstrate that the *in vitro* DNA synthesis is an elongation of the growing forks initiated *in vivo*, we did the following experiment. Cells were uniformly labeled with [^{3}H]thymidine and then incubated for the hour immediately preceding nuclear preparation in

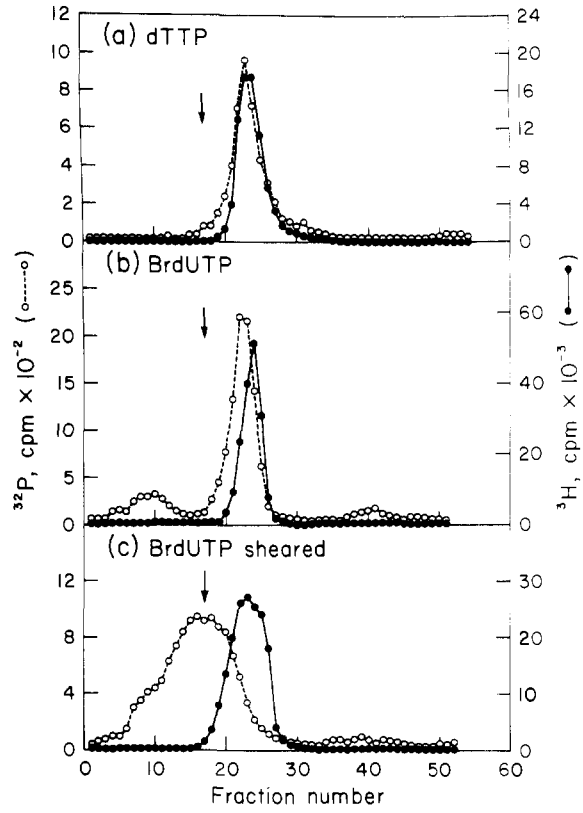

FIGURE 6: Equilibrium CsCl density gradients of the DNA made by isolated nuclei *in vitro*. Cells were grown in medium containing 0.1 μCi/ml of [^{3}H]thymidine (●) for 24 hr before the nuclei were isolated. The procedures for the reactions and gradients are described in the Experimental Section. The nuclei were incubated for 15 min in a mixture containing [^{32}P]dATP and [^{32}P]dGTP (O). (a) Control reaction mixture without a density label, sheared by forcing the DNA through a needle. In parts b and c the nuclei were incubated for 15 min in a reaction mixture containing the normal components except that 0.1 mM BrdUTP was substituted for dTTP. Following incubation, lysis, and Pronase digestion, the DNA was divided into two parts: (b) partially sheared by forcing through a needle and (c) sheared by sonication. The arrow indicates the position of purified hybrid-density DNA (in a separate gradient), generously donated by E. Stavnezer. This marker was prepared by preparative equilibrium CsCl density gradient centrifugation of DNA isolated from cells grown as described in the Experimental Section with the addition of 5×10^{-5} M [^{3}H]BrdUrd for at least one generation.

TABLE IV: Comparison of *in Vivo* and *in Vitro* Rates of DNA Synthesis During the Cell Cycle.[a]

Hr after Serum Addn	*In Vivo*		*In Vitro*[d]			
	Act.[b]	Ratio[c]	Endogenous[e]	Ratio[c]	Exogenous[f]	Ratio[c]
0	4.5	1.0	444	1.0	43,400	1.0
6	20.0	4.4	1,350	3.0	64,000	1.5
12	37.3	8.3	3,165	7.1	191,000	4.5
21	22.8	5.1	2,403	5.4	114,000	2.7

[a] The cells were turned down as described in the Experimental Section. Serum was added back to the medium (1% calf serum, 4% chicken serum) at time 0 and nuclei were isolated at the indicated time. Nuclei were stored at −70° and all samples were thawed and assayed simultaneously. [b] *In vivo* DNA synthesis rates were determined as described in Table III. [c] Ratio of activity at the time indicated to the activity at time 0. [d] The reactions were carried out under the standard assay conditions described in the Experimental Section. The values represent cpm of [^{3}H]dAMP incorporated by 2.5×10^5 nuclei in 30 min. [e] The reaction contained no exogenous DNA. [f] The reaction contained 25 μg of activated calf thymus DNA.

medium containing unlabeled BrdUrd. The isolated nuclei were incubated in a reaction mixture containing ^{32}P-labeled deoxyribotriphosphates. The DNA was isolated, sheared, and analyzed by sedimentation to equilibrium in a CsCl density gradient. Figure 7 illustrates such an experiment. Most of the *in vitro* label sediments at the hybrid density if the DNA has been sheared to a molecular weight of 2×10^6 (Figure 7b). At this molecular weight, all of the incorporated BrdUrd sediments at the hybrid density (Hallick, unpublished observations with [^{3}H]BrdUrd), as would be expected if the size of the sheared pieces was smaller than or equal to the size of the DNA made during the BrdUrd pulse *in vivo*. (If the DNA is not sheared, the [^{3}H]BrdUrd sediments as a broad peak, with most of the label at the light position and the rest between the light and the hybrid density positions.) However, this molecular weight is large relative to the size of the DNA synthesized *in vitro* (see Figure 6 and above discussion). Thus the DNA synthesized *in vitro* involves chains in the process of synthesis *in vivo*.

That the synthesis is not merely repair synthesis within the BrdUrd section of DNA is shown by shearing the product to a molecular weight smaller than or equal to that of the piece made *in vitro*. Since the *in vitro* reaction mixture contains no brominated triphosphates, the density of the newly synthesized product (^{32}P labeled) is nearly that of light or normal DNA (see Figure 7c). From the experiments of Figures 6 and 7, we conclude that the DNA synthesis observed *in vitro* is a continuation of chains already begun *in vivo* and thus involves replicative synthesis.

It may be noted that the density of DNA synthesized *in vitro* is slightly greater (1–2 fractions) than that of DNA replicated *in vivo*, even in the absence of brominated precursors (see Figures 6a and 7a). This may be due to the presence of short RNA primers attached to the newly replicated pieces, as has been reported in several other systems (Brutlag *et al.*, 1971; Sugino *et al.*, 1972; Magnusson *et al.*, 1973; Hunter and Francke, 1974b). In fact, this density difference can be increased by the addition of ribonucleotide triphosphates to the reaction mixture. Further experiments are in progress to establish whether an RNA primer is synthesized in this *in vitro* system.

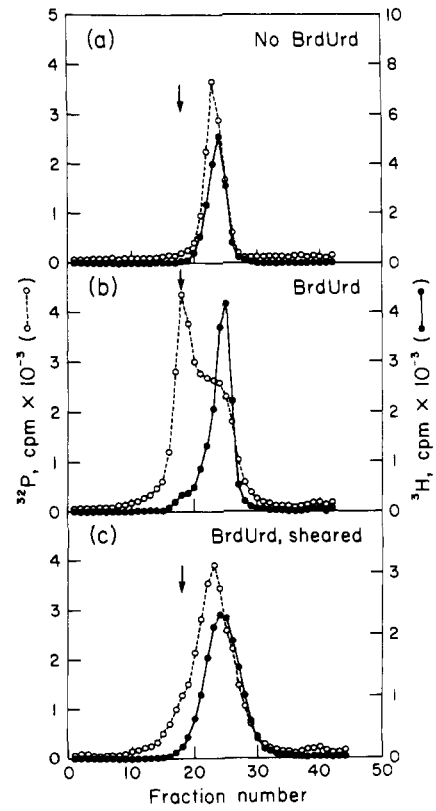

FIGURE 7: Equilibrium CsCl density gradients of the DNA made by isolated nuclei with a density label at the growing point. The cells were labeled overnight in medium containing 0.1 μCi/ml of [^{3}H]thymidine (●). The procedures for the reactions and gradients are given in the Experimental Section. The nuclei were incubated for 10 min in a mixture containing [^{32}P]dATP and [^{32}P]dGTP (○). In the control experiment without a density label (a), the DNA was partially sheared by forcing it through a needle. In the experiment shown in panels b and c the cells were incubated for 1 hr before nuclei isolation in medium containing 5×10^{-5} M BrdUrd. Following incubation, lysis, and Pronase digestion, the DNA was divided into two parts: (b) partially sheared by forcing through a needle and (c) sheared by sonicating. The arrow indicates the position of hybrid-density DNA.

Discussion

We have described a cell-free replication system which utilizes crude nuclei isolated in high yields from cultures of chick embryo fibroblasts. The endogenous rate of DNA synthesis by these nuclei is proportional to the rate of DNA synthesis *in vivo* when comparisons are made throughout the cell cycle in partially synchronized cultures. The synchronization of fibroblast cells by serum deprivation is a very gentle, reversible technique. Unlike inhibition by amethopterin or high concentrations of thymidine, serum deprivation does not appear to interfere directly with DNA precursor metabolism. Instead, it inhibits cell growth in a manner similar to that of density-dependent inhibition (Rubin and Koide, 1973) with the advantage of more convenient manipulation (*e.g.*, the cells can be used in 24–48 hr rather than waiting several days for them to become confluent). The disadvantage of this technique is that only partial synchrony is achieved. However, 10–20-fold differences in the *in vivo* DNA synthesis rates can easily be obtained, providing adequate active and inactive controls for *in vitro* systems.

Both the endogenous and the exogenous DNA-stimulated activities require ATP for maximum incorporation. ATP has been reported to stimulate nearly all crude *in vitro* replication systems—both procaryotic (Moses and Richardson, 1970; Vosberg and Hoffmann-Berling, 1971; Wickner and Hurwitz, 1972; Schaller *et al.*, 1972 and eucaryotic (Friedman and Mueller, 1968; Lynch *et al.*, 1970; Grisham *et al.*, 1972; Winnacker *et al.*, 1972; Bell, 1974). It is important to note that the activity of nuclei for DNA synthesis is inhibited by high concentrations of Mg^{2+} (for example, Friedman and Mueller, 1968; Kaufman *et al.*, 1972; Shimada and Terayama, 1972; or Figure 3, this communication), and that ATP can chelate Mg^{2+}. Raskas and Rho (1973) have shown that the Mg^{2+}:ATP ratio is extremely important in the amount of adenovirus mRNA released from nuclei *in vitro*. Similarly, in this system the extent of stimulation by ATP is dependent upon the Mg^{2+} concentration.

There have also been several reports that ATP is required for replication, but not for repair DNA synthesis (Moses and Richardson, 1970; Ganesan, 1971; Grisham *et al.*, 1972; Majumdar and Frankel, 1973). Recently Wickner and Kornberg (1973) have shown that *Escherichia coli* DNA polymerase III requires ATP to initiate DNA synthesis from an RNA-primed *single-strand* DNA template, but not to fill in *short gaps* on a template partially digested by DNase. The role of ATP in crude replication systems, however, remains to be elucidated.

We have described a technique for the isolation of nuclei which are capable of elongating DNA from preexisting growing points for at least 500–1000 nucleotides. Although repair synthesis of this length has been observed in bacteria (Cooper

and Hanawalt, 1972), the maximum lengths of repair synthesis found in eucaryotic systems by two independent techniques were 30 (Edenberg and Hanawalt, 1972) and 100 (Regan *et al.*, 1971) nucleotides. However, the fact that most of the DNA synthesized *in vitro* is immediately adjacent to the growing point is the best evidence that this synthesis is primarily replication.

This system for *in vitro* DNA synthesis with isolated nuclei offers an opportunity to understand the molecular mechanism of elongation through an analysis of the radioactive products made *in vitro*. The ultimate goal of such a system is, of course, to be able to obtain *in vitro* initiation in order to study the mechanisms whereby cells regulate the onset of DNA synthesis or the initiation of separate units of replication during the synthetic period. An *in vitro* system capable of valid replication is a necessary prerequisite to such an endeavor.

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