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Biochemistry

Initiation of replication in chromosomal DNA induced by extracts from proliferating cells

(DNA synthesis/cell-free assay/growth regulation/cell cycle/neoplastic cells)

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ABSTRACT **Addition of an extract prepared from a proliferating cell line to nuclei isolated from resting tissues such as frog liver and spleen resulted in the stimulation of DNA synthesis as assayed by [^{3}H]dTTP incorporation. This stimulated incorporation of [^{3}H]dTTP required ATP and depended on Mg^{2+} and deoxynucleoside triphosphates. Pulse-chase experiments showed that the synthesis of DNA in this system was discontinuous, resulting in the appearance of approximately 4S fragments and their ligation to yield higher molecular weight DNA. In addition, electron microscopic analysis of the DNA molecules from the reaction mixture showed that the frequency of replication "eyes" in the extract-stimulated reaction was 10-fold higher than that observed in controls. All of these results strongly suggest that the extract stimulated initiation of DNA replication in the chromatin of normally resting cells. Preliminary characterization by dialysis, heating, and enzyme treatments indicated that the activity is associated with one or more proteins of high molecular weight (>50,000).**

Comparison of the levels of stimulatory activity in extracts from various mammalian and avian sources showed that the activity was present in cells proliferating either *in vivo* or in tissue culture. In contrast, extracts from normally resting tissues and cells had no activity. The level of activity present did not appear to be directly related to the levels of DNA polymerase. These results suggest a use for this system in studying regulation of the initiation of DNA synthesis and control of the various phases of the cell cycle.

A basic feature of normal growing cells as well as transformed cells is the orderly progression leading from one generation to the next, termed the cell cycle. Research on the biochemical basis of cell cycle regulation has been focused on many aspects of cell metabolism, including nuclear DNA replication, which is the essential step for the production of progeny cells (1).

The complex process of replication can be conveniently studied in a cell-free system, and a number of such systems in which chromosomal DNA replication occurs have been described (2–5). For studies of control mechanisms, several of these systems suffer from the limitation that the nuclei show DNA replication independently and exhibit no requirement for added cytoplasmic fractions. More seriously, none of the systems is capable of *initiating* DNA replication, a stage at which one might expect important regulatory processes to operate. One exception has been described recently (6), in which extracts derived from frog eggs and early embryos were found to induce DNA replication in nuclei from frog liver.

Our interest in growth control in both normal and neoplastic cells has led us to search for a general system amenable to the study of nuclear-cytoplasmic relations at the molecular level. A consideration of the induction of cell division in normally resting cells, such as the mitogenic stimulation of lymphocytes by concanavalin A (7), has pointed up the need for a cell-free assay for activators of DNA synthesis, especially those in the stimulation pathway from the cell membrane to the nucleus. In this communication we describe a general assay adaptable to a variety of systems, particularly to the study of growth control.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; EDTA, (ethylene dinitrilo)tetraacetate; EGTA, ethylene glycol-*bis*(β-aminoethyl ether)-*N,N'*-tetraacetate.

MATERIALS AND METHODS

[*methyl*-^{3}H]dTTP (50 Ci/mmol) was from Schwarz/Mann; nucleotides and poly(dA)·$(dT)_{10}$ (20:1) were from P-L Biochemicals; and ^{32}P-labeled f1 bacteriophage and duplex, circular f1 DNA were gifts of Dr. Peter Model of The Rockefeller University.

Cell Cultures and Cell Suspensions. P388 and EL4.BU tumor cell lines were obtained from the Cell Culture Center of The Salk Institute. These cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Microbiological Associates) and 100 units of penicillin-streptomycin per ml. Extracts were routinely prepared from cultures grown to a cell density of about 10^6 cells per ml.

Fibroblast cell lines, 3T3 and SV 3T3, were gifts of Dr. Howard Green of Massachusetts Institute of Technology. Cultures of chicken embryo fibroblasts transformed by Rous sarcoma virus *ts* 68 (*ts* NY68 SRA) (8) were obtained from Dr. Hidesaburo Hanafusa of The Rockefeller University. These cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 10% tryptose-phosphate broth, and 100 units of penicillin-streptomycin per ml.

Brain, liver, and spleen tissues were obtained from 2-month-old NCS mice, and fetal livers were obtained from 17-day-old embryos of CBA/J mice (Jackson Labs). Single-cell suspensions were prepared from these tissues as described previously (9). The same procedure was used to prepare cell suspensions from spleens of frogs (*Xenopus laevis*, NASCO, Fort Atkinson, Wisc.).

Preparation of Cell Extracts and Nuclei. All operations were at 0–4°. The cells were washed once with 0.2 M sucrose–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) (pH 7.8)–5 mM KCl–0.5 mM $MgCl_2$–0.1 mM (ethylene dinitrilo)tetraacetate (EDTA)–0.5 mM dithiothreitol, resuspended (2×10^8 viable cells per ml) in the same buffer made at pH 8.5 and lacking sucrose, and allowed to stand 8 min at 0°. They were then homogenized by 20 strokes in a Dounce homogenizer. This procedure disrupted virtually all the cells, as well as some nuclei. The homogenate was centrifuged for 60 min at 45,000 rpm (2°) in a Spinco 75Ti rotor, and the supernatant (cell extract) was placed on ice.

Nuclei were prepared from frog liver according to Graham *et al.* (10). They were suspended in 0.25 M sucrose–1 mM $MgCl_2$. Nuclei were prepared from frog spleen cells by a Brij-58 lysis procedure (11). They were suspended in 0.25 M sucrose–25 mM Hepes (pH 8.0)–5 mM $CaCl_2$–2% Dextran C100 (Sigma).

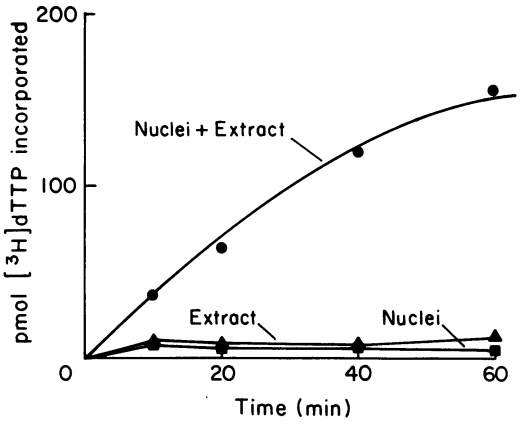

FIG. 1. Time course of [^{3}H]dTTP incorporation. Frog spleen nuclei (1.2×10^6) were incubated with 50 μl of P388 cell extract.

Assay for Chromosomal DNA Synthesis. In a final volume of 250 μl, the following were mixed at 0°: 50 μl of 0.25 M sucrose–25 mM Hepes (pH 8.0)–27.5–30 mM $CaCl_2$–2% Dextran C100; cell extract; nuclei (3×10^5 to 4×10^6); and 100 μl of incorporation mixture containing 0.5 mM each of dATP, dGTP, and dCTP, 0.2 mM dTTP (50 μCi/ml of [^{3}H]dTTP), 5 mM ATP, 12.5 mM phospho*enol*pyruvate, 10 units/ml of pyruvate kinase, 38 mM Hepes (pH 8.0), 100 mM KCl, 12.5 mM ethylene glycol-*bis*(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA), 125 mM sucrose, 12.5 mM $MgCl_2$, and 2 mM dithiothreitol. $CaCl_2$ was present in 1 mM excess over EGTA. The assay was performed at 37°. Samples were then treated with 0.1 M NaOH for 5 min at 0°, and DNA was precipitated with 5% trichloroacetic acid–0.05 M sodium pyrophosphate. Samples were filtered on Whatman GF/A discs, washed with 1 M HCl–0.1 M sodium pyrophosphate and ethanol, and radioactivity was determined.

Assay for DNA Polymerase Activity. In a final volume of 100 μl, the following were mixed at 0°: cell extract; 2.5 μl of poly(dA)·$(dT)_{10}$ at 500 μg/ml in 0.02 M KCl; and 25 μl of incorporation mixture containing 80 μM dTTP (40 μCi/ml of [^{3}H]dTTP), 61 mM Hepes (pH 8.0), 40 mM $MgCl_2$, and 4 mM dithiothreitol. The assay was performed for 60 min at 37°. After addition of 10 μg of sonicated salmon testis DNA, the samples were treated as described above for the assay of chromosomal DNA synthesis.

Discontinuous DNA Synthesis. Nuclei were incubated with cell extract in a 1-ml reaction mixture, as described above, for assay of chromosomal DNA synthesis, only [^{3}H]dTTP was at 250 μCi/ml in the incorporation mixture during the pulse for 2 min at 37°. The sample was centrifuged for 5 min at 10,000 rpm (0°) in a Sorvall SM24 rotor. For the chase, the pellet was resuspended in a fresh incubation mixture lacking [^{3}H]dTTP and incubated an additional 5 min at 37°. Pellets from both the pulse and chase were treated with 0.3 M NaOH–1 mM EDTA, applied to 5–20% linear sucrose gradients containing 0.3 M NaOH–0.7 M NaCl–1 mM EDTA formed over a cushion of 25% sucrose–6.3 M CsCl, and centrifuged 16 hr at 28,000 rpm (5°) in a Spinco SW39 rotor. Fractions were collected and treated as described above for assays of DNA polymerase activity.

Electron Microscopy. Nuclei were incubated with or without cell extract in a 250-μl reaction mixture for 60 min at 37° in the assay for chromosomal DNA synthesis. DNA was isolated directly from the incubation mixture without prior centrifugation by Sarkosyl-treatment and isopycnic banding in CsCl-gradients (6). Samples were prepared for electron microscopy utilizing the aqueous spreading technique of Davis *et al.* (12) with uranyl acetate staining and no shadowing. Grids were examined in the Philips 300 electron microscope; duplex circular DNA of bacteriophage f1 was used as a 2-μm length standard.

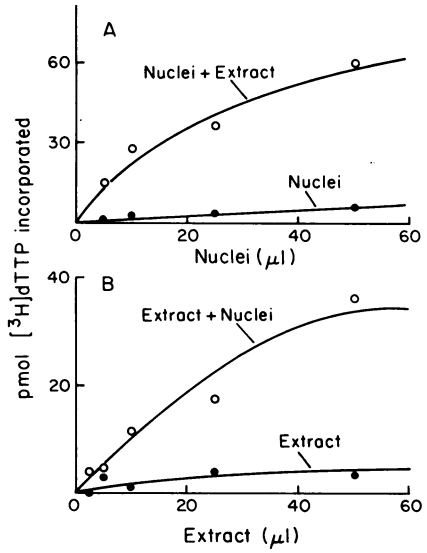

FIG. 2. Dependence of [^{3}H]dTTP incorporation on added nuclei and cell extract. (A) Nuclear dependence; (B) cell (P388) extract dependence. Frog liver nuclei were at 6×10^7/ml, and 25 μl were used in (B). In (A), 50 μl of P388 cell extract was used. Incubation was for 60 min. The value for a blank, as well as for the extract background, have been subtracted in (A), whereas for (B), the blank and the nuclei backgrounds have been subtracted.

RESULTS

An extract from proliferating cells stimulates DNA synthesis in a cell-free system

P388 is a methylcholanthrene-induced mouse lymphoma cell line grown in tissue culture. When an extract prepared from P388 cells was incubated with nuclei prepared from frog spleen cells in the presence of Mg^{2+}, ATP, and the four deoxynucleoside triphosphates, it stimulated the incorporation of [^{3}H]dTTP into acid-precipitable material. Kinetic analysis indicated that this reaction proceeded linearly for about 30 min and was essentially complete by 60 min (Fig. 1).

Results similar to those obtained using frog spleen nuclei were also obtained with nuclei isolated from frog liver cells, another population of nonproliferating cells (10). Moreover, it was found that cells from both the liver and the spleen cultured for 24 hr did not incorporate [^{3}H]thymidine. Nuclei isolated from these cells responded to stimulation of [^{3}H]dTTP incorporation by the P388 cell extract to the same extent as nuclei from their respective fresh tissues. In contrast, nuclei prepared from proliferating cells such as P388 incorporated high levels of [^{3}H]dTTP in the absence of extract, and this incorporation was not enhanced by the presence of the extract.

The extent of incorporation stimulated by the extract depended upon the amount of DNA added in the form of nuclei. A given amount of extract could be saturated with nuclei (Fig. 2A). Similarly, the reaction of a given number of nuclei was dependent upon the amount of extract added (Fig. 2B).

Addition of EDTA to the incubation mixture completely abolished the extract-stimulated incorporation of label (Table 1), indicating a requirement for Mg^{2+}. When the four deoxynucleoside triphosphates were omitted, synthesis was drastically

Table 1. Properties of the reaction

Experiments	pmol of [^{3}H]dTTP incorporated	
	Nuclei	Nuclei + extract
Exp. 1		
—	5.5	31.8
+EDTA (10 mM)	3.1	1.6
Exp. 2		
—	8.4	62.3
—dATP, —dGTP, —dCTP, +0.06 μM dTTP	0.01	0.26
—ATP (glycerol kinase, 15 min, 37°)	10.3	9.4
+ATP (glycerol kinase mock)	11.2	56.1

Assays were performed for 60 min. In Exp. 1, frog spleen nuclei (5×10^5) were incubated with 25 μl of P388 cell extract. In Exp. 2, frog liver nuclei (3×10^6) were incubated with 50 μl of P388 cell extract.

reduced (Table 1). These results strongly suggest that the observed incorporation of [^{3}H]dTTP is due to DNA synthesis, a conclusion that is further supported by the dependence of the reaction upon the number of added nuclei (Fig. 2A).

Initiation of DNA replication

It has been shown in several prokaryotic and eukaryotic systems that DNA replication is dependent upon ATP. Furthermore, DNA replication is a discontinuous process, which (in eukaryotic systems) results initially in the appearance of nascent 3–5S fragments. The progression of replication forks from the origin of replication leads to the appearance of "eyes" in the chromosomal DNA. These three criteria (3–5, 11, 13, 14) were applied to provide evidence that DNA replication was occurring and to exclude the possibility that the DNA synthesis observed in our system was due to DNA repair.

To demonstrate a requirement for ATP, it was necessary to remove the endogenous nucleotide from the incubation mixture. This was achieved by pretreating the reagents with glycerol kinase, which completely abolished extract-stimulated DNA synthesis (Table 1). In a control experiment in which a mock treatment was performed, there was no significant decrease. These results were obtained using both frog liver and frog spleen nuclei.

When the incubation was carried out for 2 min and the product of the reaction was analyzed by sedimentation in an alkaline sucrose gradient (Fig. 3A), it was found to consist of material with a sedimentation coefficient of about 4 S, as measured with respect to marker DNA. These nascent fragments were chased into fast sedimenting DNA when the incubation was continued for an additional 5 min in the absence of [^{3}H]dTTP (Fig. 3B). This demonstrates that discontinuous DNA synthesis occurred in the system.

To obtain evidence for initiation of DNA replication, we isolated DNA molecules from the reaction mixture after a 60-min incubation of liver nuclei in the presence and absence of P388 extract. These molecules were analyzed in the electron microscope and were scored for the presence of replication "eyes" (Fig. 4). In the control, the frequency of molecules possessing "eyes" was 0.20% (six "eyes" in 3020 molecules). In contrast, the frequency was 10-fold higher, 2.05% (62 "eyes" in 3030 molecules), in the DNA from the extract-stimulated reaction. Similar results were obtained using spleen nuclei.

Statistical analysis (χ^2 value of 45.6 at one degree of freedom)

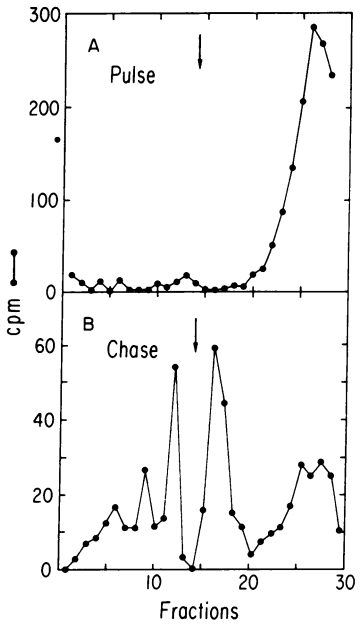

FIG. 3. Discontinuous DNA synthesis. (A) Pulse; (B) chase. Frog liver nuclei (2.5×10^7) and 200 μl of P388 cell extract were used. The arrow indicates the position of 18S marker DNA in parallel gradients in which ^{32}P-labeled bacteriophage fl was sedimented. Sedimentation is from right to left. ●—●, cpm of [^{3}H]dTTP (acid-precipitable).

indicates that it is extremely unlikely ($P \ll 0.005$) that this difference was due to chance alone. The average length of the "eyes" was found to be 0.09 μm, with a standard deviation of 0.04 and 0.02 μm for the "eyes" from the extract-stimulated and control reactions, respectively. The total length of DNA examined in the control and the extract-stimulated reaction was equivalent, and there was no evidence that the two populations differed significantly in the length of the molecules. Control experiments showed that the observed DNA was not derived from the extract.

These experiments demonstrating the ATP-dependence and the discontinuous nature of the DNA synthesis, as well as the

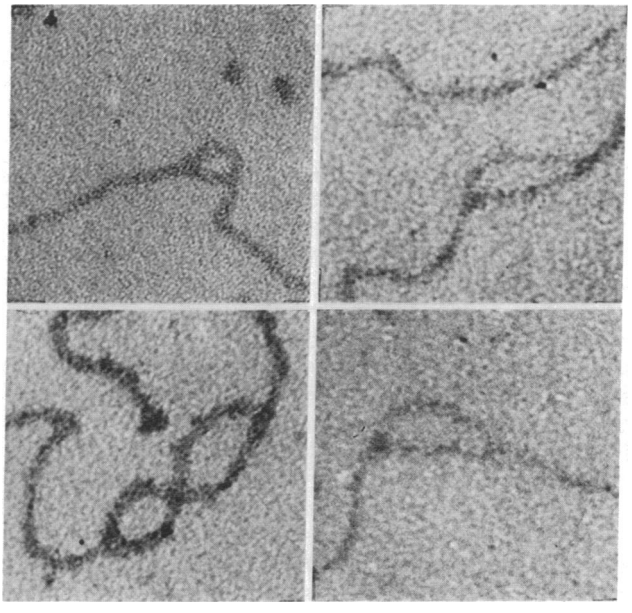

FIG. 4. Replication "eyes". Frog liver nuclei (3.6×10^6) were incubated with or without 50 μl of P388 cell extract.

Table 2. Characterization of P388 extract

Treatment of extract	pmol of [^{3}H]dTTP incorporated
Exp. 1	
—	45.7
100°, 2 min	1.5
60°, 15 min	2.2
Dialysis, 2 hr	39.6
Exp. 2	
—	30.6
Trypsin (50 μg/ml), 15 min, 37°	3.2
Soybean inhibitor (100 μg/ml)	35.5
Trypsin + soybean inhibitor	29.8

Assays were performed for 60 min. In Exp. 1, frog spleen nuclei (5×10^5) were incubated with 25 μl of P388 cell extract. In Exp. 2, frog liver nuclei (4×10^6) were incubated with 50 μl of P388 cell extract.

induction of "eyes" in the DNA after incubation with the extract, show that the synthesis resulted from DNA replication and not repair. Indeed, the appearance of replication "eyes" provides evidence that the extract is capable of initiating chromosomal DNA replication. This conclusion is supported by the fact that the nuclei used were derived from resting cells, which are not in the process of replicating their DNA. Furthermore, as described below, only extracts from proliferating cells were active.

Characterization of the activity

In order to determine the nature of the active components in the P388 extract, we subjected it to several treatments (Table 2) prior to incubation with the nuclei. Heating the extract reduced the activity to near background levels, but dialysis of the extract did not reduce activity. Most significantly, treatment of the extract with trypsin had a drastic inhibitory effect, and as shown in Table 2, this was neither due to the soybean inhibitor used to stop the action of trypsin nor to any residual tryptic activity present during the incubation with nuclei. These data indicate that the active components are most probably proteins. The activity was retained on an Amicon XM50 filter, suggesting the involvement of a protein of molecular weight >50,000. In addition, the activity was sensitive to *N*-ethylmaleimide, and therefore probably requires a free sulfhydryl group.

Distribution of the activity in various cell sources

Extracts were also prepared from a number of mammalian and avian sources. These extracts were compared in terms of their "initiation" activity using the frog liver nuclei assay and of their DNA polymerase activity using a synthetic template, poly(dA)·$(dT)_{10}$ (Table 3).

Among tissues freshly prepared from mice, adult liver and brain cells contained no detectable activity in both the "initiation" and polymerase assays. In contrast, fetal liver cells showed substantial levels of activity. The activity present in adult mouse spleen cells from freshly prepared tissue is probably due to a small number of hemopoietic cells that incorporate [^{3}H]thymidine during the first 10 hr in tissue culture (9).

When extracts were prepared from normal fibroblasts, such as 3T3 cells, whose growth had become stationary in a density-inhibited monolayer (6.1×10^4 cells per cm^2), very low levels of polymerase activity and no "initiation" activity were detected. In contrast, extracts prepared from SV 3T3 cells at a cell density four times higher than their normal counterparts showed high levels of activity, consistent with the notion that these transformed cells are less susceptible to density-dependent growth control. Similar results were also obtained with *ts*68-transformed cells. Whereas cells grown to a saturation density of 9×10^4 cells per cm^2 at the nonpermissive temperature (41°) showed no activity, cells grown to twice that density at the permissive temperature (37°), where they display the transformed phenotype, yielded appreciable activity.

Table 3. Distribution of "initiation" and DNA polymerase activities in various cell sources

Source	Units/ml "Initiation" activity	Units/ml DNA polymerase activity
P388	0.32	2.92
EL4	0.26	2.54
P388 1/2 day high-density	0.32	4.20
P388 1 day high-density	0.15	2.61
P388	0.45	3.11
3T3	0	0.06
SV 3T3	0.27	1.26
*ts*68-transformed cells (41°)	0	—
*ts*68-transformed cells (37°)	0.19	—
P388	0.44	3.70
Fetal liver	0.18	0.29
Adult liver	0	0
Adult brain	0	0
Adult spleen	0.17	0.10

The assay for "initiation" activity was for 60 min with frog liver nuclei (2×10^6). The viabilities of cells used for extract preparation were 70–100%, and extracts were made at 2×10^8 viable cells per ml. The activities in the extracts were titrated for comparison and are expressed as unit/ml, where 1 unit = 1 nmol of dTTP incorporated in 60 min at 37°.

Finally, when exponentially growing P388 cells were allowed to settle to the bottom of the culture vessel in a dense pellet, they ceased to divide although they maintained viability for at least 1 day. After 1 day of high-density growth inhibition, "initiation" activity decreased (Table 3) as compared to the levels of activity in logarithmically growing cultures of both P388 cells as well as EL4 cells, another mouse lymphoma cell line. These experiments on the distribution of activity from various sources indicates that the presence of "initiation" activity, as well as DNA polymerase activity, is restricted to growing (fetal and transformed) cells and tissues.

DISCUSSION

Our experiments define a general cell-free assay for factors involved in the initiation of chromosomal DNA replication. Incubation of an extract prepared from a variety of growing cells with nuclei from resting tissues stimulates DNA synthesis (Fig. 1, Table 3). The extent of the reaction was dependent on the presence of DNA (Fig. 2A) and extract (Fig. 2B), and the reaction required Mg^{2+} as well as the four deoxynucleoside triphosphates (Table 1). In this assay the majority of the nuclei do not remain intact during the incubation, both in the presence and absence of added extract. Inasmuch as we have not found conditions in which the bulk of the nuclei remained intact and incorporated [^{3}H]dTTP, we cannot conclude that the DNA synthesis occurs within the nuclei. Preliminary experiments indicate, moreover, that at least some synthesis can occur in the chromatin released from the nuclei.

The conclusion that the extract-stimulated synthesis is due to DNA replication rather than repair rests on three lines of evidence: (*i*) the reaction is ATP-dependent (Table 1); (*ii*) the DNA synthesis is discontinuous (Fig. 3); and (*iii*) replication "eyes" appear in the DNA (Fig. 4). The induction of replication "eyes" by the extract provides evidence that initiation of replication occurs in the chromosomal DNA.

Further support for the notion that initiation of DNA replication occurs in the system is provided by the fact that nuclei derived from resting cells that are not in the process of DNA replication responded to stimulation by the cell extract. Moreover, the "initiation" activity was present only in extracts prepared from growing cells, whereas resting cells did not possess stimulatory activity. The activity was found in mammalian and avian cells of fibroblast and lymphoid origin (Table 3). A comparison of the levels of activity in extracts of normal and transformed cells cultured under proliferative and density-inhibited conditions suggests that the activity is subject to growth control.

We have recently found that mouse lymphocytes stimulated by concanavalin A and arrested prior to the S phase by hydroxyurea accumulate "initiation" activity as assayed in our system. This suggests that resting cells can be stimulated to induce "initiation" activity. In addition, inasmuch as hydroxyurea arrests cells at the G_1/S boundary of the cell cycle, these results suggest that the "initiation" activity may first appear in the G_1 phase, a portion of the cycle in which it is suspected that many control mechanisms operate (15).

Two additional properties of the DNA synthesis reaction are worth noting. First, the lengths of the "eyes" observed in the DNA molecules suggest that they might accommodate only a few 4S DNA fragments. It is difficult, however, to compare the results of the electron microscope analysis (Fig. 4) with the results obtained by sedimentation studies (Fig. 3), since events occurring during replication are incompletely understood, and the extent of branch migration during preparation of samples for electron microscopy is not known. In a 2-min pulse, the nascent DNA sediments exclusively as 4S fragments (Fig. 3). Approximately one-third of these are ligated to yield high molecular weight DNA during a 5-min chase. Analysis of the product of a 60-min incubation reveals that the bulk of the newly synthesized DNA sediments in a peak at 4 S skewed in the direction of faster sedimenting material (up to about 10 S). This might suggest that the system is somewhat deficient in termination, re-initiation, or ligation of 4S fragments.

Second, it also appears that the level of "initiation" activity may not be directly related to the level of DNA polymerase (Table 3). In addition, a striking dichotomy is suggested by the fact that all growing cell types possess similar levels of "initiation" activity, whereas the DNA polymerase activities in the normally growing cells are about one order of magnitude lower than in the transformed cells. This suggests the possibility that, in transformed cells committed indefinitely to proliferation, synthesis of DNA polymerase and perhaps other DNA elongation factors is deregulated. If this is the case, orderly traversal of the cell cycle could still be maintained by control at the level of initiation of DNA replication.

All of these results suggest that a general system is now available for studies of the regulation of the initiation of DNA replication and particularly of its control in the various phases of the cell cycle in higher organisms.

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