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Research Article

To study the proliferative response of hematopoietic cells to growth factors at the molecular level, we developed a cell-free system for growth factor-dependent initiation of genomic DNA replication. Nuclei were isolated from the IL-3-dependent cell line NFS/N1-H7 after a 10-h period of IL-3 deprivation. Cytosolic and membrane-containing subcellular fractions were prepared from proliferating NFS/N1-H7 cells. Nuclei from the nonproliferating cells (+/- IL-3) showed essentially no incorporation of [3H]thymidine during a 16-h incubation with a mixture of unlabeled GTP, ATP, UTP, CTP, dGTP, dATP, dCTP, and [3H]dTTP. When the combination of IL-3, a cytosolic fraction, and a membrane-containing fraction from proliferating cells was added to nuclei from nonproliferating cells, a burst of [3H]thymidine incorporation into DNA began after a 12-h lag period, attained a maximal rate at 16 h, and reached a level of 860 pmol thymidine/10(6) nuclei at 24 h (corresponding to replication of approximately 56% total mouse genomic DNA). This DNA synthesis was inhibited approximately 90% by the specific DNA polymerase alpha inhibitor aphidicolin. Deletion of a single cellular component or IL-3 from the system resulted in a marked reduction of DNA replication (-membrane, 80 +/- 4%; -cytosol, 90% […]

Growth Factor–dependent Initiation of DNA Replication in Nuclei Isolated from an Interleukin 3–dependent Murine Myeloid Cell Line

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Abstract

To study the proliferative response of hematopoietic cells to growth factors at the molecular level, we developed a cell-free system for growth factor–dependent initiation of genomic DNA replication. Nuclei were isolated from the IL-3–dependent cell line NFS/N1-H7 after a 10-h period of IL-3 deprivation. Cytosolic and membrane-containing subcellular fractions were prepared from proliferating NFS/N1-H7 cells. Nuclei from the nonproliferating cells (±IL-3) showed essentially no incorporation of [^{3}H]thymidine during a 16-h incubation with a mixture of unlabeled GTP, ATP, UTP, CTP, dGTP, dATP, dCTP, and [^{3}H]dTTP. When the combination of IL-3, a cytosolic fraction, and a membrane-containing fraction from proliferating cells was added to nuclei from nonproliferating cells, a burst of [^{3}H]thymidine incorporation into DNA began after a 12-h lag period, attained a maximal rate at 16 h, and reached a level of 860 pmol thymidine/10^6 nuclei at 24 h (corresponding to replication of ~ 56% total mouse genomic DNA). This DNA synthesis was inhibited ~ 90% by the specific DNA polymerase α inhibitor aphidicolin. Deletion of a single cellular component or IL-3 from the system resulted in a marked reduction of DNA replication (⁻membrane, 80±4%; ⁻cytosol, 90%±4%; ⁻IL-3, 74±7% inhibition). This model requires a growth factor (IL-3), a sedimentable cell fraction containing its receptor and possibly additional membrane-associated components, and a cytosolic fraction. It appears to recapitulate the molecular events required for progression from early G_1 to S phase of the cell cycle induced by IL-3 binding to its receptor. (*J. Clin. Invest.* 1990. 85:300–304.) cell-free system • DNA replication • growth factor • IL-3

Introduction

The hematopoietic growth factor IL-3, a 28-kD glycoprotein secreted by activated T-lymphocytes, promotes the proliferation and differentiation of early hematopoietic stem and progenitor cells in vitro (1, 2). Along with other cytokines that bind to plasma membranes, including IL-1 (3), colony-stimulating factor-1 (4), and erythroid burst-promoting activity (5), IL-3 may play an important role in the regulation of hematopoietic cell proliferation and differentiation in vivo. Murine IL-3–dependent cell lines derived from long-term bone marrow cultures have a single class of high-affinity cell-surface receptor for IL-3 (6). Chemical cross-linking studies of IL-3 with its cell-surface receptor indicate the receptor is a 140–150-kD protein that becomes phosphorylated on tyrosine within seconds of ligand binding (7, 8). As in other growth factor–dependent cells, IL-3 is required for progression beyond a critical point in early G_1 of the cell cycle in IL-3–dependent murine myeloid cell lines, and entry into S phase occurs after a delay of 16 h after initial IL-3 exposure (9). Although recent studies suggest that the IL-3 receptor may be a tyrosine kinase (and/or activate a kinase cascade), the identity and biochemical functions of cytosolic phosphoproteins that appear within minutes of IL-3 stimulation (10), and their relationship to later biochemical events that result in progression through G_1 phase of the cell cycle and entry into S phase, are entirely unknown. In the present studies, the murine IL-3–dependent cell line NFS/N1-H7, derived from retroviral-free long-term bone marrow cultures in vitro (11), was used to develop a cell-free model of growth factor–dependent initiation of DNA replication.

Important insight into the biochemical basis of hormone- or growth factor–stimulated cell proliferation has been obtained from genetic studies of cell-cycle–deficient yeast mutants (12, 13). While the generation of temperature-sensitive cell cycle–defective mutants in mammalian cells is feasible (14), segregation into classic genetic complementation groups by cell fusion and subsequent genetic analysis is technically more difficult than in fusion yeast. Thus a cell-free system reflecting the complete sequence of essential molecular events for growth factor–stimulated proliferation of mammalian cells would provide a useful model for genetic as well as biochemical analysis.

Methods

Reagents. McCoy's 5A media and penicillin–streptomycin were obtained from Gibco Laboratories (Grand Island, NY). Agarose type VII, glycerol, DTT, aphidicolin, and bromodeoxyuridine triphosphate (BrdUTP)[1] were obtained from Sigma Chemical Co. (St. Louis, MO). FBS was from HyClone Laboratories (Logan, UT). Natural murine

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1. *Abbreviation used in this paper:* BrdUTP, bromodeoxyuridine triphosphate.

IL-3 was from Genzyme Corp. (Boston, MA). Liquid paraffin was from Serva Fine Biochemicals Inc. (Garden City Park, NY). Triton X-100 was obtained from Research Products International Corp. (Mt. Prospect, IL). dATP, dCTP, dGTP, dTTP, ATP, CTP, GTP, and UTP were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). [^{3}H]dTTP (~ 80 Ci/mmol) was from New England Nuclear Research Products (Boston, MA). Monoclonal mouse anti-BrdU and tetramethylrhodamine isothiocyanate conjugated rabbit anti-mouse IgG was from DAKOPATTS (Copenhagen, Denmark). The other reagents were of the highest commercially available grade.

Cell culture. Characteristics of the murine myeloid cell line, NFS/N1-H7, used in these studies have been previously reported (11). The cells are dependent on both (FBS) and a source of murine IL-3 for proliferation and viability. The cell line was grown in McCoy's 5A media containing 10% FBS and WEHI-3-conditioned medium as a source of IL-3. The cells were passaged twice weekly by 1:10 dilution in fresh medium adjusted to contain ≥ 500 U/ml IL-3. Cells were diluted to 10^5/ml in fresh medium 48 h before harvesting for the preparation of nuclei or subcellular fractionation as described below.

Cell cycle analysis. NFS/N1-H7 cells, before or after IL-3 deprivation as indicated in the text, were stained with propidium iodide then analyzed for DNA content by fluorescence-activated cell sorting on a cell sorter (Epics 753; Coulter Electronics Inc., Hialeah, FL) by the method of Deitch et al. (15). The percent of cells in S phase was calculated from the DNA histogram.

Preparation of encapsulated nuclei. Nuclei isolated from mammalian cells by standard techniques of cell disruption in high ionic strength buffers often display a high background rate of DNA polymerase β repair (16). Although *Xenopus laevis* spleen cell nuclei provide a more native DNA template and have been used to study potential second messengers in mitogen-stimulated lymphocyte cytosol (17), we wished to avoid the use of amphibian nuclei, which may differ importantly from mammalian nuclei because they appear not to have sequence-specific origins of replication (18). We therefore employed the method of Jackson and Cook for isolation of agarose-encapsulated nuclei under isotonic conditions (19). This method has been shown to yield mammalian cell nuclei with a highly native, supercoiled DNA template freely accessible to molecules up to 1.5×10^8 D and suitable for the study of replicative DNA synthesis (20). In brief, a 2.5% wt/vol solution of low-melting temperature agarose (Sigma type VII) was further purified by the addition of 10% wt/vol moist DEAE cellulose (DE52; Whatman Inc., Clifton, NJ) and incubation at 50°C for 30 min. The DEAE cellulose and absorbed impurities were removed by centrifugation and 1.0-ml aliquots of the supernatant agarose solution were sterilized by autoclaving.

NFS/N1-H7 cells were washed three times, resuspended in McCoy's medium containing 10% FBS but no IL-3, then incubated without IL-3 at 37°C for 10 h. The cells were then resuspended in 4 ml of McCoy's/10% FBS at 2×10^7 cells/ml and warmed to 37°C. A premelted 1.0-ml aliquot of 2.5% agarose was temperature equilibrated in a 39°C water bath, then rapidly mixed with 4 ml of the cell suspension at 37°C. 2 vol (10 ml) of liquid paraffin oil was then added to the aqueous cell suspension and the mixture was vortexed for 30 s in a sealed 50-ml round bottom centrifuge tube. The resulting emulsion was rapidly cooled in a melting ice bath and the agarose-encapsulated cells were recovered in the pellet after centrifugation at 10,000 *g* for 10 min at 10°C. The pelleted beads were resuspended in McCoy's media containing 10% FBS (no IL-3) to a final volume of 8 ml, then lysed by the addition of 3 vol (24 ml) of isotonic lysis buffer (0.5% wt/vol Triton X-100, 100 mM KCl, 25 mM EDTA, 1 mM DTT in 10 mM Tris-HCl, pH 8.0). After incubation at 4°C for 1 h, the beads containing nuclei were washed three times in isotonic wash buffer (100 mM KCl, 25 mM $(NH_4)_2SO_4$, 1 mM EDTA, 1 mM DTT, 5% glycerol in 10 mM Tris-HCl, pH 8.0). The final pellet (~ 2 g wet wt) was weighed, resuspended by addition of an equal volume of wash buffer (~ 2 ml), then divided into 0.5-ml aliquots and stored at −80°C until used in the nuclear replication assay. Since the cell density in the initial 0.5% agarose solution was 1.6×10^7/ml, the final concentration of nuclei was calculated to be 8×10^6/ml in the 1:1 final dilution of agarose beads. This assumes that 100% of the nuclei were encapsulated and may be an overestimate of the number of nuclei actually contained in the beaded agarose. Calculations of [^{3}H]thymidine incorporation per 10^6 nuclei were based on this assumption, and thus may represent underestimates of the true DNA replication rate.

Preparation of cytosolic and membrane-containing subcellular fractions. Proliferating NFS/N1-H7 cells were washed three times, resuspended at 10^8 cells/ml in 0.25 M sucrose, 40 mM Tris pH 8.0, and immediately disrupted in a melting ice bath by two 15-s bursts at 30% power from the microtip of a sonic dismembrator (model 300; Fisher Scientific Co., Pittsburgh, PA). This treatment efficiently disrupted 70–90% of the cells as judged by phase contrast microscopy. Intact cells and nuclei were removed from the sonicate by centrifugation at 1,200 *g* for 10 min. The postnuclear supernatant was then centrifuged in a swing-out rotor (SW 55; Beckman Instruments, Inc., Fullerton, CA) at 200,000 *g* for 45 min at 4°C. The supernatant fraction, referred to subsequently as the "cytosolic" fraction, was carefully aspirated and stored at −80°C in aliquots until used in the replication assay. The pellet fraction referred to subsequently as the "membrane-containing fraction," contained besides plasma membrane other sedimentable subcellular organelles such as endoplasmic reticulum, Golgi, and mitochondrial membranes. This fraction was resuspended in 0.25 M sucrose, 40 mM Tris-HCl, pH 8.0, by gentle hand homogenization in a glass homogenizer with a Teflon pestle and stored as aliquots at −80°C until used in the DNA replication assay.

DNA replication assay. Assays were initiated by the addition of 1.4×10^5 nuclei to a replication mixture containing the following components in a final volume of 80 μl: 1.25 mM ATP; 0.1 mM CTP, GTP, and UTP; 0.25 mM dATP, dGTP, dCTP, and dTTP containing 1.5 μCi [^{3}H]dTTP; 25–100 μg/ml cytosolic protein; 100 μg/ml membrane protein; 100 mM KCl; 25 mM $(NH_4)_2SO_4$; 1 mM EDTA; 1 mM DTT; 5% wt/vol glycerol; 1% penicillin–streptomycin in 10 mM Tris-HCl, pH 8.0. The mixture was incubated at 37°C in sealed 1-ml polypropylene microfuge tubes. At the indicated times the reaction was stopped by addition of 200 μl of a 2% wt/vol SDS solution and 50 μg salmon sperm carrier DNA. Samples were immediately precipitated with 10% TCA and collected by vacuum filtration on GF/C filters. The filters were washed with 10% TCA and then absolute ethanol. Radioactivity contained on the dried filters was measured by scintillation counting in Ready Value scintillation fluid (Beckman Instruments, Inc.) in a Beckman LS 6800 liquid scintillation counter.

The minimal amount of cytosolic protein capable of supporting IL-3–stimulated DNA replication was variable from one preparation to another and was determined in preliminary experiments for each cytosol preparation. In some preparations of cytosols, high amounts of cytosolic protein were capable of stimulating DNA synthesis in the absence of IL-3 and membrane protein. These high concentrations were not used in the present experiments.

Labeling of nuclei with BrdU and anti-BrdU Ab. Conditions in the DNA replication assay were identical to those described above except that 100 μM BrdUTP was substituted for dTTP. After 16 h incubation at 37°C, the beaded nuclei were smeared on glass slides and fixed with 100% methanol for 5 min at −20°C followed by denaturation with 1.5 M HCl for 30 min at room temperature. Staining with anti-BrdU Ab was essentially as described by Gratzner (21). After three washes in PBS, the fixed nuclei were reacted with mouse monoclonal anti-BrdU antibodies (DAKOPATTS) at 1:20 dilution for 1 h at 37°C. The slides were washed five times with PBS then reacted with rhodamine-labeled rabbit anti-mouse IgG (DAKOPATTS) at 1:20 dilution for 1 h at 37°C. The slides were washed again five times in PBS, mounted, and photographed under light or fluorescent microscopy.

Results

Cellular responses to IL-3 deprivation. The progressive decline in cell proliferation and cell viability after removal of IL-3 has been well documented in a variety of IL-3–dependent cell lines

(2, 9). The murine myeloid cell line used in the present study, NFS/N1-H7, also showed a loss of viability (24% viable compared with control, measured by trypan blue dye exclusion) and decline in proliferative rate (1.2% control measured by [^{3}H]thymidine uptake) after IL-3 deprivation for 24 h. As analyzed by fluorescence-activated cell sorting, the percent of cells in S phase declined from 20–25% during log growth in IL-3–containing media to 10% after a 10-h period of IL-3 deprivation. When a source of IL-3 was restored after a 10-h deprivation period, the percent cells in S phase declined further to 5% at 8 h, then increased to 65% at 12–14 h after readdition of IL-3. Thus deprivation of IL-3 for 10 h did not result in complete cell-cycle synchronization after readdition of IL-3, but it did result in a significant decline of S phase cells after deprivation, which was followed by a threefold increase in S phase cells 12 h after IL-3 restoration.

DNA synthesis in nuclei isolated from NFS-H7 cells. Nuclei prepared from NFS/N1-H7 cells that had been deprived of IL-3 for a period of 10 h were examined for DNA synthesis in the cell-free replication assay. In the presence (Fig. 1, *open circle*) or absence of 500 U/ml IL-3, these nuclei incorporated < 50 pmol [^{3}H]thymidine/10^6 nuclei from [^{3}H]dUTP after 24 h. When cytosolic and membrane-containing subcellular fractions were added to the cell-free DNA replication system in the presence of 500 U/ml IL-3 (Fig. 1, *solid circles*), incorporation of [^{3}H]thymidine remained at background levels until ~ 12 h, when [^{3}H]thymidine incorporation into DNA increased abruptly. The rate of [^{3}H]thymidine incorporation was maximal at 14–16 h and reached a level of 860 pmol/10^6 nuclei at 24 h. The 12-h lag period in the cell-free system is thus comparable to the 12–14-h lag period between addition of IL-3 and entry into S phase of intact NFS/N1-H7 cells.

As shown in Fig. 2, deletion of the cytosolic or membrane-containing fraction, or deletion of IL-3, resulted in a significant decrease in DNA synthesis in the cell-free system. Additionally the specific DNA polymerase α inhibitor, aphidicolin, caused > 90% inhibition of DNA synthesis indicating that the

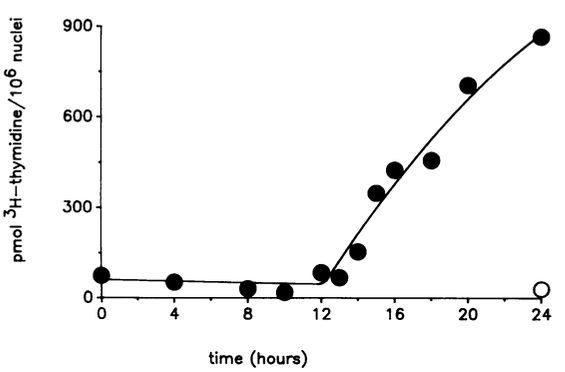

Figure 1. Time course of DNA replication in NFS/N1-H7 nuclei. Agarose-encapsulated nuclei were prepared from NFS/N1-H7 cells after a 10-h period of IL-3 deprivation and used as a DNA template in the cell-free replication assay which was stopped at the indicated times as described in Methods. The solid circles represent the mean thymidine incorporation per 10^6 nuclei, of duplicate samples that contained 1.4×10^5 nuclei, 100 μg/ml cytosolic protein, 100 μg/ml membrane protein, and 500 U/ml IL-3. The open circle at 24 h was the mean of a duplicate assay containing 1.4×10^5 nuclei and 500 U/ml IL-3 but no cytosolic or membrane protein. The time course shown is a representative one of four experiments.

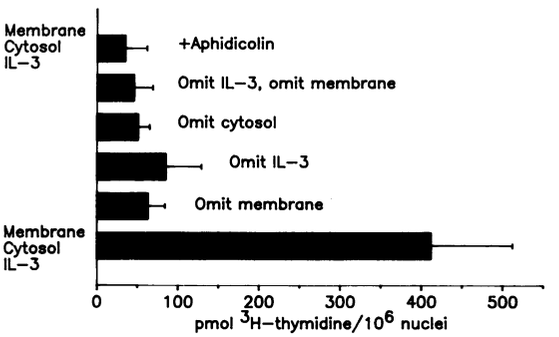

Figure 2. The essential role of IL-3, cytosolic, and membrane-containing subcellular fractions, and DNA polymerase α activity for cell-free DNA replication. Components of the cell-free system were prepared and used in the cell-free DNA replication assay as described in Methods. All replication assays were stopped at 16 h and results are mean±1 SD of three to five separate experiments. Components of the standard cell-free replication assay were omitted, as indicated, by substitution of the appropriate buffer blank. Aphidicolin was added at 40 μM final concentration at initiation of the cell-free replication assay; this concentration has been shown to inhibit DNA primase as well as DNA polymerase α activity (19). Reduction in [^{3}H]thymidine uptake by omission of components of cell-free system or addition of aphidicolin is significant with P value of < 0.005 by the paired t test.

cell-free system reflected replicative DNA synthesis by DNA polymerase α, rather than aphidocolin-insensitive DNA repair by DNA polymerase β. When nuclei were omitted from the 16-h assay, < 5 pmol [^{3}H]thymidine was recovered on the glass filter from an amount of cytosol, membrane fraction, and IL-3 equivalent to that used in a standard assay containing 1.4×10^5 nuclei.

Since mouse genomic DNA contains ~ 3×10^9 bp and 30% thymidine, the extent of DNA replication in the cell-free system represented ~ 27% of total genomic DNA at 16 h and 56% at 24 h (Figs. 1 and 2). To determine whether partial DNA replication was due to the ability of only a fraction of the isolated nuclei to replicate DNA, we examined the nuclear immunofluorescence staining pattern with rhodamine-labeled anti–BrdU antibody (21) after substitution of BrdUTP for dTTP in the cell-free system. As shown in Fig. 3, *A* and *B*, when the cytosolic fraction, membrane fraction, and IL-3 were present, > 80% of the encapsulated nuclei showed diffuse fluorescence with anti–BrdU at 16 h, although the intensity of staining was variable. In contrast, when the cytosolic and membrane-containing fractions were omitted, < 5% of the encapsulated nuclei showed any visible immunofluorescence with rhodamine-labeled anti–BrdU at 16 h (Fig. 3, *C* and *D*). Thus although DNA replication in the standard assay was incomplete, it appeared that the majority of encapsulated nuclei were capable of initiating DNA synthesis in response to IL-3 plus the cytosolic and membrane-containing cellular fractions. From the rate of DNA replication in Fig. 1, it can be estimated that complete replication of genomic DNA would require 21 h in the cell-free system, a figure that is considerably slower than the 10–12 h estimated for completion of S phase in intact NFS/N1-H7 cells.

Discussion

The present model for cell-free initiation of replicative DNA synthesis has three characteristics that are unique compared

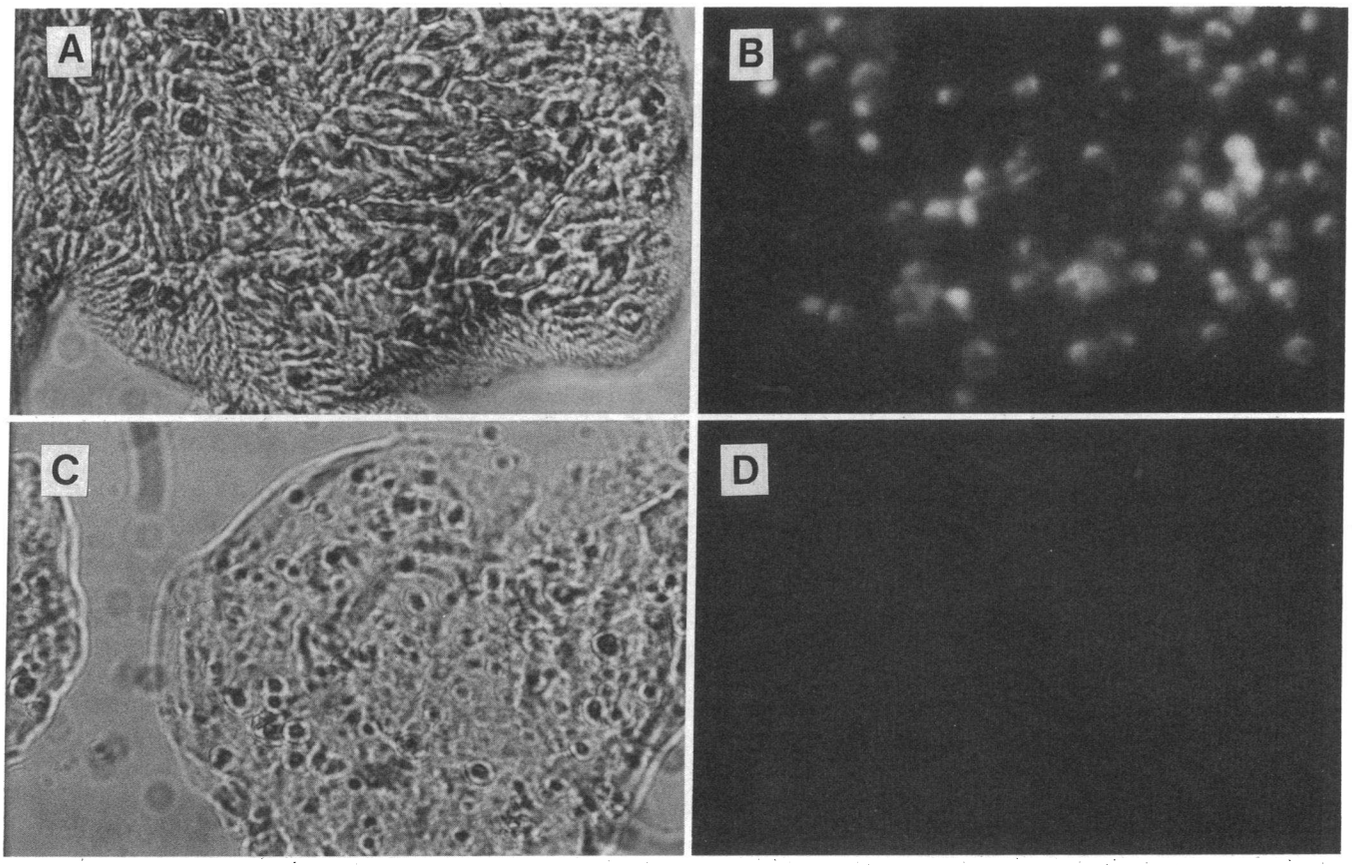

Figure 3. Incorporation of BrdU from BrdUTP into nuclear DNA. The cell-free DNA replication assay was conducted as described except that 100 μM BrdUTP was substituted for dTTP. At 16 h the encapsulated nuclei were fixed on glass slides, reacted with anti-BrdU MAb, then a second rhodamine-conjugated Ab, and photographed under light or fluorescence microscopy as described by Gratzner (21). *A* is a 100× light photomicrograph of agarose-encapsulated nuclei from a cell-free replication containing IL-3, cytosolic, and membrane-containing subcellular fractions. *B* is the same 100× field as *A* photographed by fluorescence microscopy. *C* is a 100× light photomicrograph of agarose-encapsulated nuclei from a cell-free replication assay containing IL-3 but no membrane-containing or cytosolic subcellular fractions. *D* is the same 100× field as *C* photographed by fluorescence microscopy. Individual agarose beads contain 40–60 nuclei.

with currently available systems for the study of cell-cycle regulation and DNA replication. (*a*) Components of the system are derived from a single mammalian cell type. (*b*) The system is responsive to a growth factor (IL-3) known to be an essential stimulus for progression from G_0/G_1 to S phase of the cell cycle in the intact cell. (*c*) The cell-free system allows a direct biochemical approach to study essential intermediate signals between growth factor–receptor binding at the plasma membrane and the nuclear response. The characteristics of this cell-free system thus require discussion in the context of other currently available models of cell-cycle control and DNA replication.

The replication of SV40 virus within mammalian cells has been extensively studied as a model for essential proteins, cofactors, and enzymes comprising the replicase complex for bidirectional viral DNA replication (12). Although this system requires only one protein of viral origin, large T antigen (22), as a DNA sequence-specific initiator, it is not clear whether SV40 replication mimics chromosomal replication because more than one round of SV40 replication may occur per host cell cycle (12).

Other studies have focused on growth factor–stimulated initiation of DNA synthesis by using nuclei from *Xenopus laevis* spleen cells (17) as a native DNA template. However, since *Xenopus* chromosomes may not have specific origins of replication (18), it is not clear whether stimulation of *Xenopus* nuclear replication by subcellular fractions from mitogen-stimulated mammalian cells reflects critical cell-cycle control mechanisms operative in mammalian cells.

The yeast system is probably the most powerful tool for the study of cell-cycle control and initiation of DNA synthesis in eukaryocytic cells. Through genetic studies of cell-cycle–deficient yeast, many critical genes controlling cell-cycle progression in response to external (nutrient) signals have been discovered (reviewed in references 12 and 13). Remarkably, many of the important cell-cycle regulatory yeast gene products have sequence and/or functional homology with mammalian cell counterparts (23). The ability to segregate cell-cycle–deficient yeast mutants into classical genetic complementation groups by cell fusion has been an important feature of the yeast system that, due to low fusion efficiency, cannot be duplicated in mammalian cell systems. However, the mammalian cell-free system described here is amenable to the direct assignment of genetic complementation groups without cell fusion, simply by combining complementary cell fractions directly in the nuclear replication assay. Additionally, the mam-

malian cell-free system allows the biochemical study of early or intermediate signals that may be specifically generated in response to mammalian growth factors or hormones.

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