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CHROMATIN REPLICATION IN ISOLATED NUCLEI FROM BOVINE LYMPHOCYTES

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SUMMARY: DNA replication in isolated nuclei from Concanavalin A-stimulated and resting bovine lymphocytes has been studied. Nuclei from S phase lymphocytes incorporate 4-7 times more (^{3}H)dTTP than nuclei from resting cells. The DNA synthesis was dependent on ATP, Mg^{2+} and all four deoxynucleoside triphosphates and was linear for about 60 min. The newly synthesized DNA is nuclear and DNase-sensitive and is the product of discontinuous and semiconservative replication. After limited digestion with micrococcal nuclease the in vitro replicated DNA was found to occur in nucleosomes prior to joining of primary DNA pieces. Addition of a protein extract from replicating cells stimulated the DNA synthesizing capacity of nuclei from resting lymphocytes. A preliminary characterization of this extract is given.

INTRODUCTION

Eucaryotic DNA replication in vitro has been demonstrated in lysed cells or in isolated nuclei from a variety of cell types. When these preparations are incubated with ATP and deoxynucleoside triphosphates they synthesize, under proper ionic conditions, a limited amount of DNA apparently extending polynucleotide chains in replication forks which existed in vivo before cell fractionation (1-5; review: 6). Several groups have also reported that rate and extent of DNA replication in isolated nuclei are increased when protein extracts from proliferating cells are added (1,3,7,8,9).

Most systems have been developed from permanent cell lines in culture, from HeLa cells, for instance. These cells usually proliferate continuously and must be treated, e.g. with hydroxyurea, to achieve some degree of synchronous entry into

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the DNA replicating "S-phase".
In this communication, I describe an in vitro DNA replication system developed from primary bovine lymphocyte cultures which are activated by Concanavalin A to proceed from an inactive "resting" state into a metabolically more active state leading to DNA replication and, finally, to cell division. During this transition, enzymatic activities are expressed in activated cells which are not detectable in resting cells. These enzymatic activities include a number of functions which are likely to be involved in eukaryotic DNA replication, for example: DNA polymerase (10); RNase H (11); DNA dependent ATPase (12) and nucleases (13).

As shown below, the lymphocyte system offers the interesting possibility to test the effect of these and other still unknown functions from replicating cells in nuclei from "resting" cells.

MATERIALS AND METHODS

Cells: Fresh lymphocytes were prepared from bovine retropharyngeal lymphnodes and cultured as described (14,15). One day after preparation the lymphocytes were stimulated to proliferate by addition of 7.5 ug/ml Concanavalin A.
DNA replication begins at 22-26 hours and reaches a maximal rate at about 46-50 hours after the addition of Concanavalin A. About 60% of the cells show blast formation at this time.

Preparation of nuclei: Nuclei were prepared from lymphocytes using the non ionic detergent Brij 58 essentially according to (16). As seen in the phase contrast microscope, nuclei from Concanavalin A stimulated cells are larger than those from resting cells.

Preparation of cell extracts: Between 1.5 and 2 x 10^8 cells/ml were washed with medium and resuspended in extraction buffer (20mM Hepes, pH 8.5; 5mM KCl; 0.5mM $MgCl_2$; 0.1mM EDTA; 0.5mM dithiothreitol). After swelling for 7 min at 0°C, the cell suspension was homogenized by ten strokes in a tightly fitting Dounce homogeniser. The suspension was immediately centrifuged for 1 h at 100,000 xg and 0°C. The clear supernatant was used for the experiments to be described below.

Assay of DNA synthesis in nuclei: Nuclei in 0.15 ml of a buffer composed of 250mM sucrose: 25mM Hepes, pH 8.0; 5mM $CaCl_2$ and 2% Dextran C 100 were added to 0.1 ml incorporation mixture. The incorporation mixture (16) contained: 100mM KCl; 38mM Hepes, pH 8.0; 12.5mM EGTA; 125mM sucrose; 12mM $MgCl_2$; 2mM dithiothreitol; 5mM ATP; 12.5mM phosphoenolpyruvate; 10 units/ml pyruvate kinase; 0.5mM each of dATP, dCTP, dGTP and 0.1mM (^{3}H) dTTP (25 uCi/ml). In some experiments (α ^{32}P)dATP (25 uCi/ml) was used as the radioactive tracer.

When the effect of cell extract was to be measured, nuclei in a 0.1 ml volume of the buffer described above were mixed with 0.05 ml of the cell extract and 0.1 ml incorporation mixture.

The incubation was carried out at 37°C for time periods to be indicated below. The incorporation was terminated by addition of 10% trichloroacetic acid and the precipitates were prepared for counting as described (17).

When nuclear DNA had to be analysed further, 5 x 10^6 nuclei were incubated for 16 hours at 37°C in 2 ml lysis mixture (50mM Tris-HCl, pH 7.5; 10mM EDTA; 1% Sarkosyl; 1 mg/ml pronase; 1 mg/ml proteinase K). The DNA was then extracted twice with chloroform-isoamylalcohol (24:1; v/v) and dialysed against 0.015 M NaCl, 0.0015 M sodium citrate.

The DNA was then precipitated by ethanol and resuspended in the NaCl-sodium citrate buffer.

Equilibrium centrifugation: Extracted DNA was prepared for CsCl equilibrium centrifugation by shearing. This was done by aspiration times through a 25 gauge needle. The DNA sample was then denatured in 0.1 M NaOH. To 4.5 ml of this preparation were added 4.8 g CsCl and 1 g Cs_2SO_4. Centrifugation in polyallomer tubes was performed in a Beckman 50 Ti rotor at 40,000 rev/min for 48 h at 18°C. The gradient was collected from the bottom.

Other techniques: Preparation of chromatin subunits and determination of DNA polymerase activity were described before (17).

All chemicals were obtained commercially as previously (17).

RESULTS

Characterization of the system: "S-phase" lymphocytes were harvested 48 hours after addition of Con A. "Resting" lymphocytes were cultivated for the same time in the absence of the lectin. When nuclei from stimulated (termed "S-nuclei") and unstimulated cells (termed "R nuclei") were incubated with test mixture, (^{3}H)dTTP was incorporated into acid-precipitable material for about 60 min. As expected, significantly more radioactive nucleotides are converted in S nuclei than in R nuclei. It should be noted, however, that the R nuclei are not completely inactive.

The amount of incorporated radioactivity is linearly related to the concentration of S nuclei in the range of 0.5 to 6.0 x 10^6 nuclei/assay (not shown). The optimal synthesis depends on the presence of ATP, magnesium ions and all four deoxynucleoside triphosphates (Table 1). To conclusively demonstrate the requirement for ATP, it is useful to reduce the intranuclear ATP concentration by preincubation of the nuclei preparation with

Table 1 PROPERTIES OF THE (^{3}H)dTTP INCORPORATION

Conditions	Relative activity (%)	
	S nuclei	R nuclei
complete system	100	100
+ EDTA (10mM)	4.2	2.6
- ATP; - phosphoenolpyruvate; pyruvate kinase (+ glycerol kinase, 10 min preincubated at 37°C)	9.1	3.1
- dATP, -dGTP, -dCTP, + 0.1mM dTTP	0.3	0.2
- dATP	53.5	46.5
- dGTP	58.8	49
- dCTP	42.4	43.6
+ hydroxyurea (10/ug/ml)	102	98
+ cycloheximide (20/ug/ml)	100	97

Nuclei (3 x 10^6) were incubated for 60 min and the acid-precipitable radioactivity was measured. The complete system incorporated 15.2 pmole (^{3}H)dTTP for S nuclei and 3.1 pmole (^{3}H)dTTP for R nuclei.

glycerol kinase (8). Other components of the system were not obligatory for activity, but their omission caused varying degrees of inhibition. Hydroxyurea and cycloheximide, strong inhibitors of *in vivo* DNA replication (2), had no effect on the reaction in isolated nuclei (Table 1).

The requirements for optimal incorporation are similar for R and S nuclei; although the absolute amount of incorporated radioactivity is much higher in S nuclei (Fig. 1).

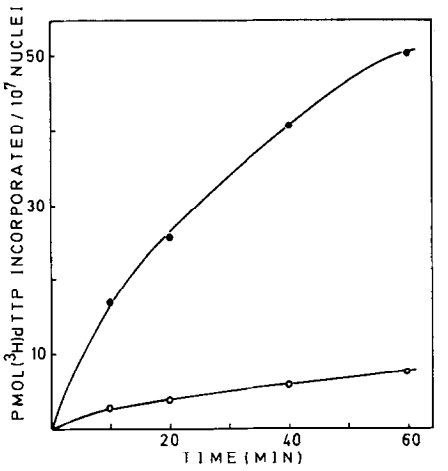

Fig. 1: INCORPORATION OF (^{3}H)dTTP IN ISOLATED NUCLEI
Nuclei (2 x 10^6) from resting lymphocytes (o) and from S-phase lymphocytes (•) were incubated under standard conditions at 37°C (see Methods).

The results of Table 1 strongly suggest that the observed incorporation of (^{3}H)dTTP is due to DNA synthesis.

Characterization of the DNA product: The DNA synthesized during a 60 min incubation was located in the nucleus and was sensitive to DNases (not shown). When in this nuclear system (^{3}H)dTTP incorporation (into S or R nuclei) was allowed for 1 min, only primary DNA pieces (4S, Okazaki pieces) were made as determined by alkaline sucrose gradient sedimentation. With increasing pulse length, the size of the DNA fragments increased. Ligation between small primary DNA pieces was also demonstrated in a pulse-chase experiment (data not shown). To distinguish whether the incorporation of (^{3}H)dTTP was due to unscheduled DNA synthesis (repair) or to a continuation of DNA replication, S nuclei were incubated for 30 min with 5-bromodeoxyuridine triphosphate, a density label, instead of dTTP and with (α^{32}P) dATP as the radioactive tracer. The incubation was stopped after 30 min and the nuclear DNA was isolated, sheared into small pieces and analysed in an alkaline $CsCl/Cs_2SO_4$ equilibrium gradient. It can be seen in Fig. 2 that nearly all *in vitro* synthesized DNA banded at a heavy position as expected. Some trailing on the "light" side of the bromodeoxyuridine-DNA peak

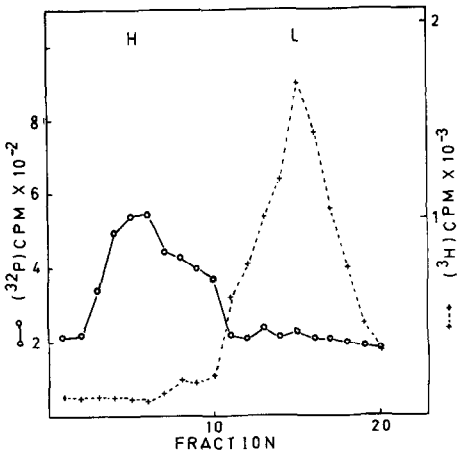

Fig. 2: EQUILIBRIUM GRADIENT CENTRIFUGATION OF IN VITRO SYNTHESIZED DNA
S nuclei(5×10^6)were incubated under standard conditions except that 0.5mM 5-bromodeoxyuridine triphosphate was used instead of dTTP and 0.025mM (α ^{32}P)dATP instead of unlabelled dATP. After 30 min at 37°C, the DNA was extracted and prepared for CsCl/Cs_2SO_4 gradient centrifugation as described. ^{3}H-labelled "light" sheared lymphocyte DNA was used as density marker. The Beckman 50 Ti rotor was used for 48 h at 18°C and 40,000 rev/min. Trichloroacetic acid-precipitable radioactivity was determined in each fraction of the gradient.

can be explained by the assumption that most DNA synthesis observed in vitro is a continuation of DNA chains which had been initiated in vivo. Therefore, some polynucleotides should be composed of "light" thymidine-containing sections covalently joined to "heavy" bromodeoxyuridine-containing sections. No distinct peak of ^{32}P labeled DNA should appear if the observed DNA synthesis were due to repair.

Nuclear DNA exists as a tightly packed nucleoprotein complex, chromatin, which is organized in a repeating subunit structure (18). To find out whether the DNA synthesized in vitro in the absence of protein synthesis was associated with chromatin subunits (nucleosomes), S nuclei, after 2 min incubation with the incorporation mixture were treated with micrococcal nuclease (17). The reaction products were analysed by sucrose gradient sedimentation. The results are shown in Fig. 3: disintegrated bulk chromatin as identified by its UV absorbance, sedimented as mono-, di-, tri-, tetrameric, etc. subunits. The measured

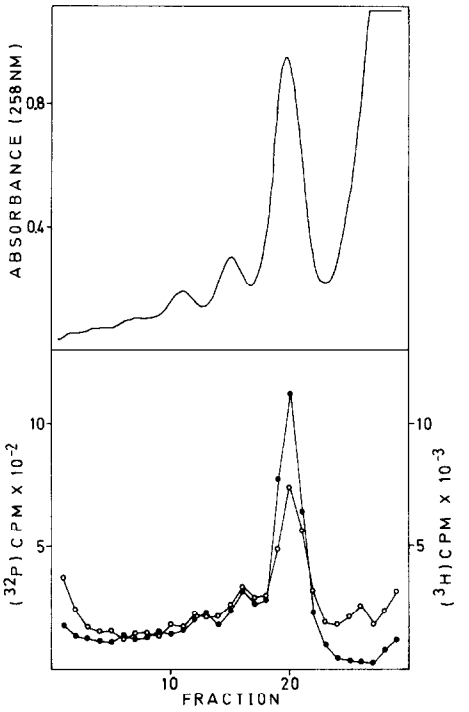

Fig. 3: DEGRADATION OF LYMPHOCYTE CHROMATIN BY MICROCOCCAL NUCLEASE
Concanavalin A activated lymphocytes were labeled *in vivo* with 1 µCi/ml (^{3}H)-thymidine for 16 hours before nuclei were prepared. 2 x 10^{7} nuclei were incubated under standard conditions with (^{32}P)dATP (0.025mM). After two min incorporation, the nuclei were quickly cooled to 0°C and collected by centrifugation. They were then resuspended in nuclease buffer (0.4 ml) and incubated with micrococcal nuclease (60 units) for 15 min at 37°C (17). After lysis of the nuclei the reaction products were analysed by sucrose gradient centrifugation (SW27 rotor 26,000 rpm, 16 h, 2°C). The gradient was pumped through a quartz cuvette to record the ultraviolet light absorbance.
(a) absorbance at 258 nm
(b) radioactivity in acid precipitate:
(^{3}H) thymidine "prelabel": o-o , (^{32}P): •-•

radioactive newly synthesized DNA sedimented with bulk chromatin subunits, mostly as monomeric nucleosomes. This finding shows clearly that *in vitro* replicated DNA (almost all primary DNA segments) is organized in chromatin subunits.

Stimulation of nuclear DNA synthesis: When R nuclei were incubated in the presence of cell extract from S-phase lymphocytes,

a considerable increase in DNA synthesis was observed. The degree of R nuclei stimulation by cell extract from S-phase cells depended linearly on the amount of extract added to the reaction mixture. Maximal activity was observed when 0.05 ml of cell extract (corresponding to about 0.09 mg protein) was added to 2 x 10^6 R nuclei under the conditions described above. The DNA synthesis in R nuclei stimulated with extract was discontinuous and found to be dependent on the presence of ATP, magnesium ions and all four deoxyribonucleoside triphosphates (not shown).

Only extracts from S-phase lymphocytes stimulated DNA synthesis in R nuclei; extracts from resting cells had no effect. The DNA synthesizing capacity of S nuclei is not further stimulated by extracts either from S-phase cells or from resting cells. On the other hand, the activity of S nuclei is not inhibited by extracts from either resting or S-phase lymphocytes. The cell extract from Con A stimulated lymphocytes showed about 7-10 times more DNA polymerase activity (predominantly DNA polymerase α) than R extract (not shown). To find out whether the enzyme activity is responsible for the stimulation of nuclear DNA synthesis the DNA polymerase activity in differently treated S extracts was determined and compared to the stimulation effect. In Table 2 some properties of the S phase cell extract are summarized. It can be seen that the stimulating activity is very heat labile. Preincubation at 65°C for 3 min essentially inactivated the stimulating activity and even preincubation at 37° for 60 min reduced the activity to 23 percentage of the control value (untreated S extract). Furthermore, all extracts prepared from cycloheximide-treated S phase lymphocytes were significantly less active than extracts from untreated stimulated cells. The data of Table 2 indicates that the stimulation of DNA synthesis could be distinguished from the DNA polymerase activity. Therefore, the high level of DNA polymerase activity in S cell extract could not be directly responsible for the enhanced DNA synthesis in nuclei from resting cells.

Table 2 SOME PROPERTIES OF THE STIMULATING ACTIVITY OF S CELL EXTRACT ON DNA SYNTHESIS IN R NUCLEI

Conditions	Stimulation activity (%)	DNA polymerase activity in S cell extract (%)
R nuclei	100	-
R nuclei + untreated S extract	453	100
R nuclei + cycloheximide S extract[a)]	272	98
R nuclei + heat-treated (3 min at 65°C) S extract	117	-
R nuclei + heat-treated (60 min at 37°C) S extract	181	104

(^{3}H)dTTP incorporation into DNA of R nuclei (2 x 10^6) was measured by incubation for 30 min. (100% corresponds to 6,8 pmol). DNA polymerase activity in cell extract was measured by incorporation of (^{3}H)dTTP into activated calf thymus DNA (17).

a) cell extract from cycloheximide treated S-phase cells (20 µg/ml) for 30 min.

DISCUSSION

According to generally accepted criteria (1-7), the observed DNA synthesis corresponds to DNA replication because the reaction is ATP-dependent; the DNA synthesis observed is discontinuous and semiconservative.

In vitro replicated DNA is associated with nucleosomes. This was found for the DNA synthesized during a 2 min incubation time (Fig. 3). Preliminary results have shown that during a 2 min incubation time, almost exclusively 4S fragments are synthesized in S nuclei. This result suggests that chain elongation and the subsequent ligation of Okazaki fragments can occur on or close to nucleosomes. These data are in a agreement with the results of replication studies *in vivo* (19).

I have shown above that an extract from S-phase lymphocytes is able to stimulate DNA replication in nuclei from resting cells. It is not yet known whether the extract induces initiation of replication or whether the residual DNA synthesis that can be observed in R nuclei preparations is enhanced by components of the extract. The results in Table 2 demonstrate that the DNA polymerase activity in S-phase extract is not (or only partially) responsible for the stimulating effect in R nuclei. In any case, the studies reported here show that the complementation of R nuclei by extracts from S-phase cells could lead to an interesting experimental approach to characterize and analyse functions involved in chromatin replication.

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