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A NOVEL SYSTEM FOR DNA SYNTHESIS IN ISOLATED NUCLEI

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1. Introduction

Isolated, intact mammalian nuclei have been employed in the study of DNA synthesis from normal [1–6] and viral infected cells [7–9]. Nuclear suspensions appear to be readily permeable to both macromolecules [1, 6] and nucleoside triphosphates with a requirement for ATP in DNA synthesis [2–4, 6, 7], analogous in this respect, to toluenized bacteria [10].

In this communication, a novel system is described for the assay of DNA synthesis in nuclei based on the cellophane disc method of Schaller et al. [11]. The activity, which reflects the physiological state of the cells, is linear for at least 2 hr in the presence of all four ribonucleoside triphosphates.

2. Materials and methods

Nuclei of a baby hamster kidney cell line (BHK 21, clone 13) were isolated by a modification of the method of Zylber and Penman [12]. Pelleted cells were gently lysed on ice by Dounce homogenization in RSB (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$) containing 0.1% Nonidet P_{40} and the lysate layered on 0.25 M sucrose in RSB-NP_{40}. After centrifugation (1250 g, 10 min, 4°C), the cytoplasm and sucrose were carefully removed by aspiration. The nuclei were resuspended in RSB-NP_{40} and treated 4 times as above. The final nuclear pellet was brought up in RSB containing 25% glycerol at a concn. of 0.2–5.5 × 10^8 nuclei/ml. Nuclei were judged to be free of cellular contamination by light microscopy.

Quantities of 1–2 μl nuclei were spread on 1 cm^2 cellophane discs [11] (kindly supplied by Kalle Aktiengesellschaft, Wiesbaden-Biebrich, Germany) on an agar salt plate (2.5% agar, 20 mM morpholinopropane sulfonic acid (MOPS), pH 7.5, 10 mM EGTA, 40% glycerol (v/v), 20 mM $MgCl_2$, 0.5 M NaCl) for 60 min at 4°C and transfered to a second agar plate (2.0% agar, 20 mM MOPS, 5 mM $MgCl_2$) on which they were partially dried [11, 13]. The discs were then placed on a prewarmed incubation mixture as described in the legend to fig. 1.

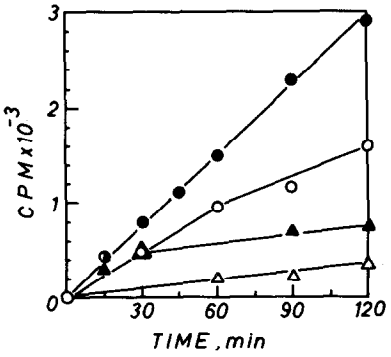

Fig. 1. Kinetics of DNA synthesis in nuclei on cellophane discs. Two μl suspensions per disc (1.6 × 10^5 nuclei) were incubated on 50 μl reaction mixtures which contained 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM ATP (Schwartz-Mann BioResearch), 40 μM each dATP, dGTP and dCTP (Schwartz-Mann), 0.11 μM [^{3}H]TTP (specific activity 11.3 Ci/mmol, 500 mCi/ml; The Radiochemical Centre, Amersham) and ribonucleoside triphosphates as listed below under symbols. The plastic Petri dish (Nunc) was covered and floated on a 37°C water bath. Incubation was terminated according to Schaller et al. [11]: discs were placed in 0.5 N NaOH, 1% SDS, 10 mM PP_i and 100 μg DNA and heated at 75°C for 5 min. After cooling, TCA was added to 20% and the precipitates collected on glass fiber filters (Whatman GF/C). They were washed with 1% TCA-10 mM PP_i, acetone dried and radioactivity determined by liquid scintillation. Points are corrected for zero time incorporation (59 cpm). Symbols: (●) all four ribonucleoside triphosphates; (○) ATP present; (▲) minus ATP; and (△) minus dATP, dGTP and dCTP.

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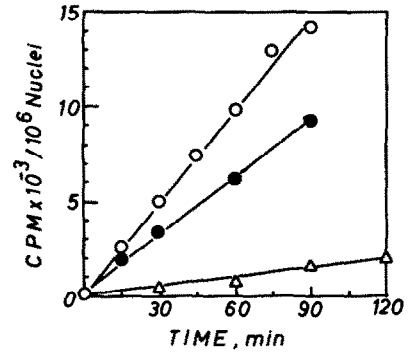

Fig. 2. DNA synthesis in nuclei from various cell densities. BHK, grown in 1 ℓ rolling bottles with a surface area of 420 cm^2, were harvested and the nuclei isolated as given in Materials and methods. Each point is the average of duplicate discs. Symbols represent the following cell densities × 10^5 per cm^2: (○) 1.2; (●) 1.6; and (△) 6.4.

3. Results

As seen in a typical experiment (fig. 1), nuclear preparations supported on cellophane discs that were exposed to high salt conditions [12] linearly incorporate [^{3}H]TTP into DNA for at least 120 min. These preparations were 2.5-fold more active than resuspending nuclei in the reaction mixture. Nuclei isolated from nonconfluent monolayers were considerably more active in DNA synthesis than contact-inhibited cells (fig. 2) similar to the observations of Winnacker et al. [7] and Freienstein et al. [14]. Identical results were also obtained with nuclei from a BS-C-1 cell line and a primary cell culture from Vervet monkey kidney.

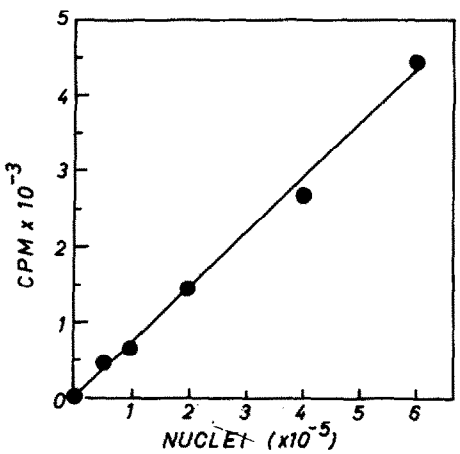

Fig. 3. DNA synthesis as a function of the number of nuclei per cellophane disc. Varying amounts of nuclei were applied in 1–2 μl quantities to 1 cm^2 cellophane discs and treated as given in Materials and methods. Duplicate discs were incubated for 60 min on reaction mixtures containing all four ribonucleoside triphosphates as stated in the legend to fig. 1.

Table 1
Ribonucleoside triphosphate stimulation of DNA synthesis in nuclei on cellophane discs.

Additions	% Activity (± S.D.)
None	24.7 ± 7.0
UTP	23.5 ± 5.2
GTP	27.1 ± 0.3
CTP	27.7 ± 4.6
ATP	58.1 ± 8.1
ATP, GTP	53.3 ± 6.2
ATP, CTP, GTP	53.8 ± 3.0
ATP, UTP, CTP, GTP	100.0 ± 3.9

Data are averages of 2–6 nuclei preparations each of which represents duplicate or triplicate determinations. "None" is the activity in the absence of any ribonucleoside triphosphates whereas "per cent activity" is expressed relative to the reaction mixture containing all four after 90 min incubation which had an average of 15 560 cpm/10^6 nuclei. The concentration of ribonucleoside triphosphates was 1 mM each when present.

Irrespective of the physiological state of the cells, nuclei were stimulated by ribonucleoside triphosphates (fig. 1) with the activity as a linear function of the number of nuclei per disc (fig. 3). The four ribonucleoside triphosphates were necessary for maximum activity; in their absence DNA synthesis gradually ceased after 30 min (fig. 1). None of them individually, nor in combination of less than all four, could optimally stimulate the system (table 1). In the presence of ATP as the sole ribonucleoside triphosphate, however, incorporation of [^{3}H]TTP continued linearly for 60 min (fig. 1) resulting in approximately half the control level (table 1). On reaction mixtures lacking dATP, dGTP and dCTP, DNA synthesis was reduced 12-fold (fig. 1), comparable to the activity with TTP alone using purified DNA polymerase [15].

Although RNA functions as a primer in the initiation of nascent DNA chains [9, 16], replication in nuclei on cellophane discs was unaffected by RNAase (1–2 μg). However, the accessibility of the replicating

complex was demonstrated by the complete elimination of activity by DNAase (10 μg). EDTA (20 mM) was also entirely inhibitory.

4. Discussion

The feasibility of DNA synthesis in nuclei supported on cellophane discs offers several advantages: i) as originally developed by Schaller et al. [11], in situ lysis circumvents dilution and denaturation of protein components required in replication; ii) activity continues linearly for prolonged periods of time; and iii) the ability to delineate the effect of small quantities of cellular or viral induced components on DNA synthesis.

From the involvement of ribonucleoside triphosphates in DNA synthesis, it would appear that the discontinuous mechanism of chain growth primed by RNA in *E. coli* [16] and viral infected bacteria [13] is applicable to mammalian systems as recently shown in SV40 [17] and polyoma infected cells [9]. The maintenance of DNA synthesis by nuclei on cellophane discs suggests a reinitiation event involving RNA. However, *in vitro* synthesis could also be a continuation of an *in vivo* process [3,7] by a semi-conservative mechanism [8] stimulated by ribonucleoside triphosphates. ATP could be involved in an ATP-dependent ligase activity found in mammalian cells [18] for DNA repair or possibly activate a hitherto unknown ATP-dependent nuclease [19] in eukaryotes.

In these nuclei preparations, DNA synthesis appears to occur semi-conservatively with the formation of single stranded regions of approx. 10–16 S (manuscript in preparation).

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