Deoxyribonucleic Acid Synthesis in Isolated Nuclei from Baby-Hamster Kidney Cells (BHK-21/C13)

CHARACTERIZATION OF THE SYSTEM

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A comparative study of some commonly employed laboratory procedures for studying DNA synthesis in isolated nuclei was carried out. Nuclei isolated from baby-hamster kidney (BHK-21/C13) cells synthesize DNA for 30–60 min at 37°C in a reaction requiring uni- and bi-valent cations, ATP and all four deoxyribonucleoside 5'-triphosphates. The addition of either ribonucleotides or cytosol from S-phase cells had no effect, but DNA synthesis was stimulated by some dextrans (mol.wt. 5×10^6). The extent of synthesis was influenced by apparently minor variations in experimental conditions. For example, DNA synthesis by nuclei in Tris/HCl, pH 7.5, was only 50% of that observed in Hepes/NaOH, pH 7.5; the presence of detergents Triton X-100, Triton N-101, Nonidet P-40, Brij 58 and Tween 80 in the incubation medium altered the amount of synthesis to different extents. Although most detergents inhibited synthesis, a stimulation occurred with Tween 80 (150% of controls). These effects were reversed on washing the nuclei, except that of Brij 58, which inhibited DNA synthesis by 90–95% irreversibly. Anomalous sucrose-density-gradient sedimentation behaviour of the DNA, and of precursor [³H]-dTTP, was observed when nuclei were lysed with solutions of sodium dodecyl sulphate/Mg²⁺ or with Sarkosyl/Mg²⁺, but consistent results, showing that the DNA synthesized *in vitro* sedimented exclusively at about 4S, were obtained when nuclei were lysed with sodium dodecyl sulphate (without Mg²⁺)/EDTA, digested with proteinase K and heated at 100°C with 11% (v/v) formaldehyde to prevent macromolecular association. These results, coupled with density-labelling studies with bromodeoxyuridine and CsCl-density-gradient analysis, showed that DNA synthesis in these nuclei was replicative and was restricted to a covalent extension of Okazaki pieces previously initiated *in vivo*. No new initiations were observed, and the DNA was not ligated into larger molecules. The cessation of DNA synthesis after about 60 min was due to the complete utilization of available primer/template DNA.

We have previously described some aspects of DNA synthesis *in vivo* in our BHK-21/C13 cell line (Hayton *et al.*, 1973a,b; Pearson *et al.*, 1976). In the present paper we describe our observations on the ability of nuclei isolated from these cells to carry out

replicative DNA synthesis, as a prelude to further studies to establish roles played by individual proteins in this complex process.

Since there are many varied claims made in the literature about the proposed ability of nuclei isolated from different cells to synthesize DNA, particularly regarding their ability to initiate synthesis *de novo*, to allow ligation of low-molecular-weight DNA into larger molecules and to respond to the addition of putative cytoplasmic factors (see Reinhard *et al.*, 1977, for summary of references), we have concentrated our attention on a comparative study of some of the laboratory procedures used in order to obtain a meaningful comparison with previous reports.

Abbreviations used: SDS, sodium dodecyl sulphate; Sarkosyl, sodium dodecyl-*N*-sarcosinate; 'activated' DNA, DNA (1 mg/ml) was digested with deoxyribonuclease I until 2% was rendered acid-soluble (Loeb, 1969); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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Experimental

Materials

Buffer A: 20 mM-Hepes, pH 7.6, 5 mM-2-mercapto-ethanol, 2 mM-MgCl₂. Buffered sucrose: sucrose dissolved in buffer A.

All chemicals were A. R. grade or the most pure available. Nucleotides were purchased from P-L Biochemicals, Milwaukee, Wisconsin, U.S.A., and Dextrans (Dextran T fractions) from Pharmacia (G.B.) Ltd., London, U.K. Nonidet P-40 (NP-40) was from Shell Chemicals Ltd., London, U.K., Tween 80 from Hopkin and Williams, Romford, Essex, U.K. Triton X-100, Brij 58 and Triton N-101 were from Sigma Chemical Co., Poole, Dorset, U.K. [6-³H]Thymidine (26 Ci/mmol), [2-¹⁴C]thymidine (50 Ci/mmol) and [methyl-³H]thymidine 5'-triphosphate (15–30 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Cell culture

BHK-21/C13 cells (Macpherson & Stoker, 1962) were used throughout this work. They were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and from Bio-Cult Laboratories, Paisley, Glasgow, Scotland, U.K. Cells were routinely grown in monolayer culture at 37°C in an atmosphere of CO₂/air (1:19) in 200 ml of ETC₁₀ medium in 2.24-litre roller bottles (House & Wildy, 1965). These were rotated at 1 rev./3 min. ETC₁₀ is Eagle's medium (Glasgow modification) containing 10% (w/v) tryptose-phosphate broth (Difco Laboratories, East Molesey, Surrey, U.K.) and 10% (v/v) calf serum (Flow Laboratories).

Cell cultures were routinely screened for bacterial and fungal contamination by plating cells on blood agar and incubating growth medium with Sabouroud liquid medium (Oxoid Ltd., Basingstoke, Hants., U.K.) and with Brain Heart infusion (Oxoid Ltd.). Cultures were routinely tested for mycoplasma contamination by three methods: (1) orcein staining (Fogh & Fogh, 1964); (2) with a test kit supplied by Flow Laboratories; (3) uridine/uracil ratio test based on the method of Schneider *et al.* (1974). Cells with a ratio of uridine/uracil incorporated of less than 100 were discarded.

Isolation of nuclei

Nuclei were isolated routinely by a method based on that previously used in this laboratory (Craig & Keir, 1975). All operations were carried out at 4°C. Cells were removed from the monolayer surface by scraping them into buffer A with a rubber 'policeman'. They were centrifuged at 500 *g*_{av} for 5 min (MSE 2L), the supernatant was discarded and the pellet re-washed in buffer A to remove traces of growth medium. This pellet was resuspended in buffer A (15 × 10⁶ cells/ml) and allowed to swell for

10 min. The suspension was then expelled forcibly three times through a 21-gauge needle, Triton N-101 was added (final concn. 0.3%, v/v) and the lysate was vigorously mixed. Buffered 2M-sucrose was added to give a final concentration of 0.32M and the suspension centrifuged for 10 min at 1000 *g*_{av} (MSE 2L) to pellet the nuclei. This was repeated and the nuclei were resuspended in buffered 0.32M-sucrose (1 × 10⁶ nuclei/50 μl). Recoveries were 70–80%. Little cytoplasmic contamination was found when these preparations were examined by electron microscopy and by enzyme assays for the cytoplasmic and mitochondrial enzymes lactate dehydrogenase and succinate dehydrogenase. The ability of nuclei to synthesize DNA was not altered by purifying them further through 2.2M-sucrose.

Nuclear DNA synthesis assay (standard assay)

For optimum DNA synthesis the assay medium contained 10 mM-ATP, 0.5 mM each of dATP, dCTP and dGTP, 1.2 μM-[³H]dTTP (958 mCi/mmol), 10 mM-MgCl₂ and 50 mM-Hepes buffer, pH 7.5. Nuclear suspension (50 μl containing 1 × 10⁶ nuclei in buffered 0.32M-sucrose) was added to start the reaction in a total volume of 200 μl. After appropriate times at 37°C DNA synthesis was stopped by adding 300 μl of ice-cold 10% (w/v) trichloroacetic acid containing 2% (w/v) sodium pyrophosphate. The solutions were kept on ice for at least 1 h before the acid-precipitable material was collected on Whatman GF/C filter discs (2.5 cm diameter), which were previously soaked with trichloroacetic acid solution. Precipitates were then washed three times with 5 ml portions of the acid solution and once with 5 ml of ethanol. Filters were dried and counted for radioactivity in toluene-based scintillation fluid (Pearson *et al.*, 1976). The efficiency of counting ³H under these conditions was about 33%.

Preparation of nuclear lysates

The conditions for lysing nuclei for subsequent centrifugation in sucrose density gradients were different for most experiments and are therefore described in the legends to Figures.

Density-gradient centrifugation

CsCl. Conditions were as previously described (Pearson *et al.*, 1976).

Alkaline sucrose. Portions (4.5 ml) of a solution containing 12.5% (w/v) sucrose, 0.7M-NaCl, 0.3M-NaOH and 1 mM-EDTA were pipetted into 5 ml polypropylene centrifuge tubes. Gradients of sucrose were then prepared by freezing and thawing each solution three times (Baxter-Gabbard, 1972). Samples (200 μl, containing up to 40 μg of DNA) were layered on each gradient with a micropipette with the end cut off to widen the bore, and gradients were centrifuged for 2 h at 37 000 rev./min and 20°C in an MSE

6×5.5ml aluminium swinging-bucket rotor. About 30 fractions were collected and counted for radioactivity as described above; the recovery was routinely 80–90%.

Extraction of DNA

DNA which was to be deproteinized with phenol was isotopically long-labelled by growing cells to late-exponential phase in medium containing [6-³H]-thymidine at 1 μCi/ml (26 Ci/mmol). Conditions for deproteinizing the DNA have been previously described (Pearson *et al.*, 1976).

Results and Discussion

Kinetics of DNA synthesis by isolated nuclei

DNA synthesis continued for about 30–60 min at 37°C and then ceased, characteristics commonly observed with other isolated nuclei (see Reinhard *et al.*, 1977). In a typical experiment radioactivity from [³H]dTTP was incorporated for about 30 min, representing 0.66 pmol of dTMP incorporated into DNA. Assuming that BHK-cell DNA contains about 30% thymidylate residues this means that 2.2 pmol of all four nucleotides, or 2.2 pmol of DNA P, was incorporated per 10⁶ nuclei. Since DNA contains 9.23% of P by weight (Hershey *et al.*, 1973) about 0.74 ng of DNA/10⁶ nuclei was synthesized. Mammalian cells generally contain about 6–12 pg of DNA per cell, that is about 6–12 μg of DNA per 10⁶ nuclei. Our nuclei were isolated from exponentially growing cells, so presumably only about 70% of them were actively involved in DNA synthesis (Howard *et al.*, 1974). Therefore, of 4–8 μg of DNA being replicated in intact cells, the isolated nuclei synthesized only 0.74 ng of DNA. Only a small proportion of the capacity for DNA synthesis was therefore utilized.

Possible explanation for the kinetics of DNA synthesis

The observed decline in DNA synthesis after about 30 min was not due to the complete utilization of substrates, because the incubation medium could support DNA synthesis with six times the normal number of nuclei, and when 'activated' calf thymus DNA was added to an incubation after 30 min at 37°C, DNA synthesis continued linearly for another 40 min. The addition of double-stranded or single-stranded DNA also promoted an increase in DNA synthesis. Another explanation for the observed cessation of DNA synthesis after 30 min at 37°C is that a required component of the nuclear replicase system decays (Hershey *et al.*, 1973), or is destroyed by endogenous proteinase (Sellwood *et al.*, 1975). However, the amount of DNA synthesized by nuclei in 30 min was unaffected by incubating them first for up to 60 min at 37°C in the absence of substrates (in

contrast with the results of Hershey *et al.*, 1973, with isolated HeLa-cell nuclei). These results suggest that DNA synthesis in BHK-cell nuclei ceases after about 30 min because of a limiting amount of available primer/template DNA, and is not due to a shortage of substrates or cofactors, or to a loss or inactivation of required enzymes or other proteins.

Conditions for isolating and incubating nuclei that affect their ability to synthesize DNA

Removal of cells from monolayer surfaces. Brown & Stubblefield (1975) reported that exposure of lysates from Chinese-hamster fibroblasts to trypsin led to an alteration of the properties of DNA polymerase isolated from these cells, and Chiu & Baserga (1975) showed that exposure of WI-38 fibroblasts to trypsin resulted in the destruction of non-histone chromosomal proteins. Although these effects of trypsin might be expected to influence the capacity of isolated nuclei to synthesize DNA, we did not observe any differences when comparing them with nuclei isolated from cells scraped from glass surfaces. Brown & Stubblefield (1974) also found that DNA synthesis was stimulated about 10-fold in Chinese-hamster fibroblast-cell lysates treated with trypsin. DNA synthesis in our BHK-cell nuclei was inhibited by 90% after exposure to trypsin.

Choice of detergent in the nuclear isolation medium. Non-ionic detergents are commonly employed in methods for isolating nuclei because they facilitate removal of adherent cytoplasm, and they permit a better yield to be obtained. However, when BHK-cell nuclei were assayed in the presence of detergents (concentrations in parentheses), Triton X-100 (0.1%, w/v), Triton N-101 (0.3%, w/v), Nonidet P-40 (0.1%, w/v), Brij 58 (2.0%, w/v) and Tween 80 (1.0%, w/v), the extents of DNA synthesis compared with those of controls incubated without detergent were 50, 10–20, 50, 5–10 and 150% respectively. The concentrations of detergent used were above the respective critical micellar concentrations, where there is equilibrium between detergent monomers and the aggregated molecules (micelles), and can thus be considered as being at saturating concentrations (Helenius & Simons, 1975). In most cases the observed inhibition was reversible if nuclei were washed free of detergent; Brij 58, however, caused irreversible inhibition of DNA synthesis.

The partially purified DNA polymerases-α and -β (for current terminology of DNA-dependent DNA polymerases see Weissbach *et al.*, 1975) were not as sensitive to these detergents as were the nuclei, except for Brij 58, which inhibited polymerase-α by 80–90%. Polymerase-β activity was 120% of control activity under similar conditions. This observation supports further work which suggests that DNA polymerase-α

catalyses the DNA synthesis observed in the isolated nuclei.

Choice of incubation buffer. The amount of DNA synthesized in 30 min at 37°C in Tris/HCl buffer was only 40–50% of that synthesized in Hepes/NaOH under otherwise comparable conditions.

Additions to the incubation medium. In an effort to increase the amount of DNA synthesized in isolated nuclei a number of additions were made to the incubation medium.

The addition (final concentrations) of 20% (w/v) sucrose, 20% (w/v) glycerol, 5 mg of bovine serum albumin/ml or 5% (v/v) calf serum had no effect, but we did observe a stimulation of DNA synthesis with some dextrans. The effect of adding 16% (w/v) dextran to BHK-cell nuclei depended on its molecular weight. Low-molecular-weight dextrans (10×10^3 , 40×10^3 and 70×10^3) inhibited DNA synthesis, whereas dextrans with a mol.wt. of 5×10^6 stimulated synthesis by about 50%. Lynch (1972) previously showed that dextrans in the mol.wt. range 13.5×10^4 – 50×10^4 caused a 60% stimulation of DNA synthesis in rat liver nuclei and suggested that they may prevent nuclear swelling and leakage of replication components from the nucleus; Brewer (1975) demonstrated a similar stimulation of DNA synthesis in nuclei from *Physarum polycephalum* by dextrans at 16% (w/v) and concluded that they stabilized the replication complex.

The addition of ribonucleotides to the BHK-cell

nuclei was without effect, although a stimulation of DNA synthesis in isolated nuclei has been reported by others (Tseng & Goulian, 1975; Krokan *et al.*, 1975; Benz & Strominger, 1975).

When cytosol from cells in the S-phase of their growth cycle was added to the incubation medium we did not observe a reproducible stimulation of DNA synthesis despite repeated attempts. This contrasts with reports that cytosol from replicating cells can stimulate the initiation of DNA synthesis in otherwise inert nuclei (Jazwinski *et al.*, 1976; Thompson & McCarthy, 1973; Kumar & Friedman, 1972; Benbow & Ford, 1975), or can simply stimulate DNA synthesis but not its initiation (Francke & Hunter, 1975; De Pamphilis & Berg, 1975).

Characterization of DNA synthesized by the isolated nuclei

Possible types of DNA synthesis *in vitro*. The DNA synthesis that we observe in isolated BHK-cell nuclei could be due to a number of processes; for example, replicative synthesis, repair synthesis, homopolymer formation such as that catalysed by a terminal nucleotidyltransferase, previously found in calf thymus (Chang, 1971) and also in plants (Srivastava, 1972), or synthesis of simple-sequence DNA like the poly(dA-dT) synthesized by calf thymus DNA polymerase- α in an assay *in vitro* containing calf thymus DNA as template (Henner & Furth, 1975, 1977).

It is unlikely that the DNA synthesized is due to

Fig. 1. *CsCl*-density-gradient centrifugation of DNA synthesized *in vitro*

Nuclei, isolated from cells long-labelled with [^{14}C]thymidine, were incubated for 30 min at 37°C in standard medium containing [^3H]dTTP (see the Experimental section). DNA synthesis was stopped by adding 1% (w/v) SDS containing 10 mM-EDTA (final concentrations). The resultant lysate, containing double-stranded native DNA, was centrifuged in *CsCl* (details described in the Experimental section). Trichloroacetic acid-precipitable ^3H (●) and ^{14}C (○) radioactivities are shown. Δ, Buoyant density.

terminal addition, because: (a) all four deoxyribonucleoside 5'-triphosphates are required for maximum synthesis (these inhibit the terminal transferase: Grisham *et al.*, 1972); (b) DNA synthesis in these nuclei is inhibited by actinomycin D (90% inhibition at 100 μ g/ml) (this binds to the DNA template and inhibits DNA polymerase, but not the terminal transferase: Keir, 1965); (c) the DNA synthesized by the isolated nuclei has the same buoyant density in caesium-salt-density gradients as DNA isolated from intact cells (1.690 g/cm³; Fig. 1), showing that it has a similar base composition. The buoyant densities of the homopolymer poly(dT) and the simple-sequence DNA poly[d(A-T)] in CsCl are 1.739 g/cm³ and 1.678 g/cm³, and these molecules would be clearly resolved from BHK-cell DNA under our conditions of centrifugation. This latter observation also confirms that the trichloroacetic acid-precipitable material resulting from the incorporation of

radioactivity from [³H]dTTP in isolated nuclei is DNA. As expected, this material is rendered acid-soluble by deoxyribonuclease I, but is resistant to NaOH (0.3M, 70°C for 10 min), ribonuclease and proteinase K.

Evidence for replicative DNA synthesis

Caesium-salt-density-gradient centrifugation. Density-labelling experiments with bromodeoxyuridine showed that the DNA synthesized in the isolated nuclei was essentially a covalent extension of DNA newly synthesized in intact cells. Fig. 2(a) shows the results obtained when late exponentially growing cells were long-labelled with [¹⁴C]thymidine and pulse-labelled for 10 min at 37°C with non-radioactive bromodeoxyuridine before isolation of the nuclei. These nuclei were then incubated in the standard medium containing [³H]dTTP before analysis of the denatured DNA in a CsCl density gradient. As expected for replicative DNA synthesis, most of the [³H]DNA made *in vitro* is of a greater buoyant density than the mature [¹⁴C]DNA, showing that it is covalently joined to the DNA made *in vivo* during the 10 min pulse with bromodeoxyuridine. The presence of [³H]DNA of normal buoyant density (fractions 22–30) could be due to some repair synthesis, to initiation of DNA strands *de novo*, or it is possibly the result of shearing the DNA such that the ³H-labelled part of a molecule is completely separated from density-labelled DNA to which it was previously covalently bound. We consider, however, because of the sucrose-density-gradient results (see below), that it is more likely to be due to DNA fragments possessing a low bromodeoxyuridine/³H ratio, since a distribution of fragments ranging from very light to very dense would be expected. In a related experiment (Fig. 2b) the density label was present *in vitro*; isolated nuclei were incubated in the presence of bromodeoxyuridine 5'-triphosphate and [³H]dATP before analysis of the denatured DNA in CsCl. As before, the buoyant density of the DNA synthesized by the nuclei was greater than that of the mature [¹⁴C]DNA, supporting our belief that this is replicative DNA synthesis and not repair synthesis. This result also suggests that there is little ligation of the DNA synthesized *in vitro* into larger molecules (see below).

Sucrose-density-gradient centrifugation of DNA synthesized *in vitro*. Newly synthesized low-molecular-weight DNA from intact cells is easily dissociated from the parental DNA under mildly denaturing conditions (Okazaki *et al.*, 1968; Wanka *et al.*, 1977) and it thus has properties of single-stranded DNA. Low-molecular-weight single-stranded DNA can easily form a complex with proteins, membranes (Infante *et al.*, 1976) and possibly with other nucleic acids (Pearson *et al.*, 1976), and can consequently lead to difficulties in interpretation of data obtained from

Fig. 2. *CsCl*-density-gradient analysis of DNA (a) Cells were long-labelled with [¹⁴C]thymidine and pulse-labelled for 10 min with bromodeoxyuridine at 50 μ M before isolation of the nuclei. Other conditions were as described for Fig. 1, except that lysates were first boiled for 5 min and then rapidly cooled in ice/water to denature the DNA before centrifugation. (b) Isolated nuclei were incubated in the standard medium, except that [³H]dTTP was replaced by bromodeoxyuridine triphosphate at 0.1 mM, and dATP was replaced by [³H]dATP (8 μ Ci/ml; 26 Ci/mmol). Other conditions were as for (a). Trichloroacetic acid-precipitable ³H (●) and ¹⁴C (○) radioactivities are shown.

Fig. 3. Alkaline sucrose-density-gradient centrifugation of DNA synthesized *in vitro*

(a) Conditions before centrifugation were as described for Fig. 1, except that the DNA was denatured by making the lysates 0.3M with NaOH before heating for 5 min at 100°C. (b) Incubation conditions were as for (a) but the nuclei were lysed with 1% (w/v) Sarkosyl NL-35 containing 5mM-EDTA (final concentrations). (c) Anomalous sedimentation of [³H]-dTTP in the presence of nuclear lysate. Nuclei isolated from cells long-labelled with [¹⁴C]thymidine were lysed by the addition of 1% Sarkosyl NL-35 containing 5mM-EDTA and 0.3M-NaOH. The lysate was then mixed with standard incubation medium containing [³H]dTTP (see the Experimental section) before it was centrifuged (without heating).

ultracentrifugation experiments (Pearson *et al.*, 1976). Because of this we conducted sedimentation studies with nuclear lysates prepared under a number of different conditions commonly used to disrupt nuclei before centrifugation in sucrose density gradients.

Nuclei lysed with sodium dodecyl sulphate. Sedimentation analysis of nuclei lysed with 1% SDS containing 10mM-EDTA indicated that there were two size-classes of DNA (Fig. 3a); one component was very small, and remained in the top three fractions under these centrifugation conditions, and the other component sedimented at about 4S [this was determined with reference to denatured poly(dT) sedimenting at 6.2S (PL Biochemicals), and from gel-electrophoretic behaviour]. If the nuclei were first incubated with deoxyribonuclease I before centrifugation of the lysate then the 4S material disappeared, but the radioactivity at the top of the gradient remained. This latter material was also observed on centrifugation of lysates obtained from nuclei which were previously incubated with [³H]dTTP in the standard medium containing 5mM-novobiocin to inhibit DNA synthesis. The amount of the material at the top of the gradient was, however, diminished by digesting the nuclear lysates with proteinase K (0.2mg/ml) for 17h at 37°C before centrifugation, suggesting that it was probably [³H]dTTP bound to protein, and only the material sedimenting at about 4S was DNA.

Nuclei lysed with Sarkosyl NL-35. When nuclear lysates prepared with 1% (v/v) Sarkosyl NL-35 containing 5mM-EDTA were centrifuged in alkaline sucrose gradients we frequently observed that some of the [³H]DNA synthesized *in vitro* sedimented with the mature [¹⁴C]DNA, and in some experiments it sedimented with an apparent *s* value greater than that of the [¹⁴C]DNA (Fig. 3b). We attributed this behaviour of the [³H]DNA to the formation of 'M-bands' caused by the co-precipitation, in the presence of Mg²⁺, of Sarkosyl together with membrane-associated protein and DNA (Tremblay *et al.*, 1969). The formation of 'M-bands'

(d) Incubation conditions were as for Fig. 1, but the nuclei were lysed by adding 1% (w/v) SDS and 10mM-EDTA (final concentrations) and the lysate was incubated overnight at 37°C with proteinase K at 0.2mg/ml. The sample was made 0.3M with NaOH, heated at 100°C for 5 min, formaldehyde was added to 11% (v/v) and the mixture was then cooled rapidly in ice/water. Samples were warmed to 20°C before layering on gradients. Trichloroacetic acid-precipitable ³H (●) and ¹⁴C (○) radioactivities are shown (all gradients).

Fig. 4. Alkaline sucrose-density-gradient analysis of DNA: aggregation artifacts

Long-labelled [^3H]DNA, deproteinized with phenol, was sonicated to decrease its molecular size and then mixed with a lysate prepared from cells long-labelled with [^{14}C]thymidine. MgCl_2 was added to the required concentration and NaOH added to 0.3M . The lysates were heated for 5 min at 100°C and rapidly cooled to 20°C before layering on gradients. Lysates contained (final concentrations): (a) 2% (w/v) Sarkosyl NL-35 and no MgCl_2 ; (b) 2% (w/v) Sarkosyl and 10mM-MgCl_2 ; (c) 2% Sarkosyl and 100mM-MgCl_2 . Trichloroacetic acid-precipitable ^3H (●) and ^{14}C (○) radioactivities are shown.

isolated nuclei might be bound to membranes, but control experiments in which nuclei were lysed with Sarkosyl, then mixed with [^3H]dTTP and finally centrifuged in sucrose gradients, showed that dTTP could also be bound in these complexes (Fig. 3c). Further experiments (Fig. 4) using DNA that had been deproteinized with phenol showed that the formation of these complexes was dependent on the concentration of Mg^{2+} , and, although low-molecular-weight single-stranded DNA was readily incorporated into such complexes (present work and other results not shown), DNA of larger molecular size also sedimented anomalously in the presence of high concentrations of Mg^{2+} (Fig. 4c).

Similar results were occasionally observed when centrifuging nuclear lysates prepared with SDS in the presence of Mg^{2+} . However, when the DNA in nuclear lysates was denatured with NaOH in the presence of 11% (v/v) formaldehyde (Pearson *et al.*, 1976) the anomalous sedimentation behaviour of the DNA was not observed (Fig. 3d).

Therefore the conditions for lysing nuclei which gave reproducible results with alkaline sucrose-density-gradient centrifugation experiments were as follows: DNA synthesis in the isolated nuclei was stopped by adding 1% (w/v) SDS and 10mM-EDTA (final concentrations), and the lysate formed was then incubated overnight at 37°C with proteinase K at 0.2mg/ml to degrade most of the protein. The sample was made 0.3M with NaOH , heated at 100°C for 5 min, formaldehyde was added to 11% (v/v) and the mixture was then cooled rapidly in a mixture of ice and water. Samples were warmed to 20°C before layering them on sucrose gradients. Results obtained by this method showed that all of the [^3H]DNA made in the isolated nuclei sedimented near the top of the gradient at about 4S (Fig. 3d). No [^3H]DNA was associated with the mature [^{14}C]DNA. This result shows therefore that the DNA synthesized *in vitro* was not due to repair synthesis carried out on parental DNA, and also that there was no ligation of newly synthesized [^3H]DNA into mature [^{14}C]DNA.

In summary, we have established the optimum conditions required for DNA synthesis by isolated nuclei from BHK-21/C13 cells and have shown that synthesis is restricted to a covalent elongation of Okazaki pieces previously initiated *in vivo*. We did not observe either initiation of synthesis *de novo* or ligation of low-molecular-weight DNA into larger molecules. Differences in the ability of isolated nuclei to synthesize DNA can in part be accounted for by differences in isolation and incubation conditions as well as by variations in subsequent procedures used to characterize the DNA synthesized *in vitro*. Even apparently minor variations, such as the means of detaching cells from monolayer surfaces (Brown & Stubblefield, 1975; Chiu & Baserga, 1975), the type of detergent used in the homogenizing

by using nuclei isolated from 3T3 cells has also been reported (Infante *et al.*, 1976). These observations led us to suppose that the DNA synthesized in the

medium or the choice of buffer used in the nuclear DNA-synthesizing medium, may influence the extent of DNA synthesis. Also, since there is a danger of artifacts occurring during centrifugation techniques, as a consequence of molecules aggregating, results obtained by these techniques, with a view to establishing initiation of DNA synthesis *de novo* or ligation of Okazaki pieces into higher-molecular-weight DNA, must be critically evaluated.

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