CALCIUM-DEPENDENT PRIMING OF DNA SYNTHESIS IN ISOLATED RAT LIVER NUCLEI*

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Received March 2, 1970

SUMMARY : Nuclei isolated from rat liver in the absence of Ca^{2+} incorporate deoxyribonucleotides into added poly d(A-T) but not into their nuclear DNA, which is of high molecular weight. On exposure to Ca^{2+} the nuclear DNA is nicked and then serves as primer for incorporation of deoxyribonucleotides, in the presence of Mg^{2+}, through the action of nuclear DNA polymerase. Incorporation of the chain-terminating arabinosyl analog araCMP[1] is a measure of Ca^{2+}-dependent production of DNA primer sites in nuclei.

Although there have been a number of studies of nuclear DNA polymerases (cf. 1,2) and of deoxyribonucleotide incorporation in isolated nuclei (3-8), little is known of the factors that control nuclear DNA replication and repair. Purified DNA polymerase from Escherichia coli initiates DNA synthesis at nicks, which provide 3'-hydroxyl primer ends in duplex DNA (9). The possibility that nuclear DNA replication is controlled by endonucleases that nick the DNA and generate primer ends is now being studied. This report describes isolation of nuclei that are unable to initiate DNA synthesis until their DNA has been nicked in a Ca^{2+}-dependent reaction.

Isolation of Nuclei - Isolation, washing and assay were carried out in Buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM tris chloride, pH 7.4) with additions as indicated. Nuclei were prepared from adult rat liver (10) with the following modifications. Livers were homogenized rapidly in at least 7 ml of Buffer A - 0.34 M sucrose, 2 mM EDTA, 0.5 mM EGTA/g of liver and the homogenate was layered on 0.33 volumes of Buffer A - 1.37 M sucrose, 1 mM EDTA, 0.25 mM EGTA and centrifuged for 15 min at 16,000 g. The nuclear pellet was dispersed in 7 volumes of Buffer A - 2.4 M sucrose, 0.1 mM EDTA, 0.1 mM EGTA, layered over an equal volume of the same buffer and centrifuged for 45 min at 75,000 g. After one wash in Buffer A -

* This work was supported by grants from the Australian Research Grants Committee and the University of Adelaide Anti-Cancer Foundation.

[1] araCMP, araCTP : arabinofuranosyl analogs of CMP and CTP; EGTA : ethylene glycol-bis-(2-aminoethyl ether)-N,N'-tetraacetic acid.

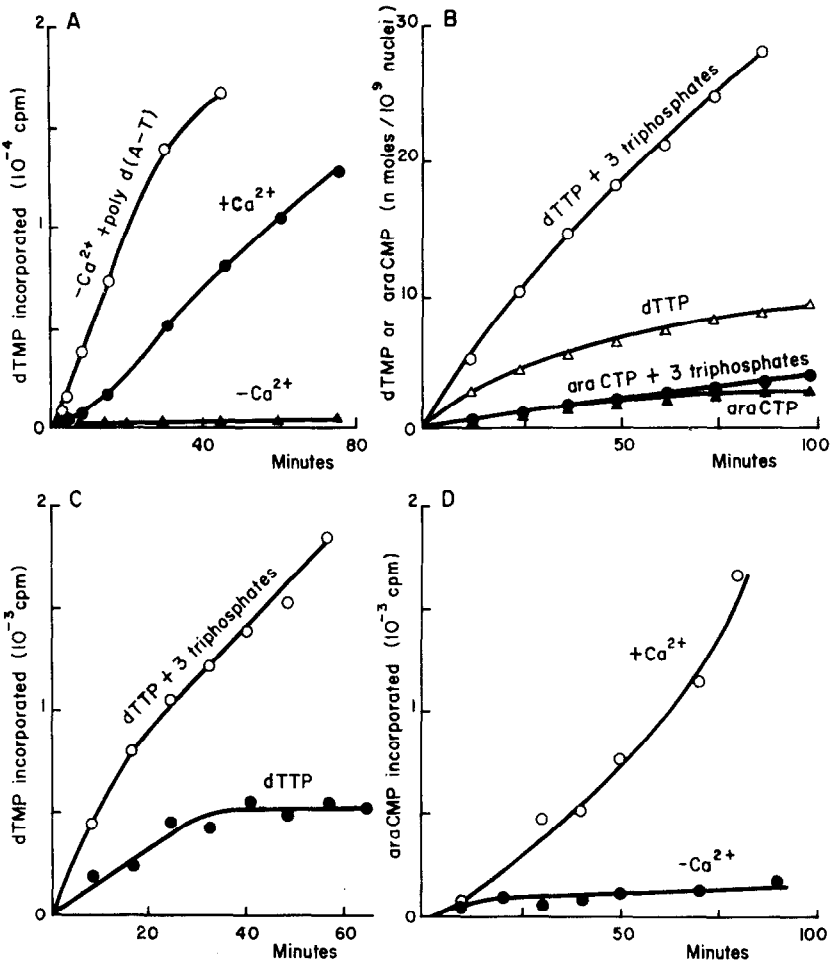

Figure 1. All assays were in 50 µl of Buffer A - 0.34 M sucrose, 2 mM phosphoenol pyruvate, 0.4 mM ATP with nucleotides as indicated. Incubations were at 37° and incorporation of ^{32}P into DNA was measured by scintillation counting after addition of 10 µl of 10 mM EDTA, 1.8 M KOH and removal of acid-soluble material by washing on paper discs with 10% CCl_3COOH; 5 times with 0.25 M Na_2SO_4, 5% CCl_3COOH, 1.5 mM KH_2PO_4; and twice with ethanol.

A. Nuclei (5.0 x 10^6/assay) in assay solution with 1 mM EDTA, 0.2 mM EGTA, 10 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP, and 16 µM α-^{32}P-dTTP (4.0 x 10^8 cpm/µmole): ▲, no additions; ○, + poly d(A-T), 90 µM in residues; ●, + 1 mM $CaCl_2$ at zero time.

B. Nuclei exposed to Ca^{2+} and Mg^{2+} during isolation (2.1 x 10^6/assay) in assay solution with 1 mM $CaCl_2$, 9 mM $MgCl_2$: ▲, + 6.5 µM α-^{32}P-araCTP (9.9 x 10^8 cpm/µmole); ●, + α-^{32}P-araCTP and 0.4 mM each of dATP, dGTP, and dTTP; ▲, + 24 µM α-^{32}P-dTTP (1.55 x 10^9 cpm/µmole); ○, + α-^{32}P-dTTP + 0.4 mM each of dATP, dCTP and dGTP.

C. Activated nuclei (2.8 x 10^5/assay) in assay solution with 1 mM EDTA, 0.2 mM EGTA, 10 mM $MgCl_2$: ●, + 27 µM α-^{32}P-dTTP (2.7 x 10^9 cpm/µmole); ○, + α-^{32}P-dTTP and 0.4 mM each of dATP, dCTP and dGTP.

D. Activated nuclei (8 x 10^5/assay) in assay solution with 1 mM EDTA, 0.2 mM EGTA, 10 mM $MgCl_2$, 0.4 mM each of dATP, dGTP and dTTP; ●, + 3.2 µM α-^{32}P-araCTP (1.2 x 10^9cpm/µmole); ○, + ^{32}P-araCTP and 1 mM $CaCl_2$.

0.34 M sucrose by centrifuging for 15 min at 16,000 g, the nuclei were dispersed for assay in the same solution (2 ml/liver). Nuclei were activated, where indicated, by suspending in Buffer A - 0.34 M sucrose, 1 mM $CaCl_2$, 5 mM $MgCl_2$ at 0-2° for 10 min. Ca^{2+} and Mg^{2+} were then removed by centrifuging through an underlay of Buffer A - 1.0 M sucrose, 2 mM EDTA, 0.5 mM EGTA. The nuclei were washed and re-suspended in Buffer A - 0.34 M sucrose.

Results and Discussion - Nuclei isolated in (Ca^{2+}, Mg^{2+}) - free solutions contained an active DNA polymerase when assayed with poly d(A-T), dATP, dCTP, dGTP, α-^{32}P-dTTP and Mg^{2+}, with EGTA to selectively chelate Ca^{2+} (Fig. 1A). Nearest-neighbor analysis showed all label in alternating d(A-T) copolymer. In these conditions added calf thymus DNA also served as primer (results not shown), but with no added primer DNA synthesis was at the limit of detection

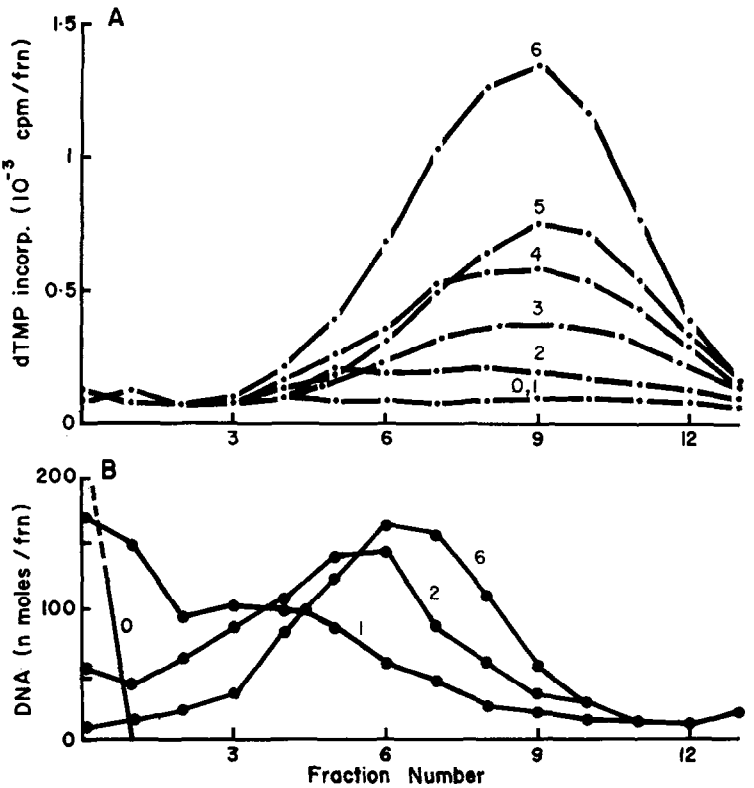

Figure 2. Nuclei (3 x 10^6/assay) in 100 µl of assay solution as for Fig. 1, with 1 mM $CaCl_2$, 9 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP and 18 µM α-^{32}P-dTTP (1.7 x 10^9 cpm/µmole). After incubation at 37° for the number of minutes indicated by the numbers on the curves, reactions were stopped with 20 µl of 10 mM EDTA, 1.8 M KOH and loaded on a 5 ml alkaline sucrose gradient (17). After centrifuging for 17 hr at 10° and 36,000 r.p.m. in an M.S.E. SW-40 rotor, 7 drop fractions were collected and acid-insoluble radioactivity (A) or total DNA (B; ref. 18) measured. The pellet ("fraction 0") contained almost all the DNA in the zero-time sample.

(Fig. 1A). When $CaCl_2$ was added, DNA synthesis was immediately initiated without added primer (Fig. 1A). Centrifugation in an alkaline sucrose gradient showed that in the presence of Ca^{2+} continuous nicking decreased the size of the DNA from its high initial value (Fig. 2B) and that incorporation of label was into DNA smaller (Fig. 2A) than the median bulk DNA.

When nuclei were exposed to 1 mM $CaCl_2$, 5 mM $MgCl_2$ during isolation and then assayed with deoxyribonucleoside triphosphates and Mg^{2+} immediate incorporation of label was observed (Fig. 1B). Omission of the three unlabeled triphosphates decreased labeling rates 2-3 fold. Labeled endogenous DNA could all be hydrolyzed to acid-soluble products by pancreatic DNase, and hydrolysis to 5' nucleotides (11) showed at least 95% of the α-^{32}P label from dATP, dCTP, dGTP and dTTP, and at least 85% from araCTP in the corresponding nucleotide. Nearest-neighbor analysis (12) showed covalent attachment adjacent to all 4 possible primer termini (13). With the labeled triphosphate as sole substrate apparent increases in nearest-neighbor frequencies of homodinucleotide sequences were: ApA, 0.033 to 0.081; CpC, 0.069 to 0.083; GpG, 0.037 to 0.051; TpT, 0.038 to 0.066 (13). Template-independent terminal transferase (cf. ref. 1) would give frequencies approaching 1.0 with single labeled deoxyribonucleotides as the only substrate, in the absence of endogenous nucleotides. However, from the results in Fig. 1C, it is unlikely that endogenous nucleotides contribute significantly to DNA synthesis. The nearest-neighbor analyses therefore indicate that little if any of the synthesis is due to terminal transferase. Nuclei activated by limited DNA nicking during brief exposure to Ca^{2+} (Fig. 1C), incorporate ^{32}P-dTMP, from ^{32}P-dTTP alone, up to a limit probably corresponding to dTMP attachment at all available primer termini. In the presence of added dATP, dCTP and dGTP, there is extensive incorporation of dTMP (Fig. 1C), well beyond the former limit.

These results are consistent with a model in which a Ca^{2+}-dependent endonuclease nicks the nuclear DNA and provides enough 3'-hydroxyl termini to account for the observed DNA synthesis. Further support for this model came from studies with araCTP. This inhibitor of DNA synthesis is a metabolite of the growth inhibitor arabinosyl cytosine and is an effective chain terminator, transferring araCMP residues mainly to terminal positions on DNA with polymerase from animal (14) and bacterial (cf. 15) sources. α-^{32}P-AraCTP did not label unactivated nuclei to a level significantly above background, but after partial activation by brief exposure of nuclei to Ca^{2+}, araCMP was attached to nuclear DNA to a limited extent (Fig. 1D), probably corresponding to "titration" of all available primer termini with chain terminator (15). Centrifugation in an alkaline sucrose gradient (Fig. 3) showed that label was in DNA of a size intermediate between that from unactivated nuclei (Fig. 2B; zero time) and the smaller product (Fig. 3) resulting from

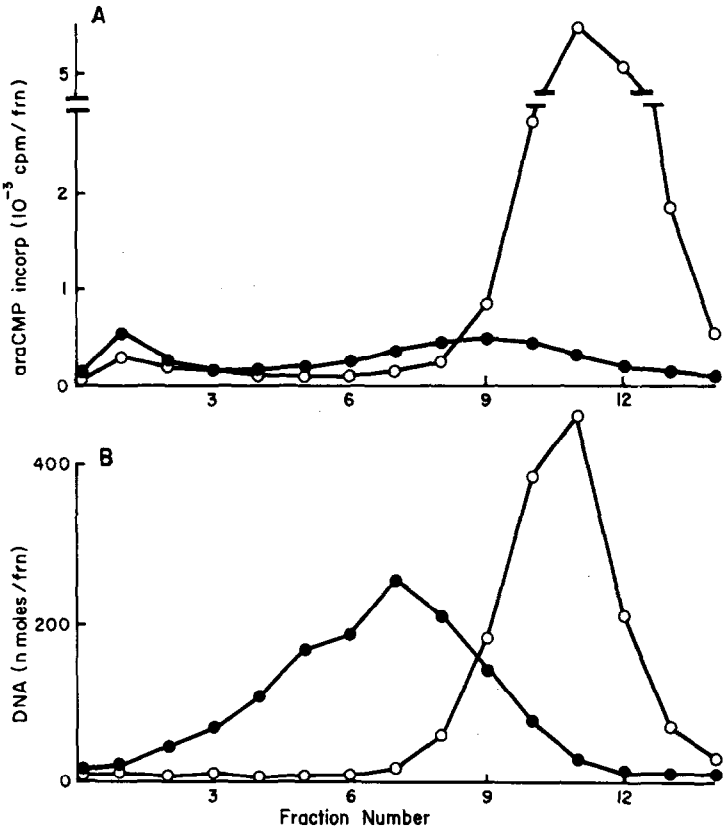

Figure 3. Activated nuclei (7.9×10^6/assay) incubated for 45 min in 100 µl assay solution (Fig. 1D) with $MgCl_2$, EDTA, EGTA, dATP, dGTP, dTTP and 23 µM α-^{32}P-araCTP (2.0×10^8 cpm/µmole). Distributions of radioactivity (A) and DNA (B), after centrifuging for 14 hr in the alkaline sucrose gradient, were measured as in Fig. 2: -●-, incubation with no added Ca^{2+}; -○-, with 1 mM $CaCl_2$.

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extensive labeling in the presence of α-^{32}P-araCTP and $CaCl_2$ (Figs. 1B & D). In each case incorporation of araCMP is a measure of Ca^{2+}-dependent production of primer sites by nicking of nuclear DNA.

Our experiments do not prove that the Ca^{2+}-dependent priming of nuclear DNA is involved in physiological initiation of DNA replication, but they do indicate that this effect must be considered in any search for factors that control nuclear DNA synthesis in vivo and in particular for endonucleases that may be involved in DNA replication (16).

The authors acknowledge the technical assistance of Mrs Donna Staunton and Mr Mario Froscio and the provision of arabinosyl cytosine by Dr. W.J. Wechter.

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