Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 27-33

EVIDENCE FOR NUCLEOSIDE CHANNELING IN VIVO: DEOXYTHYMIDINE INCORPORATION INTO RAT LIVER dTTP AND NUCLEAR MATRIX DNA

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Received October 6, 1987

Summary: Previous studies in prokaryotes and in eukaryotic cell lines have indicated the possible existence of more than one dTTP pool accessible to DNA synthesis. To investigate this possibility in eukaryotes in vivo, the incorporation of [^{3}H]deoxythymidine into nuclear matrix-attached DNA and intracellular dTTP was examined in regenerating rat liver. The labeling of matrix DNA reached a maximum after a 5 min pulse and then began to rapidly decrease. Conversely, [^{3}H]deoxythymidine incorporation into dTTP began to increase after 5 min and peaked 10 min after injection. Since the peak specific activity for [^{3}H]deoxythymidine incorporation into matrix DNA precedes that into dTTP, there seems to be channeling of exogenous thymidine directly to sites of DNA replication, bypassing existing nucleotide pools.

It has been proposed that subcellular localization and concentration, i.e., compartmentalization, of DNA precursors occurs in prokaryotic (1,2) and eukaryotic (3,4) cells. Existing data supports the idea of more than one functional triphosphate pool within the cell and at least two have been suggested (5): a large pool feeding cellular metabolic functions along with the DNA repair mechanism, and a smaller sequestered pool through which certain precursors are channeled expressly for the demands of DNA replication. The channeling of DNA precursors to replication forks is thought to be accomplished by multi-enzyme complexes of distinct, but functionally related, enzymes able to conduct a metabolite from one enzyme to another without access of the metabolite to an intracellular pool. Elucidating the nature of precursor compartmentalization may be essential for understanding allosteric regulation of nucleoside pathways during DNA replication and other biological phenomena such as the anti-metabolic effects of nucleoside analogues on dNTP biosynthesis.

Abbreviations: [^{3}H]dThd, [^{3}H-methyl]deoxythymidine; dTTP, deoxythymidine triphosphate; THF, tetrahydrofuran; HPLC, high performance liquid chromatography.

0006-291X/87 $1.50

To date, no evidence exists which indicates the presence of DNA precursor channeling *in vivo* in eukaryotic cells. This has been largely due to the difficulties associated with quickly accessing the appropriate subcellular compartments with sufficient labeled precursor and isolating relevant subnuclear fractions for subsequent analysis. In this study, we have overcome the above difficulties by investigating the kinetics of labeling nuclear matrix-associated DNA in regenerating rat liver. Total nuclear DNA can be empirically subdivided into low salt-soluble DNA (75-80%), high salt-soluble DNA (18-23%), and matrix-attached DNA which remains tightly associated with the nuclear matrix (~2%). The most newly replicated DNA is that associated with the nuclear matrix in regenerating rat liver (6,7). Since matrix-associated DNA represents the immediate product of DNA precursor metabolism, it provides a sensitive means of examining precursor incorporation kinetics in eukaryotes *in vivo*. Upon comparison of the kinetics for [^{3}H]dThd labeling of nuclear matrix-associated DNA with labeling of dTTP in regenerating rat liver, we find evidence supporting the existence of DNA precursor channeling *in vivo*.

METHODS

Male Sprague-Dawley rats (180-200 g) were partially hepatectomized by surgical removal of two-thirds of the liver. Each rat was injected with 200 uCi [^{3}H-methyl]deoxythymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) into the hepatic portal vein 22 h post-partial hepatectomy. After various pulse times, the livers were quickly excised and processed for nuclear matrix DNA isolation and/or nucleotide analysis.

Nuclear Matrix DNA Isolation: Nuclear matrix-associated DNA was isolated as previously described (6) with some modifications. Briefly, nuclei were isolated by centrifugation through 2.2 M sucrose. The purified nuclei were incubated at 37°C for 45 min (8) followed by sequential extractions with a low salt-containing buffer, a high-salt containing buffer, and 0.1% Triton X-100. The nuclear matrices so prepared contained ~2% of the total nuclear DNA as measured by the method of Burton (9). The matrix-associated DNA was removed from the nuclear matrix by treatment with 200 Kunitz units of pancreatic deoxyribonuclease I (Sigma, St. Louis, MO) per milliliter overnight at 0°C. Trichloroacetic acid was added to a final concentration of 6% and the samples cooled on ice for 10 min. The supernatant containing the nuclear matrix DNA was collected following centrifugation and the tritium content determined by liquid scintillation counting.

HPLC Analysis of Nuclear Matrix DNA: Approximately 1 mg of matrix DNA was lyophilized to dryness. The DNA was hydrolyzed to nucleic acid base components in 0.5 ml 98% formic acid at 210°C for 60 min. Bases were separated on a 4.6 x 250 mm Supelcosil C-18 column (Supelco, Inc., Bellefonte, PA) using a 20 min linear gradient from 2.5 mM KH_2PO_4 (pH 4.0), 0.1% THF to 5 mM KH_2PO_4 (pH 4.0), 0.1% THF (1 ml/min). Thymine peaks were quantitated by integration against a standard.

Nucleotide Analysis: Well-minced livers were rinsed quickly with cold 0.25 M sucrose and homogenized in 60% methanol. In cases where nucleotides and nuclear matrix DNA were isolated from the same liver, the post-nuclear supernatant was brought to a final methanol concentration of 60%. After centrifugation at 22,000 x g, 4°C for 60 min, the nucleotide-containing super-

natants were lyophilized to dryness. The residues were dissolved in 10 mM Tris-HCl (pH 6.5) and stored at -75°C.

Pyrimidine nucleotides were separated from purine nucleotides by HPLC on a 4.6 x 250 mm Partisil 10-SAX column (Alltech, Houston, TX) under conditions described by Pogolotti (10). The pyrimidine triphosphates were hydrolyzed to bases as described above and rechromatographed on a 4.6 x 250 mm Supelcosil C-18 column (Supelco, Inc., Bellefonte, PA) using 2.5 mM KH_2PO_4 (pH 4.0), 0.05% THF isocratically at a flow rate of 1 ml/min. Thymine peaks were quantitated by integration against a standard, collected, and tritium content determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Injection of [^{3}H]dThd directly into the hepatic portal vein of a rat 22 h post-partial hepatectomy results in its rapid incorporation into replicating DNA. As shown in Figure 1a, total nuclear DNA reached half maximal rate of [^{3}H]dThd incorporation by 5 min, and maximal rate was attained at 20 min. A strikingly different pattern of labeling was observed when nuclear matrix-associated DNA was analyzed during [^{3}H]dThd incorporation and compared to total nuclear DNA, Figure 1b. After 2 min of incorporation, the radiospecific activity of matrix-associated DNA was already 10-fold greater than that for total nuclear DNA. This level subsequently decreased with time and by 20 min became equal to total nuclear DNA. This pattern corresponds to the increasing amounts of [^{3}H]dThd residing in the extra-matrix DNA regions removed during salt extractions compared to the [^{3}H]dThd in the small nuclear

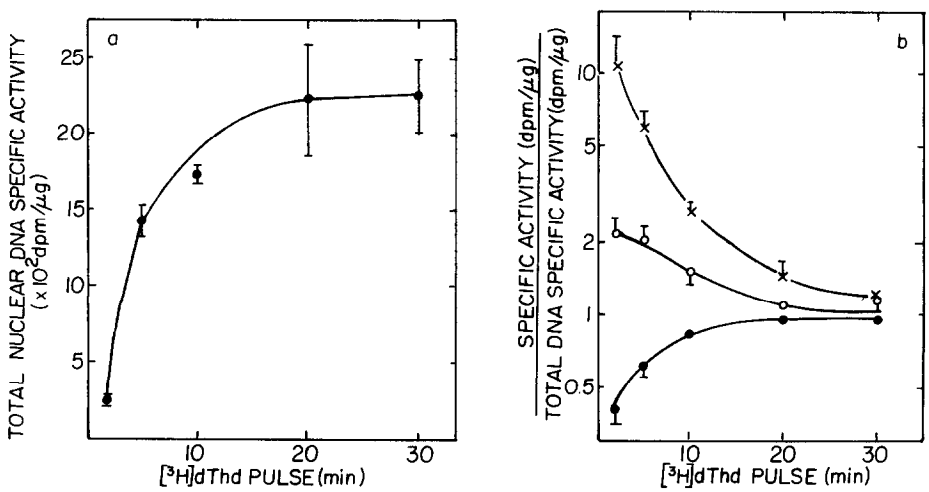

Figure 1. [^{3}H]dThd labeling of regenerating rat liver nuclear DNA after injection into the hepatic portal vein. (a) The specific activity of total nuclear DNA was calculated from the specific activities of the low salt, high salt, and matrix DNA fractions. The means ± S.D. (N=4) are shown. (b) Low salt-extractable DNA,●, high salt-extractable DNA,○, and nuclear matrix associated DNA, x , were isolated as described in Methods. The ratios of their specific activities to total nuclear DNA are plotted on a log scale. The means of at least 4 experiments ± S.D. are shown. Where not shown, error bars are less than or equal to the width of the point.

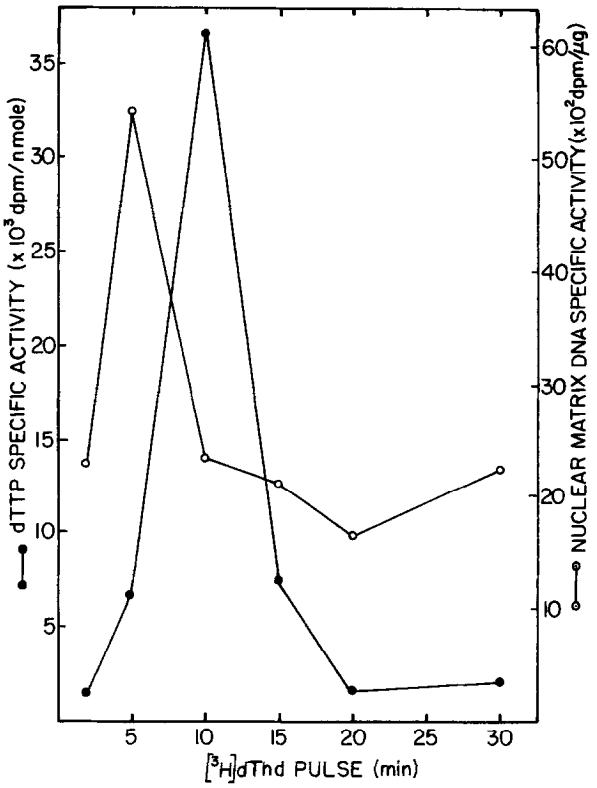

Figure 2. [^{3}H]dThd incorporation into intracellular dTTP and nuclear matrix-associated DNA in 22 h regenerating rat liver. dTTP, ●, and nuclear matrix-associated DNA, O, were isolated as described in Methods. The data shown represents the pattern from a typical experiment. The pattern obtained when nucleotides and nuclear matrix DNA were isolated from the same liver, was coincident with that obtained when nucleotides and nuclear matrix DNA were isolated from different livers.

matrix DNA compartment, illustrated in Figure 1b. Thus, by monitoring the labeling kinetics of nuclear matrix DNA, it is possible to obtain a more sensitive assessment of the radiospecific activities of dNTP pools providing precursors for DNA replication at nuclear matrix-associated replication forks.

We next compared the kinetics of [^{3}H]dThd labeling of nuclear matrix-associated DNA with the labeling of the dTTP intracellular pool. The specific activities of [^{3}H]dThd in dTTP and nuclear matrix DNA are plotted against pulse time in Figure 2. As can be seen, the peak for incorporation into newly replicating matrix DNA occurred 5 min before maximal labeling of the intracellular dTTP pool. As early as 2 min post-injection, the nuclear matrix DNA was being rapidly labeled whereas [^{3}H]dThd was only sparingly labeling dTTP. At 10 min post-injection, the dTTP pool was maximally labeled, while the specific activity of matrix DNA was decreasing. The decrease in the specific activity of nuclear matrix DNA after 5 min results from a depletion of the radioactive dTTP pool accessible to DNA replication and movement

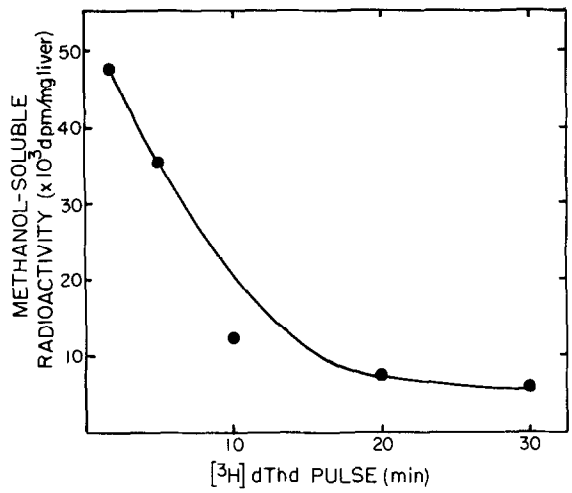

Figure 3. Methanol-soluble radioactivity after injection of 200 μCi [^{3}H]-dThd into 22 h regenerating rat liver via the hepatic portal vein. The data shown represents a typical experiment.

of the labeled DNA into salt-extractable regions of the genome (6, Figure 1b). The decrease in the dTTP specific activity after 10 min results from its incorporation into methanol-insoluble material, i.e., DNA, and intracellular degradation of [^{3}H]dThd (Figure 3).

HPLC analysis of nuclear matrix DNA was performed to determine whether variations in DNA base composition at various pulse times could account for differences in the matrix DNA kinetic pattern. Nuclear matrix DNA analyzed 2, 5, 10, 20, and 55 min after injection of [^{3}H]dThd consisted of 0.345 ± 0.019 nmoles thymine per μg DNA (11.3% base composition), slightly less than the 0.565 ± 0.024 nmoles thymine per ug total nuclear DNA (18.5% base composition). Thus, variations in nuclear matrix DNA thymine content cannot account for the pattern of [^{3}H]dThd incorporation.

The more rapid incorporation of [^{3}H]dThd into nuclear matrix DNA could theoretically result from rapid fluctuations in the rate of DNA synthesis independent of dTTP synthesis. Since there is a sufficient amount of [^{3}H]dTTP for replicational utilization by 2 min post-injection (see Figure 2), a rapid increase in the rate of DNA synthesis from 2 to 5 min followed by a rapid decrease from 5 to 10 min would result in the incorporation pattern observed. The experimental observation of such a short burst in the rate of DNA synthesis would require precise timing of the [^{3}H]dThd injection with respect to time post-partial hepatectomy. In the 22 ± 1 h regenerating liver system used in these experiments, it would not be possible to reproducibly access an event which occurs within a span of 10 min. Therefore, a more likely explanation for this pattern depends upon precursor availability to the replicational complex, i.e., channeling.

In the absence of [^{3}H]dThd channeling, one would expect the specific activity of nuclear matrix DNA to peak at the same time or later than the soluble dTTP specific activity. In such an instance, the [^{3}H]dThd would have to be phosphorylated and equilibrate with the existing dTTP pool before the maximum rate of incorporation (and therefore the peak specific activity) into DNA could be achieved. Our results show that precursor equilibration with the intracellular dTTP pool is not necessary for incorporation into newly replicating DNA. Since the peak specific activity for [^{3}H]dThd incorporation into nuclear matrix DNA precedes that into dTTP, there seems to be channeling of exogenous thymidine directly to sites of DNA replication, bypassing existing nucleotide pools.

A multi-enzyme complex responsible for metabolic channeling has been proposed from results obtained in T4 phage-infected Escherichia coli (1,11) and permeabilized Chinese hamster embryo fibroblasts (12,13). The data from regenerating rat liver presented in this study provides evidence for a similar enzyme complex responsible for nucleotide channeling in vivo. Such a complex would, by necessity, have to be proximal to DNA replication forks to affect efficient channeling. Considering that [^{3}H]dThd is channeled specifically to newly replicated nuclear matrix DNA, one would expect to find a multi-enzyme channeling complex associated with or near the nuclear matrix itself. This association seems more than likely in light of Tubo and Berezney's elucidation of 100 and 150S megacomplexes released from the nuclear matrix after mild sonication (14,15). Although only replicative enzyme activities have as yet been demonstrated by these megacomplexes, it is possible that here too exists the enzyme architecture necessary for DNA precursor channeling.

Further investigations employing this approach are under way to determine whether de novo DNA precursors are also channeled to nuclear matrix-associated replication forks, bypassing the intracellular dNTP pools.

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