DNA precursors are channelled to nuclear matrix DNA replication sites

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Studies of replicative DNA synthesis using DNA precursors have shown that the DNA that was replicated most recently is that associated with the nuclear matrix. Consequently, precursors arising via the salvage and the *de novo* metabolic pathways are first incorporated into a small percentage of the total nuclear DNA that is termed nuclear matrix-associated DNA. These results have been substantiated in cell culture, as well as in intact mammalian systems. Furthermore, when DNA precursors were injected intravenously into regenerating rat liver, a significant lag in the incorporation of orotic acid-derived nucleotides (*de novo* pathway precursors) into nuclear DNA was observed, when compared with deoxythymidine-derived nucleotides (salvage pathway precursors). This lag in incorporation kinetics was also evident at the nuclear matrix level, although, once incorporated into nuclear matrix-associated DNA, the distribution patterns of

both precursors into extra-matrix nuclear DNA fractions were identical. To determine the basis for this kinetic lag, we compared the incorporation kinetics of orotic acid and of deoxythymidine into dTTP and into nuclear matrix-associated DNA, respectively. Orotic acid-derived nucleotides entered the cytosolic dTTP pool before being incorporated into nuclear matrix-associated DNA, that is, traversing the classical metabolic route of DNA precursors. Conversely, deoxythymidine-derived nucleotides bypassed the soluble dTTP cellular pool and engaged directly in DNA synthesis at the nuclear matrix. Not only is this the first evidence for nucleotide channelling in an intact mammalian system, but it also forms direct evidence that salvage pathway DNA precursors are channelled to nuclear matrix-associated sites of DNA replication.

INTRODUCTION

Channelling of certain precursors to sites of DNA replication has been proposed, whereby intermediates in a sequence of metabolic reactions are not released into the cellular milieu, but are directly engaged in the next enzymic reaction in the sequence (Jones, 1980; Mathews and Slabaugh, 1986). Such a mechanism decreases cellular degradation of important intermediates and eliminates the limitations presented by diffusion of metabolites at low concentrations. Reddy and Pardee have provided evidence for a multi-enzyme complex termed 'replitase' that may be responsible for metabolic channelling in T4 phage-infected *Escherichia coli* (Reddy and Mathews, 1978; Chui et al., 1982) and in permeabilized Chinese-hamster-embryo fibroblasts (Reddy and Pardee, 1980, 1982).

To date, however, the phenomenon of precursor channelling has not been demonstrated in an intact mammalian system. This is primarily because actively replicating DNA represents a very small fraction of the eukaryotic genome at any given time, so that any phenomenon involving only DNA that is replicating is masked by the non-replicating majority of nuclear DNA. With the advent of methodology that fractionates DNA according to its association with the nuclear matrix, we can now specifically isolate the newly replicated DNA from the bulk of the genome (for reviews see Berezney, 1984; Nelson et al., 1986; Van Driel et al., 1991). For studying this DNA fraction in conjunction with precursor incorporation into total cellular dNTPs, a novel and sensitive approach for monitoring channelling in living tissue was employed. On comparison of the kinetics for [³H]deoxythymidine labelling of nuclear matrix-associated (NM) DNA with labelling of cellular dTTP, we have found evidence for DNA precursor channelling to NM sites of DNA replication in

regenerating rat liver. No channelling was detected for the *de novo* pathway precursor, [¹⁴C]orotic acid, thus confirming results obtained from cell-culture studies that only salvage pathway DNA precursors are channelled to sites of DNA replication *in vivo*.

MATERIALS AND METHODS

Animals

Rats weighing 180–200 g were partially hepatectomized by surgical removal of two-thirds of the liver. After 22 h post-hepatectomy, 200 μ Ci of [methyl-³H]deoxythymidine (6.7 Ci/mmol; New England Nuclear) or 125 μ Ci of [6-¹⁴C]orotic acid (40–60 Ci/mol; New England Nuclear), in a total volume of 0.2 ml of sterile water, was injected into the hepatic-portal vein about 1 cm from the liver. The rats were pulsed for the time indicated. The liver was excised immediately and was minced in ice-cold 0.25 M sucrose/Tris/MgCl₂ (TM) buffer (0.25 M sucrose, 10 mM Tris and 5 mM MgCl₂, pH 7.4). After homogenization in 10 ml of the same buffer, each sample was centrifuged for 10 min at 1000 g, at 4 °C. Analysis of soluble nucleotides was performed from the supernatant and NM DNA was isolated from the crude nuclear pellet.

Isolation of NM DNA

The fractionation of nuclear DNA according to its association with the nuclear matrix was performed as previously described (Berezney and Coffey, 1975), with some modifications. The crude nuclear pellet was resuspended in 2.2 M sucrose/TM buffer and nuclei were pelleted by centrifugation for 90 min at 40000 g. After two washes with 0.25 M sucrose/TM buffer, the nuclei

Abbreviations used: HS, high-salt; HS DNA, high-salt-soluble DNA; LS, low-salt; LS DNA, low-salt-soluble DNA; TM buffer, Tris/MgCl₂ buffer; NM, nuclear matrix-associated.

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were incubated in 1 ml of buffer at 37 °C for 45 min (Berezney and Buchholtz, 1981).

Isolation of low-salt-soluble (LS) DNA

LS DNA was extracted from the nuclei three times with a total of 2 ml of low-salt (LS) buffer (10 mM Tris and 0.2 mM MgCl_2 , pH 7.4) for 10 min at 0 °C. After each extraction, nuclei were pelleted at 750 *g* for 20 min.

The LS-DNA fraction was transferred to membrane tubing [12000–14000 molecular mass cut-off] and was dialysed against LS buffer overnight at 4 °C. Each sample was then treated with 200 units of DNAase I per ml for 90 min at room temperature, was brought to a final trichloroacetic acid concentration of 6 % (w/v), was precipitated on ice for 10 min and was centrifuged at 1000 *g* for 20 min. The supernatant was collected as the digested LS-DNA fraction.

Isolation of high-salt-soluble (HS) DNA

LS-extracted nuclei were incubated at 0 °C for 10 min with high-salt (HS) buffer (2 M NaCl, 10 mM Tris and 0.2 mM MgCl_2 , pH 7.4) in order to extract HS DNA. After each of three extractions with a total of 2 ml of HS buffer, residual nuclei were pelleted by centrifugation at 1500 *g* for 20 min. The HS DNA fraction was dialysed, digested and precipitated with trichloroacetic acid, as described above for LS DNA.

Isolation of NM DNA

Phospholipids were removed from the HS-extracted nuclear structures by incubating at 0 °C for 10 min with 1 ml of Triton/TM buffer [1 % (w/v) Triton X-100, 10 mM Tris and 5 mM MgCl_2 , pH 7.4]. After centrifugation for 15 min at 750 *g*, the supernatant was removed leaving the nuclear-matrix pellet. Each pellet was washed once with LS buffer and was resuspended in 1 ml of LS buffer. The nuclear-matrix suspension was treated with 200 units of pancreatic DNAase I (Sigma) at 0 °C overnight. After precipitation with trichloroacetic acid as described above, the supernatant was collected as the digested NM DNA fraction.

The DNA content of each sample was determined in triplicate, using the method of Burton (1968).

Nucleotide isolation

After centrifugation of the liver homogenate, the supernatant was decanted into 15 ml of cold 100 % methanol to give a final methanol concentration of 60 % (v/v). The mixture was precipitated on ice for 10 min and centrifuged at 22000 *g* for 60 min. The supernatant was lyophilized to dryness. The residue was redissolved in 2.5 ml of 10 mM Tris (pH 6.5), filtered through a cellulose-acetate filter (0.2 μm pore size) and stored at –80 °C for h.p.l.c. analysis.

H.p.l.c. analyses

LS, HS and NM DNA

An aliquot of up to 1 mg of digested DNA was lyophilized to dryness. The residue was dissolved in 0.5 ml of 98 % (v/v) formic acid and was heated in a closed aluminium block for 1 h at 210 °C. The hydrolysed samples were dried at 80–90 °C. Each LS-DNA sample was dissolved in 1 ml of h.p.l.c. running buffer, whereas the HS- and NM-DNA samples were each dissolved in 0.5 ml of running buffer.

After filtration through a nylon filter (0.2 μm pore size), base separation of the hydrolysed LS-, HS- and NM-DNA samples was carried out on a 250 mm $\times$ 4.6 mm Supelcosil C_{18} column (Supelco, Inc., Bellefonte, PA, U.S.A.). Bases were eluted isocratically with 0.9 mM KH_2PO_4 (pH 4.0) and 0.01 % (v/v) tetrahydrofuran, at a flow rate of 1 ml/min. In each case, the thymine peak was quantified by integration against a standard, the peak was collected and the radioactivity content determined by liquid-scintillation counting.

Soluble nucleotides

The nucleotide extracts were chromatographed on a 250 mm $\times$ 4.6 mm Partisil 10-SAX column (Phenomenex, Rancho Palos Verdes, CA, U.S.A.), using the procedure of Pogolotti and Santi (1982). The peak containing the pyrimidine triphosphates was collected and pooled from two runs. The pooled pyrimidine-triphosphate fraction was lyophilized to dryness. The residue was dissolved in 1.5–2.0 ml of 98 % (v/v) formic acid and was hydrolysed to bases, as described above.

Base separation of the hydrolysed pyrimidine triphosphates was performed on a 250 mm $\times$ 4.6 mm Supelcosil C_{18} column by isocratic elution with 2.5 mM KH_2PO_4 (pH 4.0) and 0.05 % (v/v) tetrahydrofuran at a flow rate of 1 ml/min. The thymine peak was quantified by integration against a standard, the peak was collected and the radioactivity content determined by liquid-scintillation counting.

RESULTS

Precursor incorporation into total nuclear DNA

Regenerating rat livers were pulse-labelled via the hepatic-portal vein with the salvage pathway DNA precursor, [^3H]deoxythymidine, for various times, after which the LS, HS and NM DNAs were isolated. As expected, there was a rapid uptake of the radiolabelled precursor into regenerating rat-liver DNA and the incorporation plateau of [^3H]deoxythymidine was attained after only 20 min (Figure 1). The incorporation of [^{14}C]orotic acid, a *de novo* pathway precursor, into total nuclear DNA in regenerating rat liver was also investigated (Figure 1). Although orotic acid is a precursor both for thymidine and for cytosine, it was necessary to consider incorporation only into thymine to compare

Figure 1 Precursor incorporation into DNA in regenerating rat liver

[^3H]Deoxythymidine (●) or [^{14}C]orotic acid (○) was injected into the hepatic portal vein and incorporation into replicating DNA was determined, as described in the Materials and methods section. Means $\pm$ S.D. are shown ($n = 3$).

Figure 2 DNA precursors are preferentially incorporated into NM DNA

$[^3\text{H}]$ Deoxythymidine (a) or $[^{14}\text{C}]$ orotic acid (b) was injected into the hepatic-portal vein of regenerating rat liver and incorporation into LS DNA (●), HS DNA (○) and NM DNA (▲) was determined, as described in the Materials and methods section. The amount of radiolabel in each DNA fraction is expressed as a percentage of the total radiolabel incorporated into nuclear DNA at a given time. Means from three experiments are shown (S.E.M. < 10% in all cases).

the incorporation kinetics with those of $[^3\text{H}]$ deoxythymidine properly. To achieve this, h.p.l.c.-isolation techniques were used to determine the specific radioactivity of thymine in each DNA sample whenever orotic acid was used. In this case, there was a significant lag before the maximum rate of incorporation of radiolabelled thymine was reached at 15–20 min post-injection and the incorporation plateau was attained only after 40 min.

Precursor incorporation into LS, HS and NM DNAs

The fraction of total ^3H label in LS, HS and NM DNAs after the injection of $[^3\text{H}]$ deoxythymidine is plotted as a function of pulse time in Figure 2(a). After a 2-min pulse, ^3H -labelled NM DNA represented 23% of the total $[^3\text{H}]$ deoxythymidine-labelled DNA in nuclei. Since NM DNA constitutes only 2% of total nuclear DNA by mass, this reinforces the original observation that NM DNA is enriched in $[^3\text{H}]$ deoxythymidine at early pulse times (Berezney and Coffey, 1975). As the pulse time increased, the fraction of label associated with the NM DNA decreased, while that associated with the LS DNA increased. The HS-DNA fraction represents an intermediate component between the two extremes exhibited by LS DNA and NM DNA. From 20 min post-injection, the distribution of radioactivity among the three DNA fractions was representative of their mass distribution in the nucleus, i.e. 75%, 23% and 2% for LS, HS and NM DNA, respectively, indicating that the $[^3\text{H}]$ deoxythymidine was randomly distributed throughout the nuclear DNA. These kinetics have been interpreted as a progressive movement of the radiolabel from NM DNA, through the HS-DNA fraction and into the LS DNA, thereby pinpointing the origin of replication to the nuclear matrix (Berezney and Coffey, 1975; Berezney and Buchholtz, 1981).

Similar incorporation kinetics were observed for the *de novo* pathway DNA precursor, $[^{14}\text{C}]$ orotic acid, with one significant difference (Figure 2b). There was a 10-min delay before the kinetic profile observed for deoxythymidine appeared, reflecting the lag observed for the incorporation into total nuclear DNA (Figure 1). We have shown that variations in DNA thymine content within the nuclear DNA fractions could not explain this kinetic shift (Panzeter et al., 1987). Indeed, the distribution of thymine in each DNA fraction paralleled the mass distribution of DNA in each fraction, indicating a largely random distribution of thymine in the DNA preparations examined. Specifically, the

relative amounts of total nuclear DNA thymine residing in the LS, HS and NM DNA fractions were $75\% \pm 8\%$, $22\% \pm 5\%$ and $3\% \pm 1\%$, respectively ($n = 4$).

While the kinetics of $[^{14}\text{C}]$ orotic acid incorporation after 10 min accurately mimicked those of $[^3\text{H}]$ deoxythymidine, the early incorporation pattern cannot be easily interpreted. As noted by Berezney and Buchholtz (1981) for $[^3\text{H}]$ deoxythymidine, it is possible that some replication sites also exist within the LS-DNA fraction, thus explaining the relatively large amount of radioactivity present in that fraction early on. Our results for $[^{14}\text{C}]$ orotic acid, shown in Figure 2(b), agree with this hypothesis. A small amount of label incorporated into the LS DNA would be the dominant signal detected at very early pulse times. As more label entered the NM-DNA fraction, a percentage decrease in the label in the LS DNA relative to that in the total labelled DNA would result. Since label in the HS-DNA fraction presumably arises from that in NM DNA (see above), its relative labelling would be dependent on that of the NM DNA. Therefore, it too would be low initially relative to LS DNA. This trend would continue until the label present in NM DNA was chased through the HS-DNA fraction into the LS DNA, when a significant rise in radioactive LS DNA would occur. This transition does indeed occur at the peak of $[^{14}\text{C}]$ orotic acid incorporation into NM DNA as this theory dictates. These early kinetics may also occur for the incorporation of deoxythymidine into DNA, however at such short pulse times that it is not observable using our techniques.

From the above and from previous data, we can conclude that all DNA precursors, arising both from the salvage and from the *de novo* synthetic pathways, are preferentially incorporated into NM sites of DNA synthesis. However, pre-matrix processing of these two precursor classes differ kinetically, which affects the incorporation rates.

Precursor incorporation into dTTP and into NM DNA

To determine whether nucleotide channelling is the source of the dissimilarity in NM incorporation kinetics that was observed for DNA precursors originating from the salvage as against the *de novo* synthetic pathways, we compared the incorporation of the respective precursors into the penultimate product, dTTP, with their ultimate incorporation into NM DNA (Figure 3). A detailed analysis of the $[^3\text{H}]$ deoxythymidine incorporation revealed that,

Figure 3 Deoxythymidine, but not orotic acid, is channelled to NM DNA replication sites

[^3H]Deoxythymidine (a) or [^{14}C]orotic acid (b) was injected into the hepatic-portal vein of regenerating rat liver and incorporation into dTTP (●) and NM DNA (○) was determined, as described in the Materials and methods section. Representative results of five experiments are shown; in each experiment, the specific radioactivity ratios of NM DNA to dTTP were > 1 for deoxythymidine incorporation and < 1 for orotic-acid incorporation, from 0 to 5 min post-injection.

after labelling for only 2 min, the specific radioactivity of thymine in NM DNA was 4-fold greater than that in soluble dTTP (Figure 3a). Even at 5 min post-injection, NM DNA retained a thymine specific radioactivity that was 2-fold higher than that in dTTP; that is, there was a clearly discernible incorporation of ^3H label into NM DNA beyond that which could have been supplied by the soluble cytosolic dTTP pool. These results provide convincing evidence for classically defined nucleotide channelling in living tissue.

The possibility of the existence of a similar channelling phenomenon to NM sites of DNA replication involving [^{14}C]orotic acid-derived thymidine was also investigated and the results are summarized in Figure 3(b). The specific radioactivity of the soluble dTTP pool progressively increased up to 50 min post-injection and then decreased. A similar incorporation pattern was observed for NM DNA but on a 10-fold lower scale. Contrary to the results with deoxythymidine, at no time was the specific radioactivity in NM DNA or the rate of ^{14}C radiolabel incorporation into NM DNA greater than that of incorporation into dTTP. This shows that orotic acid is not channelled by any classical mechanism to NM DNA replication sites in intact tissue.

DISCUSSION

We have shown that the *de novo* pathway DNA precursor, orotic acid, is incorporated into replicating DNA in regenerating rat liver more slowly than is the salvage pathway precursor, deoxythymidine (Figure 1). This lag was also clearly evident at the nuclear-matrix level of DNA analysis (Figure 2). Importantly, despite a delay in orotic-acid incorporation, the incorporation of orotic acid and deoxythymidine into LS, HS and NM DNA exhibited similar distribution patterns; both precursors preferentially labelled NM DNA before entering the extra-matrix fractions. These results support a model of nuclear matrix-associated DNA replication that is capable of using both salvage and *de novo* pathway precursors (Vaughn et al., 1990).

However, access of the injected precursors to the DNA replication machinery differed markedly (Figure 2). The incorporation of deoxythymidine into NM DNA clearly preceded that of orotic acid. This observation cannot result from differences in the access of injected precursors to the liver, since simultaneous injection of both precursors yielded the same results

(results not shown). However, the metabolic route of orotic acid to become dTTP is more complex than that of deoxythymidine. To overcome this metabolic discrepancy, the incorporation of radiolabel into the penultimate and ultimate moieties, that is, dTTP and NM DNA, respectively, was investigated. Our analyses revealed that, at early pulse times, the incorporation of exogenous deoxythymidine into newly replicated NM DNA significantly exceeded that in the extractable dTTP pool (Figure 3a). Since the specific radioactivity of thymine incorporated into NM DNA exceeded that which could have been supplied by the soluble cytosolic dTTP pool, we attribute this phenomenon to channelling in an intact mammalian system. Conversely, the orotic acid incorporated into NM DNA could easily have been provided by the soluble dTTP pool (Figure 3b), and thus is not channelled to NM replication sites.

Note that at no time did the specific radioactivities of thymine in NM DNA or in dTTP equal that of the injected precursor (Figure 3). While, under cell-culture conditions, isotope dilution may argue against efficient channelling of deoxythymidine, precursor dilution is normal in intact mammalian systems. Precursors injected into the bloodstream do not have equal access to all cells, especially during the short pulse times used in this study. Many replicating cells will not see the precursor at all and unlabelled DNA and dTTP from these cells will consequently dilute the radiolabelled DNA and dTTP from the cells that were accessed. Therefore, although the absolute specific radioactivities measured with respect to the injected precursors are small, the fact that the specific radioactivity of thymine in NM DNA exceeds that in dTTP at early pulse times verifies channelling *in vivo*.

The detection of channelling for deoxythymidine, but not for orotic acid, supports a model of compartmentalized synthesis of dNTPs in living tissue. The channelling mechanism *in vivo* appears to be extremely efficient, since nucleosides in the bloodstream are incorporated into replicating DNA within 2 min of injection (Figures 1–3). To account for this efficiency, our results suggest that the phenomenon of channelling involves transport of salvage pathway DNA precursors directly from the plasma membrane to the nuclear matrix. The most probable candidate for a membrane-associated enzyme that is responsible for deoxythymidine uptake from the bloodstream is thymidine kinase. Such an association has been proposed by Wawra (1988), who noted the rapid uptake of extracellular deoxythymidine by Swiss

3T6 mouse fibroblasts, whereas microinjection of nucleotides into cells revealed no evidence of channelling. Membrane-localized thymidine kinase would be the logical origin of deoxy-thymidine channelling to NM DNA replication sites, and a transport mechanism originating at the cell membrane would maximize the re-use of extracellular DNA precursors to help satisfy the high demand for nucleotides during DNA replication.

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