Labeling DNA with Stable Isotopes: Economical and Practical Considerations

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ABSTRACT

Labeling DNA with stable isotopes to measure cell proliferation can be a technique as effective as ^3H -thymidine labeling without the limitations imposed by using radioisotopes. Here, we investigated the relative efficiency of four nonradioactive precursors to DNA: $[1\text{-}^{13}\text{C}]$ -glycine, $[1,2\text{-}^{13}\text{C}_2]$ -glycine, $[U\text{-}^{13}\text{C}]$ -glucose, and $[U\text{-}^{13}\text{C}, ^{15}\text{N}]$ -thymidine. The efficiency of incorporation for each of these labeled precursors in HEP G2 cells in culture has been studied. When considering the actual costs of in vivo experiments in which large doses of labeled material are needed, economical constraints may play an important role in defining a practical method. Therefore, the economics of this process were also considered. Using the enrichment per dollar for whichever nucleoside had the highest incorporation in a given experiment, glycine is about five times more economical as a label than thymidine and eight times more economical than glucose in these cells.

INTRODUCTION

Incorporation of tritiated thymidine is the standard method for measuring DNA synthesis rates. The halogenated pyrimidine techniques, mostly using bromodeoxyuridine (BrUdR), were developed for flow cytometry and microscopy and have almost replaced autoradiographic techniques with ^3H -thymidine in cell kinetic studies. However, continuous exposure to BrUdR may entail a plethora of adverse effects (3). Thus, before the recent development of stable isotope-based methods (5,12,15), there had been no nontoxic, nonradioactive method to measure DNA synthesis rates in vivo.

We have developed an alternative strategy in which precursors labeled with a stable isotope (e.g., $[1\text{-}^{13}\text{C}]$ -glycine) are used to monitor DNA synthesis (5). The analytical approach to detect and quantify the stable isotope tags featuring the use of chemical reaction interface mass spectrometry (CRIMS) has been described in detail elsewhere (1). Briefly, deoxynucleoside residues obtained by digestion of DNA with DNase and phosphatases are separated by HPLC and (after elimination of the solvents) combined with a reactant gas in a microwave-induced He plasma. The result of this post-column chemical reaction is a set of stable low molecular weight compounds. For example, if O_2 is used as a reactant gas, then all the atoms of carbon are converted to CO_2 . The amount of isotopic label in these reaction products is then determined with high accuracy by an isotope ratio mass spectrometer (IRMS) connected online to the HPLC.

An alternative method, which also employs stable isotopes, has been developed by Macallan et al. (12). There, $[6,$

$6\text{-}^2\text{H}_2]$ -glucose was the metabolic probe. The deoxynucleosides liberated from DNA were derivatized, separated by gas chromatography, and the deuterium content in each nucleoside measured with a quadrupole mass spectrometer. The derivatization of the analytes and the use of conventional mass spectrometry limit the minimum detectable enrichment. They see ^{13}C abundance rise to a few percent (i.e., a relative enrichment of more than 100% from the approximately 1% natural abundance of ^{13}C with a precision of 0.3%). Measuring the $M + 2$ mass for dG that has an abundance of 0.16, this precision equates to a standard deviation of 0.0005.

The newest contribution to the field comes from Nissim et al. (15). They use 50 atom percent excess of $[U\text{-}^{13}\text{C}]$ -glucose and measure the enrichment of levulinic acid derived from all the deoxyribose species in their extracted DNA. When the levulinic acid contains 5 atoms of ^{13}C , the ratio of $[M + 5]^+$ to $[M]^+$ is 0.9%, giving them the ability to measure enrichments as low as 0.8 atom percent excess. They also use 40 atom percent excess $[^{15}\text{N}]$ -glycine and measure enrichment of the purines dG and dA in DNA.

In contrast, the use of CRIMS allows higher accuracy. Even with a conventional quadrupole mass spectrometer analyzer, a baseline isotope ratio of about 0.01 is generated, and isotopic enrichments for ^{13}C with a precision of 0.007% can be measured (10). Furthermore, with no derivatization, neither carbon nor other elements is added that contributes to the baseline isotope ratio or dilutes the enrichment observed. Most importantly, we use a multicollector IRMS to determine yet tenfold lower enrichments than with a conventional mass spectrometer (2,6,7,17).

Beyond developing a highly sensitive assay method, we are also investigating the most effective and economical method by looking at different labeled precursors of DNA. For each experiment in humans, Macallan et al. (12) used 60 g [6,6- $^2\text{H}_2$]-glucose that currently sells for \$75/g for a total cost of \$4500. This is a high-priced reagent for an assay that might be routinely used. The efficiency of any labeling procedure can limit its applicability. Four approaches to the use of stable isotope-labeled tracers to measure DNA synthesis rates are presented here: (i) and (ii) both [1- ^{13}C]- and [1,2- $^{13}\text{C}_2$]-glycine, which are precursors of de novo purine biosynthesis; (iii) glucose, which can be incorporated by de novo deoxynucleotide biosynthesis or by salvage; and (iv) thymidine, which is incorporated via a salvage pathway. We have quantified the ability of each type of precursor to label DNA in a human hepatoma cell line following a 24-h incubation. In this way, the efficiency of each type of label and the processes that it uses to incorporate in DNA are determined.

MATERIAL AND METHODS

Obtaining DNA from Cells

The method for cell culture, DNA extraction, and nucleoside purification has been previously described (5).

Briefly, HEP G2 cells (obtained from ATCC, Manassas, VA, USA) were grown continuously in Dulbecco minimal essential medium (DMEM; Sigma, St. Louis, MO, USA) supplemented per liter with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (both from Biofluids, Rockville, MD, USA), NaHCO_3 , glutamine, and pyruvate (Sigma). To find out whether or not FBS has any nucleosides that could interfere with glycine's incorporation into DNA, instead of normal FBS in the media, we used FBS dialyzed with either a 1 or 10 kDa molecular weight cutoff (Sigma). The cells were fed three times a week, and they were split once a week. Cells were grown for six days (confluency 70%–80%) and then labeled for 24 h (confluency 85%). The labels added to the medium (based on 100 mL) were: 0.32 mg [1- ^{13}C]-glycine and 0.135 mg [1,2- $^{13}\text{C}_2$]-glycine (both Cambridge Isotope Laboratories, Andover, MA, USA), 4.5 mg [U- ^{13}C]-glucose and 0.16 mg [U- ^{13}C , ^{15}N]-thymidine (both from Martek Biosciences, Columbia, MD, USA). Unlabeled cells were harvested along with the labeled cells.

Cells ($1\text{--}3 \times 10^8$) were harvested in 30 mL cell lysis buffer (PuregeneTM; Gentra System, Minneapolis, MN, USA). Proteinase K (100 $\mu\text{g}/\text{mL}$) was added overnight, then RNase (20 $\mu\text{g}/\text{mL}$ for 2 h at 37°C; both from Roche Molecular Biochemicals, Indianapolis, IN, USA). Ten milliliters of Puregene

protein precipitation solution were added, vortex mixed, and the DNA solution was placed on ice for 5 min. The solution was then centrifuged. Isopropyl alcohol (30 mL) was added to the supernatant, inverted 50 times, and centrifuged. The pellet was dried under N_2 washed with 30 mL 70% ethanol, centrifuged again, and then dried overnight at room temperature. The DNA was denatured by boiling followed by ice (5 min) and resuspension of the DNA to 0.5 mg/mL. Nuclease P1 and DNase 1 (Roche Molecular Biochemicals) were incubated with the DNA solution for 2 h at 45°C. The mixture was then incubated with phosphodiesterase followed by alkaline phosphatase (Roche Molecular Biochemicals).

As before (5), the deoxynucleosides were purified using an RCM Nova-Pak[®] HPLC column (Waters, Milford, MA, USA) with a solvent system of 5 mM ammonium acetate (pH 4.0) and 50:50 acetonitrile:water running at 2 mL/min. A 5-min hold at 0% B was followed by a linear gradient to 90% B in 5 min. In this preparative separation, deoxycytosine is lost along with impurities. The fraction containing T, dA, and dG is evaporated on a SpeedVac[®] (Savant Instruments, Holbrook, NY, USA) and reconstituted in water. This sample is analyzed with the HPLC/CRI/IRMS system using a $4.6 \times 250\text{-mm}$ SupelcosilTM LC-18-S HPLC column (Supelco, Bellefonte, PA, USA). The same solvent system was used at 1 mL/min and a linear gradient of 5%–25% B in 15 min.

Determining the Isotope Ratio

After being separated by the HPLC, the deoxynucleosides flow into the high-temperature plasma where CO_2 , among other products, is formed from each analyte. The $^{13}\text{CO}_2/^{12}\text{CO}_2$ abundances were determined from the m/z 45 and m/z 44 traces (see Figure 1) with a Delta S IRMS (Finnigan/MAT, San Jose, CA, USA) and its accompanying Isodat data system. 5-Fluorodeoxyuridine (Sigma) was used as an internal isotope ratio standard. The data are presented as the percent DNA enrichment for each deoxynucleoside by the equation $100 \times (\text{IR}_{\text{experimental}} - \text{IR}_{\text{control}}) / \text{IR}_{\text{control}}$.

Figure 1. HPLC/CRI/IRMS chromatogram of DNA nucleosides from HEP G2 cells incubated with [U- ^{13}C , ^{15}N]-thymidine. The substantial enrichment in the T peak is evident by the greater relative size of the T peak in the m/z 45 channel than in the m/z 44 channel. The injection was made at the time shown by the arrow. For clarity, the m/z 45 channel is shifted up by 500 mV.

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Table 1. Enrichment in Deoxynucleosides for Four Different DNA Precursor Species

Nucleoside		dG		T		dA	
Labeled Precursor		Isotope Ratio	% DNA Enrichment	Isotope Ratio	% DNA Enrichment	Isotope Ratio	% DNA Enrichment
[1- ¹³ C]-glycine	C	0.01178	—	0.01175	—	0.01183	—
	L	0.01211	2.8	0.01177	ns	0.01186	ns
[1,2- ¹³ C ₂]-glycine	C	0.01177	—	0.01173	—	0.01175	—
	L	0.01221	3.7	0.01169	ns	0.01186	0.9
[U- ¹³ C]-glucose	C	0.01178	—	0.01174	—	0.01180	—
	L	0.01357	15.2	0.01295	10.3	0.01293	9.6
[U- ¹³ C, ¹⁵ N]-thymidine	C	0.01181	—	0.01188	—	0.01185	—
	L	0.01190	0.8	0.03395	186	0.01213	2.4

The values are expressed as the ¹³CO₂/¹²CO₂ ratio and the percent enrichment of each deoxynucleoside in DNA. C, control; L, labeled medium; ns, not significant.

RESULTS

As in our prior work (5), the 0.4 mM glycine DMEM concentration was enriched to 9%, done here by adding 0.32 mg [1-¹³C]-glycine per 100 mL medium and incubating the cells for 24 h. The resulting DNA showed enrichment of 2.8% for dG with no significant enrichment of dA (Table 1). The specificity of incorporation of [1-¹³C]-glycine into purines and not pyrimidines was again supported by seeing no enrichment of T. After adding 0.135 mg [1,2-¹³C₂]-glycine per 100 mL medium to achieve a 4.5% enrichment (i.e., about the same amount of ¹³C as with [1-¹³C]-glycine), T still did not change, but there was an increase in DNA enrichment for dG (3.7%) and a small enrichment of dA (0.9%). Adding 4.5 mg [U-¹³C]-glucose per 100 mL medium enriched the 5 mM glucose concentration 4.5%. All three measured nucleosides showed elevated ¹³C/¹²C ratios to a greater extent than with either glycine species (Table 1). However, with [U-¹³C]-glucose, five labeled carbons appear in each deoxynucleoside compared with one or two with the glycines. The enrichments per labeled carbon from the glucose are somewhat higher for dG and much higher for dA than with glycine.

Adding 0.16 mg [U-¹³C, ¹⁵N]-thymidine per 100 mL medium made a 6.3 μM concentration. While this concentration is much lower than the glycine

or glucose concentrations, a very large increase in the enrichment of T from DNA was observed. Thymidine has 10 heavy carbons, which is another factor contributing to the high enrichment observed. Seeing enrichment of dA (Table 1) in the experiment where thymidine was the tracer was surprising, so we looked carefully at the labeled T starting material. Using the same chromatographic system but with a quadrupole MS as a detector (10), we found small amounts of ¹³C-labeled dA (0.4%) and dG (about 2%). One would have to hypothesize that dA is selectively utilized over T and dG to explain why dA is enriched (Table 1) beyond its less than 1% impurity level, while dG is less enriched than its impurity level. No known process converts T to dA.

Although replications were not done in these experiments, the constant value of isotope ratio for the four control experiments exemplifies the run-to-run consistency. For dG, T, and dA, the means and standard deviations of the isotope ratio in the control experiments are given in Table 2. Obviously, the reproducibility of the method is much better than needed to carry out experiments at this level of enrichment.

DISCUSSION

This set of results using [1-¹³C]-glycine gives enrichments that differ

substantially from our previous experiments (5). There, also using a 9% enrichment for 24 h in HEP G2 cells, we observed equal enrichments of dA and dG, and those enrichments were larger [a Δδ of 40%–45%—equivalent to a 4%–4.5% (relative) increase in isotope ratio] than those seen here: no enrichment of dA while dG rose less than 3%. The most obvious difference between our earlier experiments and the current ones is the origin and passage number of the HEP G2 cells used. In the earlier experiments, we obtained our cells from another researcher and cultured them continuously after that. For the present experiments, we obtained a new frozen aliquot of cells directly from ATCC. If a difference existed in the relative balance of salvage versus de novo DNA synthesis processes between the two cell lines, the variable results for the glycine experiments might be rationalized. The much lower response of dA compared with dG might represent more efficient salvage of dA because of its greater abundance in the DNA. We attempted to evaluate whether the lower incorporation of glycine was due to preformed nucleosides in the FBS by repeating the experiments with dialyzed FBS, which should have reduced or eliminated concentrations of nucleosides. The results were not meaningfully different, indicating that de novo purine biosynthesis was not inhibited by exogenous purines.

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Table 2. Reproducibility of Isotope Ratios in Control Experiments

Nucleoside	Mean $^{13}\text{C}/^{12}\text{C}$	
	Ratio	sd
dG	0.011785	0.000017
T	0.011775	0.000070
dA	0.011807	0.000043

Table 3. Efficiency of Labeling DNA

[1- ^{13}C]-Glycine	0%–8.7%/mg
[1,2- $^{13}\text{C}_2$]-Glycine	7%–27%/mg
[U- ^{13}C]-Glucose	2.1%–3.4%/mg
[U- ^{13}C , ^{15}N]-Thymidine	1163%/mg

The enrichments include the range of values for each label in Table 1.

Table 4. Economics of Labeling DNA

Cost of labeled precursor for 100 mL medium ^a :	
[1- ^{13}C]-Glycine	\$0.034
[1,2- $^{13}\text{C}_2$]-Glycine	\$0.042
[U- ^{13}C]-Glucose	\$1.35
[U- ^{13}C , ^{15}N]-Thymidine	\$11.20
Economic efficiency of labeling in vitro:	
[1- ^{13}C]-Glycine	82%/\$
[1,2- $^{13}\text{C}_2$]-Glycine	88%/\$
[U- ^{13}C]-Glucose	11%/\$
[U- ^{13}C , ^{15}N]-Thymidine	17%/\$

The nucleoside giving the highest enrichment was used to calculate the economic efficiency.

^a[1- ^{13}C]-glycine sells for \$105/g (Cambridge Isotopes); [1,2- $^{13}\text{C}_2$]-glycine sells for \$310/g (Cambridge Isotopes); [U- ^{13}C]-labeled glucose sells for \$300/g (Martek Biosciences); [U- ^{13}C , ^{15}N]-thymidine sells for \$70/mg (Martek Biosciences).

In planning our original set of experiments, we were not clear whether the 2-carbon of glycine would enter the 1-carbon pool to any significant extent, which is why we did not use [1,2- $^{13}\text{C}_2$]-glycine there. Here, we do not see any labeling of T with the di-labeled glycine, suggesting that any incorporation of glycine into pyrimidines from serine produced via the glycine-serine conversion is limited. Further, any contribution of $^{13}\text{C}\text{O}_2$ from the catabolism of thymidine to the biosynthesis of either purines or pyrimidines must be trivial, given the small amount of thymidine compared to other sources of the 1-carbon pool.

The data in Table 1 have been used to evaluate the relative efficiency and cost factors when performing in vitro experiments with HEP G2 cells. Labeled thymidine generates by far the greatest enrichment, but that observation is the net result of several factors. Glucose and glycine were each enriched to 4.5%–9% based on the concentration

of each species in the medium. Thymidine is not a component of the medium, so any nucleic acid species has to come from the 10% FBS. We added 0.16 mg/100 mL thymidine, an amount arbitrarily chosen to parallel the amount of [1,2- $^{13}\text{C}_2$]-glycine we had used. Under these conditions, the labeled T is not a tracer but is adding significant mass to the system. Ultimately, we must achieve a level of T that does not affect the DNA replication rate. Clearly, each milligram of labeled thymidine enriches the thymidine pool by a much larger amount than each milligram of glucose or glycine. Information on the efficiency of each precursor to label nucleosides from DNA is presented in Table 3. These enrichments combine with the magnitude of de novo and salvage pathways of DNA synthesis to establish the overall efficiency of labeling.

At 1 g/L in plasma or medium, glucose is much more concentrated than glycine (0.03 g/L), with plasma thymidine concentrations reported to be very

low—in the $\mu\text{g/L}$ range (4,16). As such, each tracer requires a specific amount to achieve a certain enrichment of the precursor pool, and this factor determines how expensive each experiment is. Table 4 shows the cost per unit enrichment of the four species examined in these experiments. Each of these values is obtained by dividing the best enrichment per milligram from Table 3 by the cost per milligram of each tracer.

The second factor governing efficiency is the mechanism of utilization of the labeled precursor. Glycine uses de novo purine biosynthesis, glucose becomes D-ribose 5-phosphate, which subsequently leads to both purine and pyrimidine nucleotides, and thymidine participates directly in salvage. As our experiments indicate, the de novo purine route may be variable, and the amount of label needed to quantify cellular replication rates may have to be several-fold higher than minimally necessary to assure the success of this scheme.

If at higher than “tracer” amounts, various rate-limiting steps could cause the labeling of DNA to be more or less dependent on the amount of labeled precursor administered. Although we did not investigate other concentrations of precursor in this study, in our first study with [1- ^{13}C]-glycine (5), we did use much higher enrichments in a preliminary experiment and found a less than proportional enrichment: a five-fold higher enrichment of dG and dA from DNA rather than the tenfold expected. In the current set of experiments, only thymidine was present at greater than tracer amounts. Even so, the use of our method to measure cellular growth rates is not concerned with the maximum enrichment achieved. Monitoring the wash out of a given level of isotopic enrichment will accurately evaluate the kinetics of DNA synthesis without having to observe a plateau in ^{13}C incorporation.

Besides cellular efficiency, another component of an in vivo method is the kinetics of the labeled precursor. Information about the half-life of each species is found in the literature. Following various doses of labeled glycine in rabbits, Nissim and Lapidot measured a $t_{1/2}$ of about 14 min that was independent of dose (14). A similar value was found in humans (11). Thymidine is

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also short-lived. In dogs, Covey and Straw measured a $t_{1/2}$ of 12–18 min (8). Moyer et al. measured a 10-min $t_{1/2}$ in mice (13). The clearance of glucose is somewhat slower, with a 42-min $t_{1/2}$ calculated from the data in Wolfe (18) for the terminal phase after a bolus in sheep.

Thus, none of these precursor species is long-lived, raising the question of how to dose animals (or humans). We are carrying out further experimentation to evaluate whether chronic infusions are required, as done by Macallan et al. (12) and Hellerstein et al. (9), or whether an acute or sub-chronic dosing schedule will provide sufficient enrichment of DNA to yield the desired results. A steady state in DNA enrichment does not have to be achieved for the wash out of label to reflect the kinetics of cellular replication.

From the data in Table 4, we conclude that glycine (in either form) is the most economical DNA precursor species in these cultured cells. Obviously, we have concern about the different enrichments obtained in our original and these repeated experiments. However, this problem is mitigated by recognizing that the peak enrichment in any one nucleoside is not the basis of the ultimate measurement, but rather serial samples to monitor the rate of decline of the enrichment will be used to quantify DNA synthesis rates. If we can achieve enrichments of a few percent (relative), the turnover of the labeled DNA can be measured. Higher enrichments provide better precision, but curve fitting a number of points as a function of time averages the errors of individual points. At the lowest levels of enrichment detectable with an IRMS, it will be necessary to have at least one nucleoside that does not incorporate the tracer species to serve as an internal isotope ratio standard. Glycine and thymidine achieve this goal, but the labeling of all nucleosides with glucose removes this possibility.

CONCLUSIONS

This work shows how each of four different labeled species incorporates into DNA in HEP G2 cells. Although the efficiency of thymidine per milligram is very high, its cost reduces

much of that advantage. Uniformly labeled thymidine is a new product, and its price would likely go down dramatically if usage increased significantly. Glycine has low efficiency, but its low cost and low concentrations in biological fluids make it the most economical. Glucose has the worst economics because the high concentration of glucose in biological fluids requires more labeled glucose for a given extent of labeling than either glycine or thymidine. These cellular efficiencies may be important considerations when carrying out these experiments in vivo, but the kinetics of each tracer will also play a major role in establishing the overall efficiency of these DNA labeling methods.

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