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CHOICE OF LABELED PRECURSORS IN SYNTHESIS OF DNA FOR MOLECULAR HYBRIDIZATION

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SUMMARY: Tritiated deoxyguanosine triphosphate, labeled in the 8 position of the purine ring, is frequently used as a precursor in the preparation of complementary DNA to be used in molecular hybridization reactions. This compound has been reported to exchange the labeled tritium with water in solution at high temperature. We have investigated the loss of label from DNA prepared with this compound, under conditions commonly used for molecular hybridization reactions. The loss of label follows first order kinetics, with a half time of 17 hrs. under the conditions used. This loss of label influences the reliability of the results of the hybridization reactions, and in some cases may confuse their interpretation. It is recommended that use of complementary DNA labeled with [8-^{3}H]-dGTP be avoided in molecular hybridization experiments.

Complementary DNA (cDNA) labeled with [8-^{3}H] deoxyguanosine is commonly used in nucleic acid hybridizations (1-7). Considerable information has been reported on the loss of tritium atoms by exchange from the 8-position of purines in acid or alkaline solutions at various temperatures or in neutral solutions at high temperatures (100^o or greater) (For review see reference 8). Little information is available concerning the stability of tritiated purines under conditions routinely used for nucleic acid hybridization studies. Harrison et al. (9) reported that an apparent decrease in the ability of globin cDNA to bind to hydroxyapatite from an initial value of 90% to 60% after 4 weeks of incubation in 0.12M phosphate buffer, pH 6.8 at 60^o was due to tritium exchange. It is not clear from this report what labeled precursors were used in the synthesis of the cDNA.

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Using DNA prepared with [8-^{3}H] deoxyguanosine we observed substantial loss of tritium from TCA-insoluble material upon incubation under conditions commonly used for hybridization. We have established that this loss can be accounted for by exchange of the tritium with the hydrogens of water.

MATERIALS AND METHODS: Reagents. The following reagents were used: Chelex 100 (Bio-Rad); dATP, dGTP and dTTP (Sigma); [8-^{3}H]dGTP, 15 Ci/mmol (Amersham/Searle); [5-^{3}H]dCTP, 20 Ci/mmol (Amersham/Searle); [α-^{32}P]dGTP, 150 Ci/mmol (Amersham/Searle); oligo (dT)$_{10}$ (Collaborative Research, Inc.); AMV RNA-dependent DNA polymenase (Life Sciences, Inc., St. Petersburg, Fla.).

PURIFICATION OF GLOBIN MESSENGER RNA: Reticulocytosis was induced in BALB/c mice as described by Gorski et al. (10). Reticulocytes were prepared and lysed according to Lebley (11) and polysomes were isolated by the method of Evans and Lingrel (12). RNA was extracted from polysomes by a modification of the procedure described by Brawerman (13), which consisted of an initial treatment with 50 µg/ml of Proteinase K. The poly (A) - containing RNA was isolated by chromatography on poly (U) - Sepharose and further fractionated on successive sucrose gradients as described by Honjo et al. (14). The RNA was assayed in the wheat germ cell-free protein synthesizing system as described by Roberts and Patterson (15).

SYNTHESIS OF GLOBIN cDNA: The synthesis of globin cDNA using either [^{3}H]dGTP, [^{3}H]dCTP, or [^{32}P]dGTP was as described by Kacian and Myers (16). The procedure was modified in some cases to include the passage of the cDNA, after alkaline hydrolysis, through a G-50 Sephadex column containing a layer of Chelex 100 (a chelating resin) in the bottom (17). This step was included to remove metal ions that may be present in the cDNA preparation, since catalytic degradation of DNA can occur at high temperatures in the presence of metal ions (18). Chelex 100 was prepared as described by Willard et al. (19).

TREATMENT OF DNA PREPARATIONS: DNA preparations were placed in 300mM NaCl, 40mM sodium phosphate, pH 6.8 and 2mM EDTA (Buffer A). Two 25 µl aliquots of each mixture were sealed in 40 µl glass capillaries which had been treated as described by Wilson et al. (20). After heating at 100° for 2 min, one aliquot was incubated at 70°, or as indicated, and the other aliquot was stored at -20°. At the indicated times each aliquot was precipitated with TCA in the presence of 100 µg of yeast RNA. The percent loss of TCA-precipitable radioactivity was determined using the aliquot stored at -20° as an unincubated control.

ANALYSIS OF TREATED cDNA PREPARATIONS ON SUCROSE GRADIENTS: Preparations of cDNA labeled with [^{32}P]dGTP, [^{3}H]dGTP or [^{3}H]dCTP were treated as described above. At the end of 20 hr. of incubation, the contents of the capillaries were expelled into 100 ul of buffer A and placed on ice. An aliquot (20 µl) of each mixture was precipitated with TCA and the remainder (100 µl) was placed on 5-20% sucrose gradients containing 10mM Tris (pH 7.5), 100mM KCl and 1mM EDTA. The gradients were centrifuged for 24 hr. at 41,000 rpm in a SW41 rotor. The gradients were fractionated into 0.45 ml fractions. An aliquot (0.2 ml) of each fraction was counted

in 5 ml of Aquasol. The remaining 0.25 ml was precipitated with TCA in the presence of 100 ug of yeast RNA, collected on membrane filters (Millipore, HA) and counted in a toluene-based scintillator containing 0.4% BBOT.

RESULTS: When mouse globin cDNA labeled with [^{3}H]dGTP was incubated under conditions routinely used for cDNA-RNA or cDNA-DNA hybridization (in buffer A at 70^o), 80% of the TCA-precipitable radioactivity was lost after 40 hr. of incubation. To insure that this loss did not result from catalytic degradation of the cDNA at high temperatures by metal ions present in the hybridization buffer, the buffer was passed through Chelex 100. The cDNA labeled with [^{3}H]dGTP was also passed through a G-50 Sephadex column containing Chelex 100 in the bottom. The cDNA preparation and a preparation of mouse spleen DNA labeled with [^{3}H] thymidine were placed in Chelex-treated buffer A and incubated as described in Materials and Methods. After 40 hr. of incubation, the TCA-precipitable radioactivity was determined. Again, 80% of TCA-precipitable radioactivity was lost for the cDNA labeled with [^{3}H]dGTP. However, less than 10% of the TCA-precipitable radioactivity was lost for the tritiated mouse spleen DNA.

The loss of TCA-precipitable radioactivity from the cDNA labeled with [^{3}H]dGTP was temperature dependent since it occurred at 70^o but not at 25^o (Table IA).

The following experiment was performed to demonstrate that the loss of TCA-precipitable radioactivity from cDNA labeled with [^{3}H]dGTP at 70^o was due to tritium exchange. Globin cDNA labeled with either [^{3}H]dGTP, [^{3}H]dCTP or [^{32}P]dCTP was synthesized. These cDNA preparations were placed in buffer A and incubated as described. After 20 hr. of incubation, and aliquot of each mixture was precipitated with TCA (Table IB). Fifty-five percent of the TCA-precipitable radioactivity was lost for cDNA labeled with [^{3}H]dGTP; however, essentially no loss occurred for cDNA labeled with [^{3}H]dCTP or [^{32}P]dGTP. An aliquot of each mixture was also placed on 5-20% sucrose

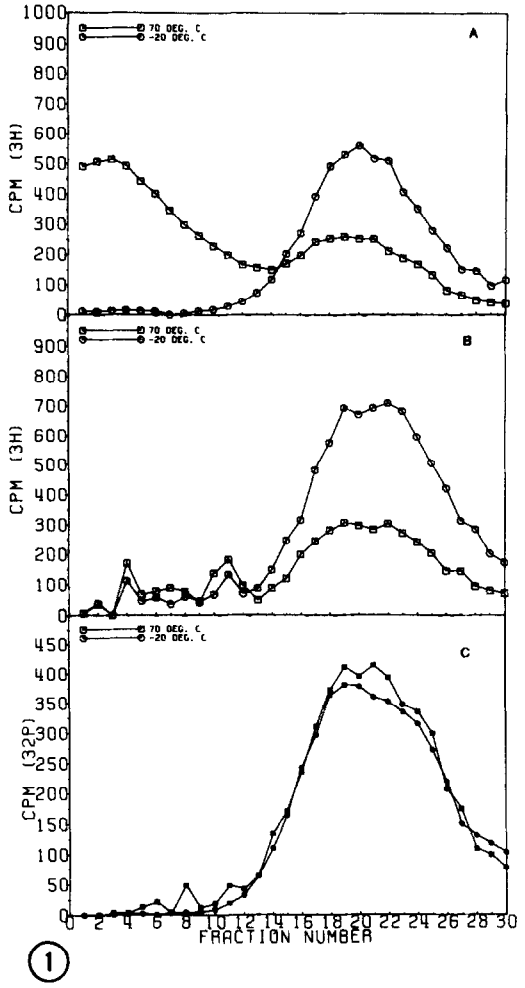
TABLE 1

STABILITY OF LABEL IN DNA UNDER VARIOUS CONDITIONS

Experiment	Label in DNA and relevent condition	Incubation Temperature	TCA-insoluble cpm	Percent Loss of label
A	12 hr incubation, buffer A, [^{3}H]dGTP	-20	1225	
		25	1220	0.4
		70	730	40
B	20 hr incubation, buffer A			
	[^{3}H]dGTP	-20	2082	
		70	1386	55
	[^{3}H]dCTP	-20	1089	
		70	1056	3
	[^{32}P]dGTP	-20	2934	
		70	3045	0
C	20 hr incubation, [^{3}H]dGTP			
	buffer A	-20	1949	
		70	849	56
	50% (v/v) formamide in buffer A	-20	1959	
		43	1927	2

gradients and centrifuged as described. The gradients were fractionated and total radioactivity was determined for part of each fraction and TCA-precipitable radioactivity for the remainder.

The majority of the radioactivity of the cDNA labeled with [^{3}H]dGTP incubated at 70° was located at the top of the gradient (Fig. 1A), and was not TCA-precipitable (Fig. 1B). This is the result that would be expected if the tritium had been exchanged with water. The remainder of the radioactivity in this gradient was located at the same position as observed for cDNA labeled with [^{3}H]dGTP that had been stored at -20°. No radioactivity was observed at the top of the gradients for cDNA labeled with [^{32}P]dGTP



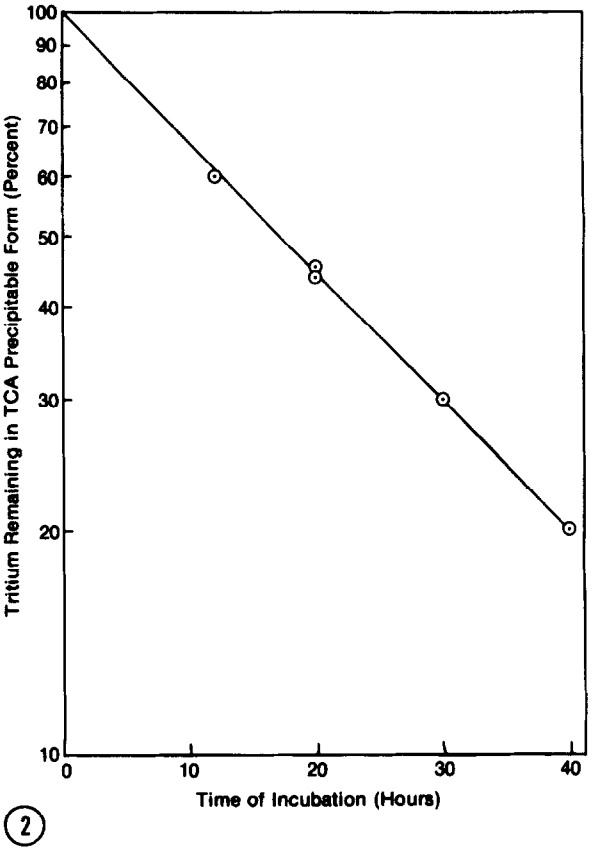


Fig. 1 Sucrose gradients (5-20%) of aliquots of labeled cDNA preparations incubated at -20° and at 70°. Sedimentation is from left to right. A: cDNA labeled with [^{3}H]dGTP (total counts). B: cDNA labeled with [^{3}H]dGTP (TCA-insoluble counts). C: cDNA labeled with [^{32}P]dGTP (total counts).

Fig. 2 Time course of loss of label from cDNA labeled with [^{3}H]dGTP and incubated at 70° in buffer A.

(Fig. 1C). Gradients of cDNA labeled with [^{3}H]dCTP and incubated as described had profiles that were essentially identical to Fig. 1C.

The time course of the loss of label from cDNA labeled with [^{3}H]dGTP is shown in Fig. 2. The reaction follows pseudo first-order kinetics, as

would be expected, since water is in excess and the rate should depend only on the concentration of labeled DNA present. The reaction has a half-time of 17 hours for the hybridization conditions described.

The loss of tritium, due to exchange, from the cDNA labeled with [^{3}H]dGTP could be avoided by altering the conditions used for hybridization analysis. McConaughy et al. (21) have used formamide to reduce the temperature of hybridization (1% formamide reduces the T_m by 0.72^o). The addition of 50% formamide to buffer A made it possible to lower the incubation temperature to 43^o (22). Under this condition, tritium exchange did not occur (Table 1C).

DISCUSSION: The use of [^{3}H]dGTP as a precursor for the synthesis of labeled cDNA should be avoided since this cDNA will be susceptible to tritium exchange under the conditions used for nucleic acid hybridization studies at high temperatures. When such DNA is used as a probe, the total amount of radioactivity in each DNA sample will be a function of the time of incubation. In extended reactions, the label remaining in DNA may fall below acceptable levels for counting. Even more serious problems might be encountered when a mixed label (e.g. [^{3}H]dGTP and [^{3}H]dCTP) is used, since label would be lost preferentially from guanosine-rich segments of the probe, so that cytosine-rich segments would be overrepresented in the hybrid detected.

On the basis of other studies concerning the stability of [^{3}H]-adenine under various conditions (Evans et al., 1970), the use of [^{3}H]dATP as a precursor should not be considered since the tritium in the 8-position will also be subject to exchange. The tritiated precursor of choice for the synthesis of DNA complementary to poly (A) - containing mRNA is dCTP, since no apparent exchange occurs when cDNA labeled with [^{3}H]dCTP was incubated in buffer A at 70^o for 20 hr.

For occasions when it is desirable to use highly labeled cDNA, cDNA can be synthesized using [^{3}H]dGTP, [^{3}H]dATP and [^{3}H]dCTP. Tritium exchange

of this cDNA, during hybridization reactions, can be avoided by using formamide and lowering the incubation temperature.

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