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Molecular Assessment of S1 Endonuclease-Resistant Snapback Hairpin Loops Generated by DNA Polymerase I During the In-Vitro Nick Translation Reaction

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ABSTRACT

The in-vitro nick translation reaction used to label DNA to high specific activity also produces aberrant DNA structures known as "snapback" hairpin loops. Hairpin structures are precluded from participating in precise DNA–DNA hybridization interactions. Three nick translation systems were all found to yield significant quantities of snapback hairpins, as determined by their resistance to S1 endonuclease digestion following denaturation. The relative quantities of hairpins produced correlated with both the mass average size of the final DNA probe product synthesized as well as the overall rate of the nick translation reaction. Decreases in the amount of exogenous DNase I used in nick translation reactions produced significant decreases in the amount of hairpin loop structures formed. Hairpins could be effectively removed from nick-translated DNAs by employing hydroxylapatite column chromatography. Strategies to reduce hairpin formation during nick translation and the removal of hairpins from nick-translated DNAs are presented.

Index Entries: Nick translation; snapback hairpin loops of DNA; S1 endonuclease, resmstance of DNA to; labeled DNA probes; hydroxylapatite chromatography, removal of snapback DNA by; DNase I, generation of snapback hairpin DNA loops in response to;

endonuclease, resistance of DNA to S1; chromatography of snapback DNA with hydroxylapatite; polymerase, generation of snapback hairpin DNA loops in response to; translation, snapback hairpin DNA loops during nick.

INTRODUCTION

The method for the in vitro labeling of DNA to relatively high specific activity by nick translation (NT)† (*1–4*) has proven very useful for the study of DNA molecules. The ability to label DNA segments or cloned DNA sequences from genomic DNA or from cDNA in plasmids (or other vectors) has been attractive for the detection, isolation, and analysis of specific DNA sequences. Consequently, nick translation is a very strategic tool used in contemporary molecular biology; the applications for the use of nick-translated DNA molecules have been numerous (*5*).

Most experimental approaches that employ nick-translated DNAs commonly include Southern blotting techniques (*6*) or modifications of other solid-phase hybridization reactions (*5,7*). DNAs labeled from moderate to high specific activities using modifications of this nick translation reaction usually provide adequately labeled probes for such approaches. However, in the preparation of certain nick-translated DNAs to be used as probes in free solution DNA–DNA hybridization reactions (*8*), which can be assayed using S1 endonuclease (S1) to monitor the degree of hybrid formation (*8,9*), we and others (*2,4,10,11*) have experienced the synthesis of nick-translated probes possessing incomplete S1 sensitivity for the zero-time hybridizing sample of the hybridization reaction. The preparation of a linear, single-stranded, S1 sensitive probe is favorable for accurate interpretations of free-solution DNA–DNA (Cot) or other DNA–RNA hybridization analyses.

Many parameters are known to affect the quality and specific activity of DNA probes prepared by nick translation using *E. coli* DNA polymerase I (Pol I) (*1,2,4,5*); these include time, temperature of the reaction, molar concentrations of both the unlabeled and labeled deoxynucleotide triphosphates, the amount of DNase I present, and the quantity and quality of the Pol I.

One aberrant DNA structure formed during the nick translation reaction includes the "snapback" hairpin loop (*2,4,10–13*), which has the property of being resistant to digestion by S1 endonuclease after DNA denaturation because of the rapid "snapback" hybridization that immediately occurs in the absence of denaturing conditions. The importance

†Abbreviations Used: BM, Boehringer Mannheim; BRL, Bethesda Research Laboratories; cDNA, complementary DNA to mRNA; CPM, counts per minute; ds, double stranded; HAP hydroxylapatite; NEN, New England Nuclear; NT, nick translation; PO_4, phosphate; Pol I, E. coli DNA polymerase I; S1, S1 endonuclease; ss, single-stranded.

of this aberrant DNA structure as a technical obstacle to free solution Cot analysis has not been emphasized or quantitatively explored. This report describes the occurrence of the snapback hairpin loops that are generated during the use of three popular nick translation methods, how the rate of DNA synthesis and product size correlate with hairpin loop formation, and strategies to prevent the formation of hairpins as well as the removal of hairpins from nick-translated DNA probes. The results allow for a better understanding of nick translation chemical events and chosing of nick translation systems that generate labeled DNA molecules to better satisfy experimental nucleic acid hybridization interactions.

METHODS

DNA Preparations

Plasmid DNA pBR322 was isolated and purified according to Norgard (*14*).

Nick Translation Systems

All *E. coli* DNA polymerase I preparations were stored at −60°C.

Nick translation reactions were carried out using the nick translation system kit (No. NEK-004) from New England Nuclear (NEN) (Boston, MA). A 1 μg quantity of pBR322 in a total volume of 40 μL was nick-translated for 90 min according to the reaction conditions described by the manufacturer.

Nick translation reactions were also carried out using a nick translation reagent kit (No. 8160) from Bethesda Research Laboratories (BRL) (Gaithersburg, MD). A 1 μg quantity of pBR322 in a total volume of 100 μL was nick-translated according to the conditions described by the manufacturer, except that the 60 min reaction was performed at 14°C instead of 15°C. As indicated, the DNase I solution (1 mg/mL) was activated and diluted 1 % 10,000 before use.

Nick translations were also performed in a 100 μL reaction mixture composed of 200 μCi of α-[^{32}P]-dCTP (predried in tube), 50 m*M* Tris-HCl (pH 7.8), 5 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 100 μg/mL bovine serum albumin (BRL enzyme grade), 2 nmol each of dATP, dTTP and dGTP (PL Biochemicals, Milwaukee, WI), 1 μg of pBR322 plasmid DNA, and 3–4 Richardson units (*15*) of *E. coli* DNA polymerase I (Boehringer Mannheim [BM], Indianapolis, IN, Grade I, nick translation grade, no. 104-485, lot 1299437). The reaction was allowed to proceed at 14°C for 1.5–2.5 h. Good consistency between various lots of this enzyme with respect to endogenous DNase levels and activities was found.

All three nick translation reaction mixtures above contained 200 μCi of α-[^{32}P]-dCTP (NEN, NEG-013X, 600-800 Ci/mmol) corresponding to about 200–300 pmol/μg pBR322 DNA. Kinetics of incorporation during

nick translation were monitored by sampling 1 μL aliquots periodically from each reaction mixture, followed by dilution in water, and using a portion in the DEAE (DE-81, Whatman) filter binding assay of Maxwell et al. (*16*). After all nick translations were completed, 4–10 μL of 0.25*M* EDTA (pH 8.0) stop buffer was added per reaction tube, followed by the addition of 5 μg of purified *E. coli* tRNA and phenol extraction. Column chromatography of the aqueous phase over Ultrogel AcA54 (LKB Instruments, Durham, NC) was performed to separate unincorporated label from the labeled DNA probe. Probes were routinely dissolved in either sterile distilled water or 10 m*M* Tris-HCl (pH 7.6), 10 m*M* NaCl, 1 m*M* EDTA. Products of nick translation were subjected to 1% agarose gel electrophoresis under denaturing conditions (glyoxal gels) (*17*) followed by autoradiography for the estimation of the mass average size of the labeled DNA fragments produced as a result of nick translation.

Analysis of Snapback Hairpin Loops by Digestion with S1 Endonuclease

The use of S1 endonuclease in free solution DNA–DNA hybridization has been described in detail elsewhere (*8*). Denatured DNA probes for assay were placed into appropriate hybridization and S1 digestion buffers for treatment with S1 endonuclease as described previously (*8*). Denatured DNA probes in the absence of allowable hybridization (i.e., zero-time values) were expected to exhibit less than 5% remaining S1-resistant [^{32}P] counts per minute (CPM) upon immediate treatment with S1 endonuclease, as assayed by the DE-81 filter binding assay (*16*). Denatured DNA probes that were resistant to the activity of S1 endonuclease represented the proportion of snapback hairpins formed during the nick translation reaction.

Separation of Single-Stranded DNA Probes from Snapback Hairpin Forms

Hydroxylapatite (HAP) column chromatography (*18*) was used to separate single-stranded DNA probe structures from snapback hairpin loop DNA forms. Briefly, an approximate 0.5 mL packed bed volume of hydroxylapatite (Bio-Rad Laboratories, Richmond, CA; DNA grade) was water-jacketed at 68°C and equilibrated with 10 m*M* phosphate buffer (pH 7.0) ([PO_4]). Nick-translated DNA probes were made 10 m*M* with respect to phosphate ion concentration, boiled for 2 min, quick chilled in an ice bath for 10 s, and immediately loaded onto the HAP column. Virtually all [^{32}P] radioactivity bound to the HAP column under these conditions. Single-stranded labeled DNA was eluted from the HAP column using 0.14*M* phosphate buffer. Double-stranded DNA forms were eluted from the HAP column using 0.4*M* phosphate buffer. Quantitative recovery from the HAP column was always 100%. All eluted DNA samples were dialyzed extensively against 10 m*M* Tris-HCl (pH 7.6), 10 m*M*

NaCl, 1 m*M* EDTA before being subjected to hybridization reactions and/or digestion by S1 endonuclease.

RESULTS

Kinetics of [^{32}P]-dCTP Incorporation, Specific Activities, Mass Average Size, and S1-Resistance for the Nick-Translated Products

Figure 1 illustrates the rate of incorporation of [^{32}P]-dCTP and the final specific activities of the nick-translated probes attained by the three nick translation systems examined. The NEN method demonstrated an extremely rapid reaction rate with the maximum specific activity being achieved after only 15 min, as opposed to the 90 min recommended incubation period. The cyclic nature of relative incorporation demonstrated for nick-translated material harvested later than 30 min is not fully understood, but was highly reproducible (see Fig. 2A). Nick translation carried out with the NEN kit gave the highest ultimate specific activities (approx-

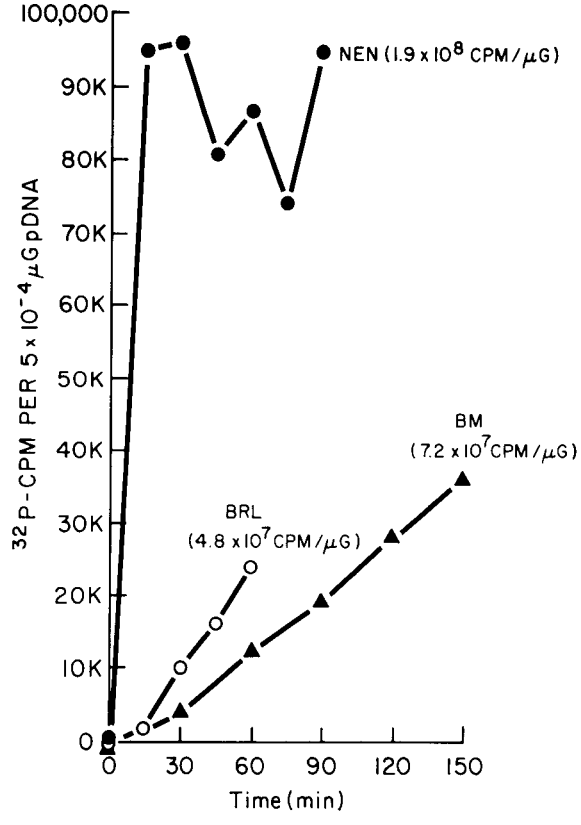

Fig. 1. Kinetics of [^{32}P]-dCTP incorporation and final specific activities of three nick-translated pBR322 DNA probes.

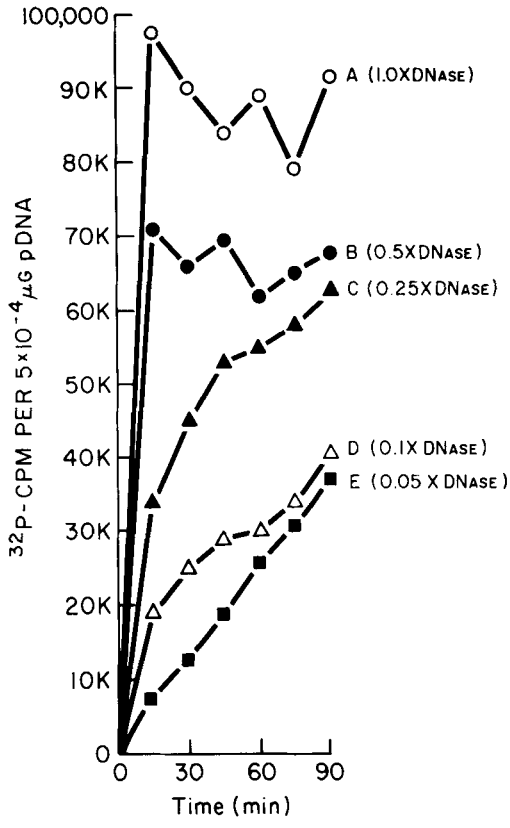

Fig. 2. Effect of DNase I concentration on the kinetics of [^{32}P]-dCTP incorporation and final specific activities using the NEN nick translation system.

imately 2 × 10^8 CPM/µg) among the three nick translation systems tested. The general nick translation method using BM Pol I produced a relatively linear rate of [^{32}P]-dCTP incorporation over a 150 min incubation period, but the resulting specific activity was only about 38% of that achieved using the NEN method. Nick translation carried out using the BRL technique showed a somewhat linear rate over a 60 min period; this rate of incorporation was more rapid than the general BM method, yielding a final specific activity of about 25% of the NEN reaction.

Other physicochemical characteristics of the nick-translated probes generated using the three nick translation systems are given in Table 1. Analysis of the mass average size of each product from individual nick translation reactions determined by electrophoresis on glyoxal gels (*17*) (not shown) revealed a majority of the NEN nick translation product to be in the average range of 260 nucleotide bases long (range = 130–390), with the probe products synthesized by the BRL or general BM methods being on the average about twice as long. Furthermore, there was a marked difference in the total proportion of S1 endonuclease-resistant snapback material found in these various probes. More than 44% of the NEN nick-translated product was resistant to the activity of S1

TABLE 1
Specific Activity, Average Size, and S1 Endonuclease Resistance of Various Nick-Translated pBR322 DNAs

Nick translation method	Final specific activity, CPM/μg	Mass average size[a] (nucleotides)	S1-resistant [^{32}P]-CPM[b]	%S1-resistant DNA
NEN	1.9×10^8	260	44,210	44.2
BRL	4.8×10^7	460	21,733	21.7
BM[c]	7.2×10^7	510	15,441	15.4

[a]Determined from glyoxal gels after autoradiography.
[b]Total input [^{32}P]-CPM per assay = 1×10^5.
[c]General method using BM DNA Pol I.

endonuclease after DNA denaturation, whereas only one-half of this resistance level (21.7%) was observed for probe material generated by the BRL method. Probe synthesized using BM Pol I possessed the least S1 resistance at a level of 15.4%.

Quantitation of Hairpin Loop Structures Found in Single-Stranded DNA Probes and Their Separation by HAP Column Chromatography

The high levels of S1 endonuclease-resistant probe material generated by nick translations suggested the formation of double-stranded (ds) snapback hairpin loop DNA structures. The amount of S1-resistant material found in nick-translated DNAs also correlated with both the small size of the nick-translated product (Table 1) and the rate of the respective nick translation reaction (Fig. 1); rapid rates of [^{32}P]-dCTP incorporation yielded significantly higher proportions of snapback S1-resistant DNA forms. The formation of this snapback S1-resistant material in single-stranded (ss) nick-translated DNA probes suggested that separation by HAP column chromatography, which exhibits differential binding affinities for ssDNAs and dsDNAs, was feasible. This possible approach was previously noted (*10*) but not quantitatively explored.

Table 2 shows the results of S1 endonuclease resistance of various nick translated probes fractionated by HAP. Individual preparations of plasmid were nick-translated using the NEN, BRL, and general BM methods, followed by chromatography of the total nick-translated product over HAP. For each nick-translated probe, 0.14*M* PO_4 and 0.4*M* PO_4 buffer eluent fractions from the HAP column were collected separately, dialyzed as described, denatured in appropriate hybridization and S1 buffers, and digested immediately with S1 endonuclease. Under the nick translation conditions outlined in Fig. 1, all nick translation systems tested yielded a majority of [^{32}P]-dCTP incorporated CPM that were

TABLE 2
S1 Endonuclease Resistance of Nick-Tranlsated Denatured pBR322 DNAs Isolated by HAP Column Chromatography

Nick translation method	$[PO_4]$ eluate, *M*	Total $[^{32}P]$-CPM eluted	$[^{32}P]$-CPM eluted, %	S1-resistant $[^{32}P]$-CPM	%S1 resistance	%S1-Resistance of total$[^{32}P]$-CPM loaded on HAP
NEN	0.14	29,433	28.7	1,589	5.4	1.5
NEN	0.4	73,112	71.3	44,013	60.2	42.9
	Totals:	102,454	100.0	45,602		44.4
BRL	0.14	41,673	41.0	2,042	4.9	2.0
BRL	0.4	59,912	59.0	19,707	32.9	19.4
	Totals:	101,585	100.0	21,749		21.4
BM	0.14	50,586	48.8	2,074	4.1	2.0
BM	0.4	53,064	51.2	13,371	25.2	12.9
	Totals:	103,650	100.0	15,445		14.9

eluted from the HAP column by 0.4*M* PO_4 buffer, indicating a relatively high proportion of snapback hairpin DNA structures (Table 2). The NEN nick-translated product was especially high in this regard, with 71.3% of the total CPM loaded on the HAP column being eluted by 0.4*M* PO_4 buffer. The 0.4*M* eluates from products of the BRL and BM procedures constituted 59.0 and 51.2%, respectively, of the total CPM applied to the HAP column. The proportion of dsDNA snapback material eluted from the HAP column by 0.4*M* PO_4 buffer correlated with the relative sizes of synthesized products and the rates of the nick translation reaction (Fig. 1), but not with the overall specific activities achieved (Fig. 1 and Table 1), since nick-translated material from the general BM method possessed a higher specific activity than that of the BRL nick-translated material. All the nick-translated probes eluted from the HAP column under the conditions of 0.4*M* PO_4 were also found to be substantially resistant to digestion by S1 endonuclease, as expected. Of the NEN product, 60.2% of the 0.4*M* PO_4 fraction was resistant to S1 activity, 32.9% of the BRL 0.4*M* PO_4 product was S1 resistant, and 25.2% of the BM 0.4*M* PO_4 product was resistant to digestion by S1 endonuclease. These results therefore showed similar correlations with both the relative size of DNA products synthesized (Table 1) and the reaction rates for the nick translation reaction (Fig. 1). The last column of Table 2 is in agreement with the total amount of S1 resistant material found in the nick-translated materials described in Table 1.

All material eluted from the HAP columns using 0.14*M* PO_4 buffer was found to be in the range of only 4–5% resistant to the activity of S1 endonuclease following DNA denaturation, indicating the effectiveness of employing HAP procedures for the ultimate removal of S1-resistant snapback hairpin structures from nick-translated probe materials. Such HAP-isolated and purified probes were found to be very useful as free solution DNA–DNA hybridization probes in experiments designed to monitor hybrid formation by S1 endonuclease resistance (*8*).

Effect of Exogenous DNase I Concentration on the Reaction Rate, Size of Synthesized Product, and Specific Activity of the Nick-Translated Product

Preliminary experiments with the NEN nick translation method suggested the presence of high levels of DNase I in the nick translation reaction mixture. Inasmuch as snapback hairpin loop formation appeared to correlate highly with the rate of the nick translation reaction and the size of the product generated, altering the levels of exogenous DNase I in the NEN reaction mixture was examined for its effect upon the rate of reaction and specific activities achieved. As expected from previous work (*1–5*), the data shown in Fig. 2 indicate that DNase I levels dramatically alter the rates of [^{32}P]-dCTP incorporation as well as the overall specific activities achieved. As little as one-twentieth (Fig. 2E) of the recom-

mended amount of DNase I was required to produce a relatively linear rate of incorporation over a 90 min period.

Effect of Exogenous DNase I Concentration on the Overall Amount of Hairpin Loop formation

Based upon previous knowledge of the nick translation reaction (*1–5*), the results of Fig. 2 were predictable. However, previous investigations have not directly implicated a relationship between the rate of the nick translation reaction (or the relative size of the final product) and the formation of hairpin loop structures, as a function of exogenous DNase I concentration. Results that demonstrate such a relationship are given in Table 3. DNA materials generated from the results shown in Fig. 2 were treated in usual fashion and assayed for their relative proportion of S1-resistant snapback hairpin components. A comparison of Fig. 2 and Table 3 reveals direct correlations between both the size of the nick-translated product and the rate of nick translation to the amount of S1-resistant material generated during nick translation. Moreover, the similar nick translation rates shown in Figs. 2E and 1 (BRL curve) also correlate very well with the overall amounts of S1-resistant hairpin structures formed (19.6% in Table 3 and 21.7% in Table 1, respectively), thus providing further evidence for such correlations. The additional correlation here between the final specific activity achieved and the amount of hairpin loop formation appears not to be correct, inasmuch as this correlation does not hold true for results presented in Fig. 1 and Table 1. It should be noted that the relative differences in the amount of hairpin loop structures formed (Table 3, 19.6–46.1%) relative to the amount of DNase I used in the nick translation reaction was striking. As also shown in Table 3, it was expected that initial stepwise decreases in the amount of DNase I used in nick translation would not significantly increase the size of the product formed proportionately, especially considering the high starting concentration of DNase I used to obtain the results in Fig. 2A.

To further illustrate the effect of exogenous DNase I on the rate of nick translation and its ultimate effect on the generation of hairpin loop structures during nick translation by Pol I, nick-translated DNAs obtained from experiments shown in Fig. 2 and Table 3 were subjected to HAP column chromatography, and the 0.4*M* PO_4 elution fractions were analyzed for their content of S1 endonuclease-resistant snapback forms. Table 4 confirms that the use of high levels of DNase I in the nick translation reaction results in the generation of a very high proportion (66.3%) of S1-resistant hairpins among the 0.4*M* PO_4 eluted fraction, as suggested by previous experiments. For the lowest dilution of DNase I tested (4×10^{-5} U/μg DNA), the proportion of snapback DNA in the 0.4*M* PO_4 HAP fraction was only 29.7%; this value corresponded to a to-

TABLE 3
Effect of Exogenous DNase I Concentration on the Size of Synthesized Product and Formation of S1 Nuclease-Resistant Hairpin Loops in Various Nick-Translated pBR322 DNAs

Proportion of recommended amount of DNase used in NT reaction[a]	Units of DNase I[b]	Mass average size (nucleotides)[c]	S1 resistant [^{32}P]-CMP[d]	% S1 resistant hairpins
1.00	8×10^{-4}	250	46,140	46.1
0.50	4×10^{-4}	290	41,919	41.9
0.25	2×10^{-4}	340	33,779	33.8
0.10	8×10^{-5}	380	23,240	23.2
0.05	4×10^{-5}	410	19,604	19.6

[a]NEN kit used according to manufacturer.
[b]Units according to Kunitz (*20, 21*)
[c]Determined from glyoxal gels after autoradiography.
[d]Total [^{32}P]-CPM per assay = 1×10^5.

tal snapback component of 19.6% in the total sample tested (Table 3, last line, last column).

DISCUSSION

This study shows that rapid rates of in vitro nick translation, which produce relatively short-sized labeled DNA products, result in the generation of a high proportion of snapback hairpin loop structures. Rapid nick translation reaction rates and short products formed as a result of

TABLE 4
Effect of Exogenous DNase I Concentration on the Proportion of S1 Endonuclease-Resistant Hairpins Formed During Nick Translation[a]

Proportion of recommended amount of DNase used in NT reaction[a]	Units of DNase I[b]	[PO_4] eluate from HAP column	S1 resistant [^{32}P]-CMP[c]	% S1 resistant hairpins
1.00	8×10^{-4}	0.4 M	66,283	66.3
0.50	4×10^{-4}	0.4 M	56,917	56.9
0.25	2×10^{-4}	0.4 M	39,945	39.9
0.10	8×10^{-5}	0.4 M	33,490	33.5
0.05	4×10^{-5}	0.4 M	29,676	29.7

[a]NEN kit used according to manufacturer.
[b]Units according to Kunitz (*20, 21*)
[c]Total [^{32}P]-CPM per assay = 1×10^5.

relatively high concentrations of DNase I used during nick translation greatly increased the production of a high complement of snapback material. The appearance of snapback DNA components among nick translated DNA molecules has been previously recognized (*2,4,10,11*), but such formation as a direct consequence of exogenous DNase I levels has not been described. The data presented here clearly show that limiting the nick translation reaction rate, resulting in longer copy product, can significantly reduce the amount of snapback DNA formed in a nick-translated probe.

A reduction in the synthesis of snapback hairpin structures found within a nick-translated probe preparation is important to both the quantity and quality of labeled probe. Under the conditions of high DNase I concentration during nick translation, as much as 50% of the total material generated can be resistant to the activity of S1 endonuclease, and therefore not useful as probe material in free solution hybridization protocols that employ S1 endonuclease to monitor hybrid formation (*8,9*). The quality in terms of fidelity of copy may also be compromised (*4*). The conditions for nick translation should therefore be adjusted to provide a relatively uniform rate of label incorporation over an approximate 2-h period; this approach has been found to provide copies of adequate mass average size and with a low proportion of snapback material. This is especially important for the synthesis of probe material to be used in free solution hybridization employing S1 endonuclease to assess the degree of DNA–DNA or DNA–RNA hybrid formation, where the zero-time hybridization value should be below 5% hybridization for effective use of the probe.

Irrespective of the degree of snapback hairpins present in a nick-translated probe preparation, the results presented here also show that HAP column chromatography is effective for the removal of such hairpin structures from the more desirable ssDNA probe components. Results from Table 2 indicate that probe preparations containing as much as 44% (or more) S1 endonuclease-resistant material can be reduced to a preparation possessing only 5% or less of these structures, following a single purification passage over HAP and elution with 0.14*M* PO_4 buffer. It may be possible to employ even more restrictive and discriminatory HAP elution conditions, such as the use of 0.10–0.12*M* PO_4 buffer, provided that other conditions (such as the temperature) are also considered (*10,18*). Furthermore, because it is now possible to label DNA to high specific activity using very high specific activity labels in nick translation, the apparent detrimental loss of as much as 50% of the labeled nick-translated product after HAP column chromatography may be insignificant, assuming that these double-stranded snapback regions are randomly located in the DNA probe.

Much is currently known about the nick translation reaction, and extensive discussions on the technique have been presented (*1–5*). However, no precise explanation has yet been given to the mechanisms re-

sponsible for the occurrence of snapback hairpins formed by Pol I. Several possible explanations exist, including the role of strand displacement (*12,13*) through template switching, which has also been implicated as a result of loss of the 5′- to 3′-exonuclease activity of Pol I (*2,19*), allowing for the synthesis of intrastrand complementary regions. Nick translation reaction temperatures above 22°C may also facilitate the formation of snapback forms (*12*), since this temperature was near a threshold for that observed in ϕX174 nick-translated DNA (*12*); the reason for this temperature effect is not known. There also remains the possibility that the fidelity of synthesis activity for Pol I is in some way overwhelmed by the excess number of internal strand nicks, or by the fact that many more nick translated copies may be initiated from a higher molar concentration of 3′-hydroxyl termini (*2*). This study did not address the precise mechanism(s) of snapback DNA formation, but defined newly recognized parameters that affect its formation.

Based upon findings reported here, Fig. 3 diagramatically summarizes the types of nick-translated products formed during the nick translation reaction and defines some of their properties following denaturation. Form I is the desired structure, which is a relatively long (ca. 500 nucleotide bases), uniformly labeled, single-stranded DNA structure possessing no hairpin loop component. This form provides an excellent probe for any type of hybridization, including those that employ S1 endonuclease to monitor hybrid formation. Form I can be isolated in the 0.14*M* PO_4 eluent fraction upon HAP column chromatography. Nick-translated Forms II and III possess a relatively short haipin loop, but may contain either a short or long single-stranded DNA copy; these structures have only minor effect upon zero-time background hybridization values in free solution hybridization experiments because of the relatively small

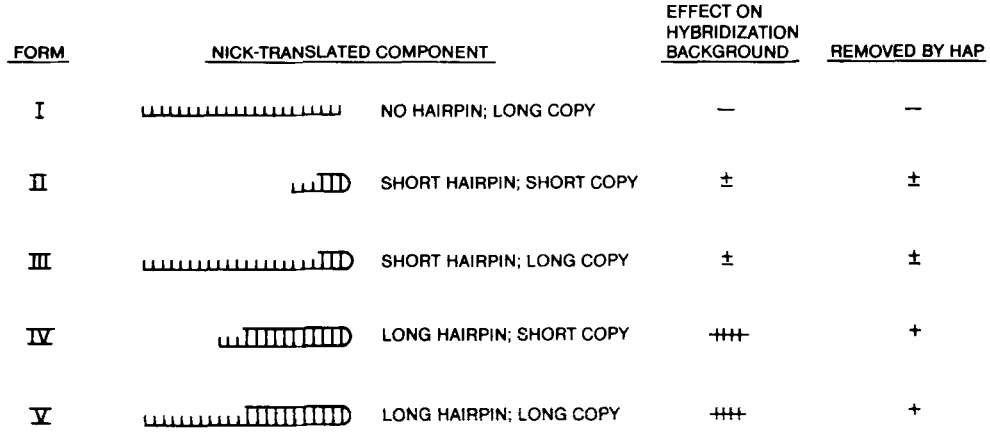

Fig. 3. Summary of DNA structures formed during the activity of DNA Pol I in the nick translation reaction. A (+) effect on hybridization background indicates an overall resistance of DNA probe molecules to the activity of S1 endonuclease following denaturation.

amount of label present in the short hairpin loop. Forms II and III may or may not be retained on a HAP column following elution with 0.14*M* phosphate buffer, depending upon the relative size of the double stranded hairpin structure component. Forms IV and V are the most detrimental obstacles to the efficient synthesis of desireable probe for free solution hybridization interactions. Each contain long hairpin structures, and may possess either short or long single-stranded copies of the DNA. Both forms IV and V bind as dsDNA to HAP, and are therefore retained by the HAP column following elution with 0.14*M* phosphate buffer, and are thereby separated from the more desirable forms I–III. Forms IV and V greatly interfere with the interpretations of free solution Cot analysis (*8*) using S1 endonuclease and therefore should be removed from the probe preparation before use in such systems.

Data provided in this study allow for a more precise identification of the factors that influence hairpin loop formation during nick translation by Pol I, and thus provide a better basis for the selection of strategies to avoid hairpin formation through adjustment of relevant reaction conditions. The use of HAP column chromatography also provides a useful approach for the removal of aberrant hairpin loop structures from the more desirable labeled probe material.

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