Determination of the 3' terminal nucleotide of DNA fragments

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ABSTRACT

A new method for determining the 3'-terminal nucleotide of a DNA strand is presented. Use is made of the fact that one (and only one) 2',3'-dideoxyribonucleotide can be added to the 3'-end of a DNA fragment with calf thymus terminal transferase. Addition of more than one nucleotide analog per strand is impossible due to the absence of a 3'-terminal hydroxyl group. If the terminating dideoxyribonucleotide contains an [$\alpha^{32}\text{P}$] label, the resulting 3'-blocked strand can be digested by "nearest neighbor" techniques and the original 3'-endgroup determined. Picomole quantities of DNA strands can be labeled and the 3'-end determined.

INTRODUCTION

A general method is available for the determination of the 5'-terminal nucleotide of a given strand of RNA or DNA (1). Picomole quantities can be labeled at the 5'-end with polynucleotide kinase and [$\gamma^{32}\text{P}$] ATP. The ^{32}P -containing terminal nucleotide at the 5'-end is readily identified following hydrolysis by snake venom phosphodiesterase and separation of the resulting mononucleotide by electrophoresis.

Methods generally employed for the identification of 3'-terminal nucleotides require a quantity of DNA sufficient to permit detection of the 3'-terminal nucleoside by UV absorbance after spleen phosphodiesterase hydrolysis. It would be desirable to analyze picomole quantities of a given oligodeoxyribonucleotide for 3'-terminal nucleotides. Several techniques are available which label the 3'-terminus with radioactivity. Radioactive acetic anhydride can be used to acetylate the 3'-hydroxyl residue providing the 5'-nucleoside is phosphorylated (2). The acetyl group is, however, quite labile during subsequent enzymatic hydrolysis and chromatography.

The 3'-terminus can also be labeled by the addition of [$\alpha^{32}\text{P}$] ATP with terminal deoxynucleotidyl transferase (3). Be-

cause more than one AMP residue may be added, the oligomer is treated with alkali and alkaline phosphatase before the terminal nucleotide can be identified. A recent modification of this method allows chromatography of the digested strand without the removal of the added ribonucleotides (4).

A procedure is presented here in which as little as one picomole of a DNA strand can be used in identification of the 3'-nucleotide terminus. [$\alpha^{32}\text{P}$] Dideoxyadenosine triphosphate (ddATP) was added to the 3'-terminus of a DNA fragment using terminal deoxynucleotidyl transferase. After separation, the addition product was hydrolyzed with enzymes to 3'-nucleotides, thus transferring the [^{32}P] label to the adjacent or original 3'-end. Identification of the labeled nucleotide was made by electrophoresis.

MATERIALS AND METHODS

Enzymes and nucleotides. Enzyme units were the same as described by the supplier. Calf thymus DNA, micrococcal nuclease and spleen phosphodiesterase were obtained from Worthington-Biochemical Corp. DEAE cellulose paper was Whatman DE81 from Reeve Angel. Polynucleotide kinase was supplied by P-L Biochemicals. Terminal deoxynucleotidyl transferase from calf thymus was purified as described (5), except that the final hydroxylapatite chromatography was omitted. The enzyme from Sephadex G-100 chromatography was further purified by iso-electric focusing (6). No detectable exo- or endonucleolytic activity was found under our conditions of use. Nonradioactive 2',3'-dideoxyadenosine 5'-triphosphate was synthesized as described (6). [$\alpha^{32}\text{P}$] 2',3'-Dideoxyadenosine 5'-triphosphate was synthesized from 2',3'-dideoxyadenosine (7) according to the method of Symons (8). The synthesis of the following oligodeoxyribonucleotides used in this paper are described in the indicated publications, and were provided by the authors for analysis. d(pA-A-G-A-C-A-G-C-A-T-A-T) (9), d(pT-T-A-A-T-C-C-A-T-A-T-G C) (10), d(pT-G-T-C-T-T-T-C-A-A-A-T) (11), d(pA-T-G-G-A-A-A-C-T-G-C-G-G-C) (12), d(pT-T-A-G-C-A-G-C-C-G-C-A-G) (13), d(pT-G-C-T-A-A-A-T-T-T-G-A) (14), d(pT-T-T-C-C-A-T), and d(pG-G-A-T-T-A-A) were synthesized by classical procedures and will be reported elsewhere.

Synthesis of [$5'-^{32}\text{P}$]d(pT)₅. The 5'-phosphate was removed from 20 nmole of d(pT)₅ in 0.1 ml of reaction mixture containing

5 μmol of Tris (pH 7.6 and 5 μg of calf alkaline phosphatase (Sigma Chemical Co., Type VII). After incubation at 37° for 1 hr, the reaction was heated 3 min at 100° to inactivate the phosphatase. The reaction was brought up to 0.3 ml with 3 μmol MgCl_2 , 2 μmol dithiothreitol, 30 nmol [$\alpha^{32}\text{P}$] ATP (2×10^4 cpm per nmole) and 0.6 units of polynucleotide kinase. After 1 hr at 37°C, the reaction was applied to a DEAE paper strip (3.0 cm wide) and subjected to descending chromatography with 0.5 M triethylammonium bicarbonate (pH 7.6) for 20 hr to separate [$5' - ^{32}\text{P}$]d(pT)₅ from [$\alpha^{32}\text{P}$] ATP. The [$5' - ^{32}\text{P}$]d(pT)₅ was extracted from the paper with 1 M triethylammonium bicarbonate buffer (pH 7.6).

Addition of ddAMP to oligodeoxyribonucleotides. 0.8 nmole Oligodeoxyribonucleotide was incubated in a volume of 200 μl containing 200 mM potassium cacodylate pH 7.2, 5 mM 2-mercaptoethanol, 8 mM MgCl_2 , 7.6 nmoles [$\alpha^{32}\text{P}$]ddATP (0.7×10^6 cpm per nmole) and 13 U terminal deoxynucleotidyl transferase. After 16 hr at 37°C the reaction was applied to a 3 cm wide DEAE paper strip followed by descending chromatography with 0.5 M triethylammonium bicarbonate buffer (pH 7.6) for 4 hr. The strip was cut up and counted in a liquid scintillation counter. The oligonucleotide, located several centimeters from the origin, was eluted with 1M triethylammonium bicarbonate (pH 7.6).

Identification of the 3'-terminal nucleotides. The above eluted oligonucleotide, containing [^{32}P]-labeled ddAMP at its 3' terminus, was concentrated to dryness and dissolved in a solution containing 50 mM triethylammonium bicarbonate (pH 8.6), 2 mM CoCl_2 , 0.9 A₂₆₀ calf thymus DNA and 60 U micrococcal nuclease. The digestion proceeded for 4 hr at 37°C in a volume of 200 μl . The reaction was adjusted with 2 M acetic acid to a pH of 6.5 followed by the addition of 2 μmole KH_2PO_4 , pH 6.5, and 0.2 U spleen phosphodiesterase. Incubation continued for 2 hr at 37°C. A portion of the digestion mixture was applied to Whatman 3 MM paper and together with appropriate 3'-nucleotide markers, subjected to electrophoresis for 2.5 hr at 87 v/cm, using 0.05 M ammonium formate, pH 3.9. The nucleotides containing 3'- ^{32}P were identified by counting in a liquid scintillation counter.

RESULTS

Figure 1 shows that, after addition of a dideoxyadenylate residue with terminal deoxynucleotidyl transferase, the resulting

product traveled a shorter distance on DEAE-paper. This indicates that the substrate had been lengthened, as expected. The absence of any radioactivity in the position expected for the substrate

Fig. 1. Addition of ddAMP to $[5'\text{-}^{32}\text{P}]\text{d(pT)}_5$. The reaction was carried out with enzyme (— —) and without enzyme (—) as described in Materials and Methods. The separation was as described except the DEAE paper was developed 20 hr.

$[5'\text{-}^{32}\text{P}]\text{d(pT)}_5$ shows that close to 100% of the oligomer was terminated with the dideoxyadenylate under the conditions used.

The analysis of a number of oligodeoxyribonucleotides synthesized in conjunction with the chemical synthesis of a minigene (12) is shown in Table 1. To identify the 3'-terminal nucleotide, $[^{32}\text{P}]$ -labeled ddAMP was added to each of the strands indicated. The labeled oligomers were subjected to nearest neighbor analysis to detect the original 3'-terminating nucleotide. The results were as expected except for three of the strands, which contained a contaminating 3'-nucleotide. In two of the strands the impurity was minor, but in the third strand it was significant. These impurities are believed to represent a failure to separate the immediate chemical precursor from the final condensation product.

In each of these three condensations, a tetramer was added to the immediate precursor. As can be seen, the immediate precursor (the sequence shown in Table 1 minus four residues from the 3'-terminus) would yield the contaminating nucleotide actually encountered.

Table 1. 3'-Nucleotide analysis of synthetic deoxyribooligonucleotides.

<u>Strand</u>	dAp	dCp	dGp	dTp
d(pT-T-A-A-T-C-C-A-T-A-T-G-C)		95		5
d(pA-A-G-A-C-A-G-C-A-T-A-T)		3		97
d(pT-G-T-C-T-T-T-C-A-A-A-T)				100
d(pA-T-G-G-A-A-A-C-T-G-C-G-G-C)		69	31	
d(pT-G-C-T-A-A-A-T-T-T-G-A)	100			
d(pT-T-A-G-C-A-G-C-C-G-C-A-G)			100	
d(pG-G-A-T-T-A-A)	100			
d(pT-T-T-C-C-A-T)				100

Table 2 demonstrates that this method can be used with picomole quantities of DNA strands. Using 3 pmol (3×10^{-8} M) of d(pT)₉, all strands were blocked with [³²P]-labeled ddAMP. This complete incorporation was obtained with a low substrate concentration (10^{-5} M ddATP). Thus, a high specific activity of the triphosphate could be used.

Table 2. Addition of ddAMP to d(pT)₉. Reaction same as in Materials and Methods except only 1 nmol [³²P]ddATP was used in 100 μ l of reaction mixture.

<u>dT(pT)₈</u>	<u>[³²P]ddAMP incorporated</u>
<u>p moles</u>	<u>p moles added</u>
3	3.2
3	3.2
6	6.4
6	6.4

DISCUSSION

2',3'-Dideoxyribonucleoside 5'-triphosphates are potent in-

hibitors of DNA synthesis, blocking chain extension after their addition due to the absence of a 3'-hydroxyl linkage group. Likewise, DNA and DNA oligomers, after addition of a dideoxyribonucleotide, are resistant to the pyrophosphorolysis and 3'-exonuclease functions of DNA polymerase (6,15). In contrast to the addition of ribonucleotides to DNA oligomers with terminal transferase (3), only one molecule of a dideoxyribonucleotide can be added. Finally, using a ddATP concentration of 10^{-5} M and a primer concentration of 3×10^{-8} M it is possible to obtain 100% incorporation at a high enough specific activity to permit the analysis of DNA strands the size of certain bacteriophages.

These facts and the supporting data demonstrate that [$\alpha^{32}\text{P}$]-labeled ddATP and calf thymus terminal transferase can be used as a sensitive method for 3'-endgroup analysis of DNA strands as well as a procedure for determining the concentration or length of strands present.

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REFERENCES

- 1 Richardson, C. (1965) Proc. Nat. Acad. Sci. U.S.A. **54**, 158.
- 2 Stuart, A. and Khorana, H. (1964) J. Biol. Chem. **239**, 3885.
- 3 Kossel, H. and Roychoudury, R. (1971) Eur. J. Biochem. **22**, 271.
- 4 Bertazzoni, U., Ehrlich, S., and Bernardi, G. (1973) Biochim. Biophys. Acta **312**, 192.
- 5 Chang, L. and Bollum, F. (1971) J. Biol. Chem. **246**, 909.
- 6 Olson, K., Gabriel, T., Michalewsky, J., and Harvey, C. (1975) in press.
- 7 Russell, A.F., Greenberg, S., and Moffatt, J.G. (1973) J. Amer. Chem. Soc. **95**, 4025.
- 8 Symons, R. (1974) in Methods in Enzymology, vol. XXIX, pp 102-115, Academic Press, New York.
- 9 Poonian, M., Nowoswiat, E., and Nussbaum, A.L. (1972) J. Amer. Chem. Soc. **94**, 3992.
- 10 Cook, A., Heimer, E., Holman, M., Maichuk, D., and Nussbaum, A.L. (1972) J. Amer. Chem. Soc. **94**, 1334.
- 11 Harvey, C., Wright, R., Cook, A., Maichuk, D., and Nussbaum, A.L. (1973) Biochemistry **12**, 208.
- 12 Poonian, M., Nowoswiat, E., Tobias, L., and Nussbaum, A.L. (1973) Biorg. Chem. **2**, 322.

- 13 Cook, A., DeCzekala, A., Gabriel, T., Harvey, C., Holman, M., Michalewsky, J., and Nussbaum, A.L. (1973) Biochim. Biophys. Acta 324, 433.
- 14 Heimer, E., Ahmad, M., Roy, S., Ramel, A., and Nussbaum, A.L. (1972) J. Amer. Chem. Soc. 94, 1707.
- 15 Atkinson, M., Deutscher, M., Kornberg, A., Russell, A. and Moffatt, J. (1969) Biochemistry 8, 4897.