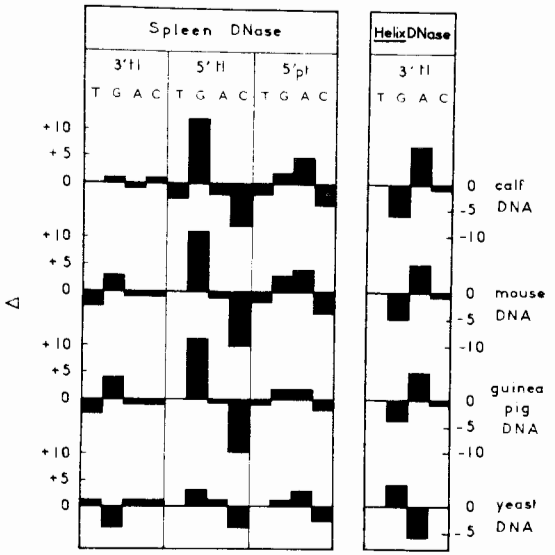

FIG. 9. Differences between the percentages of 3′ terminal, 5′ terminal, and 5′ penultimate nucleotides formed from eukaryote DNA's and the corresponding values for bacterial DNA's. In the case of yeast, nuclear DNA was used. From S. D. Ehrlich and G. Bernardi, *J. Mol. Biol.*, to be submitted for publication.

It should be pointed out that the sequences which are split are seen only through the termini they release, and that a great part of the possible differences in K_m and V_{max} values associated with individual sequences are lost through the averaging of the compositions of the termini released. In the only case where the sequences which were split could be estimated, the case of poly(dAT:dAT), A was found to form 80% and 87% of the 3′ termini released by the spleen and the snail enzyme, respectively, indicating that both enzymes have different K_m and/or V_{max} for the two equally abundant sequences ATAT and TATA.[10,20] This finding stresses the fact that the frequencies of the termini as determined by our analysis are only *apparent* frequencies and should be clearly distinguished from the *real* frequencies of the termini recognized by the enzymes.

The empirical relationships of Fig. 7 do not hold for DNA's having different distributions of the frequencies of nucleotide sequences split by the enzymes compared to bacterial DNA's, as shown by the deviation plots (Figs. 8 and 9) of satellite DNA's from mouse and guinea pig, mitochondrial DNA from yeast,[20] and the DNA's from eukaryotes.[21]

[20] J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.

[21] S. D. Ehrlich and G. Bernardi, *J. Mol. Biol.*, to be submitted for publication.

In conclusion, the analysis of termini formed by DNase is a new method for characterizing and comparing nucleotide sequences in DNA's, the deviation plots of Figs. 8 and 9 being a novel approach to the study of "repetitive" nucleotide sequences. It should be noted that, when applied to the 3′ terminal, the 5′ terminal, and the 5′ penultimate nucleotides, the method, as described here, requires 100 μg of DNA. Radioactive labeling of the 3′ terminals with [α-^{32}P]ATP, using the terminal nucleotidyltransferase,[22] and the 5′ ends with polynucleotide kinase (work in progress) should lead to a considerable reduction in scale.

[22] U. Bertazzoni, S. D. Ehrlich, and G. Bernardi, this volume [28]. Also *Biochem. Biophys. Acta*, in press.

[28] Analysis of Labeled 3′ Terminal Nucleotides of DNA Fragments

By UMBERTO BERTAZZONI,[1] STANISLAV D. EHRLICH, and GIORGIO BERNARDI

We describe here a procedure for the analysis of 3′ terminal nucleotides of DNA fragments. The procedure is based on (a) the labeling of 3′ ends of oligo- or polydeoxyribonucleotides by the addition of 2 residues of [^{32}P]AMP catalyzed by terminal deoxyribonucleotidyltransferase using [α-^{32}P]ATP as a donor[2]; (b) the separation of terminally labeled DNA fragments from excess ATP; (c) the digestion of the fragments with spleen acid DNase and exonuclease; (d) the separation of the four labeled terminal nucleotides on DEAE-cellulose columns, under conditions permitting the simultaneous separation of labeled-AMP.[3] Figure 1 summarizes the two enzymatic steps involved in the procedure.

Materials and Methods

3′-Hydroxy oligonucleotides were prepared by degradation of calf thymus DNA by spleen acid DNase followed by dephosphorylation of denatured DNA fragments.[4] Their average chain length (average degree of polymerization, $\bar{P}_n$) and the composition of 3′-terminal nucleotides were determined

[1] Euratom scientific agent. This publication is contribution No. 869 of Euratom Biology Division.

[2] H. Kössel and R. Roychoudhury, *Eur. J. Biochem.* **22**, 271 (1971).

[3] U. Bertazzoni, S. D. Ehrlich, and G. Bernardi, *Biochim. Biophys. Acta*, in press.

[4] C. Soave, J. P. Thiery, S. D. Ehrlich, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.

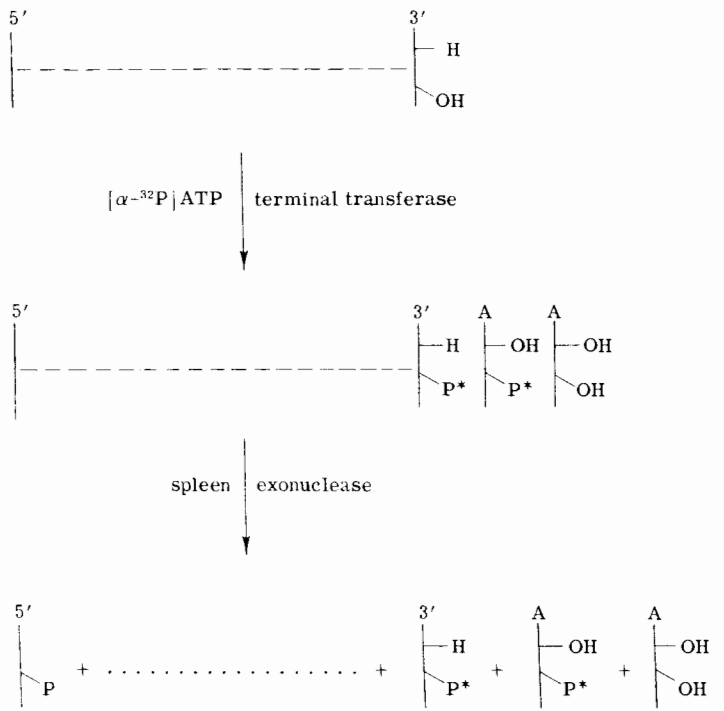

FIG. 1. Scheme of 3′ terminal nucleotide determination.

according to Carrara and Bernardi[5] as modified by Soave *et al.*[4] and by Thiery *et al.*,[6] respectively.

Enzymes. Acid DNase B, acid exonuclease, and acid phosphomonoesterase B were obtained from hog spleen.[7–9] Terminal transferase was purified from calf thymus according to a modification of the method of Yoneda and Bollum.[3,10] The final specific activity was 14,000 units[10] per A_{280} unit of protein when tested for deoxyadenylate polymerization on oligonucleotides ($\bar{P}_n = 6$) prepared by pancreatic DNase degradation of calf thymus DNA followed by removal of mono- and dinucleotides on a DEAE-cellulose column.

[α-32P]ATP had a specific activity of 1080–1330 mCi/mmole (C.E.A., Saclay, France).

[5] M. Carrara and G. Bernardi, *Biochemistry* **7**, 1112 (1968).
[6] J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.
[7] G. Bernardi, A. Bernardi, and A. Chersi, *Biochim. Biophys. Acta* **129**, 1 (1966).
[8] A. Bernardi and G. Bernardi, *Biochim. Biophys. Acta* **155**, 360 (1968).
[9] A. Chersi, A. Bernardi, and G. Bernardi, *Biochim. Biophys. Acta* **246**, 51 (1971).
[10] M. Yoneda and F. J. Bollum, *J. Biol. Chem.* **240**, 3385 (1965).

DEAE-Cellulose Chromatography of Nucleotides. DEAE-cellulose (Whatman DE-32) was washed, in succession, with 0.5 *M* HCl, water, 0.5 *M* NaOH, water, and 1 *M* ammonium acetate, pH 5.5; fines were removed by decantation. A suspension of wet exchanger and 1 *M* ammonium acetate, pH 5.5 (1:1; v/v) was deaerated in a rotary evaporator and gently sucked into columns of 0.5 cm diameter fitted with an extension tube. Columns were packed at a flow rate of 20 ml/hr to the height of 15 cm and washed with 1 *M* ammonium acetate, pH 8.7, followed by 50 m*M* ammonium acetate, pH 9.3. Elution was performed with this buffer; in order to obtain an optimal degree of separation of nucleotides, some slight pH adjustment (0.1–0.2 unit) were sometimes required. A step of 0.15 *M* ammonium acetate, pH 8.9, was then used for the elution of deoxyguanylate. The same column could be reequilibrated with the starting buffer and reused many times.

The ultraviolet absorbancy of the column effluent was monitored using a Uvicord (LKB, Stockholm, Sweden) equipped with a 0.3 cm cell. Fractions were collected in 1.5 × 5 cm polystyrene tubes (Greiner, Bischwiller, France) fitting the counting vials. Counting of ^{32}P aqueous samples and column chromatography eluates by Cerenkov radiation effect was made by setting the Intertechnique (Plaisir, France) counter lower window at 0 and the upper one at 675.

Experimental Procedure

*Preparation of Oligonucleotides Carrying p*Ap*A at Their 3′ Ends*

The incubation mixture (0.05 ml, final volume) was: 1–10 μ*M* in 3′ ends of oligonucleotides (0.025–0.250 A_{260} unit; $\bar{P}_n = 54$); 0.15 m*M* in [α-32P]ATP (of specific radioactivity of 1 Ci/mmole); 0.2 *M* in potassium cacodylate, pH 7; 8 m*M* in $MgCl_2$; 1 m*M* in 2-mercaptoethanol and contained 45 units of terminal transferase. The enzymatic reaction was carried out for 12–14 hours at 37° in sealed glass microtubes and was terminated by shaking for 5 minutes with 0.2 volume of a chloroform-isoamyl alcohol mixture (24:1, v/v).

The unreacted, labeled ATP was separated from the radioactive product by gel filtration of the reaction mixture on a Sephadex G-25 column equilibrated with 50 m*M* ammonium acetate pH 5.5. As estimated from the G-25 chromatograms, two AMP residues were added to about 25% of 3′ ends, whereas a 50% yield was obtained when 0.5 m*M* [α-32P]ATP was used.

*Degradation of Oligo(dN)p*Ap*A to 3′-Deoxynucleoside Monophosphates*

The fractions from G-25 chromatography corresponding to the oligo-(dN)p*Ap*A were pooled; 1.2 A_{260} units of calf thymus DNA were added.

The material was lyophilized, dissolved in 20 m*M* ammonium acetate, pH 5.5 (final volume 0.1 ml), and digested at room temperature with 1 μl of spleen DNase (600 units/ml) and 10 μl of spleen exonuclease (60 units/ml). Hydrolysis was followed in duplicate by measuring the amount of label rendered acid soluble as a function of time[11] and was complete after 1 hour; after inactivation of the enzymes, the pH of the sample was raised to 8.5–9 by adding 1 *M* NH_4OH.

*Procedure for the Removal of [α-^{32}P]ATP and Degradation of Oligo(dN)p*Ap*A on a Microscale*

Ten microliters of terminal transferase mixture were spotted on a 0.5 cm-diameter glass fiber disk (Whatman GF/C); the radioactivity of the wet disk was measured by Cerenkov counting in a plastic tube fitting

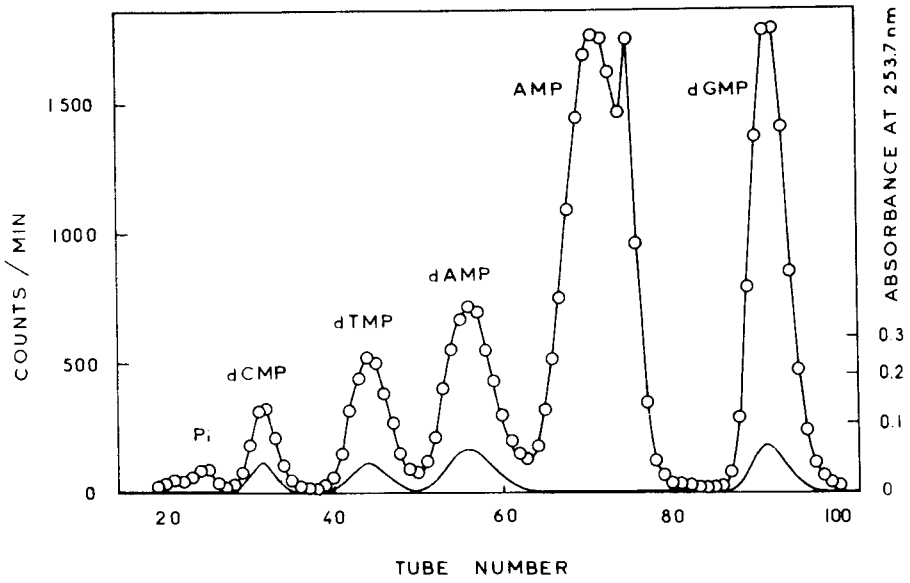

FIG. 2. Chromatography on DEAE-cellulose of ^{32}P-labeled mononucleotides. Twenty thousand counts per minute of ^{32}P-labeled 3′ nucleotides (obtained after complete digestion of oligo(dN)p*Ap*A by spleen DNase and exonuclease) in 50 μl of 50 m*M* ammonium acetate buffer (adjusted after digestion to pH 8.5–9 by adding 2 μl of 1 *M* NH_4OH) were loaded on a DEAE-cellulose column (0.5 × 15 cm) equilibrated with 50 m*M* ammonium acetate, pH 9.3. Elution was started with the same buffer using a flow rate of 12 ml per hour; a step of 0.15 ammonium acetate pH 8.9 was applied after fraction 63, in order to elute dGMP. The solid line refers to UV monitoring; the circles to Cerenkov counting.

[11] F. J. Bollum, *in* "Procedures in Nucleic Acids Research" (G. L. Cantoni and D. R. Davies, eds.), Vol. 1, p. 296. Harper, New York, 1966.

3′ TERMINAL NUCLEOTIDES OF DEPHOSPHORYLATED DNA FRAGMENTS[a]

3′ Nucleotide	Nucleoside analysis	Labeled nucleotide analysis
dC	5	6
dT	14	14
dA	24	24
dG	58	56

[a] 3′ Terminals of oligonucleotides ($\bar{P}_n$ 54), obtained by controlled degradation of calf thymus DNA by spleen DNase (see Materials and Methods) were determined by the nucleoside analysis according to Carrara and Bernardi[5] as modified by Thiery *et al.*[6] and by the present method, respectively. All values are given in percentage.

the counting vial; 2 ml of 5% TCA–1% sodium pyrophosphate were added to the disk which was transferred after 15 minutes to a sintered-glass funnel and washed under suction successively with 2–5 ml of 5% TCA, ethanol, and ether. Ten microliters of a mixture of spleen DNase (40 units/ml), spleen exonuclease (40 units/ml), and BSA (0.01%) in 0.05 *M* ammonium acetate, pH 5.5 were added to the dry disk; the radioactivity on the disk was measured by Cerenkov counting, and the extent of AMP incorporation into the oligonucleotides was calculated from the ratio of radioactivity before and after the acid washing. As shown by control experiments, the digestion was complete after 30 minutes at room temperature. The disk was transferred then to the top of a 0.5 × 15 cm DEAE-cellulose column, and labeled mononucleotides were separated as described below.

Analysis of Labeled 3′ Terminal Nucleotides from 3′-Hydroxyoligodeoxyribonucleotides

Figure 2 shows the separation of the labeled 3′ terminal nucleotides from 3′-hydroxyoligodeoxyribonucleotides on the DEAE-cellulose column. The elution volumes of nucleotides derived from the carrier DNA were determined by UV monitoring of the eluate; the radioactivity of the four deoxynucleotides and of AMP was measured by Cerenkov counting. The radioactivity was found associated with mononucleotides only; the amount of inorganic phosphate was very low (Fig. 2); ^{32}P-labeled ATP, ADP, or oligonucleotides, which could be eluted from the column by a 1 *M* ammonium acetate step, were practically undetectable.

The composition of 3′ terminal nucleotides from oligonucleotides released by spleen DNase from calf thymus DNA as determined by the technique described above, is compared in the table with data obtained by analysis of the 3′ terminal nucleosides.[5,6] An excellent agreement between the two sets of values is observed.