tides (in contrast to trinucleotides and trinucleoside diphosphates, which could be subjected to the method[9,15]); this limitation is based on the requirement of terminal transferase for a minimum primer chain lengths of three nucleotides. As to the upper chain lengths, undecanucleotides of specific sequences,[16] and the homopolymers poly[d(T)$_{\overline{32}}$], poly[d(A)$_{\overline{100}}$] and poly[d(A)$_{\overline{600}}$][15] have been tested successfully so far. It seems therefore likely that also polynucleotides with specific sequences of higher chain length can be labeled by the method described here, as long as contaminating nucleases are excluded from the reactions.

Oligodeoxynucleotides carrying a 3′-phosphomonoester group are not accepted as primers for terminal transferase; such limitation, however, may be easily circumvented by phosphatase treatment of the oligonucleotide to be labeled, before the terminal addition reaction is carried out. In this case care must be taken not to carry phosphatase into the terminal addition reaction, as ribonucleoside triphosphates are readily degraded by phosphatase.

In special circumstances (for instance for studies with 3′-specific phosphatase), it may be necessary to have oligodeoxynucleotides available with ^{32}P-labeled phosphomonoester groups at the 3′ ends. For this purpose, elimination of the ribonucleoside residues is possible by periodate and amine treatment[8]; by this reaction for instance (A-C-C-A)$_d$ [^{32}P]pA is converted to (A-C-C-A)$_d$ [^{32}P]p.

Partial Sequence Analysis. One crucial point of this method consists in meeting the proper conditions for the partial digestion of the oligonucleotide to be analyzed with snake venom phosphodiesterase. In fact, unsuccessful experiments had to be encountered a few times owing to insufficient digestion or to overdigestion of the oligonucleotidic material. Since the priming activity necessary for the terminal addition reaction decreases with the chain length of the primer,[21] the ideal digestion condition would be the one in which more molecules of smaller oligonucleotides are produced with very few undigested molecules remaining. Thus, the extent of digestion has to be balanced carefully by variation of enzyme concentration or incubation time.

Partial sequence determination on semimicroscale is possible with as little as 0.8 A_{260} units of the synthetic octanucleotide (A-C-C-A-T-C-C-A)$_d$, whereby only the two nucleotides toward the 5′-end remain unidentified (Fig. 3).

Partial sequence determination on microscale can be carried out with as little as 5×10^{-3} A_{260} units of the same oligonucleotide (Fig. 5); in this case, however, three nucleotides from the 5′ end remain unidentified. It should be pointed out that the fingerprint analyses of labeled oligonucleotide mixtures allow additional information in respect to mononucleotide residues sequentially released by snake venom phosphodiesterase; thus, according to the fingerprint rules[17,19] the relative position of spot IV to V of Fig. 5 evidences the removal of one thymidylic acid residue positioned at the fourth place counted from the 3′ end of the original oligonucleotide; likewise the relative positions of spots II to III and III to IV indicate the removal of cytidylic acid residues. This information confirms or even supplements the results obtained from the identification of the 3′-terminal nucleotides of the individual labeled oligonucleotides by spleen phosphodiesterase degradation. In contrast to this, partial sequence determination on semimicroscale is based exclusively on the identification of the 3′-terminal nucleotides by spleen phosphodiesterase degradation, since separation of the labeled oligonucleotides in this case is carried out by DEAE-cellulose column chromatography (Fig. 3), not by the fingerprint technique.

Acknowledgment

This work was supported by grants from the Deutsche Forschungsgemeinschaft, which are gratefully acknowledged. H. Kössel wishes to thank Drs. F. Sanger, B. Griffin, and V. Ling for introduction into the two-dimensional fingerprint technique. Valuable technical assistance is credited to Miss S. Baars.

[27] A New Approach to the Study of Nucleotide Sequences in DNA: the Analysis of Termini Formed by DNases

By Giorgio Bernardi, Stanislav D. Ehrlich, and Jean-Paul Thiery

We describe here a method developed in our laboratory for characterizing and comparing nucleotide sequences in DNA's. The method is based upon the fact that DNases are sequence-specific; that is, they are able to recognize the nucleotides near the phosphodiester bonds which they split. We have demonstrated this point for at least the three DNases we have investigated so far: spleen acid DNase, snail acid DNase, and pancreatic DNase. The spleen enzyme, for instance, is able to recognize at least the two nucleotide pairs lying on each side of the breaks; we call *termini* the terminal and penultimate nucleotides, W X Y Z (Fig. 1).

Since DNases are sequence-specific, the analysis of termini, namely, the determination of the base composition of the termini released from the sequences split by DNases, provides information on the frequency of these sequences in the DNA's analyzed. The analysis of termini is characterized

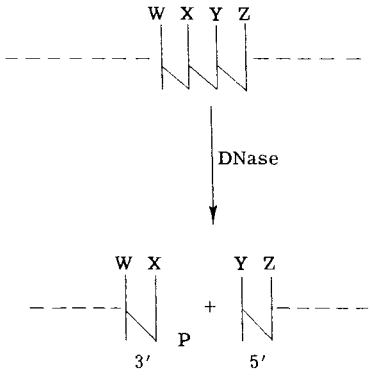

FIG. 1. A scheme of degradation of DNA by a DNase. The phosphate is arbitrarily put at the newly formed 3′ end.

by two features, which distinguish it from a determination of the frequency of the sequences split by the enzymes (Fig. 2): (a) the analysis of termini provides the composition of each terminus as derived from all the split sequences; (b) since different nucleotide sequences can be split with different k_m and V_{max} values, the analysis of termini is related to an apparent frequency, not to the real frequency, of the split sequences.

It can be expected, and it is experimentally found, that (a) the composition of each terminus released from bacterial DNA's having different base compositions is linearly related to their G + C contents; (b) the composition of termini released from DNA's containing "repetitive"

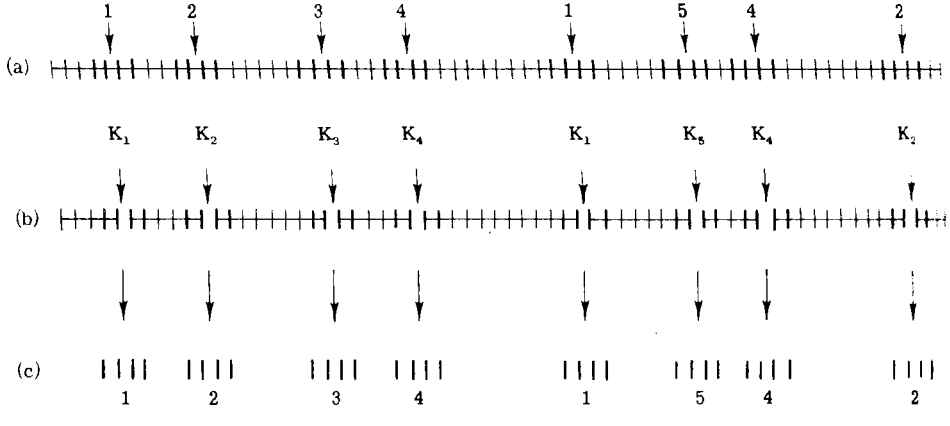

FIG. 2. A scheme of the analysis of termini: (a) Sequences 1 to 5 are recognized and split by the enzyme. (b) Ends are formed from sequences 1–5 proportionally to constants K_1 to K_5, which depend upon the k_m and V_{max} values for sequences 1–5. (c) Termini deriving from each position of the terminal doublets of all sequences split are isolated, and their composition is determined.

nucleotide sequences deviates, by excess or by defect, from that expected for "nonrepetitive" DNA's having the same G + C contents. The deviation plots which can be thus obtained represent a novel way of characterizing "repetitive" DNA's.

Materials and General Procedures

DNA samples to be submitted to the analysis of termini were routinely purified by chromatography on hydroxyapatite columns from contaminating RNA, oligoribonucleotides, and ultraviolet-absorbing materials.[1]

Enzymes. Spleen DNase B, spleen phosphomonoesterase B, spleen exonuclease, and snake venom exonuclease were preparations obtained according to described procedures.[2–5] Snail DNase[6] was a preparation obtained following a method to be published. Pancreatic DNase was a commercial preparation (code D; Worthington, Freehold, New Jersey). For the definition of enzyme units see references cited in footnotes.[2–5,7]

DNase digestions were routinely performed at room temperature on native DNA (A_{260} = 8 or 0.8). In the case of spleen and snail DNases, DNA solutions in 50 m*M* ammonium acetate–1 m*M* EDTA, pH 5.5 were used; enzyme concentrations in the incubation mixture were 0.1–1 unit/ml. In the case of pancreatic DNase, digestions were carried out in 50 m*M* Tris·HCl–10 m*M* $MgCl_2$ (or $MnCl_2$), pH 7.6, with 0.02–0.2 μg of enzyme per milliliter of incubation mixture. The use of different DNases implying different digestion conditions as far as pH, ionic strength, and metal ions are concerned, the resulting oligonucleotides were dialyzed against running distilled water (see below) before further treatments.

The termini of oligonucleotides released by the snail and pancreatic DNase do not vary with the digestion level; some variation takes place during digestion by the spleen DNase, an enzyme which hydrolyzes DNA according to two different mechanisms (diplotomic and haplotomic[8]). In this latter case, which was investigated in detail,[9,10] termini released from

[1] G. Bernardi, this series, Vol. 21, p. 95. Also, *in* "Procedures in Nucleic Acids Research" (G. L. Cantoni and D. R. Davies, eds.), Vol. 2, p. 455. Harper, New York, 1971.
[2] G. Bernardi, A. Bernardi, and A. Chersi, *Biochim. Biophys. Acta* **129,** 1 (1966).
[3] A. Chersi, A. Bernardi, and G. Bernardi, *Biochim. Biophys. Acta* **246,** 51 (1971).
[4] A. Bernardi and G. Bernardi, *Biochim. Biophys. Acta* **155,** 360 (1968).
[5] S. D. Ehrlich, G. Torti, and G. Bernardi, *Biochemistry* **10,** 2000 (1971).
[6] J. Laval and C. Paoletti, *Biochemistry* **11,** 3604 (1972).
[7] J. Laval, J. P. Thiery, S. D. Ehrlich, C. Paoletti, and G. Bernardi (1973). *Eur. J. Biochem.*, submitted for publication.
[8] G. Bernardi, *Advan. Enzymol.* **31,** 1 (1968). Also, *in* "The Enzymes" (P. Boyer, ed.), 3rd ed., Vol. 4, p. 271. Academic Press, New York.
[9] C. Soave, J. P. Thiery, S. D. Ehrlich, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.
[10] J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.

different DNA's were always compared at identical oligonucleotide size levels. A convenient way to prepare the oligonucleotides having the desired average chain length ($\bar{P}_n$) is to use the relationship existing between the hyperchromic shift (HS) undergone by DNA and the reciprocal average size ($\bar{P}_n^{-1}$) of the oligonucleotides. For the spleen and snail DNases a linear relationship is obtained[9]; using the digestion conditions described above, at 22°,

$$\bar{P}_n^{-1} = 0.0065 \cdot \mathrm{HS} \cdot (\mathrm{G} + \mathrm{C}) + 0.01$$

where HS is expressed as percentage value and G + C as molar fraction; this equation is valid for $\bar{P}_n^{-1}$ values comprised between 0.02 and 0.1.

For pancreatic DNase the equivalent equation[11] determined on calf thymus DNA is

$$\bar{P}_n^{-1} = 0.0028 \cdot \mathrm{HS} + 0.025$$

which is valid for $\bar{P}_n^{-1}$ values comprised between 0.035 and 0.1.

Dialysis of Oligonucleotides.[5] Visking $^8/_{32}$-inch tubings were boiled successively in 10% sodium carbonate–0.1 *M* EDTA, 0.05 *M* ammonium acetate buffer, pH 5.5, and water. Dialysis was done at 4° against water flowing through a glass tube having a diameter only slightly larger than the dialysis tubings. Several oligonucleotide samples (2 A_{260} units at a concentration of 1–10 A_{260} units/ml) were dialyzed simultaneously against 10–25 l of distilled water for 8–14 hours. Under these conditions, even dinucleotides, when present in less than 2–3% amounts, were quantitatively retained.

DEAE-cellulose (Serva, Heidelberg, Germany; 0.57 meq/g) was washed in succession with 0.1 *N* NaOH, water, 0.1 *N* HCl, water, and 2 *M* ammonium acetate, pH 5.5. Only the finest particles, obtained by decantation and forming 1–2% of the material, were used. The column was packed from a suspension of DEAE-cellulose in 2 *M* ammonium acetate (about 1:1, v/v), and was washed with 2 *M* ammonium acetate and water immediately before use.

Average chain length or average degree of polymerization, $\bar{P}_n$, of the oligonucleotides obtained by DNase digestion was determined as described in the following section.

Nucleoside analyses were done on BioGel P-2 columns,[12] or Sephadex G-10 (Pharmacia, Uppsala, Sweden) column,[13] or, more recently, by the following modification[10] of the method of Uziel *et al.*[14]: 0.03 A_{260} unit of

[11] S. D. Ehrlich, U. Bertazzoni, and G. Bernardi, *Eur. J. Biochem.*, to be submitted for publication.

[12] G. Piperno and G. Bernardi, *Biochim. Biophys. Acta* **238**, 388 (1971).

[13] S. D. Ehrlich, J. P. Thiery, and G. Bernardi, *Biochim. Biophys. Acta* **246**, 161 (1971).

[14] M. Uziel, C. K. Koh, and W. E. Cohn, *Anal. Biochem.* **25**, 77 (1968).

nucleosides in 5 μl of 0.4 ammonium formate, pH 4.7, were loaded on a 0.2 × 5 cm column of Aminex A6 (Bio-Rad, Richmond, California), equilibrated with the same solvent; elution was done at room temperature using a flow rate of 0.75 ml per hour; a typical separation required about 90 minutes; the detection and evaluation of the nucleoside peaks were done as already described.[13]

Methods for the Determination of Terminal and Penultimate Nucleotides

Determination of 3′ Terminal Nucleotides[10,15]

Figure 3 shows the principle of the method as applied to 3′-P oligonucleotides. Oligonucleotides are first dephosphorylated, then hydrolyzed by spleen exonuclease, an enzyme which degrades oligonucleotides, starting from their 5′ terminal ends and releasing 3′-mononucleotides. The 3′ terminals, liberated as nucleosides, are separated from nucleotides, and analyzed. The same procedure can be applied to 5′-P oligonucleotides; in this case, dephosphorylation has the purpose of allowing spleen exonuclease to act. A typical determination is described below.

Dephosphorylation. Ammonium acetate 1 *M*–EDTA 20 m*M*, pH 5.5, is added to dialyzed oligonucleotides to reach 10–20 m*M* concentration of acetate. Digestion is performed with 0.1 unit/ml (final concentration)

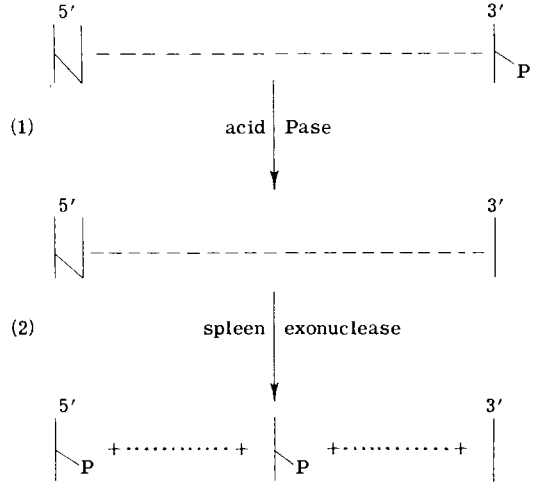

FIG. 3. A scheme of determination of the 3′ terminal nucleotide composition.

[15] M. Carrara and G. Bernardi, *Biochemistry* **7**, 1121 (1968).

of spleen acid phosphomonoesterase at room temperature for 14 hours. The enzyme is inactivated by shaking the solution vigorously for 5 minutes with 1/10 to 1/2 volume of a chloroform–isoamyl alcohol (CA) (24:1; v/v) mixture.

Exonuclease Digestion. This is done in a quartz cuvette in order to follow continuously the A_{260} increase; 0.2 unit of spleen exonuclease is added per A_{260} unit of oligonucleotide. Digestion takes about 1 hour at room temperature; the end of reaction is indicated by a plateau of hyperchromicity. In the case of oligonucleotides having an average chain length higher than 15, 0.2 unit of spleen acid DNase is added; this is necessary to insure the complete degradation of oligonucleotides which are resistant to exonuclease.

Separation of 3′ terminal nucleosides is accomplished on either a DEAE-cellulose or a QAE A-25 Sephadex column. Spleen exonuclease digests are loaded on 0.5 × 10 cm DEAE-cellulose column previously washed with 10 ml of 2 *M* ammonium acetate, pH 5.5, and then with 10 ml of water using a 20 ml per hour flow rate. UV absorbance of the column effluent is monitored. Nucleosides are washed out with 3–5 ml of water using a flow rate of 6 ml per hour; nucleotides are eluted by a step of 1 *M* ammonium acetate, pH 5.5, at a 20 ml per hour flow rate. Alternatively, the separation can be accomplished on 0.4 × 7 cm QAE Sephadex columns, washed before use for 2 hours with 2 *M* NH_4OAc–7 *M* urea and then for 3 hours with water at a 6 ml per hour flow rate. QAE-Sephadex is more suitable for work with oligonucleotides of average chain length greater than 50, since its higher capacity permits loading of greater amounts of exonuclease digests.

The average oligonucleotide chain length, $\bar{P}_n$, is taken as equal to the ratio $(A_{271N_t} + A_{271N_s})/(A_{271N_s})$ where N_t and N_s indicate nucleotides and (terminal) nucleotides, respectively.

Nucleoside analysis of 3′ terminals is accomplished as described in the previous section.

Determination of 5′ Terminal and 5′ Penultimate Nucleotides[5]

The procedure is shown in Fig. 4. Oligonucleotides are dephosphorylated, treated with pancreatic DNase in order to decrease their average size, and digested with venom exonuclease. This enzyme degrades oligonucleotides starting from the 5′ end and splits off one 5′ nucleotide at a time. 5′ Terminal dinucleoside monophosphates, being very resistant to digestion, accumulate in the digestion mixture and can be isolated by DEAE-cellulose chromatography. They are then split with spleen exonuclease to 3′ nucleotides (corresponding to the 5′ terminals of oligonuclootides) and nucleosides (5′ penultimates of oligonucleotides). These are subsequently separated and analyzed. A typical experiment is described.

Dialysis and dephosphorylation are done as described above except that

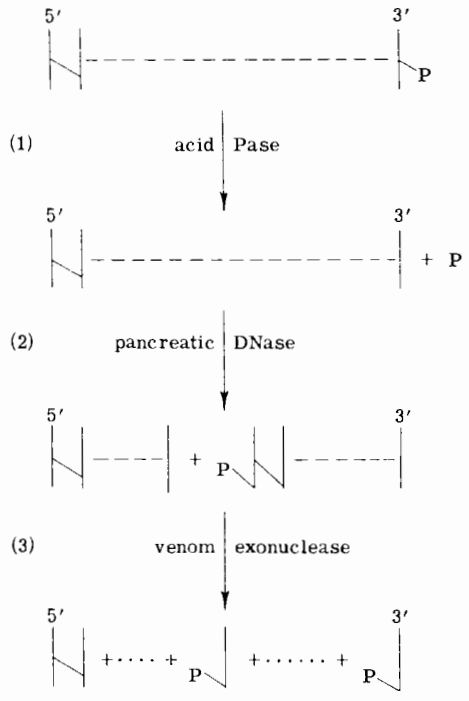

FIG. 4. A scheme of determination of the 5′ terminal and 5′ penultimate nucleotide compositions.

6 A_{260} units of oligonucleotides in 0.8 ml of buffer are treated and that the phosphatase digestion is done in 1 m*M* Tris·acetate, pH 5–5.5.

Pancreatic DNase Treatment. Dialyzed, dephosphorylated oligonucleotides, 0.2 ml, are lyophilized and dissolved in 0.25 ml of 5 m*M* $MgCl_2$. The pH is adjusted to 7.5–8 with 1 *M* Tris; 2 μl of pancreatic DNase solution (0.2 mg/ml in 10 m*M* $MgCl_2$) are added, and the sample is incubated at 37° for 10 minutes.

Venom Exonuclease Digestion. The pH of pancreatic DNase digest is adjusted to 8.8–9.0 with 1 *M* Tris. The sample is transferred to a quartz cuvette (0.1 cm optical path) and incubated at 37° with 20 μl of venom exonuclease (2 units/ml). The increase of A_{260} is followed; it is rapid in the first phase of digestion, then becomes progressively slower. When the slow phase of A_{260} increase has been reached, the yield of dinucleoside monophosphates is close to 90%; if digestion is continued, the yield decreases as some hydrolysis of dinucleoside monophosphates takes place. The reaction is stopped by diluting the sample with 0.5 ml of 4 m*M* acetic acid and shaking vigorously for 5 minutes with 0.2 ml of CA.

Isolation of Dinucleoside Monophosphates. Exonuclease digests are loaded on 0.5 × 10 cm DEAE-cellulose columns, washed previously with 1 *M* ammonium acetate for 1 hour and then with water for 2 hours at a flow rate of 6 ml per hour. The ultraviolet absorbance of column effluent is monitored. Nucleosides are eluted with water and then a linear gradient of ammonium acetate, pH 7.6, is started (0–1 *M*, total volume 100 ml). As soon as dinucleoside monophosphates are eluted, the slope of the gradient is increased by reducing the total gradient volume to 15 ml. To achieve the separation, it is essential to use the finest particles, forming less than 5% of DEAE-cellulose. These can be prepared by sieving or by decantation.

Spleen Exonuclease Digestion. Dinucleoside monophosphates are lyophilized after chromatography on DEAE-cellulose, dissolved in 0.6 ml of 17 m*M* ammonium acetate and digested at 22° with 10 μl of spleen exonuclease (40 units/ml). The A_{260} increase is followed; its plateau indicates the end of the reaction (about 10 minutes).

DEAE-cellulose chromatography of spleen exonuclease digest is done on 0.4 × 7 cm DEAE-cellulose columns, washed previously for 1 hour with 1 *M* ammonium acetate and then for 1.5 hours with water using a flow rate of 6 ml/hour. Nucleosides (5′ penultimates) are washed out with water; nucleotides (5′ terminals) are subsequently eluted with a step of 0.4 *M* ammonium acetate.

Dephosphorylation of Nucleotides. Nucleotides are lyophilized in order to get rid of excess of salt and dissolved in 15 m*M* ammonium acetate, pH 5.5, 10 μl of a 0.5% bovine serum albumin solution in water and 0.5–1 unit of acid phosphomonoesterase are added. Dephosphorylation is carried out for 12–18 hours at 22°. Nucleosides are isolated from the digestion mixture by a DEAE-cellulose column chromatography performed as described in preceding paragraph.

Nucleoside analysis is accomplished as described in the preceding section.

Determination of 3′ Penultimate Nucleotides[16]

The isolation and analysis of this terminus was performed only on spleen acid DNase digests of calf thymus DNA. We will briefly mention here the two methods used to determine it. The first one (Fig. 5), which can be used for any 3′-P-ended oligonucleotides, was to split the spleen DNase digest with pancreatic DNase in the presence of Mn^{2+}; the dinucleoside triphosphates, which represent about half of the products originated from

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

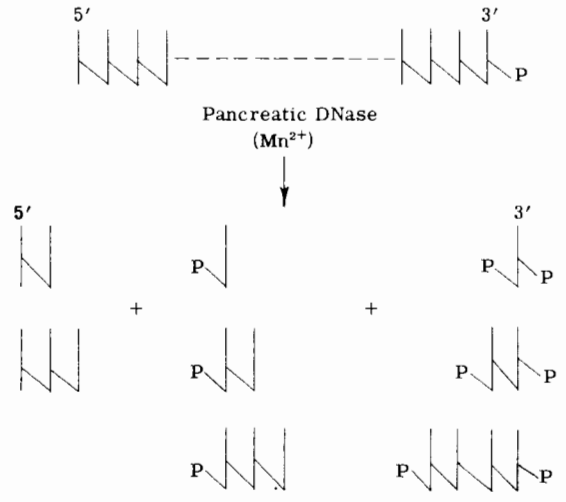

FIG. 5. Products formed by digestion of spleen DNase oligonucleotides with pancreatic DNase in the presence of Mn^{2+}. Dinucleoside triphosphates which were subsequently analyzed are underlined. From A. Devillers-Thiery, S. D. Ehrlich, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.

the 3′-phosphate ends of the fragments, were isolated, dephosphorylated, and analyzed as described above for dinucleoside monophosphates.

The second approach consisted in quantitatively splitting, with pancreatic DNase in the presence of Mn^{2+}, the tetranucleotides into doublets; these were subsequently isolated and analyzed. It should be mentioned that the analysis of the 3′ terminal, 5′ terminal, and 5′ penultimate nucleotides of tetranucleotides which can represent as much as 25% of the isostichs in acid DNase digests, showed values in agreement with those obtained on total digests ($\bar{P}_n = 15$); suggesting that the 3′-penultimate nucleotides of tetranucleotides are representative of those of total digests. The results obtained by this method were in agreement with those obtained by the first one.

Specificity of DNases[7,10,16,17]

Tables I and II show the compositions of the termini released by spleen and snail DNases, respectively, from three bacterial DNA's of different G + C contents. The data obtained for each terminus strongly differ from those expected from a random degradation, in which case the composition of each terminus considered should be equal to the average DNA

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

TABLE I
TERMINI RELEASED FROM DIFFERENT DNA'S BY SPLEEN ACID DNASE[a]

DNA	Nucleotide	3′ Terminal	5′ Terminal	5′ Penultimate
Hemophilus	G	37	30	22
influenzae	T	22	15	18
(38% G + C)	C	8	34	11
	A	33	22	49
Escherichia coli	G	47	32	26
(51% G + C)	T	17	12	14
	C	11	39	15
	A	25	17	45
Micrococcus luteus	G	56	44	42
(72% G + C)	T	12	5	8
	C	17	44	18
	A	15	7	32

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

composition. The possibility exists, however, that some of the differences merely reflect the composition of the nearest neighbors of the termini actually recognized by the enzymes. Such a possibility can be checked by comparing the experimental results with those expected from the nearest-

TABLE II
TERMINI RELEASED FROM DIFFERENT DNA'S BY SNAIL ACID DNASE[a]

DNA	Nucleotide	3′ Terminal	5′ Terminal	5′ Penultimate
Hemophilus	T	15	25	36
influenzae	G	9	32	19
	A	74	21	27
	C	2	22	18
Escherichia coli	T	16	22	29
	G	15	38	25
	A	66	13	24
	C	3	27	23
Micrococcus luteus	T	14	8	15
	G	26	46	43
	A	58	5	9
	C	2	41	33

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

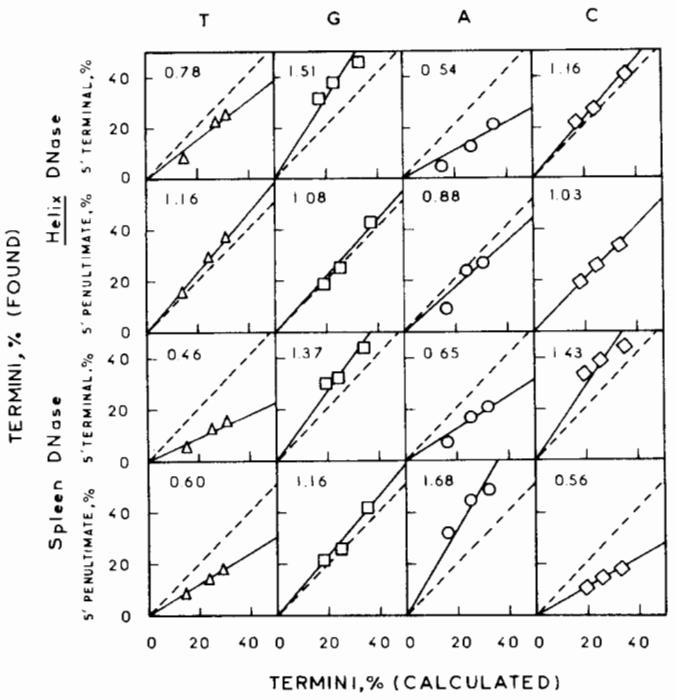

FIG. 6. The observed compositions of the 5′ terminal and 5′ penultimate nucleotides released from bacterial DNA's by the spleen and the snail DNase are plotted against the compositions calculated for the nearest neighbors of the 3′ and 5′ terminal nucleotides, respectively. The points are aligned on the broken line of slope 1, if the enzyme makes no selection. The slopes of lines through the points are shown in the left-hand upper corner of each frame. From J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication; and S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.

neighbor data. Figure 6 shows that the composition of the 5′-terminal and penultimate nucleotides obtained by spleen DNase digestion is quite different from that expected from nearest-neighbor data; in contrast, in the case of the snail enzyme, this is true only for the 5′ terminal nucleotide, whereas the composition of the 5′ penultimate nucleotide is practically identical with that expected from nearest-neighbor data. Since the 3′ terminal nucleotides have a composition different from that expected for the nearest neighbors of the 5′ terminal nucleotides (Table II), it should be concluded that the spleen DNase recognizes sequences of at least three nucleotides and the snail DNase sequences of at least two nucleotides. In fact, the analysis of the 3′ penultimate nucleotide released by the spleen

TABLE III
AVERAGE COMPOSITION OF SEQUENCES SPLIT BY ACID DNASE IN CALF THYMUS DNA AT $\bar{P}_n = 15$[a,b]

	3′-P penultimate		3′-P terminal			5′-OH terminal			5′-OH penultimate	
Residue	Exp.	Calc.	Calc.	Exp.	Calc.	Calc.	Exp.	Calc.	Calc.	Exp.
T	22	(31)	(29)	20	(32)	(29)	11	(29)	(29)	14
G	16	(21)	(22)	43	(21)	(23)	43	(23)	(19)	26
A	46	(30)	(29)	29	(30)	(29)	18	(30)	(31)	52
C	16	(19)	(20)	8	(17)	(18)	28	(18)	(20)	8

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

[b] Values in parentheses indicate the composition of each terminus as calculated from its nearest neighbor(s).

DNase from calf thymus DNA strongly indicates that this nucleotide is also recognized by the enzyme (Table III). Finally, it should be mentioned that recent experiments have shown that pancreatic DNase recognizes a sequence of at least three nucleotides.[11]

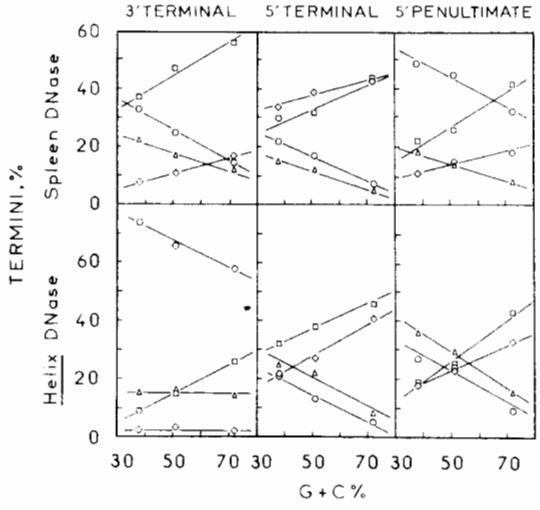

FIG. 7. Plot of the percentage of A(○), G(□), C(◇), and T(△) in the 3′ terminal, 5′ terminal, and 5′ penultimate nucleotides formed by the spleen and the snail DNase from bacterial DNA's as a function of their G + C contents. From J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication; and S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, submitted for publication.

Relationships between the Composition of Termini and the Base Composition of DNA's

If the data of Tables I and II are plotted against the G + C contents of the DNA's used, linear relationships are obtained (Fig. 7). This is not surprising if one considers that (a) both enzymes recognize a large number of sequences, as indicated by the fact that the average size of the final digests are of the order of 4–6, which means that about 20% of all internucleotide bonds can be broken by the enzymes; (b) the termini deriving from all the sequences which were split are averaged out; (c) the observed doublet frequencies show a linear relationship with the frequencies predicted for random association,[18,19] indicating a common type of doublet distribution in the bacterial DNA's examined.

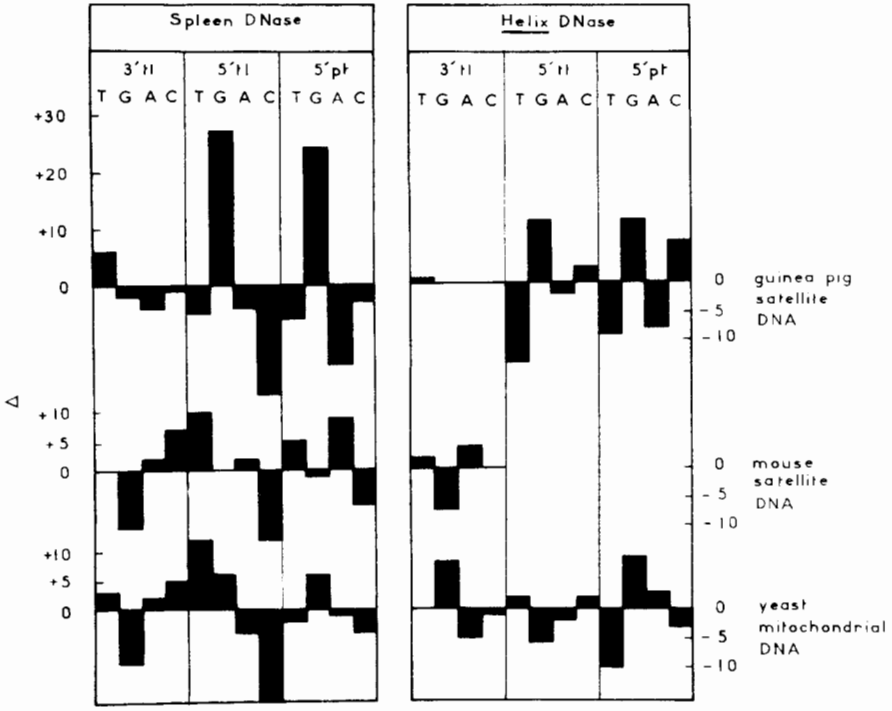

FIG. 8. Differences between the percentages of 3′ terminal, 5′ terminal, and 5′ penultimate nucleotides formed from "repetitive" DNA's and the corresponding values for bacterial DNA's. From J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, and G. Bernardi, *Eur. J. Biochem.*, to be submitted for publication.

[18] A. D. Kaiser and R. L. Baldwin, *J. Mol. Biol.* **4,** 418 (1962).

[19] M. N. Swartz, T. A. Trautner, and A. Kornberg, *J. Biol. Chem.* **237,** 1961 (1962).

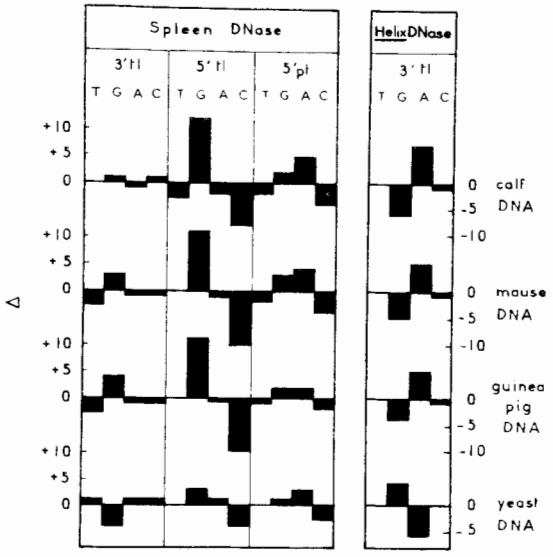

FIG. 9. Differences between the percentages of 3′ terminal, 5′ terminal, and 5′ penultimate nucleotides formed from eukaryote DNA's and the corresponding values for bacterial DNA's. In the case of yeast, nuclear DNA was used. From S. D. Ehrlich and G. Bernardi, *J. Mol. Biol.*, to be submitted for publication.

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.