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The Specificity of Pancreatic Deoxyribonuclease

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The kinetics of degradation of calf thymus DNA by pancreatic DNAase has been investigated with the main purpose of providing the background for studying the specificity of the enzyme and its use in investigations of nucleotide sequences in DNA. The relationships between the hyperchromic shift or acid solubility and the reciprocal average degree of polymerization ($\bar{P}_n^{-1}$) of the hydrolysate have been established; these can be used in order to prepare oligonucleotides of desired average chain length simply by measuring the increase of absorbance or of acid solubility of the digest during DNA degradation.

The specificity of the pancreatic DNAase was studied by determining the base compositions of 3′-terminal, 5′-terminal and 5′-penultimate nucleotides of oligonucleotides released by the enzyme. The 3′-terminal nucleotide composition was found not to vary in the $\bar{P}_n$, or average size, range 150 to 8 and 30 to 8 for digestions of calf thymus DNA in the presence of magnesium and manganese ions, respectively, indicating that no change in the specificity takes place during digestion. The compositions of 3′-terminal, 5′-terminal and 5′-penultimate nucleotides differ from those expected on the basis of random splitting of DNA. The conclusion is drawn that pancreatic DNAase recognizes sequences at least three nucleotides long.

Recent investigations carried out in our laboratory have led to the identification of specific patterns in the termini[1] of oligonucleotides released from different DNAs by spleen DNAase [1—4], snail DNAase [5, 6] and *Escherichia coli* endonuclease I [7]. The conclusion was drawn that these enzymes recognize nucleotide sequences in DNA. These findings allowed us to develop a new method for characterizing and comparing nucleotide sequences in DNAs by determining the base composition of termini released from the sequences split by DNAases [4, 6, 8].

In the present work we have investigated: (a) the changes of hyperchromicity, acid solubility and average chain length of the digest, during degradation of calf thymus DNA by pancreatic DNAase; (b) the termini of oligonucleotides released from different DNAs. The main findings are: (a) the demonstration that pancreatic DNAase recognizes sequences at least three nucleotides long: (b) the invariance of the specificity of the enzyme during the course of DNA degradation; (c) the demonstration that a linear relationship exists between the relative amounts of 3′-terminals released from different bacterial DNAs and their (dG + dC) contents; in contrast, the 3′-terminals obtained from calf thymus DNA do not fit the relationships established with bacterial DNAs. These results are similar to those found for the spleen and the snail DNAase [3—6] and *E. coli* endonuclease I [7]; they extend to pancreatic DNAase the conclusion that DNAases (a) recognize nucleotide sequences and (b) can be used to study nucleotide sequences in DNA.

[1] With this term we designate, conventionally, the 3′-terminal, 5′-terminal and 5′-penultimate nucleotides.

Abbreviations. $\bar{P}_n$ average degree of polymerization, average size or average chain length of oligonucleotides.

Enzymes. Deoxyribonuclease from bovine pancreas (EC 3.1.4.5); acid deoxyribonuclease from porcine spleen (EC 3.1.4.6); acid exonuclease from porcine spleen (EC 3.1.4.1); acid phosphomonoesterase from porcine spleen (EC 3.1.3.2); snake venom exonuclease from *Crotalus adamanteus* (EC 3.1.4.1); terminal deoxyribonucleotidyl transferase from calf thymus (EC 2.7.7.7); acid deoxyribonuclease from *Helix aspersa*, Müll. (EC 3.1.4.—); endonuclease I from *E. coli* (EC 3.1.4.—).

MATERIALS AND METHODS

DNAs

Calf thymus DNA was preparation CTR 2 [9].

E. *coli* and *Haemophilus influenzae* DNAs were preparations already described [4].

Enzymes

Pancreatic DNAase (code D) was purchased from Worthington (Freehold, N. J.). It is known that the

commercial enzyme preparation contains several components [10,11]; in this work we did not attempt to examine them separately. Spleen DNAase [12], spleen exonuclease [13], spleen phosphomonoesterase [14] and venom exonuclease [2] were prepared according to Bernardi *et al.* Terminal deoxyribonucleotidyl transferase was prepared by a modification of method of Yoneda and Bollum [15,16].

Pancreatic DNAase Digestions

These were performed at 25 °C on native DNA, using solutions having an $A_{260} = 8.0$ in 0.05 M Tris-HCl pH 7.6, $MgCl_2$ or $MnCl_2$ was 0.01 M. Absorbance at 260 nm was followed during digestion; at different times aliquots were withdrawn and the reaction stopped by shaking the samples with 0.1—0.5 volumes of chloroform—isoamyl alcohol mixture (24:1, v/v).

Size and Terminals of Oligonucleotides

Average size of oligonucleotides (average chain length, $\bar{P}_n$), and compositions of 3′-terminal, 5′-terminal and 5′-penultimate nucleotides were determined as previously described [4,9,16,17] on samples dialyzed against running water [2]. DEAE-cellulose-7 M urea chromatography [18] of samples showed that upon dialysis there was no loss of oligonucleotides even as small as dinucleotides. Acid solubility of the digest was determined as described earlier [9].

RESULTS

Kinetics of DNA Degradation

Fig. 1 shows the time course of the hyperchromicity shift of calf thymus DNA during digestion by pancreatic DNAase in the presence of magnesium ions. As for the spleen [9] and the snail [5] DNAase, three distinct phases were observed; these were characterized by: (a) an initial lag time during which the DNA size decreases, but very little melting of fragments takes place; melting was shown to be responsible for the increase of absorbance accompanying digestion of DNA [9]; (b) a linear increase of hyperchromicity; (c) a progressive slowing down, probably because of exhaustion of the preferred, double-stranded substrate [9]. Replacing magnesium with manganese did not change this pattern.

Fig. 2 shows a plot of the relative absorbance of the pancreatic DNAase hydrolysate against its reciprocal average size ($\bar{P}_n^{-1}$). A sigmoidal relationship was obtained, differing from those obtained for spleen [9], snail [5] and *E. coli* [7] DNAases; the corresponding relationships for these enzymes lack the first non-linear part. The part of the curve shown in Fig. 2 comprised between $\bar{P}_n^{-1}$ values of 0.035 and 0.1 can be approximated by the equation.

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

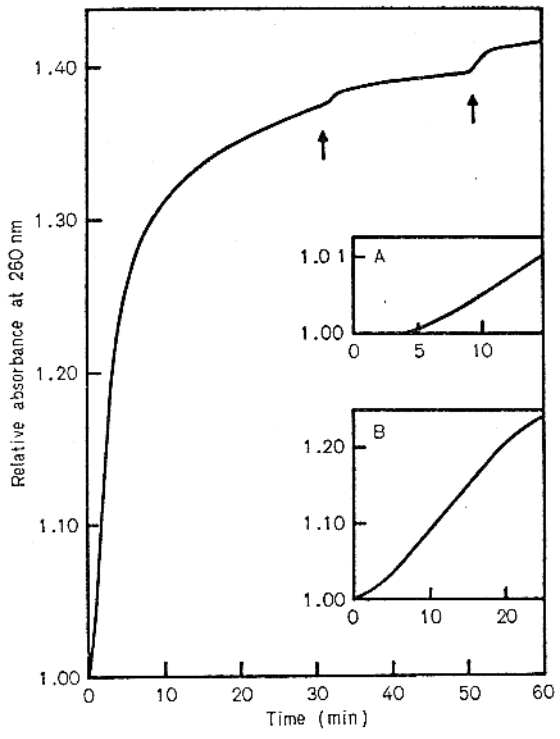

Fig. 1. *Hyperchromic shift of calf-thymus DNA during pancreatic DNAase digestion.* The values refer to digestion of DNA solution, $A_{260} = 8.0$ in 0.05 M Tris-HCl, 0.01 M $MgCl_2$, pH 7.6 at 25 °C with 1 μg/ml (main figure), 0.015 μg/ml (insert A), 0.17 μg/ml (insert B), respectively, of pancreatic DNAase. Arrows indicate further enzyme additions (1 μg/ml)

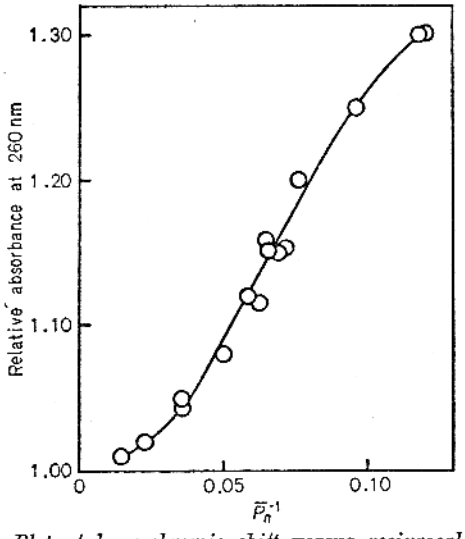

Fig. 2. *Plot of hyperchromic shift* versus *reciprocal average chain length of oligonucleotides.* Conditions of digestion and $\bar{P}_n^{-1}$ determinations were as described in Materials and Methods

where HS is the hyperchromic shift at 260 nm expressed in percentage. This equation can be used in order to prepare oligonucleotides of desired $\bar{P}_n$ simply by measuring the increase of absorbance during digestion of calf thymus DNA.

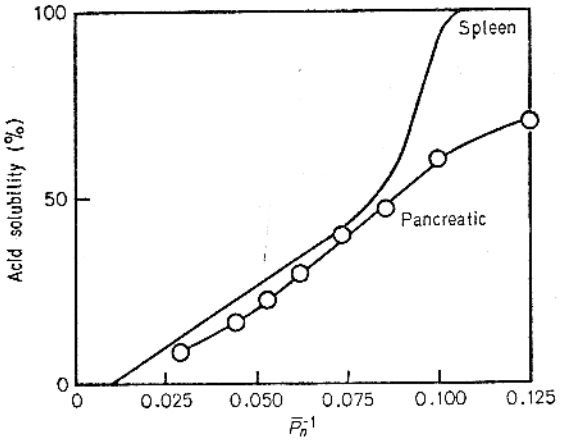

Fig. 3. *A plot of the relative acid solubility of pancreatic DNAase digest against its reciprocal size.* The results correspond to the digestion of 25 ml of calf thymus DNA solution having $A_{260} = 8.0$ in 0.05 M Tris-HCl pH 7.6, 0.01 M $MgCl_2$, with 0.2 μg/ml of pancreatic DNAase at 25 °C. $\bar{P}_n^{-1}$ values were calculated from the hyperchromic shift determinations using the relationship of Fig. 2. The spleen DNAase data [9] are shown for the purpose of comparison

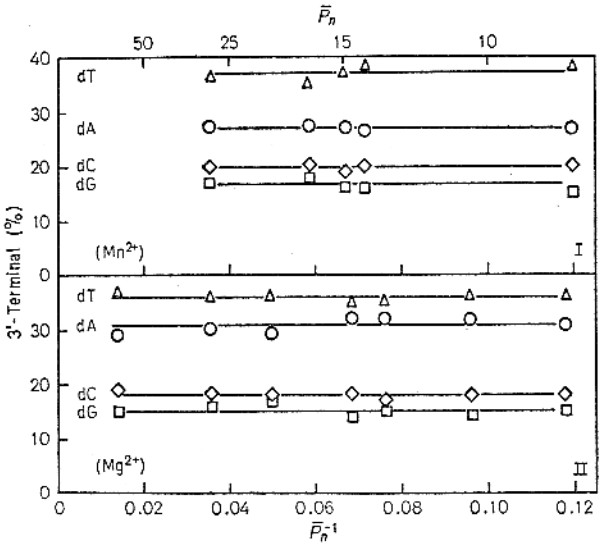

Fig. 4. *3'-Terminal nucleotides obtained at different degradation levels of calf-thymus DNA by pancreatic DNAase in the presence of (I) manganese and (II) magnesium ions*

It should be stressed that the increment of relative absorbance per broken bond [which is proportional to the constant factor of Equation (1)] is found to be very close to those obtained with spleen DNAase [9], snail DNAase [5] and *E. coli* endonuclease I [7]. The lower increment observed with the venom exonuclease and micrococcal nuclease [19] is clearly due to the exonucleolytic mode of action of these two enzymes, an exonucleolytic break providing much less (if any) melting, as compared to the endonucleolytic break.

Fig. 3 shows a plot of the acid solubility of the pancreatic DNAase digest against its reciprocal average size. Results obtained with the spleen DNAase [9] are shown for the sake of comparison. Some differences can be seen during the early phase ($\bar{P}_n^{-1} < 0.09$), the pancreatic DNAase relationship being slightly sigmoidal while that of the spleen DNAase is linear. The major difference takes place at the $\bar{P}_n^{-1} \approx 0.09$; the acid solubility of the spleen DNAase digest increases exponentially to completion at a $\bar{P}_n^{-1} = 0.1$, while that of the pancreatic DNAase slows down and is close to 75% at a $\bar{P}_n^{-1} = 0.125$. These differences can be explained by the greater preference of the spleen, as compared to the pancreatic, DNAase for native DNA fragments; the former enzyme preferentially splits the longest fragments still having the native structure, thus reducing their size below the threshold acid-solubility size level [9].

Composition of Termini

Fig. 4. shows the relative amounts of 3'-terminal nucleotides released by the pancreatic DNAase

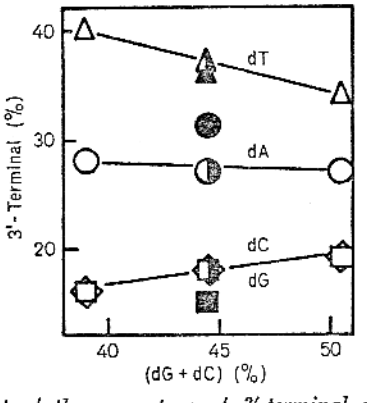

Fig. 5. *Plot of the percentage of 3'-terminal nucleotides as function of DNA (dG + dC) contents.* (△) Thymidine; (○) deoxyadenosine; (□) deoxyguanosine; (◇) deoxycytidine. Open, half-filled and filled symbols correspond to terminals released from bacterial DNAs, mixture of bacterial DNAs and calf thymus DNA, respectively

from calf thymus DNA in the presence of manganese and magnesium ions, respectively, plotted against the reciprocal average size of the digest. The compositions are invariant in both cases, at least at the size levels studied ($\bar{P}_n$ between 30 and 8 for manganese and between 80 and 8 for magnesium). 3'-Terminals formed in the presence of magnesium were also determined at a much higher size level, ($\bar{P}_n \approx 150$) by terminal labeling of oligonucleotides as described by Bertazzoni *et al.* [16]; their composition (36% thymidine, 33% deoxyadenosine, 14% deoxyguanosine and 18% deoxycytidine) was in excellent agreement with the values indicated in the Fig. 3.

Table 1. *Average composition of termini released by pancreatic DNAase from calf-thymus DNA in the presence of magnesium and manganese*
Values in parentheses indicate the composition of each terminus as calculated from its nearest neighbor(s)

Ion	Nucleotide	3′-Terminal		5′-Terminal			5′-Penultimate	
		Expt	Calcd	Calcd	Expt	Calcd	Calcd	Expt
		%						
Mg^{2+}	dT	36	(30)	(29)	38	(30)	(29)	13
	dG	15	(20)	(21)	22	(20)	(20)	36
	dA	31	(30)	(29)	15	(30)	(31)	30
	dC	18	(20)	(20)	25	(19)	(20)	21
Mn^{2+}	dT	37	(36)	(29)	39	(30)	(29)	14
	dG	15	(20)	(21)	22	(20)	(21)	32
	dA	27	(30)	(29)	17	(30)	(29)	31
	dC	20	(21)	(21)	22	(20)	(21)	23

Table 2. *3′-Terminals released by pancreatic DNAase from two bacterial DNAs and their mixture in the presence of magnesium*

Nucleotide	Terminals in DNA		
	H. influenzae (dG + dC = 38%)	Mixture (dG + dC = 44.5%)	*E. coli* (dG + dC = 51%)
	%		
dT	40	37	34
dG	16	18	19
dA	28	27	27
dC	16	18	19

These results show that the specificity of pancreatic DNAase does not vary during digestion.

Table 1 shows compositions of 3′- and 5′-terminal and 5′-penultimate nucleotides released from calf thymus DNA by pancreatic DNAase in the presence of magnesium and manganese ions. In both cases the results obtained differ from (a) the base composition of the DNA (dG + dC = 44%); (b) the compositions calculated from the nearest neighbor frequences [20]. These results indicate that the pancreatic DNAase recognizes sequences at least th ee nucleotides long. The termini released in the presence of magnesium and manganese ions are slightly, but significantly, different; this result shows that the pancreatic DNAase recognize different nucleotide sequences in the presence of different metal ions.

Table 2 shows the 3′-terminals released by the pancreatic DNAase from two bacterial DNAs (*H. influenzae* and *E. coli*) and the 1:1 mixture of the two, the use of which is justified by previous results [4]. As for the spleen [4] and the snail [6] DNAases, a linear relationship is found to hold between the percentage of terminal released and the (dG + dC) contents of the bacterial DNAs as shown in Fig. 5. The composition of 3′-terminals formed by digestion of calf thymus DNA deviate from that relationship.

The results obtained with different DNAs (Tables 1 and 2) show that the compositions of termini reported here cannot be compared directly with those obtained by Weiss *et al.* [21] with T7 DNA. The 3′-terminal composition found by these authors is nevertheless not too far from those reported here; 5′-terminals differ more, thymidine being, however, predominant in both cases.

DISCUSSION

The compositions of termini released by the pancreatic DNAase (Table 1) differ from those expected on the basis of random breakage of DNA; in this case the composition of each terminus should be equal to the DNA base composition. The possibility exists, however, that some of these differences merely reflect the compositions of nearest neighbors of the position actually recognized by the enzyme. Such a possibility can be checked by comparing the experimental results with those expected from the nearest neighbors data. Table 1 shows that the compositions of all three positions are different from those expected, indicating that pancreatic DNAase recognizes sequences at least three nucleotides long. The sequence specificity was also found for the spleen, and snail DNAases and *E. coli* endonuclease I [4—7], as well as for the restriction endonucleases [22,23]. This latter class of DNAases differs from the former in two respects: (a) it recognizes a much smaller number of sequences and (b) these have a larger size. It is therefore very likely that specificity towards nucleotide sequences such as those shown by the enzymes investigated so far is a general property of DNAases.

A linear relationship was found to hold between (dG + dC) contents of bacterial DNAs and the relative amounts of 3′-terminals formed by pancreatic DNAase. This linearity is expected for the reasons already discussed in the case of spleen and snail DNAase [4,6]; namely: (a) the distribution of fre-

quences of nucleotide sequences of the same type in the bacterial DNAs studied, as indicated by the nearest-neighbor analysis [24,25]; (b) the enzyme recognizes a large number of sequences; (c) the termini deriving from the sequences which are split are averaged out by our analytical methods. The terminals released from the calf thymus DNA show a deviation from the linear relationship for bacterial DNAs (a result already obtained for the spleen and the snail DNAases [4,6]), the reason being that the distribution of frequences of nucleotide sequences is of a different type for the calf thymus DNA as compared to bacterial DNAs [20]. The observed deviations for the calf thymus DNA, though small, are significant since a precision of $\pm 1\%$ was regularly obtained in the determination of 3′-terminal nucleotide in this $\bar{P}_n$ range.

The results reported here answer several questions raised by previous investigations [26]. They unequivocally show that the specificity of pancreatic DNAase does not vary during DNA digestion (Fig. 3), in agreement with results obtained on poly-[d(A-T)] [27]. They also show (Table 1) that different ions can influence the sequence specificity of pancreatic DNAase, a result in agreement with the work of Bollum [28] on the degradation of biosynthetic polymers. The different initial kinetics of DNA degradation with manganese and magnesium ions [29] might be somehow related to this change in specificity. The results reported by Murray [30], namely that the oligonucleotides released by pancreatic DNAase have different terminals but appear in highly reproducible amounts in the digestion mixture, are also easy to understand, taking into account our conclusion that this enzyme recognizes nucleotide sequences in DNA.

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