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PARTIAL PURIFICATION AND PROPERTIES OF A CHROMATIN-BOUND DEOXYRIBONUCLEASE FROM THE EMBRYO AXES OF GERMINATING PEA

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Key Word Index—*Pisum sativum*; Leguminosae; pea; chromatin; DNA; DNase.

Abstract—A deoxyribonuclease associated with chromatin extracts of the embryo axes of germinating pea seeds has been partially purified. The enzyme rapidly catalyses the hydrolysis of denatured DNA and has low activity on native DNA substrates. The enzyme can be partially purified from chromatin by solubilization with ammonium sulphate followed by anion-exchange chromatography and gel filtration. The purified DNase is inhibited by 8 mM Ca^{2+} and has a M_r of *ca* 18 000. The enzyme hydrolyses the synthetic duplex alternating copolymer poly(dA-dT): poly(dA-dT) at a 10-fold higher rate than the duplex copolymer poly(dG-dC): poly(dG-dC). This, and the limited extent of hydrolysis of native DNA, suggests that the sites of action of the DNase in native DNA are in regions exhibiting 'structural breathing', i.e. transient single-strandedness or local single-stranded regions caused by the presence of mismatched bases.

INTRODUCTION

Enzymes having a degradative activity on nucleic acids in general, and on DNA in particular, are widespread throughout all living organisms. While some of these enzymes have clearly defined physiological roles, for example, in the recycling of deoxyribonucleotides from senescing cells or in DNA repair, there are many deoxyribonucleases (DNases) to which no physiological roles have been assigned. DNase enzymes are likely to take part in several of the important processes involving DNA such as repair, recombination and degradation [1–3], while a role in replication has also been suggested (e.g. ref. [4]). However, progress to identify and characterise the enzymes has been slow. In an earlier paper [5], we demonstrated the presence of a chromatin-bound deoxyribonuclease in pea seedlings. The crude enzyme exhibited enhanced activity in the presence of Ca^{2+} ions, and during germination, its activity increased at about the same time as DNA replication was initiated. Preliminary evidence for the existence of an inhibitor of enzyme activity was also presented. In this paper we report a partial purification of the chromatin-bound endodeoxyribonuclease followed by a more detailed characterization. The results are discussed in relation to our earlier data and to postulated roles of endodeoxyribonucleases.

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RESULTS

Optimal assay conditions

Total DNase activity was previously assayed [5] using double-stranded high -M_r calf thymus DNA as substrate in the presence of 8 mM Ca^{2+}. However, there is markedly higher activity with a heat-denatured (single-stranded) substrate than with a double-stranded substrate (Fig. 1). The activity of fraction 1 DNase (i.e. crude chromatin) on heat-denatured DNA is 25-fold greater than the activity of the enzyme on native DNA. Heat denaturation of a double-stranded substrate *after* enzyme assay did not release any further acid-soluble material indicating that the enzyme does indeed have a strong preference for single-stranded DNA. This was further confirmed by the treatment, prior to assay, of double-stranded DNA with S1 nuclease to remove any minor single-stranded regions. This treatment rendered double-stranded DNA almost completely resistant to the pea DNase.

The presence of 8 mM Ca^{2+} ions is found to stimulate Fraction I DNase single-strand degrading activity by 40% (Fig. 2) which is similar to our earlier finding that 8 mM Ca^{2+} stimulated the double-strand degrading activity by 60%. However, at all further purification steps, it is found that 8 mM Ca^{2+} inhibits the total DNase activity. There is 45% inhibition of solubilized, de-salted, i.e. fraction II DNase (results not shown) and 80% inhibition of fraction III DNase (i.e. after DEAE-cellulose) activity by 8 mM Ca^{2+} with single-stranded DNA as substrate (Fig. 2); thus the purer the enzyme, the greater the degree of inhibition by Ca^{2+}.

In the light of these findings, all DNase assays were subsequently performed using heat-denatured (single-

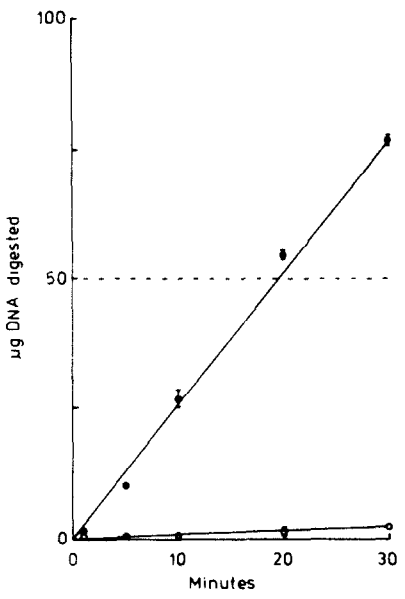

Fig. 1. Activity of fraction I DNase (i.e. crude chromatin) on double-stranded and single-stranded DNA, assayed by conversion of ^{3}H-labelled, pea DNA to an acid-soluble form. ●—●, single-stranded DNA: ○—○, double-stranded DNA. Points are means of two replicates from one experiment of several which gave similar results. Bars show the range of the two replicates. Some bars are hidden by the points. The horizontal dashed line indicates hydrolysis of 10% of the input substrate.

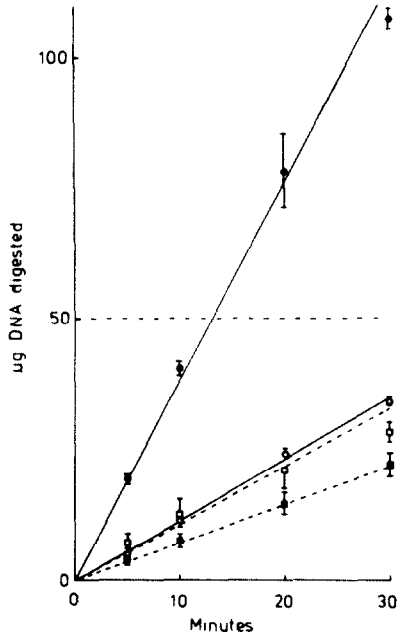

Fig. 2. Effect of 8 mM Ca^{2+} ions on DNase activity. Assay and replication of data as in Fig. 1. ○, ●, –, Crude enzyme (Fraction I); □, ■, ----, after DEAE-cellulose chromatography (Fraction III). ○, □ = −Calcium; ●, ■ = +Calcium. The horizontal dashed line indicates hydrolysis of 10% of the input substrate.

stranded) DNA as substrate except where otherwise indicated; calcium was omitted from the assay.

Enzyme purification

The purification procedure for the isolation of chromatin-bound pea DNase from the embryo axes of germinated peas is summarized in Table 1. There are several points that need to be made about the purification of the DNase enzyme. Firstly, the starting purity of 1.0 is based on the crude chromatin; this in itself represents a several-fold purification over unfractionated homogenates. Secondly, there is apparently a five-fold increase in the total activity (i.e. yield) of the enzyme after solubilization with ammonium sulphate followed by de-salting. This indicates the possibility of the loss of an inhibitor of DNase activity from the crude preparations, especially as there is no significant increase in yield during the next step of

Table 1. Purification of pea chromatin DNase

Purification step	Total protein (mg)	Units*	Specific activity (units/mg *protein*)	Apparent yield (%)	Apparent purification (fold)
I : Chromatin	11.2	0.6	0.055	100	1.0
II : Solubilized, de-salted	4.75	3.25	0.685	542	12.5
III : After DEAE-cellulose	3.95	3.4	0.86	567	15.6
IVa : After Sepharose 6B (no glycerol)	1.2	0.215	0.175	35.8	3.2
or					
IVb : After Sepharose 6B (run in presence of glycerol)	1.2	1.52	1.26	253	22.9

* µg single-stranded DNA hydrolysed per min.

purification. Thirdly, despite the long term stability of the enzyme at −18°, there is a marked decrease in the specific activity of the enzyme during gel filtration on Sepharose 6B. This may be ascribed to loss of activity during the long period of time at 2–4° taken to complete the gel filtration. In long term incubations of the enzyme in gel filtration buffer at 2–4°, 50% of activity was lost in 1 hr and 70% in 6 hr, confirming the instability at this temperature. Inclusion of 20% (v/v) glycerol in the buffer stabilized the enzyme and gel filtration in the presence of glycerol, although slow, ameliorated the loss of activity during this step (Table 1). The further purification of the enzyme afforded by gel filtration on Sepharose 6B was confirmed by PAGE under denaturing conditions. Before gel filtration, the enzyme preparation yielded many protein bands; after gel filtration, only one, somewhat diffuse band, with an M_r in the range 16–19000, was apparent (Bryant, J. A. and Fitchett, P. N., unpublished data). Finally, anion-exchange chromatography of solubilized, de-salted DNase (fraction II) gave variable results: in the majority of cases, one peak of DNase activity was eluted from the column in the wash volume (Fig. 3); in about 20% of our experiments two peaks of activity were observed, one in the wash volume and a second eluting with 0.4 M potassium chloride (data not shown). When two peaks of DNase activity were observed, the activities of each were rather low. In addition, re-chromatography of the material eluting in the wash volume on DEAE-cellulose under the same conditions as before, also gave rise to the same two peaks of DNase activity. In all other respects the activity eluting at 0.4 M potassium chloride had very similar properties to the activity eluting in the wash volume. These data suggest that the second peak of activity is derived from the first.

M_r

The M_r of the enzyme was determined by gel filtration on Sepharose 6B. Enzyme solubilized from crude chromatin (= solubilized fraction I) or enzyme eluted from DEAE-cellulose (fraction III) was chromatographed on the column which had previously been calibrated with blue dextran, catalase, alkaline phosphatase, ovalumin, chymotrypsinogen A, myoglobin and ribonuclease A (Fig. 4). The M_rs of solubilized fraction I DNase and of fraction III DNase were calculated as 18 500 ± 3000 and 16 000 ± 1000 respectively.

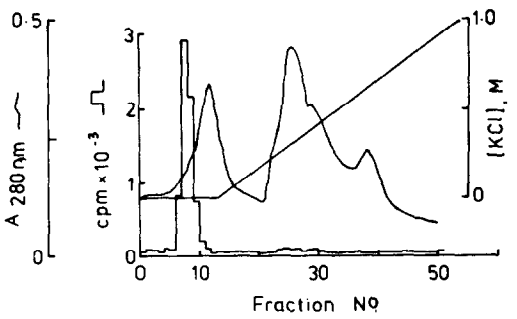

Fig. 3. DEAE-cellulose anion-exchange chromatography of fraction II DNase. Assay as in Fig. 1. Histogram: DNase activity. Solid line: A_{280}. Diagonal: KCl concentration in gradient.

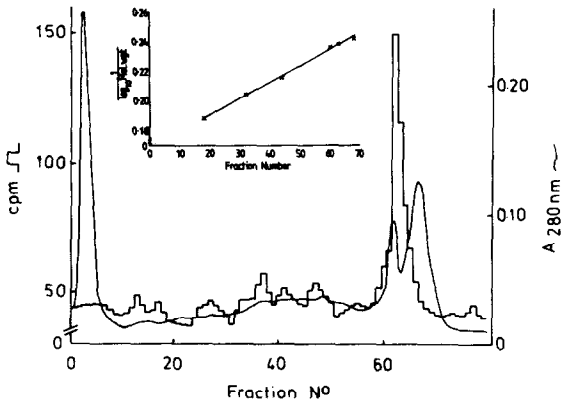

Fig. 4. Gel filtration on Sepharose 6B of solubilized fraction II DNase. Assay as in Fig. 1. Histogram: DNase activity. Solid line A_{280}. Inset shows calibration graph of marker proteins with position of DNase marked by the open circle. Marker proteins: catalase, alkaline phosphatase, ovalbumin, myoglobin, RNase A.

Activity of purified DNase on deoxyhomopolymers and copolymers

The activity of Fraction III DNase on various deoxyhomopolymers and copolymers is shown in Fig. 5. The DNase has greatest activity on the purine analogue polymer poly(dI); the hydrolysis of this polymer reaches a maximum after 10 min when just under 50% of the input DNA has been converted to acid-soluble fragments. Poly(dA) and poly(dT) are also hydrolysed extensively with 43 and 46% of the input DNA being solubilized in 1 hr respectively, although the initial rates are lower than with poly(dI). In contrast, only 15% of the poly(dC) is hydrolysed over 1 hr. In terms of hydrolysis rates therefore, the order of preference with homopolymers is poly(dI) > poly(dA) > poly(dT) > poly(dC), i.e. purines are preferred over pyrimidines. With double-stranded substrate, Fraction III DNase shows a high degree of preference for poly(dA–dT): poly(dA–dT) as compared with poly(dG–dC): poly(dG–dC); percentage hydrolysis rates of the input DNA over the 1 hr assay period are 25 and 2.5% respectively.

DISCUSSION

Pea chromatin-bound DNase shows a high preference for single-stranded rather than double-stranded DNA. This is similar to several other plant DNases so far isolated including those from mung-bean sprouts [6], wheat seedlings [7] and rye [8]. Pea DNase is not calcium-dependent; although the presence of 8 mM Ca^{2+} ions stimulates the crude total DNase activity by 40%, there is marked inhibition of purified DNase by calcium. This suggests that crude preparations contain at least one other DNA hydrolysing activity which is calcium-stimulated or calcium-dependent. Most other nuclease enzymes show either a non-stringent requirement for Ca^{2+} or are stimulated by Ca^{2+}. Pea chromatin DNase is therefore unusual in this respect. It has been reported, however, that the acid DNase of *Euglena gracilis* is inhibited by high concentration of Ca^{2+} ions [9].

The finding that pea chromatin DNase hydrolyses duplex poly (dA–dT) at ten times the rate of hydrolysis of

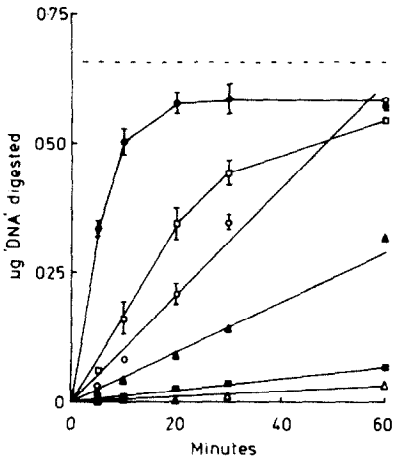

Fig. 5. Activity of Fraction III DNase on deoxyhomopolymers and copolymers. Replication of data as in Fig. 1. The horizontal dashed line indicates hydrolysis of 50% of the input substrate. ●—●, poly(dI); ○--○, poly(dT); ■—■, poly(dC); □—□, poly(dA); ▲—▲, poly(dA–dT): poly(dA–dT); △—△, poly(dG–dC): poly(dG–dC).

duplex poly (dG–dC) suggests that the residual activity seen with native DNA as substrate is due to cleavage at A–T rich sites, almost certainly because these sites are transiently single-stranded due to structural breathing [10]. The further lowering of the activity when double-stranded DNA is pre-treated with S1 nuclease is also consistent with this view. Other single-strand-specific DNases from plants have been shown to have similar action: for example, the DNases from mung-bean sprouts [11] and from the soluble fraction of pea embryo axes homogenate [12].

The data for the purification of the DNase suggests that there is loss of an inhibitor, or that the enzyme is activated, during ammonium sulphate solubilization. Preliminary evidence for the presence of an inhibitor in the chromatin of pea embryo axes was presented in an earlier paper [5]. Pea chromatin DNase exists in at least two forms as shown by the presence of either one or two peaks of activity being eluted during anion-exchange column chromatography. However, the two forms of the DNase are not consistently seen and when they do appear, they are usually of low activity. This raises the possibility that partial proteolysis of the pea chromatin DNase occurs under certain (unknown) conditions during the latter stages of purification. The slight decrease in apparent M_r (from 18000 to 16000) during purification may also indicate partial proteolysis. Inclusion of protease inhibitors during enzyme extraction and purification reduces significantly but does not abolish the occurrence of the second peak of activity (Bryant, J. A. and Fitchett, P. N., unpublished data), again indicating the role of partial proteolysis in generating the second peak. The M_r of the pea chromatin DNase is approximately 18000. The enzyme is therefore somewhat smaller than the reported M_rs of the few plant DNases that have been determined: the sugar-non-specific nuclease of *Petunia hybrida* has a M_r of 32000 [13], that for the pea cytosolic DNase 42000 [14], and the M_r of the single-strand-specific nuclease from the nucleoplasm of rye germ nuclei is 45000 [8]. DNases of M_rs below 20000 are typically repair endonucleases [15] such as those from *Micrococcus luteus* [16], barley leaves [17] and rat liver [18]. The M_r of the pea chormatin DNase is therefore consistent with its being involved in DNA repair although, clearly, several other criteria are need to confirm this idea.

The correlation between the increase in activity of the DNase and the onset of DNA replication, previously taken as suggestive of a role in replication [5], could just as easily indicate a role in repair, particularly post-replicative repair. The enzyme's probable action on double-stranded DNA: selective action at sites which are locally single-stranded, is consistent with a role in either process. However, the assignment of two possible replication functions to DNase, namely generation of 3'OH for DNA polymerase and nicking of one strand to relieve supercoiling, has been somewhat superceded by what is now known about primase and topoisomerase activities [19, 20]. The most probable role then for the enzyme described here is in repair, but a good deal of work is necessary to establish that role.

EXPERIMENTAL

Germination and growth of plants. Pea seeds (*Pisum sativum* L. cv. 'Feltham First') were soaked in running tap water for *ca* 4 hr. For embryo axes germinated for longer than 4 hr, the soaked seeds were planted in moist vermiculite and grown in the dark at 22°. The start of imbibition was taken as the onset of germination. All assays were performed using enzyme preparations from 48 hr germinated peas unless otherwise stated.

Extraction of crude chromatin DNase(s). Crude chromatin was routinely extracted from the embryo axes of 50 germinating seeds or from the shoot apices of older seedlings. Tissue was homogenized in 11 ml of homogenization buffer at 4°. (Homogenisation buffer is: 50 mM MOPs–KOH buffer, pH 6.7, 25 mM sucrose, 10 mM 2-mercaptoethanol.) The homogenate was filtered through one layer of 25 μm nylon mesh and made up to 15 ml with homogenization buffer. Crude chromatin was pelleted by centrifugation at 1500 *g* for 45 min at 4°. The pellet was used as the source of enzyme and designated fraction I DNase. For assays of fraction I DNase activity, the pellet was re-suspended in 10 ml MOPS–KOH buffer, pH 6.7.

Solubilization and fractionation of crude chromatin. The crude chromatin pellet was detached from the wall of the centrifuge tube with a small (8 mm) magnetic stir bar. Crude chromatin was then solubilized by stirring for 3 hr with 1 ml solubilization buffer (50 mM MOPS–KOH buffer pH 6.7, 25 mM sucrose, 10 mM 2-mercaptoethanol, 2 M $(NH_4)_2SO_4$). After centrifugation at 25000 *g* for 1 hr at 4°, the supernatant was retained and de-salted by passage through a pre-packed Sephadex G25 column that had been washed and equilibrated with 50 mM MOPS–KOH buffer, pH 6.7, 10 mM 2-mercaptoethanol, 20% (w/v) glycerol. Protein was eluted from the column as a sharp peak with the same buffer. Fractions (1.0 ml) were monitored for absorbance at 280 nm and the absorbing fractions (15 ml) were pooled. The pooled fractions were frozen at −18° before further purification. Enzyme preparations were stable for at least 6 weeks in this state. The pool was termed fraction II DNase.

DEAE-cellulose anion-exchange-chromatography. 10 ml of fraction II DNase were loaded onto a DEAE-cellulose (DE52) column (85 × 29 mm) which had previously been washed and equilibrated with 50 mM MOPS/KOH, pH 6.7, 10 mM 2-mercaptoethanol, 25% (w/v) glycerol. The column was washed with 30 ml of the same buffer and then a 120 ml O M to 1 M KCl gradient was applied. At the end of the gradient the column was

washed through to fraction 50 with the original buffer. Fractions of 3.0 ml were collected automatically at a flow rate of *ca* 40 ml/hr throughout the chromatography procedure. The fractions were monitored for absorbance at 280 nm and assayed for DNase activity. DNase activity so obtained was pooled (12 ml) and designated fraction III DNase.

Assay of DNase activity. DNase activity was assayed by monitoring the formation of radioactive acid-soluble fragments from [^{3}H]thymidine-labelled pea DNA substrate. DNase was assayed in a volume of 1.5 ml containing 0.5 ml assay buffer (50 mM MOPS–KOH buffer, pH 6.7, 25 mM sucrose, 10 mM 2-mercaptoethanol, 24 mM $CaCl_2$), 0.5 ml [^{3}H]-labelled pea DNA (*ca* 20 000 cpm/ml) and 0.5 ml enzyme preparation. After incubation at 37°, reactions were stopped by the addition of 1.5 ml ice-cold 10% TCA. The mixtures were cooled on ice and 0.5 ml bovine serum albumin (2 mg/ml) was added. The mixtures were incubated at 4° overnight and then acid-insoluble DNA was pelleted by centrifugation at 3000 rpm for 20 min at 4°. The supernatants were decanted into clean test tubes and 1 ml aliquots of the supernatants were mixed with 10 ml scintillation fluid (PPO–POPOP–toluene–Triton-X100) and assayed for radioactivity. For assay of single-strand specific DNase activity the same procedure was used except that the DNA was first denatured by heating in a boiling water-bath for 20 min and then cooled rapidly on ice prior to the assay.

Labelling and extraction of pea DNA. The embryo axes of 72 hr germinated peas were excised and washed free of vermiculite. DNA was labelled by placing 20–30 embryo axes into a mixture consisting of 15 ml dist. H_2O, 380 μl streptomycin (1 mg/ml), 300 μl chloramphenicol (1 mg/ml) and 100 μCi [Me-^{3}H]-thymidine (70–90 Ci/mmol). The embryo axes were allowed to take up label for *ca* 24 hr at room temp. The axes were washed free of excess labelling mixture in dist. H_2O, dried and then homogenized thoroughly in a pestle and mortar in 25 ml SDS soln (4% w/v). The homogenate was transferred to a 50 ml conical flask and lysis was allowed to proceed overnight. The lysate was deproteinized with phenol (150 ml dist. H_2O, 500 g phenol, 70 μl *m*-cresol, 0.5 g 8-hydroxyguinoline), and nucleic acids were then pptd from the aq. phase by the addition of two vol. of EtOH followed by incubation at −18°. Pptd nucleic acids were pelleted by centrifugation and dried. The pellet was resuspended in 2 ml saline sodium citrate (SSC), pH 5.2 (0.015 M sodium citrate buffer pH 5.2, 0.15 M NaCl). 10 μl RNase A (20 mg/ml) and 400 μl RNase T1 (1000 units/ml) were added per ml of SSC used. RNase treatment was allowed to proceed for 4 hr at 37°; then the mixture was again deproteinized with phenol mixture. DNA was pptd from the aq phase by the addition of two vol of EtOH and incubation overnight at −18°. The DNA was pelleted, dried and resuspended in 5 ml 0.5 M NaCl. The soln was dialysed against 0.5 M NaCl overnight at 4° and the DNA reprecipitated with EtOH for storage. Prior to use the DNA was dissolved in 1 ml MOPS–KOH buffer, pH 6.7, and scanned from 200 nm to 320 nm (to check concentration and purity) and a 0.1 ml aliquot was counted in scintillant to determine its radioactivity. For DNase assays, the DNA was diluted to give *ca* 20 000 cpm/ml.

Gel filtration on Sepharose 6B. The M_r of pea chromatin DNase was determined by gel filtration through a Sepharose 6B column (21 × 105 mm) previously calibrated with proteins of known M_r. Either solubilized, but not desalted, fraction I DNase or fraction III DNase (i.e. after ion-exchange chromatography) was used for this. For both, washing and elution of the column was with 50 mM MOPS–KOH buffer, pH 6.7. The flow rate was 20 ml/hr and fractions of 4.0 ml were collected automatically. Fractions were monitored for absorbance at 280 nm and assayed for DNase activity.

Protein determinations. Protein was determined by the method of ref. [21].

Activity of purified pea DNase on synthetic deoxyhomopolymers and copolymers. The action of fraction III DNase was tested on the ^{14}C-labelled deoxyhomo-polymers poly (dI), poly (dT), poly (dC) and poly (dA) (2.2–2.8 mCi/mmol each) and also on the alternating copolymers poly (dA–dT): poly (dA–dT) and poly (dG–dC): poly(dG–dC) (2.5 mCi/mmol). Assay mixtures (0.3 ml) contained 0.1 ml enzyme, 0.1 ml assay buffer (no calcium) and 0.01 μCi ^{14}C-labelled deoxyhomopolymer or copolymer. Reactions were incubated at 37° and stopped at various time intervals by the addition of 0.5 ml 10% (w/v) TCA. Mixtures were cooled on ice 0.2 ml BSA (1.5 ml/ml) added. Pptn of undigested DNA was allowed to proceed overnight at 4°; then ppts were pelleted acid-soluble DNA in 0.1 ml aliquots of the decanted supernatants was determined by scintillation counting.

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