Biochimica et Biophysica Acta, 312 (1973) 192–201

BBA 97708

RADIOACTIVE LABELING AND ANALYSIS OF 3′-TERMINAL NUCLEOTIDES OF DNA FRAGMENTS

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(Received January 15th, 1973)

SUMMARY

3′-Hydroxy oligodeoxyribonucleotides (average chain length 54 nucleotides), prepared by digestion of calf thymus DNA with spleen acid deoxyribonuclease followed by dephosphorylation, were used as primers for the addition of two ribonucleotides, using terminal deoxyribonucleotidyl transferase and [α-^{32}P]ATP as donor. Conditions for the optimal incorporation of labeled nucleotides were investigated.

A method for the separation of the four deoxyribonucleotides and of AMP on DEAE-cellulose columns was devised. This permitted the analysis of 3′-terminal deoxyribonucleotides after spleen exonuclease digestion of the ^{32}P-labeled oligodeoxyribonucleotides, thus avoiding the alkaline splitting of the inter-ribonucleotide bond and the phosphatase digestion which would otherwise be necessary. The results obtained by this procedure were found to be in excellent agreement with the analysis of the 3′-terminal nucleosides released from the same DNA fragments, thus showing that the use of terminal transferase provides a valid method for determining the composition of 3′-terminal nucleotides.

INTRODUCTION

The composition of the 3′-terminal nucleotides in 3′-hydroxy oligodeoxyribonucleotides is usually determined by the analysis of 3′-terminal nucleosides liberated by spleen exonuclease[1,2]. Recently an enzymatic reaction has been described which permits the ^{32}P-labeling of oligodeoxyribonucleotides at their 3′-terminal position[3]. The reaction is based on the addition, catalyzed by terminal deoxyribonucleotidyl transferase[4], of one or two ribonucleotide residues to the 3′-hydroxy ends of oligodeoxyribonucleotides, using ribonucleoside triphosphates as donors. The original tech-

Abbreviations: Oligo (dN), oligodeoxyribonucleotides; Oligo(dN)p*Ap*A, product of diaddition of ^{32}P-labeled riboadenylate to oligo(dN); P_n, average degree of polymerization.

* Euratom scientific agent. Recipient of a short-term EMBO fellowship (1972). This publication is contribution No. 868 of the Euratom Biology Division.

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nique[3] involved the incorporation of AMP from [α-^{32}P]ATP into synthetic oligonucleotides of known sequence (average chain length 6–8), transformation of diaddition into monoaddition products by alkali treatment and dephosphorylation; labeled 3'-terminal deoxyribonucleotides were then released by spleen exonuclease degradation. These results prompted us to investigate the possible use of this method for the determination of 3'-terminal nucleotides composition of DNA fragments, such as those produced by deoxyribonucleases.

The addition of two ribonucleotide residues to DNA fragments was investigated at different nucleoside triphosphate and primer concentrations in order to find optimal conditions for the labeling of the fragments. In order to analyze the labeled products, we have devised a procedure for the chromatographic separation of the four deoxyribonucleotides and of AMP on DEAE-cellulose columns. This permits the analysis of 3'-terminal deoxyribonucleotides, after spleen exonuclease digestion of the ^{32}P-labeled oligodeoxyribonucleotides to which two riboadenylate residues were added, thus avoiding alkali and phosphatase treatments. When applied to the determination of 3'-terminal nucleotides in oligodeoxyribonucleotides (average chain length 54), prepared by digestion of calf thymus DNA with spleen acid deoxyribonuclease, the new procedure gives results in excellent agreement with the analysis of the 3'-terminal nucleosides released from the same DNA fragments. Fig. 1 shows a scheme of the two enzymatic steps involved in the procedure.

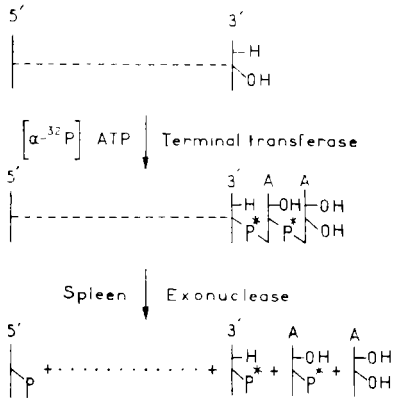

Fig. 1. Scheme of 3'-terminal nucleotide determination

MATERIALS AND METHODS

Enzymes. Acid deoxyribonuclease B, acid exonuclease, acid phosphomonoesterase B and acid ribonuclease were obtained from hog spleen[5–8]. Pancreatic ribonuclease and deoxyribonuclease were purchased from Worthington (Freehold, N. J.). Polynucleotide kinase was prepared according to a procedure to be published (A. M. Bopp, A. Bernardi, A. Devillers-Thiery). Terminal deoxyribonucleotidyl transferase (terminal transferase) was purified from calf thymus according to the method of Yoneda and Bollum[4] with the following modifications: the 35 to 55 % $(NH_4)_2SO_4$ fraction was dialyzed against 0.1 M potassium phosphate pH 7.8, prior to hydroxyapatite column chromatography; the enzyme was eluted from the column at 0.185 M

by a linear gradient (0.1–0.5 M) of potassium phosphate pH 7.8 well separated from acid deoxyribonuclease (elution molarity 0.115)[9]. Active fractions were precipitated at 70 % of $(NH_4)_2SO_4$ saturation, dissolved in 0.1 M KH_2PO_4–1 mM mercaptoethanol and dialyzed for 14 h at 4 °C against the same buffer. The white precipitate so formed was discarded by centrifugation and the supernatant was loaded on a G-100 column equilibrated with 0.1 M KH_2PO_4–1 mM mercaptoethanol. Active fractions were pooled and dialyzed for 20 h against 0.05 M potassium phosphate, (pH 7.1)–0.1 M KCl–1 mM mercaptoethanol in 45 % glycerol. The final specific activity was 14 000 units[4] per $A_{280\,nm}$ unit of protein when tested for deoxyadenylate polymerization on oligonucleotides (average size $\bar{P}_n \simeq 6$) prepared by pancreatic deoxyribonuclease degradation of calf thymus DNA, followed by removal of mono- and dinucleotides on a DEAE-cellulose column. The incorporation of [^{32}P]AMP on DNA fragments was followed as described by Bollum[10]. The paper discs were counted in a liquid scintillation spectrometer (Model SL30, Intertechnique, Plaisir, France) with the aid of 10 ml of toluene containing 0.4 % Omnifluor, obtained from NEN Chemicals (Frankfurt, Germany).

Nucleoside triphosphates. [α-^{32}P]ATP (1080–1330 Ci/mole) and [α-^{32}P]UTP (650 Ci/mole) in 50 % ethanol were purchased from C.E.A. (Saclay, France). Before use, ethanol was eliminated by rotary evaporation and the products redissolved in water. Unlabeled ATP and UTP were products of Boehringer (Mannheim, Germany) and P.L. Biochemicals (Milwaukee, Mich.), respectively.

3'-hydroxy oligonucleotides. These were prepared as follows. 2 μl of spleen deoxyribonuclease (400 units/ml) were added to 15.0 ml of calf thymus DNA ($A_{260\,nm}$ 8.0) in 0.05 M ammonium acetate–0.001 M EDTA, pH 5.5. After 15 min of digestion at 22 °C, the hyperchromic shift at 260 nm having reached 2 %, the reaction was stopped by shaking the reaction mixture for 5 min with 1.0 ml of chloroform–isoamyl alcohol (24 : 1, v/v). The digest was then dialyzed against running water[11], brought to 0.05 M NaOH for 2 min in order to insure complete denaturation, and adjusted to pH 5.5 with 1 M acetic acid. Denatured 3'-phosphate oligonucleotides were dephosphorylated with 0.2 units/ml of acid phosphomonoesterase B at 37 °C for 14 h. Control experiments using the same oligonucleotides, ^{32}P-labeled with polynucleotide kinase, showed that under these conditions more than 95 % of terminals were dephosphorylated.

The average chain length or average degree of polymerization, $\bar{P}_n$, of the unlabeled oligonucleotides obtained by spleen deoxyribonuclease digestion, and the composition of 3'-terminal nucleotides were determined according to Carrara and Bernardi[1] as modified by Soave *et al.*[12] and by Thiery *et al.*[2], respectively.

Nucleotides. 3'-Deoxyribonucleotides were prepared by digestion of calf thymus DNA with spleen deoxyribonuclease and exonuclease as described previously[13]. 3'-Ribonucleotides were obtained by digestion at 37 °C of yeast total RNA (Sigma, St. Louis, Mo.; $A_{260\,nm}$ 10.9) with 30 units of pancreatic ribonuclease and 3 units of spleen exonuclease[6] in a total volume of 0.5 ml of 0.05 M ammonium acetate, pH 5.5. The reaction was followed by hyperchromic shift and was complete after one hour. The enzymes were inactivated with a chloroform–isoamyl alcohol mixture (24 : 1, v/v) as described above.

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; fine particles 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/h to the height of 15 cm and washed with 1 M ammonium acetate, pH 8.7, followed by 0.05 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 re-equilibrated with the starting buffer and re-used many times.

The ultraviolet absorbance 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 cm × 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 counter lower window at 0 and the upper one at 675.

RESULTS

Incorporation of ribonucleotides into DNA fragments by terminal transferase

This was studied at an ATP–oligonucleotide molar ratio higher than 10, allowing therefore only diaddition products to be formed[14]. Figure 2a shows the time course of [^{32}P]AMP incorporation into 3'-hydroxy oligonucleotides ($\bar{P}_n$ 54) at increasing ATP molarities. In all cases the reaction was biphasic and slowed down under the conditions used after about 2 h, a fact not due to enzyme inactivation since the reaction rate could not be increased by further enzyme addition. A real end point

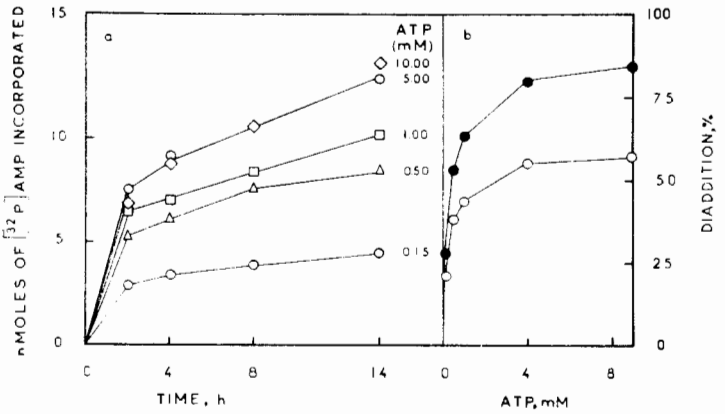

Fig. 2. Kinetics of AMP incorporation. The data presented in (a) refer to 0.1 ml of incubation mixtures containing: 7.9 μM 3'-hydroxy oligodeoxynucleotides primer ($\bar{p}_n$ 54); 0.05 M potassium cacodylate, pH 7.0; 7.5 mM $MgCl_2$; 1 mM 2-mercaptoethanol; the increasing concentrations of [α-^{32}P]-ATP (from 0.01 to 1 Ci/mmole); and 90 units of terminal transferase. The reaction was carried out at 35 °C; 5 μl of incubation mixtures were withdrawn at different times, spotted on paper discs and treated for acid-insoluble radioactivity (see Materials and Methods). The ATP saturation curves of (b) were done by plotting the results obtained after 8 (○–○) and 14 h (● ●) of incubation.

of the reaction was never attained. The initial rate was increased by increasing the ATP concentration, at least up to 1 mM. A similar but stronger effect could be seen in the slow phase up to 5 mM ATP (Fig. 2a). The yield in the diaddition product was found to be strongly dependent upon the ATP concentration and reached saturation at a 5 mM level (Fig. 2b).

The effect of temperature on the reaction (not shown) was studied at 20 and 40 °C at 5 mM ATP. The biphasic pattern was found in both cases. The initial rate increased with increasing temperature; the yield after 6 h of reaction was 20 % higher at the higher temperature.

Fig. 3 shows the incorporation of AMP as obtained at increasing primer concentrations. The relative yield in the diaddition product increased with decreasing primer concentration, reaching a 100 % value only at very low concentration of 3'-OH ends.

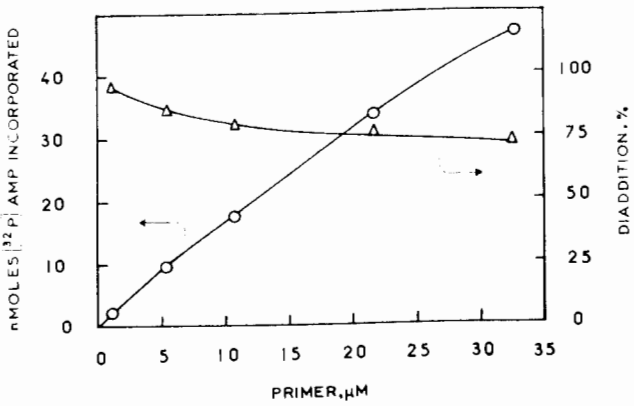

Fig. 3. Primer saturation curves at 5 mM ATP. The data refer to 0.05 ml of incubation mixtures containing: 0.05 M potassium cacodylate, pH 7.0; 8 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 5 mM [α-^{32}P]ATP (30 Ci/mole); 45 units of terminal transferase; and increasing concentrations of 3'-hydroxy oligodeoxynucleotide primers ($\bar{p}_n$ 54). The reaction was carried out at 35 °C for 14 h; 5 μl of the incubation mixtures were treated for acid-insoluble radioactivity (see Materials and Methods). ○–○, AMP incorporation; △–△, formation of diaddition product.

Conditions leading to 100 % incorporation, which are only needed for estimating chain length from labeling data, are impractical for the routine determination of the composition of 3'-terminal nucleotides, because of the relatively low specific radioactivity of commercial [α-^{32}P]ATP (see following section).

The incorporation of [^{32}P]UMP was studied with different bivalent ions as activators (Fig. 4). The highest rate was observed with Co^{2+}, as it occurs for the polymerization of deoxyribopyrimidine triphosphate on oligodeoxyribonucleotides[15] or on ribonucleotide-terminated primers[14]. The formation of labeled product was, however, on the average, 2–3 times lower than that obtained with the same concentration of ATP, which was thus used in our standard procedure. The UMP incorporation data presented here are of possible value if separation of labeled nucleotides is done by paper electrophoresis.

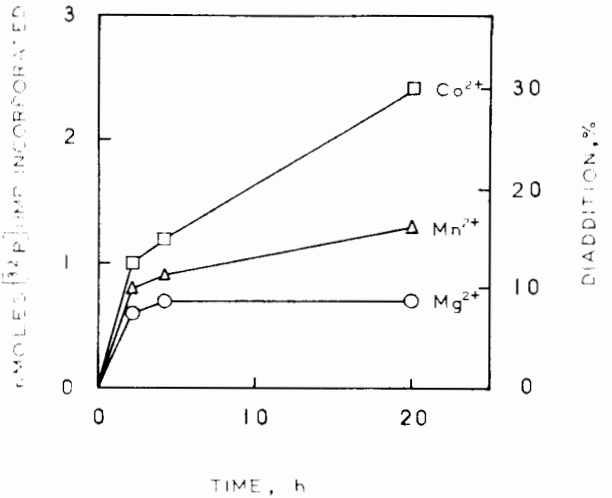

Fig. 4. Kinetics of UMP incorporation. The data refer to 0.1 ml of incubation mixtures containing 4.15 μM 3'-hydroxy oligodeoxynucleotide primer ($\bar{P}_n$ 60); 0.05 M potassium cacodylate, pH 7.0; 1 mM 2-mercaptoethanol; 0.5 mM [α-^{32}P]UTP (1 Ci/mole); 90 units of terminal transferase; and the following concentrations of divalent ions: ○–○, 8 mM $MgCl_2$; △–△, 1 mM $MnCl_2$; □–□, 1 mM $CoCl_2$. The reaction was carried out at 35 °C. 10 μl of incubation mixtures were withdrawn at different times and treated for acid-insoluble radioactivity (see Materials and Methods).

*Preparation of oligonucleotides carrying p*Ap*A at their 3'-ends*

The incubation mixture (0.05 ml, final volume) was: 1–10 μM in 3'ends of oligonucleotides (0.025–0.250 $A_{260\,nm}$ units; $\bar{P}_n$ 54); 0.15 or 0.5 mM in [α-^{32}P]ATP (of specific radioactivity of 1 and 0.3 Ci/mmole, respectively); 0.2 M in potassium cacodylate, pH 7; 8 mM in $MgCl_2$; 1 mM in 2-mercaptoethanol and contained 45 units of terminal transferase. The enzymatic reaction was carried out for 12–14 h at 37 °C in sealed glass micro-tubes and was terminated by shaking for 5 min with 0.2 volumes 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 0.05 M ammonium acetate pH 5.5 (Fig. 5). As estimated from the G-25 chromatograms at the two ATP concentrations used, two AMP residues were added to about 25 % and 50 % of 3'-ends, respectively.

*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 (first peak of Fig. 5) were pooled and 1.2 $A_{260\,nm}$ units of calf thymus DNA were added. The material was lyophylized, dissolved in 0.02 M ammonium acetate, pH 5.5 (final volume 0.1 ml), and digested at room temperature with 1 μl of spleen deoxyribonuclease (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[10] and was complete after one hour. After inactivating the enzymes the pH of the sample was raised to 8.5–9, by adding 1 M NH_4OH.

It should be noted that attempts to split the inter-ribonucleotide bond, in-

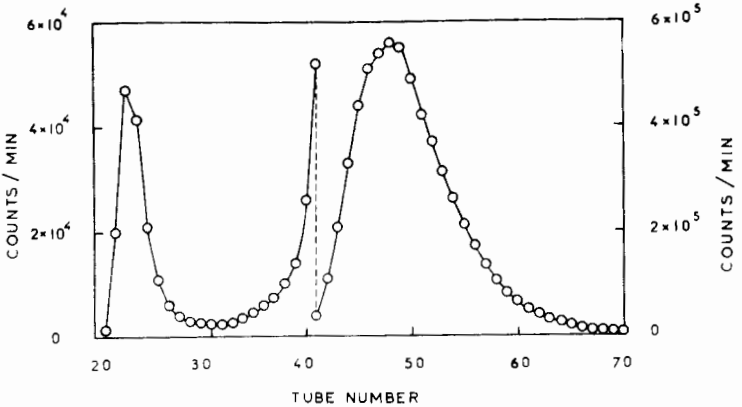

Fig. 5. Chromatography on Sephadex G-25. The terminal transferase incubation mixture was loaded on a Sephadex G-25 (1 cm × 35 cm) column equilibrated with 0.05 M ammonium acetate, pH 5.5, satured with chloroform. Gel filtration was carried out with the same buffer at a flow rate of 20 ml/h. Fractions of 0.5 ml were collected in polystyrene tubes and counted by Čerenkov radiation. Fractions 21–40 left-hand scale; Fractions 41–70 right-hand scale.

volving rA or rU, by spleen or pancreatic ribonuclease, respectively, completely failed. We were not able to detect any decrease in acid-insoluble radioactivity and in the amount [^{32}P]AMP released after prolonged incubations of diaddition products with large quantities of these enzymes followed by the acid phosphatase treatment.

Analysis of labeled 3′-terminal nucleotides from 3′-hydroxy oligodeoxyribonucleotides

Fig. 6 shows the results of the chromatographic procedure developed in order to afford the analysis of 3′-terminal deoxyribonucleotides in the presence of AMP and/or UMP. Under the experimental conditions used, it was also possible to obtain

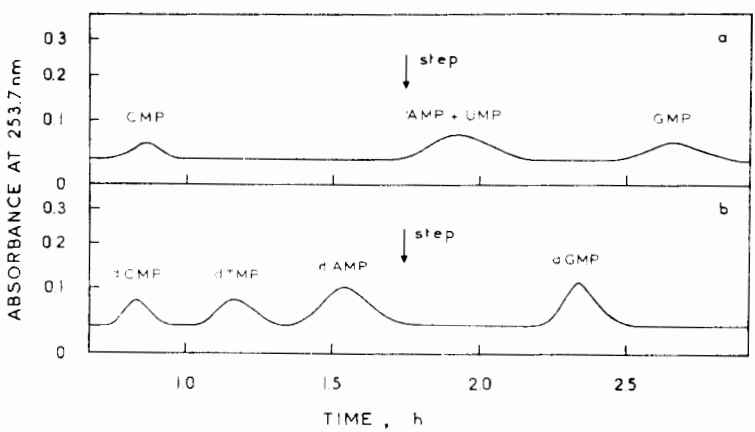

Fig. 6. Chromatography of mononucleotides on DEAE-cellulose columns. Elution was carried out with 0.05 M ammonium acetate, pH 9.3; followed by a step of 0.15 M of the same buffer, pH 8.9. (a) Load was 1.4 $A_{260\ nm}$ units of ribonucleoside 3′-monophosphate in 100 μl of 0.05 M ammonium acetate, pH 8.5 (b) Load was 2.0 $A_{260\ nm}$ units of deoxyribonucleoside 3′-monophosphates in 20 μl of 0.05 M ammonium acetate, pH 8.5.

complete separations 3'-dGMP from 3'-GMP, 3'-AMP from 2'-AMP (the latter being eluted at the position of the 3'-dAMP) and 3'-dGMP from 5'-dGMP (this being eluted at the position of 3'-GMP); cyclic AMP and inorganic phosphate were eluted at essentially the same elution volume before 3'-dCMP. Other possible uses of this method were not investigated.

Fig. 7 shows the analysis of the labeled 3'-terminal nucleotides from 3'-hydroxy oligodeoxyribonucleotides on the DEAE-cellulose column. [^{32}P]Orthophosphate was practically negligible in amount when carrier DNA was present during the digestion step. ^{32}P-labeled ATP and ADP, when present at trace levels in the incubation mixture, could be eluted by washing the column with 0.5 M ammonium acetate, pH 8.5.

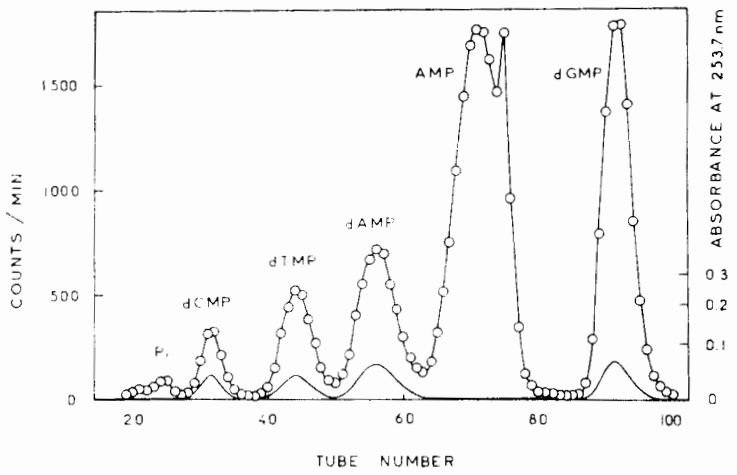

Fig. 7. Separation of ^{32}P-labeled mononucleotides on a DEAE-cellulose column. 20 000 cpm of ^{32}P-labeled 3'-nucleotides (obtained after complete digestion of oligo(dN)p*Ap*A by spleen deoxyribonuclease and exonuclease) in 50 μl of 0.05 M ammonium acetate buffer (adjusted after digestion to pH 8.5–9 by adding 2 μl of 1 M NH_4OH) were loaded on a DEAE-cellulose column (0.5 cm × 15 cm) equilibrated with 0.05 M ammonium acetate, pH 9.3. Elution was started with the same buffer using a flow rate of 12 ml/h; a step of 0.15 ammonium acetate pH 8.9, was applied after Fraction 63, in order to elute dGMP. ——, ultraviolet monitoring; ○–○, Cerenkov counting.

TABLE I

3'-TERMINAL NUCLEOTIDES OF DEPHOSPHORYLATED DNA FRAGMENTS

3'-Terminals of oligonucleotides ($\bar{p}_n = 54$), obtained by controlled degradation of calf thymus DNA by spleen acid deoxyribonuclease (see Materials and Methods) were determined by the nucleoside analysis according to Carrara and Bernardi[1] as modified by Thiery *et al.*[2] and by the present method, respectively. The nucleoside analysis concerns the totality of the 3'-ends.

3'-Nucleotide	*Nucleoside analysis*	*Labeled nucleotide analysis*	
		0.15 mM ATP	*0.5 mM ATP*
dC	5 %	6 %	6 %
dT	14 %	14 %	13 %
dA	24 %	24 %	24 %
dG	58 %	56 %	57 %

The composition of 3′-terminal nucleotides from oligonucleotides released by spleen deoxyribonuclease from calf thymus DNA as determined by the technique described above, is compared in Table I with data obtained by analysis of the 3′-terminal nucleosides[1,2]. An excellent agreement between the two sets of values is observed, at both ATP levels used in the incubation mixture.

DISCUSSION

The strong decrease in reaction rate observed in [^{32}P]AMP incorporation and the failure to attain the end point of the reaction (Fig. 2a) might be due to: (a) formation of tri-or multiaddition products; (b) formation of new 3′-OH ends by contaminating deoxyribonucleases present in the terminal transferase preparation; (c) presence of dATP contamination in the unlabeled ATP used; (d) hindered access of terminal transferase to the 3′-OH ends of a number of DNA fragments.

The first explanation can be ruled out because the amount of label in AMP was close to 50 % of total label incorporated, as expected for the exclusive formation of diaddition products, and in agreement with the report[13] that tri- or multiaddition products never appear to be formed in the reaction. The presence of a 5′-phosphate forming deoxyribonuclease or of a combination of a 3′-phosphate forming deoxyribonuclease and a phosphatase in the terminal transferase preparation is very unlikely since the composition of the 3′-terminal nucleotides obtained would be changed owing to the different specificity of the contaminating deoxyribonuclease, whereas our results show an excellent agreement with that determined by direct 3′-terminal nucleoside analysis (Table I); even if the contaminant deoxyribonuclease had the same specificity as the spleen deoxyribonuclease (a possibility worth considering in view of the vry great similarity of thymus and spleen deoxyribonuclease[16]), the further degradation of DNA fragments would have led to a different composition of terminals because the spleen enzyme releases different 3′-terminal nucleotides from shorter DNA fragments[2]. The presence of dATP contamination in the unlabeled ATP used also appears to be ruled out because it would lead to an increase of [^{32}P]dAMP (preferentially incorporated dAMP acting as a new 3′-OH end) in the terminal nucleotides[15,17], a result which was not obtained at any of the two ATP concentrations used. Finally, all the three phenomena discussed above would have led to a [^{32}P]-AMP incorporation higher than 100 % diaddition; in fact, this was never found (Fig. 3). The last possibility, namely that the secondary structure near the 3′-ends of a number of fragments might indeed hinder the access of the enzyme appears to be the most likely explanation. Three observations are interesting in connection with this: (a) the strong dependence of the reaction rate upon ATP concentration (Fig. 2b) might be due to the denaturing effect of ATP on the secondary structure of the oligonucleotides[18,19]; (b) the effects obtained by increasing the temperature of the incubation mixture, and therefore by changing the secondary structure of the oligonucleotides, are also compatible with this explanation; (c) in spite of the incomplete labeling of 3′-ends (25 % and 50 % at the two ATP concentrations used) [^{32}P]AMP incorporation was completely random; this is not surprising in view of the enormous number of different oligonucleotides having different secondary structures present in the incubation mixture. For this reason the concentrations of ATP in the reaction can be kept

much lower than saturating; this avoids dilution of the radioactive ATP and leads to a higher labeling of oligonucleotides.

In conclusion we have shown that terminal transferase has no preference for any 3'-end of DNA fragments. The labeling of 3'-terminal nucleotides of DNA fragments by terminal transferase has two advantages over the classical procedure, the possibility of scaling down considerably the amount of oligonucleotides needed for the analysis and of determining the composition of 3'-terminal nucleotides of very large DNA fragments.

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