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Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase

Ranajit Roychoudhury, Ernest Jay* and Ray Wu+

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca NY 14853, USA.

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ABSTRACT

Terminal deoxynucleotidyl transferase, which requires a single-stranded DNA primer under the usual assay conditions, can be made to accept double-stranded DNA as primer for the addition of either rNMP or dNMP, if Mg^{+2} ion is replaced by Co^{+2} ion. The priming efficiency in the presence of Co^{+2} ion with respect to initial rate tested with 2 single-stranded primer, is 5-6 fold higher than that observed with Mg^{+2} ion. In the presence of Co^{+2} ion, the primer specificity is altered so that all forms of duplex DNA molecules can be labeled at their unique 3'-ends regardless of whether such ends are staggered or even. Thus, using ribonucleotide incorporation, we have for the first time employed this reaction for sequence analysis of duplex DNA fragments generated by restriction endonuclease cleavages. Furthermore, by using Co^{+2} ion, it is possible to add a long homopolymer tract of deoxyribonucleotides to the 3'-terminus of double-stranded DNA. Therefore, without prior treatment with λ exonuclease to expose the 3' terminus as single-stranded primer, this reaction now permits insertion of homopolymer tails at the 3'-ends of all types of DNA molecules for the purpose of *in vitro* construction of recombinant DNA.

INTRODUCTION

The enzyme terminal deoxynucleotidyl transferase catalyzes polymerization of deoxynucleotide residues at the 3' termini of single-stranded DNA or oligodeoxynucleotides[1]. For catalytic activity, the enzyme requires a free 3'-OH group and a minimum of three nucleotide residues in a single-

Abbreviations: dNTP, deoxynucleoside triphosphate, rNTP, ribonucleoside triphosphate; $\overset{*}{p}$ denotes a [^{32}P]phosphate and the phosphodiester linkages are represented by a hyphen. Thus, rN preceded by a hyphen (-rN) indicates ribonucleotide addition. dNp, a deoxynucleoside 3'-phosphate.

stranded configuration[1]. The enzyme also catalyzes a limited polymerization of ribonucleotides at the 3'-end of oligodeoxynucleotides[2]. Such ribonucleotide addition has been shown to be useful for primer extension[2-5], 3'-end labeling[6,7] and DNA sequence analysis[8-11]. These applications would be more valuable if the techniques can be employed for terminal labeling and chain extension of duplex DNA molecules or duplex DNA fragments generated by restriction endonucleases. However, double-stranded structure near the 3'-ends inhibits the transferase reaction. The problem is more severe where the 3'-OH terminus is adjacent to a complementary strand with protruding 5'-ends, such as those found in the cohesive ends of λDNA and DNA fragments generated by Eco RI endonuclease digestion. For an efficient homopolymeric extension on EcoRI treated SV40 or λdvgal DNA for the purpose of in vitro construction of recombinant DNA molecule, it was necessary to digest away the 5' terminal protruding nucleotides with λ exonuclease to expose the single-stranded region adjacent to the 3'-OH terminus[12,13].

We have recently noticed that the priming efficiency of oligodeoxynucleotides is remarkably improved if the reaction mixture contains Co^{+2} ion instead of Mg^{+2} ion. Under these conditions, the enzyme catalyzes not only an efficient nucleotide addition to the primer, but also the addition of several ribonucleotides at the 3'-end of oligodeoxynucleotides. In this communication, we wish to report a new application of this reaction for labeling of duplex DNA molecules or fragments with either ribonucleotides (for sequence analysis) or deoxynucleotides (for insertion of homopolymeric tails). Furthermore, such labeling can be performed with DNA molecules or fragments which have protruding 5'-ends; thus eliminating the need for the λ exonuclease step in the insertions of homopolymeric tails at the 3'-ends of DNA molecules for in vitro construction of recombinant DNA[12-14].

EXPERIMENTAL PROCEDURE

Materials

Nucleotides--[α-^{32}P]rGTP, [α-^{32}P]rCTP, [α-^{32}P]rUTP, [α-^{32}P]rATP, [α-^{32}P]dATP and [α-^{32}P]dTTP (specific activities 50-130 Ci/mmole) were obtained from New England Nuclear Corporation. [γ-^{32}P]rATP (50-125 Ci/mole) was purchased from ICN Pharmaceuticals.

Enzymes--Terminal deoxynucleotidyl transferase (specific activity, 20,000 units/mg) was isolated as described earlier[3]. E. coli DNA polymerase, isolated according to Jovin et al.[15] had a specific activity of 5000 units/mg with calf thymus DNA as template-primer. Polynucleotide kinase (specific activity 20,000 units/mg) was obtained from Biogenics Re-

search Corporation. Restriction endonuclease Hind (a mixture of Hind II and Hind III) was isolated according to Smith and Wilcox[16] and further purified to obtain Hind III completely free from Hind II and other contaminating nucleases. The endonuclease Hae III was purified according to Roberts et al.[17]. The restriction endonuclease Eco R1, purified according to Greene et al.[18] was a gift of R. Roberts or C.D. Tu.

Oligonucleotides and DNA--d(T-T-T-T-T-T) was a gift of Dr. C.P. Bahl. Covalently closed circular SV40 DNA was isolated as described by Wu et al.[19]

Intact λDNA was isolated from purified phage as described earlier[20]. $(dT)_{\overline{30}}$ was prepared as described earlier[21].

Methods

Digestion of SV40 DNA with Eco R1, Hind restriction endonuclease, Hind III endonuclease, isolation of fragments and redigestion with Hae III, and phosphorylation of d(T-T-T-T-T-T) with [γ-^{32}P]rATP and polynucleotide kinase were performed according to procedures described by Wu et al.[19]. Other procedures are mentioned in the legends. DEAE-cellulose TLC homochromatography was carried out using homo-mixture VI of Jay et al.[11]. Acid insoluble radioactivity was determined by using filter paper disc method of Bollum[22].

RESULTS

Replacement of Mg^{+2} ion with Co^{+2} ion increases the priming efficiency of oligonucleotide

All four common ribonucleotides are incorporated at the 3'-end of oligodeoxynucleotides in the presence of calf thymus terminal transferase and Mg^{+2} as the divalent cation[3]. We have recently noticed that the rate of limited polymerization of ribonucleotides at the 3'-end of oligodeoxynucleotides is remarkably improved if Co^{+2} is used as divalent cation instead of Mg^{+2}. The incorporation of [α-^{32}P]rCMP with $(dT)_{\overline{30}}$ as primer shows an initial rate approximately 6-fold higher in the presence of Co^{+2} ion as compared to that observed in the presence of Mg^{+2} ion (Fig. 1).

The above experiment, however, does not show the extent of conversion of primer into ribonucleotide terminated products and the nature of products formed. In order to compare polymerization characteristics of all four common ribonucleotides under identical conditions, and to make a direct measurement of the amount of primer distributed into different ribo-addition products, the primer d($\overset{*}{p}$T-T-T-T-T-T) was extended with unlabeled ribonucleotides. The extended products d($\overset{*}{p}$T-T-T-T-T-T)rN, d($\overset{*}{p}$T-T-T-T-T-T)-rN-rN, etc., can be clearly resolved by homochromatography[23]. In the

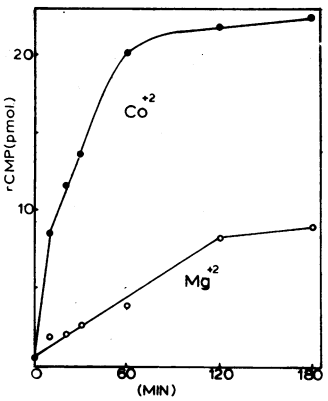

Fig. 1. The addition of rCMP to the end of $(dT)_{30}$ in the presence of Mg^{+2} or Co^{+2} ion. The reaction mixture (50 µl) contained cacodylate buffer system*, 1 mM $CoCl_2$ or 10 mM $MgCl_2$, 100 µM dithiothreitol, 26 pmoles of $(dT)_{30}$, 200 pmoles of [α-^{32}P]rCTP (15,000 cpm/pmole) and 0.4 µg of terminal transferase. At intervals, aliquots (5 µl) were assayed for acid insoluble radioactivity.

*1.4M K-Cacodylate, 0.3 M Tris (base), 1 mM dithiothreitol, 10 mM $CoCl_2$, final pH 7.6. Mixing of $CoCl_2$ is done last with constant stirring during slow addition in order to avoid precipitation. During incubation, this buffer is diluted 10 fold and the pH becomes 6.9.

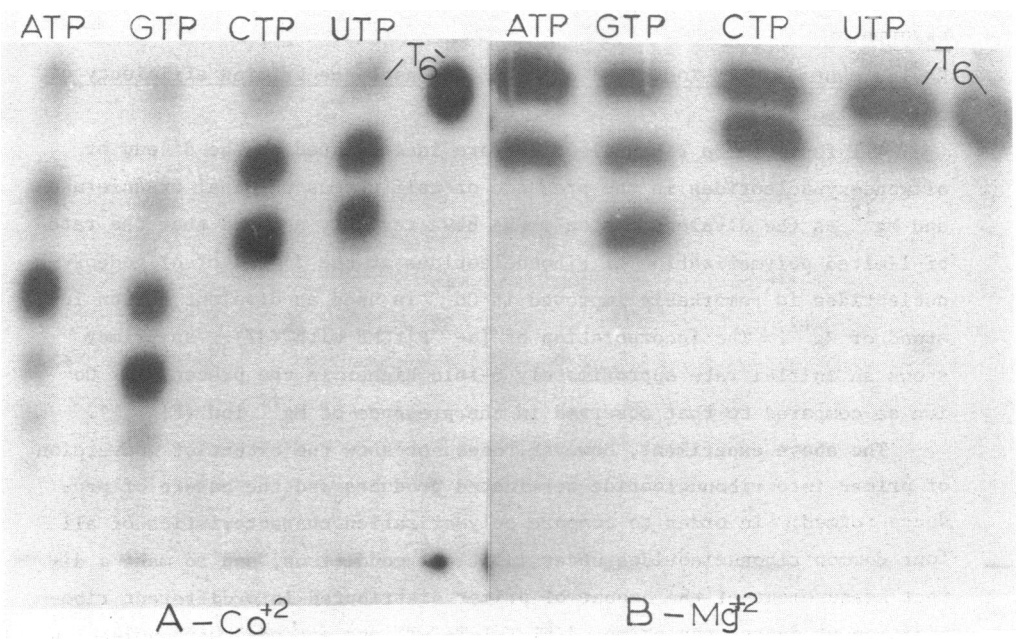

Fig. 2. The nature of products formed after addition of different ribonucleotides at the 3'-end of $(dpT)_6$. The incubation conditions were those described under legend to Table I.

presence of Co^{+2} ion and a 10 fold excess of unlabeled rNTPs, more than 90% of the radioactivity appeared in the ribo-addition products and several (five with rATP) ribonucleotides were added when purine rNTPs were used (Fig. 2A). The conversion of primer was much lower with Mg^{+2} ion and mainly single terminal addition (except with rGTP) was obtained with different rNTPs (Fig. 2B).

A quantitative analysis of radioactivity in different products is shown in Table 1. The spots shown in Fig. 2A and 2B were scraped out and counted. The highest conversion of primer was noticed with rGMP additions, whereby 66% and 96% of the primer was utilized in the presence of Mg^{+2} and Co^{+2}, respectively. In the presence of Mg^{+2}, the order of primer utilization

Table 1. The distribution of radioactivity in products after rNMP additions

The reaction mixture (15 µl) contained 10 pmoles of d($\overset{*}{p}$T-T-T-T-T-T), 100 pmoles of rNTPs and 0.8 µg of terminal transferase. After 5 hours at 37°, aliquots of samples (5 µl in Mg^{+2} experiment and 3 µl in Co^{+2} experiment) were applied on DEAE-cellulose thin layer plates and developed in Homo-mixture 6. The spots detected by radioautography were scraped out and counted. The results are expressed as percent ^{32}P cpm/spot.

No. of rNMP added	rATP		rGTP		rCTP		rUTP	
	Mg^{+2}	Co^{+2}	Mg^{+2}	Co^{+2}	Mg^{+2}	Co^{+2}	Mg^{+2}	Co^{+2}
0	75.5%	7.2%	33.2%	3.7%	47.9%	7.5%	86%	10.8%
1	21.3%	14.7%	25.6%	5.6%	52.1%	42.2%	14%	47.5%
2	3.2%	68.8%	38.1%	31.2%		50.3%		41.7%
3		6.9%	3.1%	52.8%				
4		2.4%		6.7%				
Total counts	29,455 (100%)	19,548 (100%)	29,170 (100%)	20,106 (100%)	32,607 (100%)	22,766 (100%)	31,630 (100%)	17,267 (100%)
Primer conversion	24.5%	92.8%	66.8%	96.3%	52.1%	92.5%	14%	89.2%

of [α-^{32}P]rUMP into duplex DNA fragments generated by the action of restriction endonucleases Hind on SV40 DNA molecule and their isolation by gel filtration (Fig. 4B). Of the total radioactivity (1450 pmol) and 165 with different rNTPs shows: rGTP>rCTP>rATP>rUTP. In the presence of Co^{+2} ion, all four rNTPs show close to 90% or greater conversion of primer.

For 3' terminal labeling of specific DNA fragment or oligonucleotide isolated from biological sources, it may sometimes be necessary to work on micro-scale with as little as 0.1 pmole (in terms of 3'-OH ends) of material. As shown in Fig. 3, the use of 0.15-0.9 pmole of $(dT)_{\overline{30}}$ resulted in 72,000 - 199,500 cpm (0.38 - 1.05 pmole) of [^{32}P]rCMP incorporation. The incorporation curve is non-linear because while keeping the concentration of [^{32}P]rCMP constant with increasing amounts of $(dT)_{\overline{30}}$, the substrate to primer ratio decreased as the concentration of primer increased. We notice that several ribonucleotides are added with all four rNTPs in the presence of Co^{+2} ion when a large excess of rNTP is used (results not shown). Using our incubation conditions, Sekiya, Van Ormondt and Khorana recently reported incorporation of four rGMP residues at the 3'-end of an oligodeoxynucleotide primer[5].

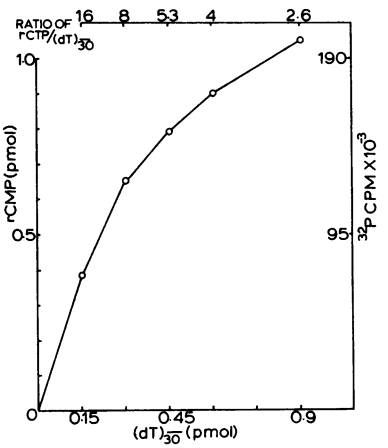

Fig. 3. Microscale labeling of oligodeoxynucleotide. The reaction mixture contained 2.4 pmoles of [α-^{32}P]rCTP (118 ci/mmole, or 190,000 cpm/pmole), 26 ng of terminal transferase and indicated amounts of $(dT)_{\overline{30}}$ in a final volume of 2 µl. Incubation was carried out in sealed siliconized glass capillary tubes for 6 hours at 37°. The contents were directly applied on Whatman orange ribbon paper and subjected to chromatography in 95% ethanol - 1M NH_4-OAc, pH 7.0 (3:1 v/v). The point of application of samples was previously treated with large excess of unlabeled rCTP and EDTA. This eliminates non-specific binding of [^{32}P]rCTP to the origin. A control incubation without enzyme gave 78 cpm in the origin. The origin $[(dT)_{\overline{30}}(rN)_n]$ was cut out and counted.

Incorporation of ribonucleotide at the 3'-ends of duplex DNA fragments generated by restriction endonucleases.

Terminal transferase provides an unique opportunity for enzymatic 3' terminal labeling of single-stranded DNA or oligonucleotides[2]. So far, labeling of duplex DNA or duplex DNA fragment using terminal transferase has been found to be inefficient[12,13]. With the use of Co^{+2} ion, we have now succeeded in labeling duplex DNA fragments generated by restriction endonuclease cleavages whether or not the 3'-OH terminus is present in a duplex DNA with even or staggered end. Fig. 4 shows the kinetics of incorporation

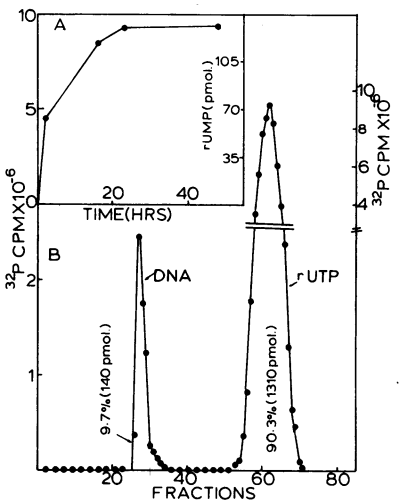

Fig. 4. 3' terminal labeling of SV40 DNA Hind fragments with [^{32}P]rUMP. The reaction mixture (100 µl) in cacodylate buffer system contained 0.5 A_{260} unit of SV40 Hind fragments (82.5 pmoles of fragments containing 165 pmoles of 3'-ends), 1450 pmoles of [α-^{32}P]rUTP (specific activity 55 Ci/mmole) and 4 µg of terminal transferase. Aliquots (2 µl) of the reaction mixture was monitored for acid insoluble radioactivity (Fig. 4A). Other aliquots (10 µl) at different time points analyzed by gel electrophoresis. The remaining sample was loaded onto a column (0.8 x 96 cm) of Sephadex G-50 and eluted with 50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA. Fractions of 20 drops (300 µl) were collected in siliconized tubes (10x50 mm). The tubes were inserted into scintillation vials and directly counted for Cerenkov radiation in a Beckman LS 230 counter.

pmol of 3'- ended DNA fragments applied to the column, 140 pmol of radioactive DNA fragments was isolated which amounted to a 84% conversion of primer (Fig. 4B). Using [α-^{32}P]rCTP or [α-^{32}P]rGTP, much higher conversion (close to 100%) of primer is obtained.

Analysis of labeled fragments by gel electrophoresis

When an aliquot of reaction mixture from the rUTP labeling experiment (Fig. 4) was subjected to electrophoresis in 1.4% agarose slab gel (Fig.5A)

followed by radioautography (Fig. 5B), it became apparent that all the bands were not labeled with equal intensity. In order to ascertain which bands are more heavily labeled, polyacrylamide gel electrophoresis was per-

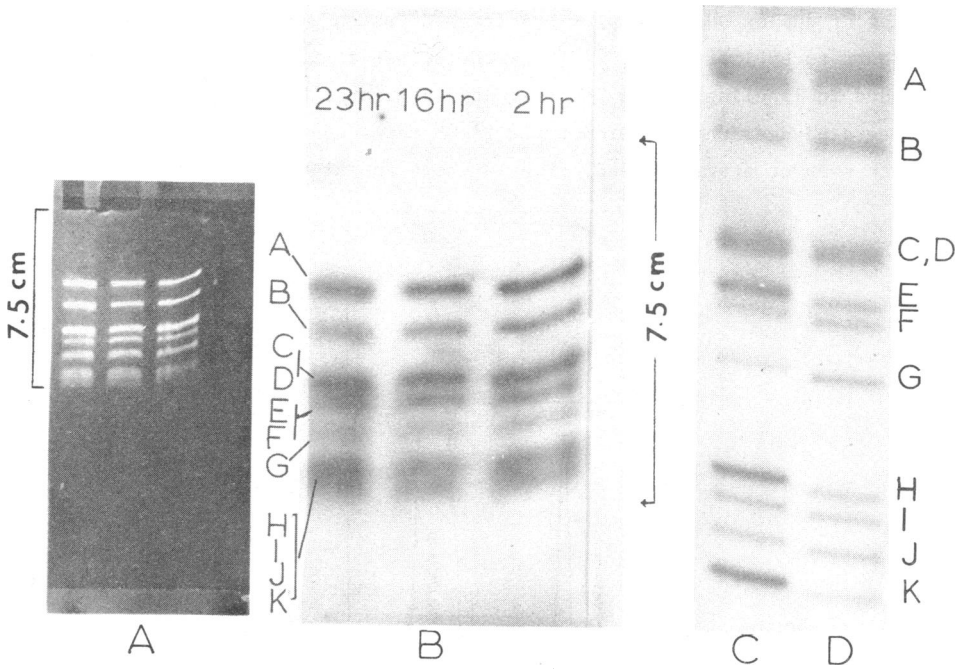

Fig. 5. Ribonucleotide incorporation in relation to the nature of restriction endonuclease cleavage site. Aliquots (10 µl) of reaction mixture from incubation described in Fig. 4 were withdrawn at intervals of 2, 16 and 23 hr followed by electrophoresis on 1.4% agarose slab gel containing ethidium bromide and visualized under ultraviolet lights (A), or by radioautography (B). Hind fragments labeled with rCMP were subjected to electrophoresis in 5-10% polyacrylamide gradient gel (C) together with uniformly labeled fragments (D).

-ormed in a different experiment with [^{32}P]rCMP labeled Hind fragments. For comparison, uniformly labeled Hind fragments were run side by side (Fig. 5D). In the unformly labeled DNA fragments, the radioactivity is proportional to the number of nucleotide residues. In the terminally labeled fragments the radioactivity is expected to be equal in different fragments. As shown in Fig. 5C, the bands A, C+D, E, H and K are more intensely labeled with [^{32}P]rCMP as compared to the bands B, F, G, I and J of Hind fragments of SV40 DNA. Bands generated by Hind III cuts on both ends

(A, E and K) are always more intensely labeled. From the nucleotide sequence recognition site of Hind III,

5' pA-G-C-T-T.....3'
3' HO-A.....5'

we know that an A-T pairing occurs at the 3' terminus of Hind III fragments. The terminal "breathing" of the 3' terminus probably makes it more accessible to terminal transferase, an enzyme prefering single-stranded DNA as primer. As expected, when only Hind III digested DNA fragments are used for 3' terminal labeling, all the fragments are labeled with equal intensity (Fig. 6). Besides terminal 'breathing', the enzyme bound cobalt ion probably alters the physical state of the

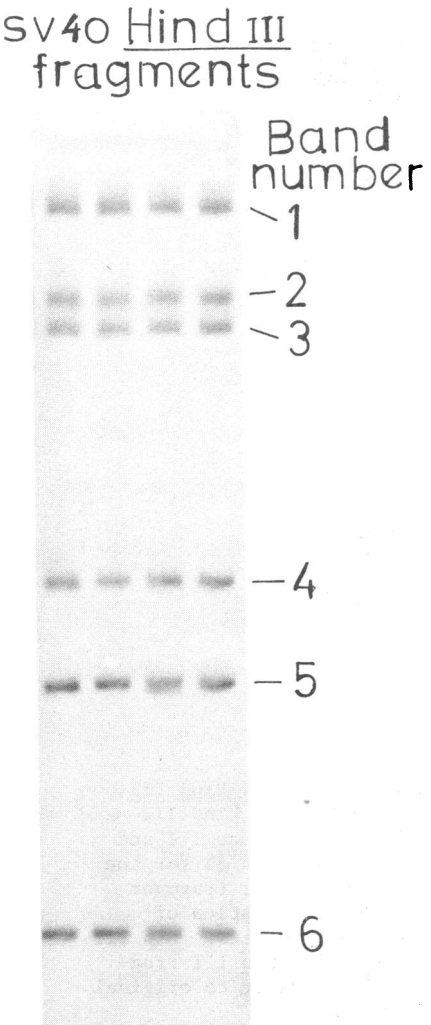

Fig. 6. Separation of [^{32}P]rCMP labeled Hind III fragments. Hind III fragments of SV40 DNA were labeled with [^{32}P]rCMP and transferase as described in Fig. 4. The labeled DNA fragments were fractionated by electrophoresis in 4% polyacrylamide gel, and the bands were visualized by radioautography. To avoid overloading, the same material was applied in several gel slots.

double helix whereby the binding of the enzyme at the 3'-OH terminus is improved. Unique labeled ends can be obtained after redigestion of the fragments with a second restriction endonuclease as shown in Fig. 7.

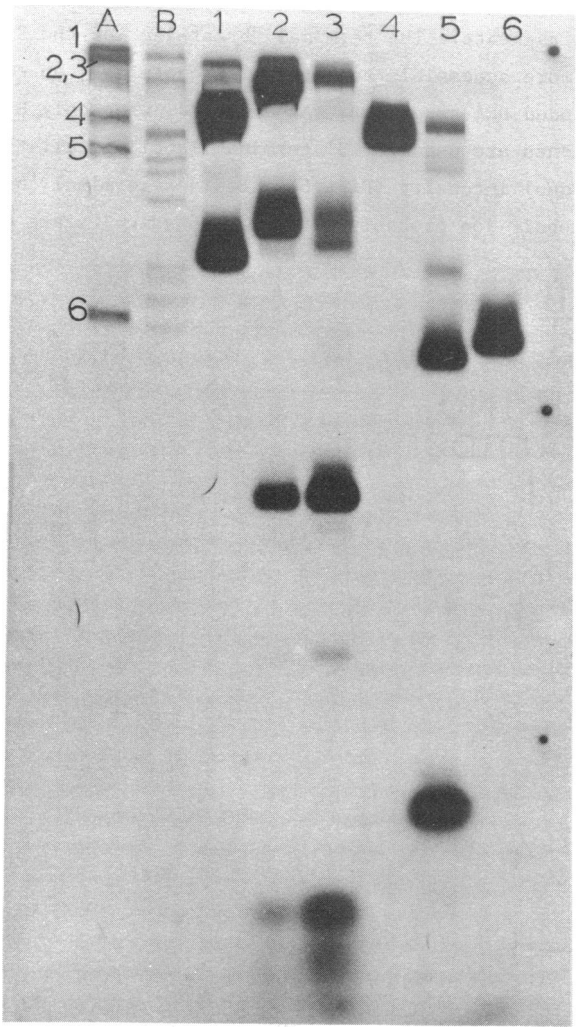

Fig. 7. The separation of unique [^{32}P]rCMP labeled 3'-ends of Hind III fragments after redigestion with Hae III. Terminally labeled Hind III fragments were extracted from each gel band, redigested with Hae III and subjected to electrophoresis on a polyacrylamide step gel with 5% for the top two-thirds and 10% for the lower one-third. The Hin III-2 fragment was contaminated with a small amount of Hin III-3. The redigestion of Hin III-3 was incomplete thus giving some large partial products. Hin III-4 was not digestable by Hae III. For comparison, Hind and Hin III fragments were run side by side (7A, B). Bands numbered 1-6 refer to original [^{32}P]rCMP labeled Hind III fragments before redigestion.

during ribonucleotide incorporation encouraged us to study the feasibility of inserting deoxyhomopolymer blocks at the 3'-ends of duplex DNA molecules. This is an important step for <u>in vitro</u> construction of recombinant DNA[12,13]. As shown in Fig. 8, the polymerization of dAMP and dTMP with intact λ DNA as primer proceeds without lag in the presence of Co^{+2} ion and the extent of polymerization is remarkably higher than that observed with Mg^{+2} ion. With Co^{+2} ion, the initial rate of dAMP addition is 10-15 fold and that for dTMP addition is 50-60 fold higher than that observed with Mg^{+2} ion. Thus,

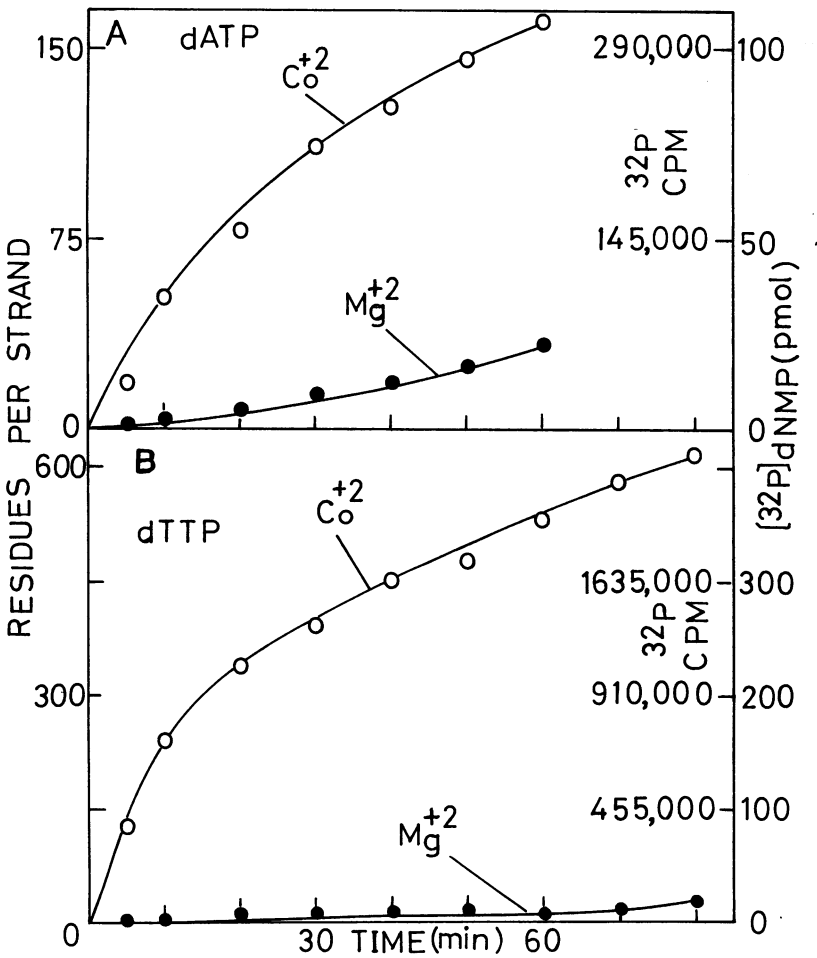

Fig. 8. Polymerization of deoxynucleotides at the 3'-ends of λ DNA. The reaction mixtures (200 µl) contained 0.22 A_{260} unit (0.66 pmol 3'-OH ends) of intact λ DNA in cacodylate buffer system, 1375 pmol of dATP (2900 cpm/pmol) or 1450 pmol of dTTP (4550 cpm/pmol) and 2 µg of terminal transferase. At intervals indicated aliquots (10 µl) were acid washed and counted. From this, the total incorporation as shown in the figure was calculated.

even with duplex λ DNA which contains 5' protruding single-stranded tails, 12 nucleotides in length[25], the addition of poly(dA) or poly(dT) tails to the 3'-ends can be accomplished without the use of λ exonuclease to remove the 5' protruding tails. Lobban and Kaiser have reported the addition of nucleotide residues at the 3'-ends of phage P22 DNA molecules (which con-

Fidelity of 3' terminal labeling

For the purpose of sequence analysis it is essential that the labeling takes place at the expected 3' terminal position in the DNA molecule. This was determined by nearest-neighbor analysis of the Hind III fragments labeled by two independent methods, i.e. labeling with terminal transferase and labeling with E. coli DNA polymerase. Since the 3' terminal deoxynucleotide at the end of Hind III cleavage site is known[24], one would expect $d\overset{*}{A}p$ as the nearest-neighbor of the incorporated nucleotide. Accordingly, Hind III fragments were labeled with [α-^{32}P]rUTP and terminal transferase (Fig. 4) or with [α-^{32}P]dATP and E. coli DNA polymerase as described earlier[19]. As shown in Table 2, all the isolated fragments show the cor-

Table 2. Nearest-neighbor analysis of isolated labeled Hind III fragments

The labeling, separation by polyacrylamide gel electrophoresis, extraction and nearest-neighbor analysis were carried out according to Wu et al (19). The results are expressed as percentages of radioactivity used for analysis.

Fragment number	Terminal transferase labeled				DNA polymerase labeled			
	Ap	Gp	Tp	Cp	Ap	Gp	Tp	Cp
1	82	10	1	7	97	1	1	1
2	88	5	3	4	99	(*)	(*)	(*)
3	86	8	2	4	99	(*)	(*)	(*)
4	89	5	3	3	Not done		-	-
5	88	5	3	4	98	(*)	(*)	(*)
6	94	1	1	4	99	(*)	(*)	(*)

(*) less than 1%

rect $dA\overset{*}{p}$ as the nearest neighbor. Furthermore, the sequence analysis of each separated ends as shown in Fig. 7, revealed unique sequences, confirming the high fidelity of 3' terminal labeling (not shown).

Incorporation of deoxynucleotides: Insertion of homopolymer block at the 3'-end of duplex DNA molecules

The increased priming activity in the presence of Co^{+2} ion observed

Insertion of homopolymeric tails at the EcoRI site of SV40 DNA molecule

Since it is possible to insert homopolymer blocks at the cohesive ends with as many as 12 protruding nucleotides (Fig. 8) we felt that homopolymer extension can be made at any cohesive restriction endonuclease cleavage site. We selected EcoRI cleavage site which produces highly specific staggered end:

5' pA-A-T-T-C.....3'
3' OH-G.....5', with a G-OH at the 3'-end[18]. To check the nature of the tail formation with respect to length, correctness of 3' terminal addition and the degree of homogeneity in the extended products, we selected SV40 DNA for the ease of separation by agarose gel electrophoresis, and EcoRI site because it will produce $dG\overset{*}{p}$ as the nearest neighbor.

Fig. 9 shows polymerization of [α-^{32}P]dAMP residues at the EcoRI site of SV40 linear DNA. An aliquot of the labeled DNA was completely digested[19] for nearest-neighbor analysis. Of the aliquot (409,000 cpm) used for analysis, 3480, 680 and 390 cpm were detected in $dG\overset{*}{p}$, $dT\overset{*}{p}$ and $dC\overset{*}{p}$ respectively, indicating that a covalent chain extension mainly from the correct 3' terminus (dG-OH) had taken place. As expected, the bulk of radioactivity (404,000 cpm) appeared in $dA\overset{*}{p}$. On the basis of this result, it appeared that an average ($dA\overset{*}{p}/dG\overset{*}{p}$) of 116 residues were added as homopolymer tails. Similar results were obtained with [^{32}P]dTMP addition (not shown).

DISCUSSION

In the presence of Co^{+2} ion, the labeling of duplex DNA molecules with terminal transferase is efficient with either an even-duplex or a staggered-duplex with protruding 5'ends. As little as 0.1 pmole (0.05 μM) of primer and 1 μM concentration of [α-^{32}P] rNTP can be used for close to 100% conversion of the primer. This highly efficient labeling reaction makes DNA sequence analysis easier. Thus, the reaction is ideally suitable for labeling fragments generated by restriction endonucleases. Using [^{32}P]rNTPs of high specific activity as much as 40 million cpm can be obtained from 100 pmoles (in terms of 3'-OH ends) of DNA fragments. Such high radioactivity is often necessary in order to process many fragments up to the stage of final se-
tains even ends) in the presence of Mg^{+2} ion. However, after λ exonuclease

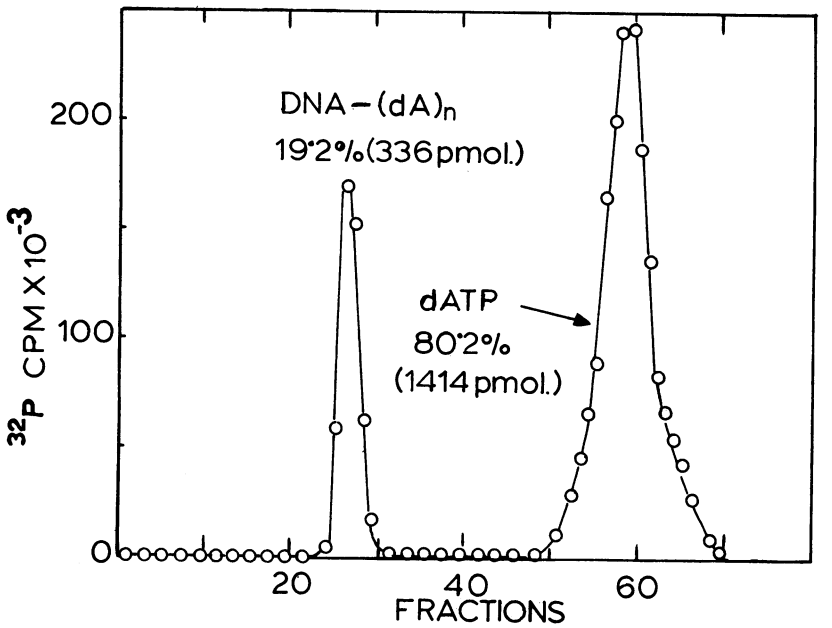

Fig. 9. Insertion of homopolymeric tails at the EcoRI site of SV40 DNA. The reaction mixture (100 µl) contained 0.28 unit (4.2 pmoles) of EcoRI treated linear SV40 DNA, 1750 pmoles of [α-^{32}P]dATP and 2 µg of terminal transferase. After 26 hours at 37° the labeled DNA was isolated as described in Fig. 4.

treatment to expose the 3'-end as single-stranded primer, the rate of addition was appreciably improved[13].

λ DNA labeled with [α-^{32}P]dATP for 10 min in the presence of Co^{+2} ion was subjected to nearest-neighbor analysis[19]. Of the total radioactivity distributed in $\overset{*}{Gp}$, $\overset{*}{Tp}$ and $\overset{*}{Cp}$, 790 cpm were found in Gp and less than 200 cpm found in $\overset{*}{Tp}$ and $\overset{*}{Cp}$. Since a G-OH residue occurs at the 3'-ends of both the strands of λ DNA[25], the above results indicate that the insertion of homopolymer blocks took place mainly in the expected position.

quence analysis. For single terminal addition or for chain termination at a desired point, [α-^{32}P]di-deoxyadenosine triphosphate may be used[26].

Since it is also possible to carry out deoxynucleotide polymerization at the 3'-ends of duplex DNA molecules with staggered breaks (Fig. 8 and 9) without any prior enzyme treatment to expose the 3'-ends as single-stranded primers, this method of labeling simplifies the procedure for inserting

homopolymer tails at the 3'-ends. Inserting similar homopolymer tails with a complementary deoxynucleotide to another type of DNA molecule with a defined genetic information, it is possible to construct a recombinant DNA in vitro after annealing the complementary tails followed by gap filling with E. coli DNA polymerase I and ligase joining[12-14]. For example, SV40 EcoRII G fragment containing the DNA replication site can be joined to EcoRI treated-linear SV40 DNA to yield a recombinant DNA with two replication sites. Such DNA molecules can be used for studying some fine points of replication in vitro. In this way, any DNA fragment generated by any type of restriction endonuclease can be joined to another DNA molecule. Furthermore, DNA molecules with homopolymer tails cannot self anneal and thereby when two types of DNA with two different homopolymer tails are used, the chances of desired annealing are increased.

This is paper XXVI in a series on "Nucleotide Sequence Analysis of DNA." Paper XXV is by R. Wu, G. Ruben, B. Siegel, E. Jay, P. Spielman and C.D. Tu (submitted). This work was supported by research grants CA-14989 and GM-18887 from the National Institutes of Health, and MBS 73-01859 A02 from the National Science Foundation.

*Present address: Department of Chemistry, University of New Brunswick, Fredericton, NB. Canada

+To whom correspondence should be sent.

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