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An improved procedure for utilizing terminal transferase to add homopolymers to the 3' termini of DNA

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ABSTRACT

Terminal deoxynucleotidyl transferase (E.C.2.7.7.3.1.) from calf thymus was used to add homopolymer tails to duplex DNA with 3' protruding, even, or 3' recessive ends. A gel electrophoresis method was employed to analyze the tail length and the percent of DNA with tails. In all the tailing reactions, dA, dT, and dC tails from $CoCl_2$-containing buffer were longer than those from $MnCl_2$- or $MgCl_2$-containing buffers, whereas dG tails from $MnCl_2$-containing buffer were the longest. By varying the ratio of dNTP over DNA terminus and the concentration of terminal transferase, optimal conditions were found for adding dG or dC tails of 10-25 nucleotides in length and dA and dT tails of 20-40 nucleotides in length to duplex DNA with all types of 3' termini.

INTRODUCTION

Terminal deoxynucleotidyl transferase catalyzes the polymerization of deoxynucleotides at the 3' ends of single-stranded DNA[1,2]. Lobban and Kaiser[3] and Jackson et al.[4] made use of the terminal transferase to add homopolymer tails to duplex DNA for cloning P22 DNA fragments and SV40 DNA fragments respectively. In this procedure, poly dA tails were added to one species of DNA and poly dT tails to a different species of DNA. By annealing the complementary tails, these two species of DNA can be joined *in vitro* to form a recombinant DNA molecule. DNA molecules with even ends were found to work inefficiently as primers for the terminal transferase-catalyzed tailing reaction. Efficiency of tailing was appreciably increased when the DNA molecules were treated with λ exonuclease to remove a short stretch of nucleotides from the 5' end[3,4]. Since the λ exonuclease reaction[5] is difficult to control, and highly purified λ exonuclease was not generally available, Roychoudhury et al.[6] adopted a Co^{+2}-containing buffer that allowed terminal transferase to add tails to duplex DNA with all types of 3' termini (even end, 3' protruding end and 3' recessive end) without prior λ exonuclease treatment. Brutlag et al.[7] and Humphries et al.[8] chose a low

ionic strength buffer containing Mg^{+2} to add tails to the 3' ends of duplex DNA. The different conditions for tailing have been reviewed recently[9,10]. However, the efficiency of adding homopolymer tails to duplex DNA with different types of termini has not been thoroughly studied.

The advantages of the terminal transferase-catalyzed tailing method include the possibility of joining almost any double-stranded DNA fragment to a cloning vehicle. Furthermore, since each species of DNA carries the same type of homopolymer tail, it cannot hybridize with another molecule of the same species but can hybridize only with the cloning vector carrying the complementary tail. Thus, after cloning, each transformant should represent the desired recombinant DNA. Several disadvantages of this method include the fact that in most cases the useful restriction enzyme recognition sites are eliminated by the addition of tails and cloning. Furthermore, the tailing efficiency may be low for DNA with even ends or 3' recessive ends, and, where the efficiency is high, the length of added tails may be too long to give stable clones. Therefore, to make this procedure more useful, one must be able to control the tailing reaction to give relatively short tails and to make it efficient with all types of DNA molecules.

Most investigators have calculated the tail length by counting the labeled DNA after acid precipitation. This method depends on the assumption that 100 percent of the DNA molecules have been tailed. However, this assumption may not be correct since the percent of tailed molecules can be low and can vary depending on the conditions. Nearest neighbor analysis of the DNA carrying a 3' terminal nucleotide different from that of the radioactive homopolymer tail can give an average length of the tail. However, even when this method is used, it cannot determine the distribution of tail lengths. In this communication, we have developed a gel electrophoresis method for analyzing the efficiency of the tailing reaction and the length of the homopolymer tail. By applying gel analysis, we have established the optimal conditions for adding homopolymer tails to duplex DNA with all types of 3' termini. We have also found the conditions for adding dG or dC tails of 10-25 nucleotides in length, and dA or dT tails of 20-40 nucleotides in length to duplex DNA.

MATERIALS AND METHODS

Nucleotides

dATP, dTTP, dCTP and dGTP were obtained from P-L Biochemicals, Inc.

[α-^{32}P]dATP, [α-^{32}P]dTTP, [α-^{32}P]dCTP, [α-^{32}P]dGTP (specific activities: 410 Ci/mmole) and [γ-^{32}P]ATP (2000-3000 Ci/mmole) were purchased from Amersham Corporation.

Enzymes

Terminal deoxynucleotidyl transferase was obtained from P-L Biochemicals. Restriction endonucleases Pst I, Bgl I, Pvu II, Eco RI, Hind III, Bam HI, Sal I and Hinf I were purchased from New England Biolabs, Inc. and Hinc II from Bethesda Research Laboratories, Inc. Polynucleotide kinase was obtained from Biogenics, Inc. and bacterial alkaline phosphatase from Bethesda Research Laboratories, Inc.

Tests for contaminating nuclease activities in the terminal transferase preparation[11]

A covalently closed circular duplex DNA, pBR322, was used as substrate to determine the contaminating endonuclease activities in the terminal transferase preparations. After incubation of 0.5 μg of DNA in 10 μl with 5 units of transferase (30°C, 2 hours), and electrophoresis, less than 5% of the covalently closed circular DNA was converted to a nicked open-circle configuration.

Hinf I digested pBR 322 DNA was used for the assays of contaminating exonuclease activities in the terminal transferase preparations. The Hinf I-DNA fragments were first repaired with [α-^{32}P]dATP to incorporate a single nucleotide per strand of DNA, and incubated with the transferase. The reaction was carried out according to the conditions for the tailing reaction which will be discussed below. After the reaction was terminated, the mixture was passed through a pipet tip containing DE-52. By using the counting method mentioned below, the exonuclease activity could be calculated from the ratio of the counts of the liberated [α-^{32}P]dAMP to the total counts from the original DNA fragment. From this assay, we found less than 4% of [α-^{32}P] dAMP released by the transferase preparations (lot 11). Thus, it is suitable for the tailing reaction.

Digestion of DNA with restriction endonucleases

For Pst I, Bgl I, Pvu II, Hinc II, Hind III, Sal I and Hinf I digestion, the reaction mixtures contained 7 mM Tris-HCl (pH 7.4), 60 mM NaCl, 7 mM $MgCl_2$, 0.3-0.5 mg/ml pBR 322 DNA and 0.3-0.5 unit/ml of a restriction enzyme. For Eco RI digestion, the reaction mixture contained 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.3 mg/ml pBR 322 DNA and 0.3 unit/ml of enzyme. For Bam HI, the mixture contained 6 mM Tris-HCl (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$, 0.3 mg/ml pBR 322 DNA and 0.3 unit/ml of enzyme.

After incubation at 37°C overnight, the completeness of digestion was checked by agarose gel electrophoresis. Then the reaction mixtures were extracted once with phenol, once with chloroform-isoamyl alcohol (24:1) and precipitated twice with ethanol.

Labeling of DNA at the 5' end

Bam HI-cut DNA fragments or Sal I-cut fragments of pBR 322 (final concentration of 5' ends 200 pmoles/ml) were incubated with 10 mM Tris-HCl (pH 8.5), 100 mM NaCl, bacterial alkaline phosphatase (360 units/ml) at 64° for 30 min to remove the 5' phosphate. The samples were extracted three times with phenol, once with chloroform-isoamyl alcohol and precipitated twice with ethanol. The dephosphorylated DNA (10 pmoles 5' ends) was dissolved in 30 µl of a mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothrietol, 0.1 mM spermidine, 50 pmoles of [γ-^{32}P]ATP and 3 units of polynucleotide kinase. The mixture was incubated at 37°C for 1 hour and extracted once with phenol, once with chloroform-isoamyl alcohol and precipitated twice with ethanol. The 5'-labeled pBR 322-Sal I fragment was digested with Bgl I to get 3' protruding ends, or with Bam HI to get 3' recessive ends, and the 5'-labeled pBR 322-Bam HI fragment was digested with Hinc II to get blunt ends.

Buffer systems for the addition of homopolymer tails

Three different buffer systems for adding homopolymer tails to the 3' ends of DNA molecules were used. (1) The $CoCl_2$-containing buffer: 100 mM K cacodylate, (or a different concentration as specified) pH 7.0, 1 mM $CoCl_2$, 0.2 mM dithiothrietol; (2) the $MnCl_2$-containing buffer: 100 mM K cacodylate, (or a different concentration as specified), pH 7.1, 2 mM $MnCl_2$, 0.1 mM dithiothrietol; and (3) the $MgCl_2$-containing buffer: 40 mM K cacodylate, pH 7.1, 4 mM $MgCl_2$, 0.1 mM dithiothrietol.

Addition of homopolymer tails to 3' ends of DNA

Double-stranded DNA fragments with 3' protruding ends were obtained from Pst I or Bgl I digestion of pBR 322, fragments with blunt ends from Pvu II or Hinc II digestion, and fragments with 3' recessive ends from Eco RI, Sal I, Hind III or Bam HI digestion.

Each DNA fragment (50-60 pmoles 3' ends/ml) was incubated in one of the buffer systems mentioned above with one of the four triphosphates at a 20-200 fold excess over the concentration of DNA ends, and terminal transferase from P-L Biochemicals, Inc. (lot 11, 250-2000 units/ml). The mixture (5 µl) was incubated at 30°C for 30-60 min. and terminated by adding EDTA (to 12 mM) and chilling to 0°C. [α-^{32}P]dNTPs were used for incorporation

into unlabeled DNA fragments, and unlabeled dNTPs were used for addition of tails to the 3' ends of the 5'-labeled DNA fragments.

Determination of the incorporation of nucleotides to 3' ends of DNA

After the addition of [α-^{32}P]dNTP (diluted by the same kind of unlabeled dNTP to a specific activity of 5-20 Ci/mmole) to the 3' ends of DNA according to the conditions already mentioned, we passed the reaction mixture through a pipet tip, containing about 100 µl of DE-52 ion-exchange cellulose, and eluted the [^{32}P]dNTP with 0.35 M ammonium formate (600 µl). We counted the pipet tip which carried the labeled DNA and also the eluate which contained the unreacted [^{32}P]dNTP by Cerenkov counting in a scintillation counter. The ratio of the counts of the pipet tip to the total counts gave the percent incorporation of [^{32}P] dNMP into the DNA (this assay was developed by Robert Yang). If we assume that 100% of the DNA molecules have added homopolymer tails, the average tail length can be calculated.

Determination of the length of homopolymer tails by gel electrophoresis

After adding tails to 3' ends of 5'-labeled DNA fragments, the labeled DNA was denatured in 0.3 N NaOH and the samples loaded on a 3.5% or 5% polyacrylamide gel in 8 M urea. The ratio of Bis to acrylamide was 1:29, the gel size was 350 x 400 x 1.5 mm and the running buffer was 50 mM Tris-borate (pH 8.1), 1 mM EDTA. Electrophoresis was carried out at 20-25 mA (160-400 V) for 20 hours. The gel was exposed to X-ray film (Kodak XR-5) for 1-5 days and developed. According to the image on the film, the gels were cut into 2 or 4 pieces, one containing the original fragment and the others containing slower moving fragments that contained homopolymer tails (Fig. 1, 2 and 3). The gel fragments were counted in a scintillation counter for Cerenkov radiation. From these counts, we calculated the percent of DNA molecules that contain homopolymer tails. The average length of the tails and the distribution of the tail lengths also were estimated either from the autoradiogram of the gel or from counting the gel fragments.

RESULTS AND DISCUSSIONS

Addition of homopolymer tails to DNA using different amounts of terminal transferase

For tailing reactions, the amounts of transferase used by different investigators are vastly different[9,10]. We first determined the optimal amount of transferase that can give the tail length suitable for hybridiza-

tion and cloning of the DNA molecules. These lengths are at least 10 nucleotides for dC and dG tails, and at least 20 nucleotides for dA and dT tails. We varied the amounts of transferase from 250 units/ml to 2000 units/ml and found that (data not shown) the longest tails were obtained with 500 units/ml of transferase. At this level, the concentration of transferase molecules was ten times more than the concentration of 3' ends of the DNA molecules. Somewhat higher percent of DNA molecules with tails was obtained with around 1000 units/ml of transferase. Beyond the optimal level, higher levels of transferase reduced the tail length or percent of tailed DNA. Thus, all the experiments reported below were carried out with 500 units/ml of transferase.

Addition of homopolymer tails at different temperature

We varied the incubation temperatures around 37°C for adding different tails to different types of 3' end and found in some cases the percent incorporation was similar between 30°C and 42°C, or slightly higher at 42°C. On the other hand, the incorporation of dT or dA to Sal I cut pBR322 DNA in Co^{+2}-containing buffer was two-fold higher at 30°C than that at 37°C or 42°C. Thus, all the reactions reported in this communication were carried out at 30°C.

Addition of homopolymer tails in different buffer systems

The most common divalent metal ions in the buffer systems for the terminal transferase reaction are Co^{+2} (1 mM) and Mg^{+2} (4 or 8 mM)[9,10]. We now compared the efficiencies of $CoCl_2$, $MgCl_2$ and $MnCl_2$ in supporting the addition of tails to DNA molecules with 3'-protruding end (Pst I cut DNA), even end (Pvu II cut DNA), and 3'-recessive end (Eco RI cut DNA). As shown in Table 1, in all three types of tailing reactions, dA, dT and dC tails from $CoCl_2$-containing buffer were longer than those from $MnCl_2$ or $MgCl_2$ containing buffer (with 40 mM or 100 mM K cacodylate), whereas dG tails from $MnCl_2$-containing buffer were the longest. Since the tailing efficiency in 2 mM $MnCl_2$ was about 30% higher than that in 1 mM or 4 mM (data not shown), we chose 2 mM.

The tailing reaction was also affected by the type and concentration of anions in the buffer[12,13,14]. At 100 mM of K cacodylate buffer, or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)-NaOH buffer, or Tris-HCl buffer, the incorporation of labeled nucleotides into DNA was the highest in K cacodylate buffer, while those in Tris-HCl buffer were about 10-fold lower. Using different concentrations of K cacodylate in the buffer for the tailing reaction, we found that the tails incorporated in

TABLE 1

Tail length from reactions using different divalent metal ions

pBR 322 DNA cut with	Tails	Calculated tail length		
		$MnCl_2$	$CoCl_2$	$MgCl_2$
Pst I (3' protruding end)	dA	5	13	2
	dT	21	29	1
	dC	27	27	4
	dG	32	23	17
Pvu II (even end)	dA	4	6	2
	dT	3	33	1
	dC	10	21	1
	dG	30	16	4
Eco RI (3' recessive end)	dA	3	4	0.5
	dT	15	37	0.4
	dC	13	23	3
	dG	36	22	4

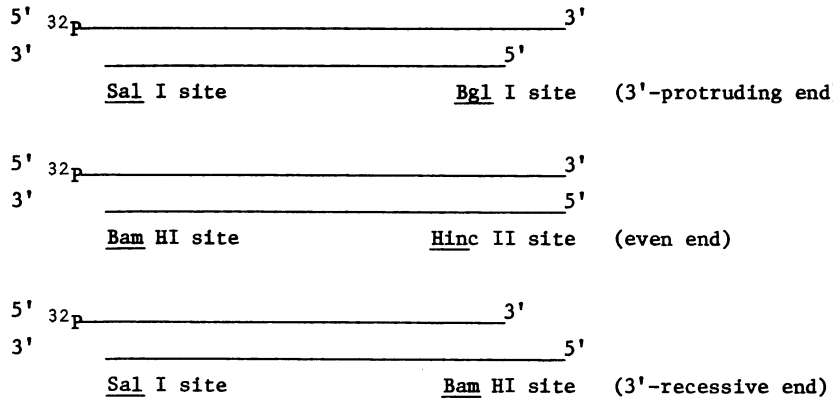
Addition of radioactive dA, dT, dC, or dG homopolymer tails to pBR 322 DNA (50 pmoles of 3' ends/ml) cut by Pst I, Pvu II or Eco RI, respectively, was carried out with different [^{32}P]dNTPs (100-fold excess over 3' ends of DNA). The transferase reactions were carried out at 30°C for 60 minutes. The tail length was calculated by incorporation assuming that tails were added to all the DNA molecules.

100-140 mM K cacodylate buffer were twice as long as those in 25-50 mM buffer.

Adding tails to DNA molecules with 3'-protruding, even, or 3'-recessive ends

Terminal transferase requires single-stranded DNAs as primers[1,2]. Thus, the incorporation efficiency was high for DNA with 3' protruding ends[3,4]. However, for DNA molecules with blunt ends or 3' recessive ends, the reactions were less favorable[3,4,6]. Replacing $MgCl_2$ with $CoCl_2$ increases the efficiency of these reactions[6]. Most investigators have used the incorporation method to estimate the tail length, which assumes that 100 percent of the DNA molecules were tailed. But this assumption is not valid under most conditions.

The following experiments were designed to measure more exactly the efficiencies of adding homopolymer tails to DNA molecules with 3'-protruding, even, or 3'-recessive ends. The DNA fragments with different types of 3' ends used in this investigation are shown below.



The 3' end of the upper strands are 3'-protruding, even or 3'-recessive, respectively. The 5' ends of the upper strands were ^{32}P-labeled beforehand (as described in Materials and Methods). Although the 3' ends of both upper strands and lower strands can accept homopolymer tails, only the pre-labeled upper strand was detected after the tailing reaction and strand separation by electrophoresis.

We then determined more precisely the efficiency of adding tails to DNA with different types of 3' ends by measuring the percent of DNA molecules with tails and the average length of the tails. We selected gel electrophoresis for analysis since it gives much more quantitative data than the methods used previously. The results in Table 2 show that the longest tail length and the highest percent of DNA with tails were obtained in DNA with 3' protruding ends except for dA and dT reactions in $CoCl_2$-containing buffer. The average length of dA and dT tails in $CoCl_2$ was the highest for DNA with even ends, but the percent of DNA with tails was low.

We expected even-ended DNA to be more efficiently tailed (higher percent with tails) than DNA with 3' recessive ends. However, this was not true in certain cases, such as the addition of dC tails to Hinc II fragment as compared to Bam HI fragment in both types of buffers (Table 2). Results from this table also show that although the percent DNA with added dA and dT tails was low, the tailing reactions were more efficient in $CoCl_2$ than in $MnCl_2$-containing buffer for DNA with even or 3' recessive ends. Whereas, for addition of dC or dG tails to blunt or 3' recessive end, the reactions were more efficient in $MnCl_2$-containing buffer (Table 2, Panel B of Fig. 1 and Fig. 2). That means a higher percent of tailed molecule can be obtained in $MnCl_2$ buffer.

The distribution of the tail length is as follows. For example, for

TABLE 2

The efficiency of tailing reactions using DNA with different types of 3' ends

Tails	^{32}P-pBR322 DNA cut with	Types of ends	$MnCl_2$-containing buffer		$CoCl_2$-containing buffer	
			Average tail length	% of DNA with tails	Average tail length	% of DNA with tails
dA	Bgl I	3'-protruding	48	72	76	91
	Hinc II	even	--	<1	141	17
	Bam HI	3'-recessive	40	2	40	4
dT	Bgl I	3'-protruding	78	89	100	90
	Hinc II	even	42	15	167	27
	Bam HI	3'-recessive	50	14	109	28
dC	Bgl I	3'-protruding	74	91	72	89
	Hinc II	even	23	30	43	20
	Bam HI	3'-recessive	35	79	45	47
dG	Bgl I	3'-protruding	46	96	42	92
	Hinc II	even	25	95	19	64
	Bam HI	3'-recessive	42	72	21	15

Homopolymer tails were added to 5' labeled pBR322 DNA (60 pmoles of 3' ends/ml) cut by Bgl I, Hinc II or Bam HI, with unlabeled dATP, dTTP, dCTP or dGTP (100-fold excess over 3' ends of DNA). The transferase reactions were carried out at 30°C for 60 min. Average tail length and percent of DNA molecules with tails were estimated from gel electrophoresis analysis as described under "Materials and Methods." There are two cuts in pBR322 DNA by Hinc II digestion; data of only the shorter Hinc II fragment are shown.

DNA with 3' protruding ends (Panel A, Figure 1), the length of the majority (over 90% of the molecules) of the dA tails was from 30 to 65 nucleotides (to be recorded as an average tail length of 48 in Table 2) in the Mn^{+2} buffer and 58 to 95 in the Co^{+2} buffer; that of the dT tails was from 50 to 106 in the Mn^{+2} buffer and longer in the Co^{+2} buffer; that of the dC tails was from 40 to 108 in the Mn^{+2} buffer and 51 to 92 in the Co^{+2} buffer; that of the dG tails was from 36 to 56 in the Mn^{+2} buffer and 32 to 52 in the Co^{+2} buffer. For DNA with 3' recessive ends (Panel B of Fig. 1), the length of the dC tails was from 28 to 42 and that of the dG tails was from 36 to 48 in the Mn^{+2} buffer, whereas for DNA with blunt ends (Fig. 2), the length of dC tails was from 15 to 30 and that of dG tails from 14 to 33 in Mn^{+2} buffer. Thus, the Mn^{+2} buffer is recommended for the addition of

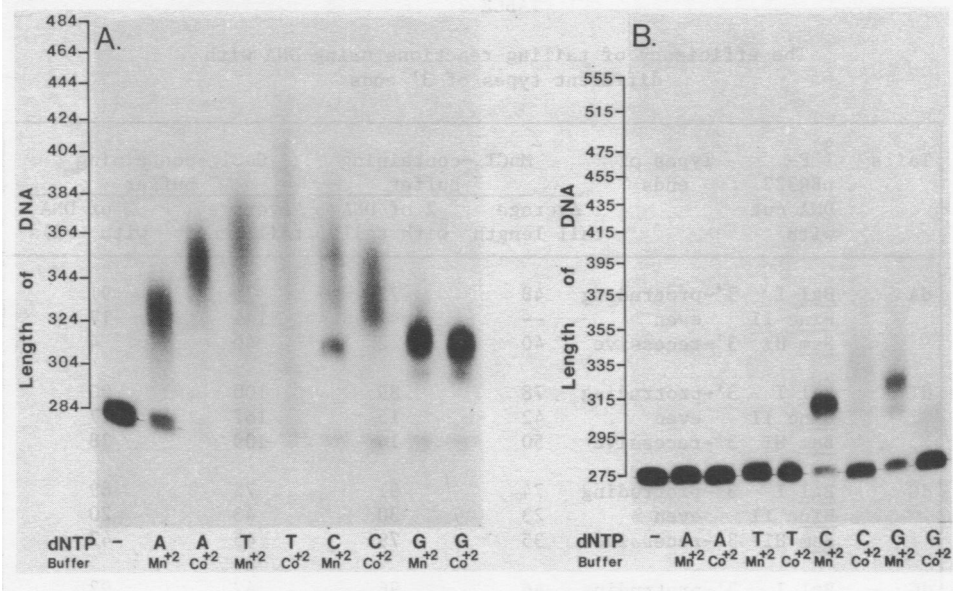


Figure 1. Addition of homopolymer tails to 3' ends of DNA fragments cut by Bgl I or Bam HI enzymes. Homopolymer tails were added to the 3' ends of 5' ^{32}P-labeled pBR 322-Bgl I or pBR 322-Bam HI DNA fragments (final concentration: 60 pmoles of ends/ml for each DNA). The reaction mixture contained either the Mn^{+2} buffer or the Co^{+2} buffer, one unlabeled dNTP (dTTP, dCTP, dATP or dGTP, 100-fold excess over the 3' end of DNA) and terminal transferase (500 units/ml). Incubations were carried out at 30°C for 60 min. The labeled DNA was precipitated by ethanol, denatured in 0.3 N NaOH, and loaded on a 5% polyacrylamide gel in 8 M urea. Only the short restriction fragment of each experiment is shown in the Figure. A. Tailing reactions for pBR 322-Bgl I fragment (284 nucleotides in length with 3' protruding ends). B. Tailing reactions for pBR 322-Bam HI fragment (275 nucleotides in length with 3' recessive ends).

these tails to DNA with blunt or 3' recessive ends.

Adding tails to DNA fragments containing different sequences at the even ends

For adding labeled rNTP to the 3' end of double-stranded DNA, earlier work from this laboratory has indicated that labeling efficiency is related to the number of dG:dC base pairs adjacent to the exposed end of the duplex DNA. The higher the number of dG:dC pairs at the 3' end up to 6-8 base pairs, the lower the labeling efficiency[15]. In the experiments shown in Table 3, we used two pBR 322 Hinc II fragments which contained different sequences at their 3' ends, which are shown below:

```
5'                          3'
d(.....-G-G-A-G-A-G-C-G-T-C)        The short Hinc II fragment
d(.....-C-C-T-C-T-C-G-C-A-G)
5'                          3'
d(.....-A-T-C-C-C-G-T-G-T-T)        The long Hinc II fragment
d(.....-T-A-G-G-G-C-A-C-A-A)
```

The first base pair at the exposed end of the short fragment is a dG:dC pair, whereas that of the long fragment is a dA:dT pair. For the first four base pairs from the exposed ends, the short fragment has three dG:dC pairs, whereas the long fragment has only one dG:dC pair.

The percent of DNA with tails for the two Hinc II fragments with different sequences is shown in Table 3. It is obvious that the efficiencies are much higher in adding tails to the dA:dT rich sequence (the long fragment). This result is consistent with earlier observations and interpreta-

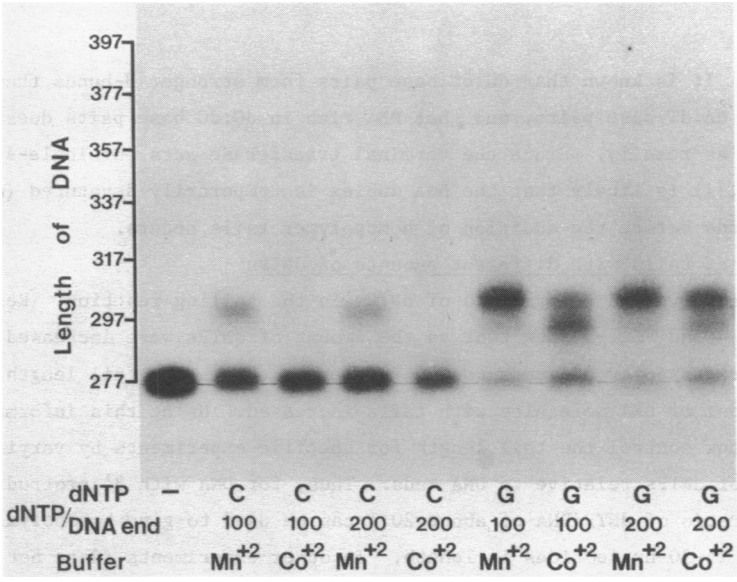


Figure 2. Addition of homopolymer tails to 3' ends of Hinc II fragment. The 5' labeled pBR322-Hinc II fragment (60 pmoles ends/ml) was incubated with dCTP or dGTP (100 or 200 folds excess over 3' ends of DNA), and terminal transferase (500 units/ml) at 30°C for 60 min. Reactions were carried out either in $MnCl_2$ or in $CoCl_2$ containing buffer. Electrophoresis was performed as described in legend of Fig. 1 except that a 3.5% polyacrylamide gel was used.

TABLE 3

The efficiency of tailing reactions in DNA fragments with different sequences adjacent to the blunt ends

Tails	Fraction of DNA with tails (%)	
	dG:dC rich end	dA:dT rich end
dA	17	81
dT	27	72
dC	20	80
dG	95	95

Homopolymer tails were added to 5'-labeled pBR322-Hinc II fragments (60 pmoles of 3' ends/ml) with two different sequences adjacent to the blunt ends: one with 3 dG:dC pairs out of 4, the other with 3 dA:dT pairs out of 4. Four tubes each with a different dNTP (100-fold excess over 3' ends) were incubated with terminal transferase at 30°C for 30 min. For the dA, dT, dC tailing reactions, $CoCl_2$-containing buffer was used, whereas for the dG tailing reaction, $MnCl_2$-containing buffer was used. Results were calculated from gel electrophoresis data.

tions[15]. It is known that dG:dC base pairs form stronger H-bonds than those of dA:dT base pairs, and that DNA rich in dG:dC base pairs does not denature as readily. Since the terminal transferase acts on single-stranded DNA only, it is likely that the DNA duplex is temporarily denatured (melted) at the ends before the addition of homopolymer tails occurs.

Addition of tails with different amounts of dNTPs

We also varied the amounts of dNTPs in the tailing reaction. Results in Table 4 and Fig. 3 show that as the amount of dNTPs were increased from 20-fold excess over DNA ends to 200-fold excess, both the tail length and the percent of DNA molecules with tails increased. Using this information, one can now control the tail length for specific experiments by varying the amounts of dNTPs relative to DNA ends. Thus, for DNA with 3' protruding ends, a ratio of dNTP/DNA of about 20:1 can be used to give homopolymer tails of 15-30 nucleotides in length. In other experiments (data not shown) using DNA with even ends and 3' recessive ends, a ratio of dNTP/DNA of about 100:1 was found to be best. It is clear from Fig. 2 that a higher percent of tailed molecules cannot be obtained by increasing the amounts of dNTPs from 100 to 200 folds excess for tailing reaction of even end.

Restoration of the recognition sequence of restriction enzymes

After tailing, in order to recut the DNA fragments from the recombinant

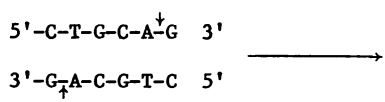
TABLE 4

Tail length and percent of DNA with tails using different amounts of dNTPs

Tails	dNTP/DNA 3' ends	Tail length	Fraction of DNA with tails
	mole/mole	nucleotides	%
dA	20	25	65
	40	42	82
	50	57	86
	100	65	89
dT	20	30	75
	40	50	84
	50	64	86
	100	109	90
dC	20	18	66
	40	34	85
	100	82	90
	200	112	90
dG	40	17	87
	50	27	90
	80	29	91
	100	38	91

The 5' labeled pBR322-Bgl I fragment with 3' protruding ends (60 pmoles of ends/ml) was incubated with different amounts of dNTPs and terminal transferase in $CoCl_2$-containing buffer at 30°C for 60 min. The results were analyzed by the gel electrophoresis method.

DNA after cloning we should reconstruct the restriction sequences at the 3' end of either the DNA fragments to be cloned or the cloning vector. For DNA fragments cut by Pst I, the recognition sequences and the digestion product are:



Therefore, if a dG tail is added to the 3' end of a Pst I cut DNA fragment, the restriction sequence is restored after cloning[16-19]. For DNA fragments cut by Eco RI, Hind III, Bam HI, Sal I, etc., we recommend the use of reverse transcriptase and the required dNTPs to first repair the 3' recessive ends to produce blunt ends, and then add the required tails to restore the recognition sequences (Table 5).

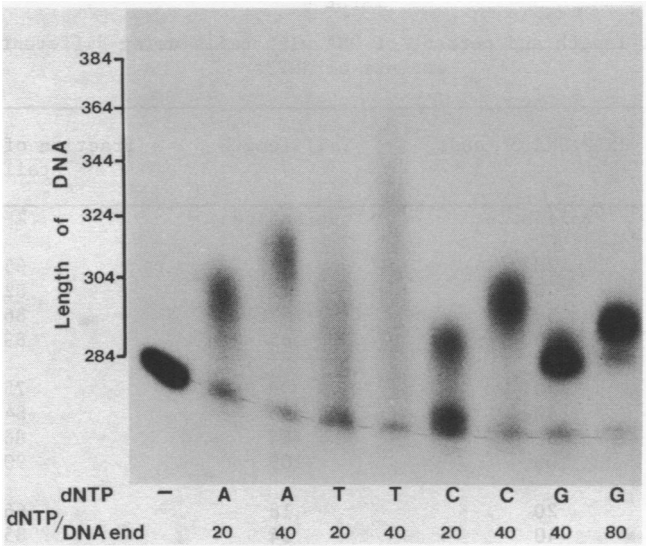


Figure 3. Addition of homopolymer tails to 3' ends of Bgl I fragment using different amounts of dNTPS. The 5' labeled pBR322-Bgl I fragment (60 pmoles ends/ml) was incubated with dNTPs and terminal transferase (500 units/ml) in $CoCl_2$-containing buffer at 30°C for 60 min. Electrophoresis was performed according to legend of Fig. 1.

CONCLUSIONS

Using a gel electrophoresis method for determining the tail length and the percent of DNA molecules with tails, we have analyzed and optimized the efficiency of the transferase-catalyzed reaction. For DNA with 3' protruding ends, the percent of DNA that can be tailed is usually higher than 70 percent (Table 2). To avoid unnecessarily long tails (e.g. greater than 50), a lower dNTP/DNA ratio (such as 20) could be used. For DNA with even ends or 3' recessive ends, the percent of DNA with tails is much lower and it is dependent on the number of G:C pairs at the 3' ends as well as on the type of tails. For example, the addition of dA tails to the Bam HI fragments (3' recessive ends) shown in Table 2 was very inefficient since only 4% of the DNA was tailed. On the other hand, 28% of the same DNA fragments was tailed with dT. In order to maximize the yield of the recombinant DNA carrying complementary tails, two strategies may be considered. First, dG and dC tails are preferred when dA or dT is not needed to restore the restriction site. When dA or dT tails are needed (e.g. in DNA cut with Bgl

TABLE 5

Required tails for restoration of the recognition sequence of restriction enzymes

Enzyme	Recognition sequence	Desirable tail
Bam HI	5' G↓G-A-T-C-C 3' C-C-T-A-G↑G	dC
Bgl II	5' A↓G-A-T-C-T 3' T-C-T-A-G↑A	dT
Eco RI	5' G↓A-A-T-T-C 3' C-T-T-A-A↑G	dC
Hind III	5' A↓A-G-C-T-T 3' T-T-C-G-A↑A	dT
Hinf I	5' G↓A-N-T-C 3' C-T-N-A↑G	dC
Hpa II	5' C↓C-G-G 3' G-G-C↑C	dG
Sal I	5' G↓T-C-G-A-C 3' C-A-G-C-T↑G	dC
Taq I	5' T↓C-G-A 3' A-G-C↑T	dA

After repair synthesis, the required tails for restoring the recognition sequence of several of the common restriction enzymes that give 5' protruding ends are listed.

II or Taq I), the dA tail should be added to the repaired cloning vehicle (more abundant DNA), and the more efficiently added dT tail should be added to the DNA to be cloned (less abundant DNA). A 30 minute incubation time is recommended.

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