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# DNA polymerase fluorescent substrates with reversible 3′-tags

(Nucleotide sequencing; primer extension; gel; genome; termination; modified nucleotides)

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SUMMARY

We have synthesized 3′-substituted-2′-deoxyribonucleotide-5′-triphosphates corresponding to A, T, G and C. The 3′ position was esterified by a separate anthranylic derivative (3′-tag) giving specific fluorescent properties to each nucleotide (nt). These nt acted as substrates with several DNA polymerases leading to chain termination. Upon alkali or enzymatic treatment of the terminated DNA chain, free 3′-hydroxyl groups were recovered and found able to undergo chain extension when incubated with a mixture of dNTPs and a DNA polymerase. Because each tag has different fluorescent properties in itself, i.e., as a free acid, it theoretically is possible, after removal and characterization of the tag, to infer which nt has been inserted. Reiteration of the process can then be used to determine a nt sequence with a non-gel-based method amenable to automation.

## INTRODUCTION

DNA sequencing has revolutionized the speed and depth of our understanding of complex molecular biology processes. Presently classical sequencing techniques were introduced around 1977 (Sanger et al., 1977; Maxam and Gilbert, 1977). Dideoxy sequencing (Sanger et al., 1977) has gained wide acceptance and is now the method of choice for determining a nt sequence from a single-stranded (ss) DNA template. During the four enzymatic chain elongations, dideoxy nt are randomly inserted in place of the corresponding deoxy nt. Sequencing reactions generate a complex mixture which is subsequently resolved by polyacrylamide-gel electrophoresis.

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Abbreviations: AMV-RT, avian myeloblastosis virus reverse transcriptase; An, anthranyloyl; bp, base pair(s); BSA, bovine serum albumine; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; RTa, reversibly tagged; ss, single strand(ed).

We wanted to reconsider the basic features of the dideoxy sequencing method, in particular the enzymatic reaction itself where most of the complexity of the process is generated and analysed during the following steps of resolution and data acquisition, i.e., gel electrophoresis and sequence reading, respectively. Indeed, we thought that we could take advantage of the molecular recognition performed at the polymerase active site after a single nt incorporation. This would considerably reduce product diversity generated by an extension reaction in the presence of classical deoxy and dideoxy nt. Thus, a canonical sequencing reaction would produce a single adduct in a controled fashion, allowing identification of the added nt, before iteration of the process, leading to a stepwise, real-time sequence determination. Protection of the 3′-end of the extending DNA molecule would have the desired properties for such a reaction, provided that a free 3′-hydroxyl group could be eventually regenerated.

As a first step toward such a method, we describe such deoxy nt derivatives and their use by different DNA polymerases. We show using a end-labelled primer and a gel assay that these nt are chain terminators which, once incorporated, can be converted to regular, functional

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3′-ends. We describe the basic steps of a putative cycle (namely complete incorporation, deprotection and re-incorporation), as a contribution towards a new nt sequencing method that circumvents gel electrophoresis and the use of radio-isotopes.

## RESULTS AND DISCUSSION

The aim of this work was to design 3′-modified 2′-deoxynucleotides 5′-triphosphate substrates for DNA polymerases, such that the 3′-moiety would be different for each base G, A, T or C, be easily identified (e.g., fluorescent), and be removed under conditions compatible with DNA stability to restore an unprotected 3′-hydroxyl end.

### (a) Synthesis of nt analogs

We synthesized 3′-anthranyloyl 2′-deoxy-nucleotide-5′-triphosphates derivatives and evaluated them as substrates for several DNA polymerases. For each of the four dATP, dGTP, dTTP and dCTP, the 3′-hydroxyl group was esterified by a distinct anthranylate or fluorescein-based fluorescent residue using the corresponding anhydride or isothiocyanate (Fig. 1). This resulted in different spectrofluorometric properties for each 3′-Reversibly Tagged-dNTP (3′-RTa-dNTP), as a consequence of the respective free acid counterpart (Table I) and allowed discrimination between them thanks to their different fluorimetric absorption or emission spectra. Nuclear magnetic resonance assignments are available upon request.

TABLE I

Spectral properties of uncoupled fluorophores[a] and their corresponding nt

| Anthranylic derivative[b] | | | | Wavelength (nm) | | Coupled to |
|---|---|---|---|---|---|---|
| R1 | R2 | R3 | R4 | $\lambda_{max}$ absorption | $\lambda_{max}$ emission | |
| H | H | H | H | 315 | 396.5 | dATP |
| $CH_3$ | H | H | H | n.d.[c] | 416.5 | dGTP |
| H | $CH_3$ | H | H | 312 | 403 | dTTP |
| H | H | $CH_3$ | H | 317 | 409 | dCTP |
| H | H | H | $CH_3$ | 289 | 403 | dCTP |
| Fluorescein | | | | 494 | 523 | dTTP |

[a] **Methods:** Absorption spectra were recorded at 25 C in a double-beam spectrophotometer in the presence of 50 mM Tris·HCl (pH 8.0). Fluorescence emission and excitation spectra were measured at 25 C in a LS50B Perkin-Elmer fluorescence spectrophotometer, using a 2-ml cuvette or a 16-µl liquid chromatography flow cell from Perkin-Elmer. All compounds were excited at their absorption maxima. The slit widths for excitation and emission were 2.5 nm.

[b] See Fig. 1 for compound 1 with groups R1–R4.

[c] n.d., not determined.

### (b) Incorporation of 3′-RTa-dNTPs

Since these nt analogs did not contain a 3′-hydroxyl group, their incorporation into an elongating DNA strand resulted in chain termination. This point was assessed using the solid-phase assay described in Methods in the legend to Fig. 3. A primed ss oligo (Fig. 2, substrate 31-G,A,T,C) was incubated with the complementary 3′-RTa-dNTP adjacent to the primer and various DNA polymerases, and the resulting product was

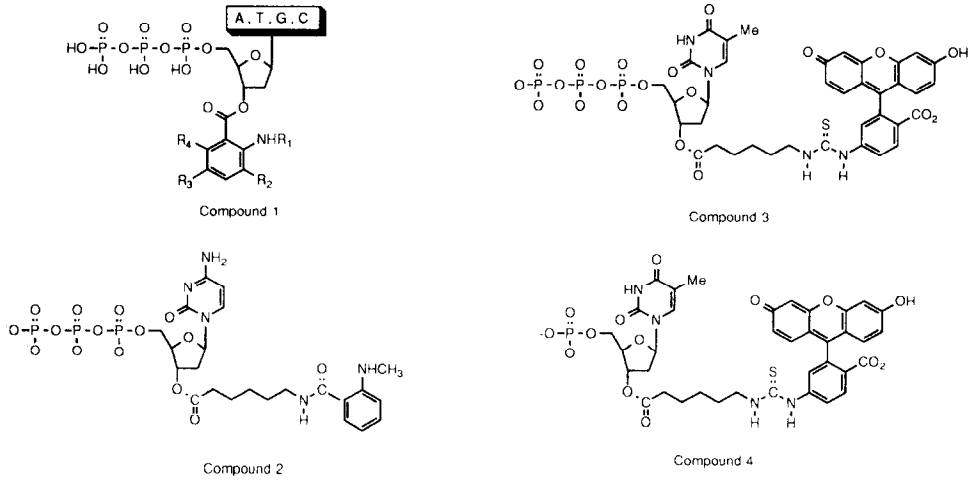


Fig. 1. Chemical structure of 3′-RTa-2′-deoxy-nucleotides. **Methods:** An-dATP (Sarfati et al., 1990) was prepared from dATP and isatoic anhydride essentially by the same procedure as (Hiratsuka, 1982) for the synthesis of An-ATP. *N*-methyl-An-dGTP, 6-methyl-An-dTTP, 3-methyl-An-dCTP and 5-methyl-An-dCTP were synthesized, purified and characterised by the same procedure. 3-Methyl, 5-methyl and 6-methyl-isatoic anhydrides were prepared by the procedure described by Erdmann (1899) for isatoic anhydride. Synthesis of compounds 1, 2, 3 and 4 will be described elsewhere (R.S.S., T. Berthod, C. Guerreiro and B.C., data not shown).

5'-Bio-ATACTTTAAGGATATGTATCC
TATGAAATTCCTATACATAGGNTTTTTTTTT **31-G,A,T,C**

5'-$^{32}$P-ATACTTTAAGGATATGTATCC
TATGAAATTCCTATACATAGGCCCCC **26-C**

Fig. 2. Diagram of the system used to measure enzymatic insertion of 3'-modified nt. The synthetic primer is either biotinylated in 5' (substrate 31-G,A,T,C) or $^{32}$P-end-labelled (substrate 26-C) and annealed to a complementary template.

assayed for free 3'-hydroxyl groups available for further extension with a chase containing a radio-labelled deoxy nt (Fig. 3A). Typically, this assay indicated that some incorporation of the 3'-modified nt might have occurred, as higher counts were always found in the control (unblocked) relative to the various enzyme/substrates tested. Several DNA polymerases could have extended the primer with a 3'-modified deoxy nt to some extent, the Sequenase and the M-MuLV reverse transcriptase being respectively the most and the least efficient under the experimental conditions tested here. Unmodified T7 DNA polymerase, *Taq* polymerase and Klenow fragment of DNA polymerase I were also able to use such substrates (data not shown).

However, the observed blocking was not complete under some of these conditions (Fig. 3A), and such assays did not tell us what may have happened at the molecular level.

Consequently, AMV-reverse transcriptase, *Taq* DNA polymerase and modified T7 DNA polymerase were selected for further studies using a $^{32}$P-end-labelled primer extension assay followed by denaturing polyacrylamide-gel electrophoresis. Fig. 3B shows the result of such an assay in conjunction with its corresponding solid-phase assay. Modified T7 DNA polymerase was able to incorporate very rapidly 3'-RTa-dGTP in front of its cognate base up to a plateau value dependent on the concentration of the modified nt, and comparison of Fig. 3A and B showed that the solid-phase assay correlated well with the primer extension assay when relative band intensities were determined upon densitometric analysis (data not shown). The equilibrium was not exclusively dependent on nt concentration, since addition of more enzyme was able to displace this equilibrium towards further incorporation. The plateau value did not come from a rapid inactivation of the enzyme, since a chase of classical deoxy-nt lead to rapid extension of the remaining 21-mer up to 31-mer (data not shown). In Fig. 3B, concentrations of 3'-RTa-dGTP above 1 mM were able to displace this plateau value to nearly 100% incorporation in less than 1 min. Although 3'-RTa-dNTPs did act as chain terminators, they did not compete out significantly ddNTPs in classical Sanger dideoxy sequencing reactions even with a fivefold molar excess relative to ddNTPs. For example, addition of 40 μM of 3'-RT-dCTP to a M13 sequencing ddC termination mix made of 80 μM of each dGTP, dATP, dCTP and dTTP, 8 μM ddCTP and 50 mM NaCl did not lead to significant shortening of sequencing products upon examination of polyacrylamide gel autoradiograms obtained with commercial T7 sequencing kits. Taken with the fact that high concentrations of 3'-RTa-dNTPs were needed to reach high incorporation levels, this may indicate that these modified nt are not very efficient chain terminators, a result awaiting precise determination of their $K_m$, $V_{max}$ and $k_{cat}$ (work in progress). The DNA polymerases used here had different kinetic behaviors ranging from very slow (e.g., AMV-RT, data not shown), to moderate (e.g., *Taq* DNA polymerase), to instantaneous incorporation (e.g., Sequenase) up to the plateau value. For example, one can compare the Sequenase enzyme in Fig. 3B with the *Taq* DNA polymerase in Fig. 5A. We do not know if this kinetic behavior is related to the processivity of these polymerases. No products smaller in size than the primer were detected, except when DNA polymerases having a 3' to 5' exonuclease activity were used, a finding which indicates that such activity should not be present when one expects a single addition product.

These results show that, despite a relatively bulky 3'-group, these modified nt are still accepted by the enzyme. However, the nature of the 3'-substitute played a key role in the incorporation level, as shown in Fig. 4.

A spacer arm was esterified in the 3' position of dTTP and dCTP, allowing facile coupling of *N*-methyl-anthranylic and fluorescein derivatives (compounds 2 and 3), respectively (R.S.S., T. Berthod, C. Guerreiro and B.C., unpublished results). This led to very slow and incomplete incorporation for the 3'-fluorescein derivative of dTTP, no matter which enzyme was used, but had a dramatic effect for the *N*-methyl-anthranylic derivative (Fig. 4A and not shown). Indeed, 500 μM of this 3'-substituted nt were sufficient to drive the incorporation reaction close to completion with modified T7 DNA polymerase. To our surprise, two band products in equilibrium with one another were obtained. The fact that this was again independent of the enzyme used (Sequenase, AMV-RT or *Taq* DNA polymerase) suggested that these two products were probably conformers or differed only by a net electric charge under the electrophoretic conditions used here, but this awaits further characterization of the addition products.

**(c) The tags can be chemically or enzymatically removed**

Chemical or enzymatic removal of such a tag is shown in Fig. 5. Panel A shows incorporation of 3'-RTa-dGMP in front of its cognate dC base (template 26-C of Fig. 2),

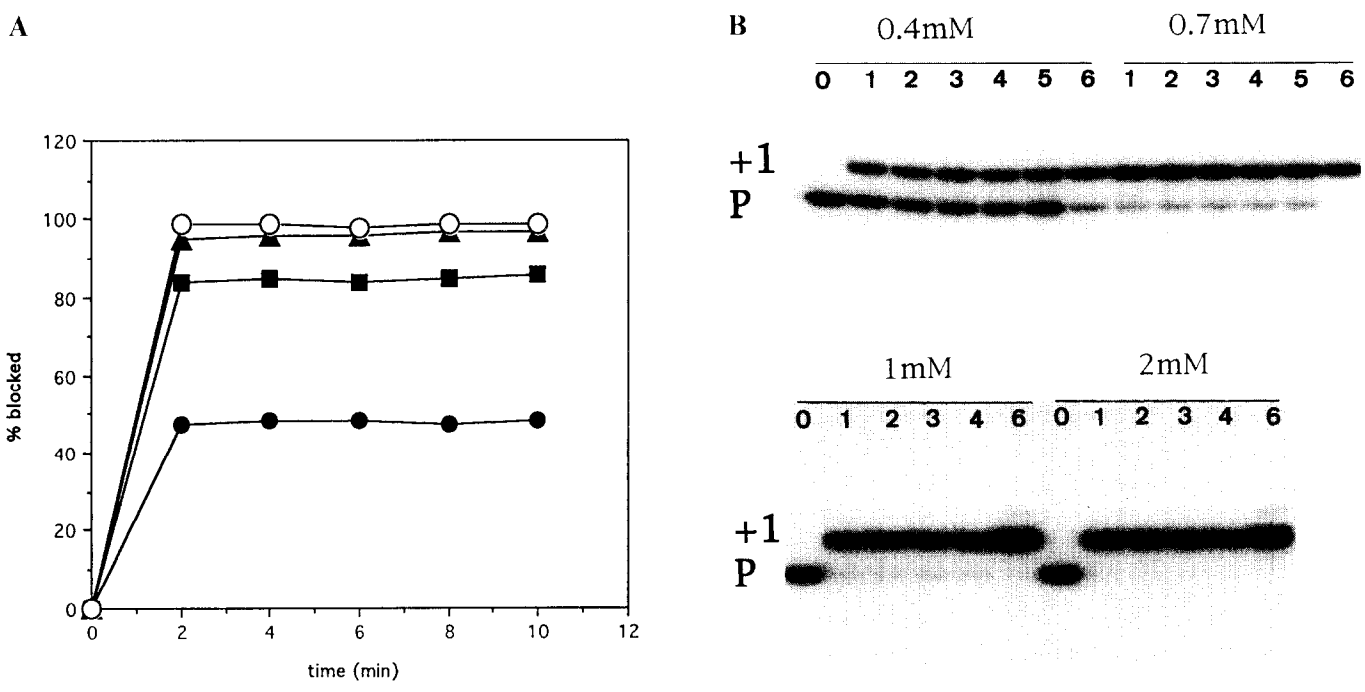


Fig. 3. Incorporation assay into DNA. (**A**) Incorporation of 3′-RTa-dNTPs with a solid-phase assay. **Methods:** Approx. 2 pmol of 5′-biotinylated 21-mer (5′-Bio-ATACTTTAAGGATATGTATCC) were bound to M-280 Dynabeads as described by the manufacturer and hybridized to an excess (50 pmol) of a complementary oligo presenting a 5′ tail (Fig. 2). Annealing was for 1 h at room temperature in the presence of 1 M NaCl/5 mM Tris·HCl pH 7.5/0.5 mM EDTA. After removal of the unbound oligo, washed beads were suspended in 50 mM Tris·HCl pH 8.0/10 mM $MgCl_2$/10 mM dithiothreitol/100 μg BSA per ml and incubated in the presence of one 3′-RTa-dNTP at various concentrations and a DNA polymerase at 37 C. The reaction was terminated with 20 mM EDTA/0.01% Triton X-100, the beads washed and their concentration determined under the microscope with a hematocyter cell before being assayed for free 3′-hydroxyl group with radiolabel incorporation as follows: beads carrying the hybridized oligos were incubated in the same buffer as for 3′-RTa-dNTPs supplemented with a mix of dNTPs containing [α-$^{35}$S]dATP and AMV-RT at 37 C. The beads were washed until radioactive counts reached background level in the supernatants and the concentration of the beads was determined in an aliquot as above. The beads were then dispersed in scintillation counting cocktail (Aquasafe 300, Zinsser Analytic). The amount of radiolabel was estimated relative to the unblocked control in pre-set channels for the corresponding isotopes in a scintillation counter. Concentration of 3′-RTa-dGTP: (■) 0.4 mM; (●) 0.7 mM; (▲) 1 mM; (○) 2 mM. (**B**) End-labelled primer extension and gel assay. Simple standing start reactions were performed exactly as described (Boosalis et al., 1987) using the primer:template 26-C of Fig. 2 and 5 units of modified T7 DNA polymerase. Incubation was for 0 min (lane 0), 2 min (lane 1), 4 min (lane 2), 6 min (lane 3), 8 min (lane 4), 10 min (lane 5). After 10 min, 5 units were added and the incubation extended to 20 min (lane 6). P: primer (21-mer). The reaction products were subjected to electrophoresis through a 15% denaturing polyacrylamide gel, which was subsequently autoradiographed.

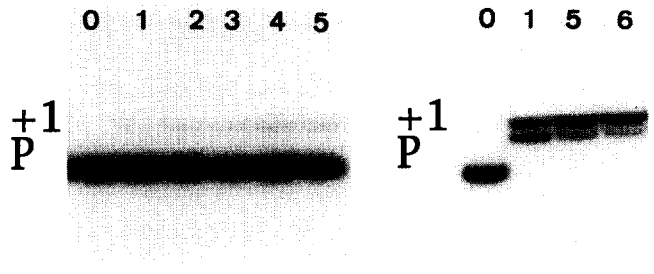


Fig. 4. Incorporation of compound 2 (2 mM, left panel) and compound 3 (0.5 mM, right panel) into primer:template 31-A and 31-G of Fig. 2, respectively, using 5 units of modified T7 DNA polymerase. Incubation times are 0, 1, 2, 3, 4, 5 and 10 min for lanes 0, 1, 2, 3, 4, 5 and 6, respectively. P, primer (21-mer). The reaction products were subjected to electrophoresis through a 15% denaturing polyacrylamide gel, which was subsequently autoradiographed.

saponification with 0.1 M NaOH, neutralization, re-annealing and re-incorporation of ddAMP using *Taq* DNA polymerase. In both incorporations, a single-nt adduct is detected, at the expexted level, indicating the easy deprotection of the 3′-end of the growing DNA chain (lane 6), to give a functional 3′-hydroxyl end. Omission of the alkaline treatment did not allow a second primer extention (lane 5).

Fig. 5B shows a thin-layer chromatogram ot the time-course of compound 4 reacted with proteinase K. After 2 h, complete removal of the tag was obtained, and absence of a ninhydrin positive spot indicated that the ester linkage was indeed the cleaved bond.

### (d) The chemical nature of the 3′ bond is important for a convenient re-incorporation

Of particular importance was the nature of the chemical bond between the ribosyl moiety and the anthranyloyl substituents. Ethers or esters are both expected to restore a hydroxyl group upon deprotection. We reasonned that ether bonds would be hard to cleave under mild conditions compatible with DNA chemical stability, whereas chemical deprotection using alkali has the present disad-

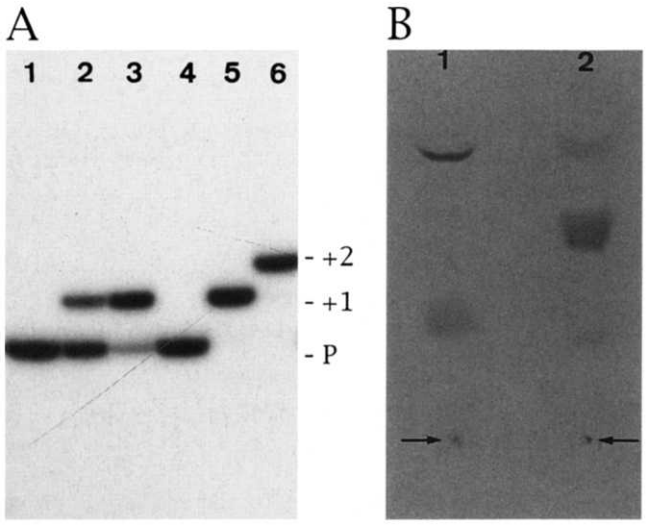


Fig. 5. Removal of the 3′-tag. (**A**) Chemical cleavage of the 3′-ester after incorporation of 3′-RT-dGMP using primer:template 26-C and 5 units of *Taq* DNA polymerase. Incubation time was 0 min (lane 1 and 4), 5 min (lane 2), 10 min (lane 3). After 15 min, 200 μM of ddATP was added. Half of the mixture was incubated for another 10 min (lane 5) and the other half was treated with 0.1 M NaOH for 10 min at 37°C, neutralized with HCl, ethanol precipitated and dried under vacuum. The oligos were reannealed for 2 min at 60°C and left at room temperature for at least 30 min. Re-incorporation was with 200 μM ddATP and 5 units of *Taq* polymerase. (**B**) Enzymatic cleavage of the 3′-ester. 3′-RT-TMP (5 mM) was dissolved in Tris·HCl buffer (50 mM, pH 8) and incubated at 50°C in the presence of 100 μg of proteinase K per ml. Reaction products were separated by thin-layer chromatography on silica gel plates using isopropanol/ammonium hydroxide/water (7:1:2) or dichloromethane/ammonium hydroxide/methanol (65:35:10) as eluent and detected upon UV examination. Incubation time was 120 min (lane 1) and 0 min (lane 2). Arrows indicate the origin of migration.

vantage of melting the primer-template duplex. However, we foresee several ways of remediating to this problem, such as 'locking' the primer covalently by means of a cross-linking agent, or by ligating a 5′-phosphorylated 'hairpin-like' primer to the 3′-end of the ss template. In both cases, alkali denaturation followed by neutralization would lead to immediate intra-molecular reannealing compatible with cycling of the process. Hence, the ester bond is a very attractive candidate to fullfill appropriate conditions for attachment of the tag, and it is amenable to reaction with hydroxyl groups through several activated forms. Moreover, esterases of broad specificity are ubiquitous in nature, and we anticipated that it would be possible to find such an enzymatic activity in the wide group of serine enzymes (Fersht et al., 1985). This point is illustrated with our results using proteinase K. Although deprotection on a free 3′-esterified-dNMP was complete after 2 h of incubation, we propose that it is not out of reach to optimize this time of removal in the growing DNA chain, or to find a better adapted enzyme, or to esterify the 3′ position with a tag carrying a spacer arm specifically designed to be recognized quickly and efficiently removed by an enzyme.

**(e) Conclusions**

(*1*) Taken together, these results clearly indicate that 3′-anthranyloyl-deoxy nt can be used as reversible chain-terminators in a stable and reproducible fashion. Indeed, they act as substrates for several DNA polymerases to extend a DNA primer at its 3′ end by only one nt. Many chain-terminating nt analogs are substrates for different DNA polymerases. Proper base pairing of the nt substrate with its template DNA strand and formation of the phosphodiester bond has been shown to occur even with β-L-ribosides enantiomers (Van Draanen et al., 1992), indicating that binding of the sugar portion by the enzyme was probably not specific.

(*2*) Upon chemical or enzymatic hydrolysis, the 3′ substituent can be removed, and the 3′-hydroxyl regenerated with a good yield. This 3′-hydroxyl end can now be used as a new DNA primer. It is important to note that the tag released by hydrolysis is specific of the incorporated nt. Its identification would mean the identification of the nucleobase corresponding to the DNA template using standard rules of base pairing, and thus provide a very easy and rapid way for determining a nt sequence in a single tube or column, provided the process could be efficiently cycled. In this respect, each step of the process should be performed with a good yield and be compatible with the next step. A full cycle would include incorporation, tag removal and tag identification. Our results show that it is possible to reach high incorporation levels required to perform several cycles in a row. Modified T7 DNA polymerase shows a versatile ability in using 3′-modified substrates at a very high rate (Tabor and Richardson, 1989; this work), and it is of great interest that the presence of an ester at the 3′-position of a given dNTP does not slow down dramatically the incorporation reaction, making total chain termination within reach in less than 1 min. However, this is clearly not the case for all 3′-esters, as exemplified by the bulky fluorescein moiety, and this finding might be of interest in the fine mapping of functional domains of DNA polymerases.

(*3*) Solid-phase sequencing reactions on magnetic beads utilize 2 pmol of immobilized template (Hultman et al.,1991). The quantitative release of 2 pmol of fluorophore can be characterized with a classical, commercially available spectrofluorimeter and liquid chromatography detection cell (see Methods in the legend to Table I). Thus, signal-to-noise ratio should not be a problem, considering that detection of a single fluorescent molecule is under way (Davis et al., 1991). Moreover, as fluorescence chemistry is presently a rapidly growing field, we predict that the spacer arm of our 3′-derivatized nt will allow the coupling (through its primary amine) of powerful fluorophores that are compatible with DNA polymerases. This should lead to convenient identification of tags present in microtiter wells.

Sequencing technology is under intense study, mainly due to the development of genome projects. Most improvements are directed to the dideoxy method at several of its steps (Prober et al., 1987; Venter et al., 1992; Mathies and Huang, 1992). Emerging new approaches, such as sequencing by hybridization (Strezoska et al., 1991) or scanning tunneling microscopy (Driscoll et al., 1990) may become widespread if elimination of the gel electrophoresis is replaced by a cost-effective and simple alternative.

We have synthesized 3′-modified-dNTPs substrates for DNA polymerases. They can go through three distinct reactions, (namely incorporation, deprotection and re-incorporation), that can be extended as a new non-gel-based, non-radioactive method to determine a nt sequence from a point mutation to a whole DNA fragment.

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