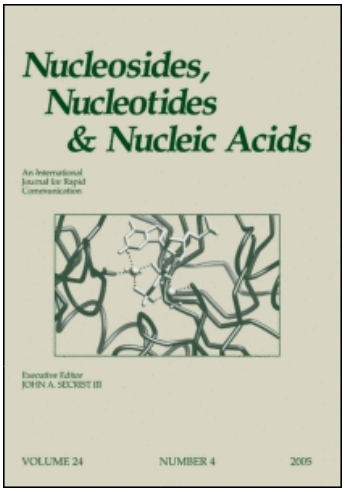

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SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING THE ABASIC SITE MODEL COMPOUND 1,4-ANHYDRO-2-DEOXY-D-RIBITOL.

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ABSTRACT.

An improved approach for the synthesis of phosphotriester and phosphoramidite derivatives of the 1,4-anhydro-2-deoxy-D-ribitol is presented. The incorporation of these compounds in synthetic DNA and the insertion of purine deoxyribonucleotides opposite the reduced abasic site by DNA polymerase is described.

INTRODUCTION

Abasic (apurinic/ apyrimidinic) sites are generated in DNA as a result of the cleavage of the N-glycosidic bond between a base, usually a purine, and its deoxyribose moiety. Some base modifications, including alkylation of purines and fragmentation of the heterocyclic ring, accelerate their formation. Furthermore some damaged bases are enzymatically excised by specific DNA glycosylases, leading also to the formation of abasic sites (1,2).

It is described that, in vivo, abasic sites are recognized and removed by specific nucleases (3). It is also

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well known that abasic sites are susceptible to hydrolysis which is accelerated by the presence of alkali and amines (1).

We are interested on the introduction of stable abasic sites into synthetic oligonucleotides in order to have a model assay system to investigate DNA repair, abasic lesion bypass, and DNA synthesis fidelity studies in vitro and to study lesion bypass at selected template sites using transfection assays in vivo.

In this communication, we report on the synthesis and the characterization of synthetic oligonucleotides containing 1,4-anhydro-2-deoxy-D-ribitol (3). This compound can be suitably protected, and, using standard methods, inserted into unique positions in DNA. We show that this reduced abasic site is stable and behaves in a similar manner to natural abasic sites with respect to the efficiency of inserting purine deoxyribonucleotides by DNA polymerase.

RESULTS AND DISCUSSION

The synthesis of 1,4-anhydro-2-deoxy-erythro-D-pentitol, or simply 1,4-anhydro-2-deoxy-D-ribitol (3) has been achieved by acid-catalyzed dehydration of 2-deoxy-D-ribitol (2) which was prepared by borohydride reduction of the commercially available 2-deoxy-D-ribose (see FIG. 1). This synthetic scheme is based on early observations on the acid-catalyzed dehydration of pentitols. In particular, it has been shown that compound 3 is the only product of the dehydration of the 2-deoxy-D-ribitol (4,5).

Using this approach, compound 3 was obtained, after purification by vacuum distillation, in a 54% yield. Spectroscopical properties of the purified product are identical to the product obtained using a different synthetic route (6). Since partial decomposition of 3 was observed during its purification by vacuum distillation, we decided to use both purified and unpurified compound 3 in the reaction of dimethoxytritylation. The reaction mixture

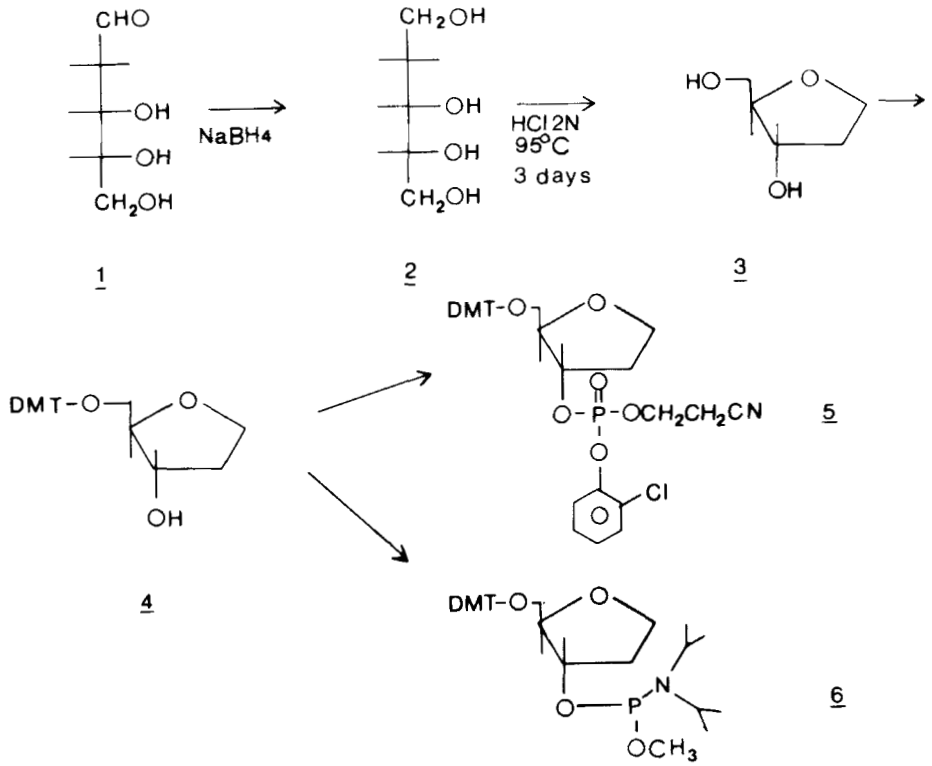

Fig. 1. Synthetic scheme used for the preparation of the phosphotriester and phosphoramidite derivatives of the 1,4-anhydro-2-deoxy-D-ribitol.

obtained from the unpurified compound reaction contains two minor impurities, that we assign to the dimethoxytritylation products of the uncyclized compound 2. However, the separation of these compounds is easily accomplished by silica gel chromatography, obtaining in this case, a higher overall yield (72% from 1 to 4). This synthetic scheme represents a clear improvement over previously described preparations of compound 4 (6,7).

The phosphotriester (5) and the phosphoramidite (6) derivatives of compound 4 were prepared using standard methods (8,9).

The oligonucleotides shown in FIG. 2 were prepared by conventional solid-phase methods.

A 5'CGGXGGC3'	I 5'A_9XCC-primer
B 5'CTCCTGXGGAGAAGTCTGC3'	J 5'A_9XTC-primer
C 5'CCCGTGCCGXACGCCGCGCC3'	K 5'A_8GXC-primer
D 5'T_9XAGA-primer	L 5'C_8GXA-primer
E 5'T_{20}XAGA-primer	M 5'T_8GXA-primer
F 5'A_8XGG-primer	N 5'T_8GXC-primer
G 5'A_9XAC-primer	O 5'T_8GXG-primer
H 5'A_9XGC-primer	P 5'T_8GXT-primer

primer : ACGTCGTGACTGGGA3' X: 1,4-anhydro-2-deoxy-D-ribitol

FIGURE 2 : Sequences of the oligonucleotides containing 1,4-anhydro-2-deoxy-D-ribitol prepared.

Oligonucleotide A was synthesized on a large scale using the phosphotriester method (10). The synthesis was controlled by measuring the absorbance of the dimethoxytrityl cation released during the deprotection steps. The overall yield of the synthesis was 80% and the incorporation of the ribitol derivative was achieved in 96% yield. After removal of the protecting groups (10), the oligonucleotide was purified on a DEAE-Sephadex A-25 column (see figure 4). NMR studies of this oligonucleotide are currently being undertaken.

The remaining oligonucleotides were prepared on a small scale using phosphite-triester techniques (8). The incorporation of compound 6 proceeded with yields similar to those obtained with standard phosphoramidites (> 99%). After HPLC purification, the homogeneity of the purified oligonucleotides was checked by gel electrophoresis. We observed in all the oligonucleotides containing 1,4-anhydro-2-deoxy-D-ribitol an anomalous high mobility on gel electrophoresis (see for example FIG. 3a). This characteristic feature can be explained by the fact that the presence of the analog adds a full negative charge with only

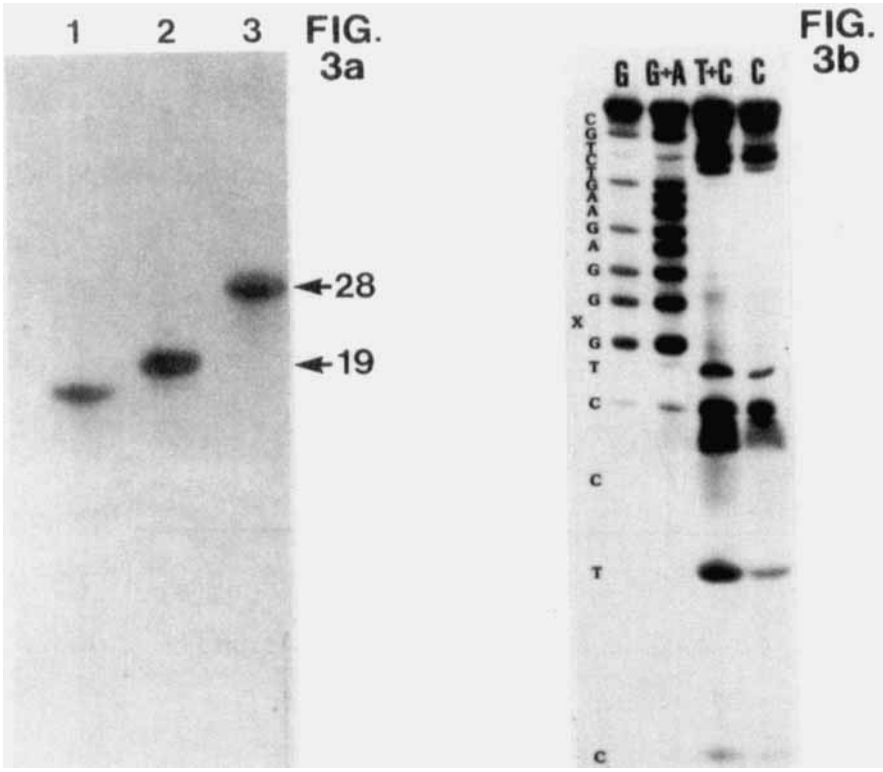

FIG. 3. a) Gel electrophoresis (20% acrylamide, 7M urea) of 1) oligonucleotide C (20 mer); 2) marker, 19 mer; 3) marker, 28 mer stained with Stains-all. b) Maxam-Gilbert sequencing gel of oligonucleotide B.

a small increase in the molecular weight of the oligomer. Finally, the modified oligonucleotides were analyzed by the Maxam-Gilbert method (11). As it can be seen in FIG. 3b, the position of the 1,4-anhydro-2-deoxy-D-ribitol is characterized by the absence of radioactivity in the four specific reactions. This result shows the stability of the phosphate bond at the modified position.

The introduction of a single abasic site at a specific site provides a novel system to study the fidelity with which DNA polymerases copy templates containing non-coding lesions. FIG. 5 is an example of gel data for the insertion

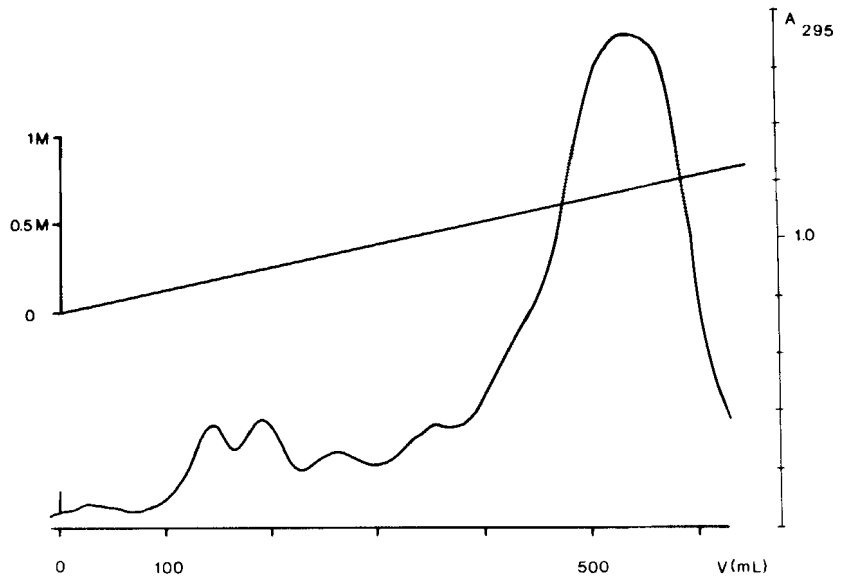

FIG. 4. DEAE-Sephadex A-25 purification of CGGXGGC.

of dATP and dGTP at the abasic site by Klenow fragment using the template 3'...AGAXT$_9$ (FIG. 6).

Comparison of band intensities for insertion of dATP (lanes 1-3) and dGTP (lanes 4-6) opposite the abasic site, shows dATP to be incorporated much more frequently than dGTP. In lanes 1-3 it appears that with increasing time there is less incorporation of dATP into the abasic site. This is expected since Klenow fragment contains a 3'-5' exonuclease activity which preferentially removes nucleotides incorrectly base paired (12,13). In lanes 4-6, the exonuclease activity of Klenow fragment appears less active in the removal of dGMP apposite the abasic site. In lane 7, the poor insertion of pyrimidines (dCTP and dTTP) opposite the abasic site is demonstrated by the absence of an abasic gel band. This is in agreement with earlier studies using "natural" abasic sites. Purines were found to be inserted opposite abasic sites more readily than

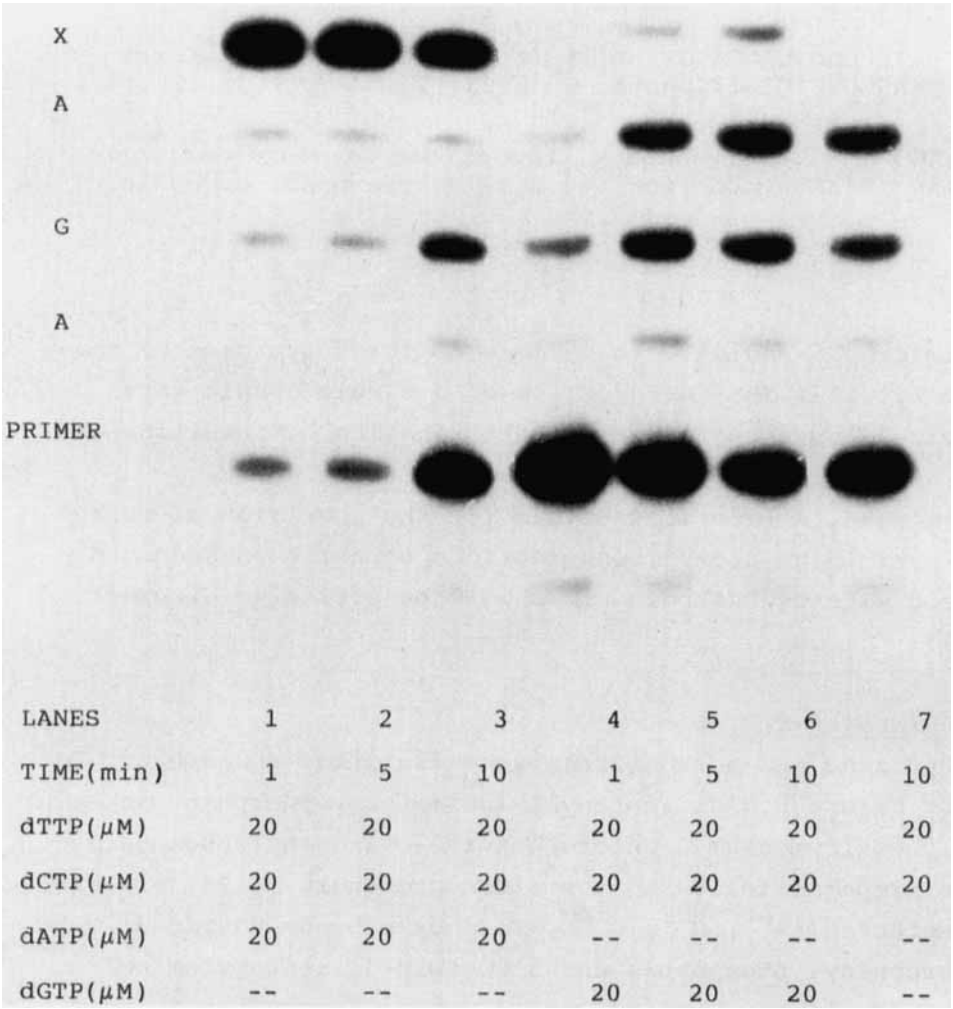

FIG. 5. Gel data for the insertion of dATP and dGTP at the abasic site by DNA Polymerase I-large fragment on template $AGAXT_9$(see FIG.6). Individual lanes in the gel are labelled by numbers from 1 to 7. The time and the concentration of the triphosphates used in each particular primer extension reaction are expressed underneath the lane number. More detailed reaction conditions are described under "Experimental part". Row labels are : primer, unextended primer; A, primer molecules extended by one nucleotide incorporated opposite template base A; G, primer molecules extended by two molecules (opposite G and A); A, primer molecules extended by three molecules (opposite A,G and G); X, primer molecules extended by four nucleotides (opposite abasic site, A, G and A).

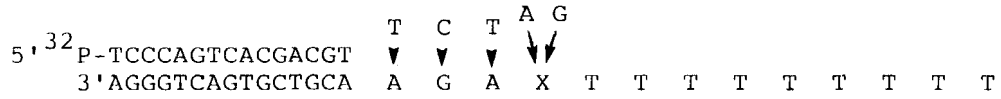

FIGURE 6. Illustration of the primer-template designated $AGAXT_9$. "X" symbolizes the abasic site model compound.

pyrimidines, and dATP to be more efficiently inserted than dGTP (14,15). The introduction of a single abasic site provides a system to measure the kinetics of insertion of any nucleotide opposite the abasic site using DNA polymerase. A kinetic analysis for the insertion of purine and pyrimidine deoxyribonucleotides opposite the reduced abasic site by DNA polymerase will be presented elsewhere (16).

EXPERIMENTAL PART

Pyridine and acetonitrile were distilled and stored over CaH_2. Chloro N,N-diisopropylamino methoxy phosphine and cyclohexylammonium 2-chlorophenyl 2-cyanoethyl phosphate were prepared following described protocols (8,9). 5'-O-DMT-N-protected (A^{Bz}, C^{Bz}, G^{ibu}, T) deoxyribonucleoside 3'-O-2-chlorophenyl phosphates and 3'-O-(N,N-diisopropylamino) methoxy phosphoramidites were purchased from Bachem and American Bionetics respectively. Oligonucleotide syntheses were done in a Bachem manual synthesizer and in an automatic Microsyn 1460 synthesizer (Systec Inc). All nonradioactive nucleotides were purchased from P-L Biochemicals. [γ-^{32}P]ATP was purchased from ICN Radiochemicals, s.a.= 4082 Ci/mmol. DNA Polymerase I-large fragment was purchased from New England Bio-Labs. T4 polynucleotide kinase was purchased from U.S. Biochemicals.

1,4-anhydro-2-deoxy-D-ribitol (3)

2-Deoxy-D-ribose (2.1 g, 15 mmol) was treated with $NaBH_4$ (215 mg) in a 0.1 mM NaOH solution. After 4 hrs of magnetic

stirring at room temperature, the reaction mixture was negative to the Fehling test and the reaction was stopped by addition of a 50% acetic acid solution until pH 4. The solution was desalted with a mixed-bed ion-exchange resin (Bio-Rad AG501 x 8(D) 20-50 mesh) and evaporated to an oil. The resulting product was treated with HCl 2N at 95 ^{o}C for 72 hours and the solution evaporated. The residue was vacuum distilled, obtaining 1.0 g (8.5 mmol) of compound 3. Yield 54%. $[\alpha]_D^{20}$ =51.3 (c=0.67, H_2O) (Lit. (3)$[\alpha]_D^{20}$ =48.1 (c=1.18, H_2O)). ^{1}H-NMR and ^{13}C-NMR identical to the previously described (6). Alternatively unpurified 1,4-anhydro-2-deoxy-D-ribitol (without vacuum distillation) was used in the next step (see below).

5-O-(4,4'-dimethoxytrityl)-1,4-anhydro-2-deoxy-D-ribitol (4)

Compound 3 (0.4 g, 3.4 mmol) was treated with 4,4'-dimethoxytrityl chloride (1.2 g, 3.7 mmol) in anhydrous pyridine for 2 hrs. After standard work up (17), the oil was purified on a silica gel column eluted with dichloromethane obtaining compound 4 in a 90% yield. When the dimethoxytritylation reaction was carried out directly with unpurified 3 (without vacuum distillation), we observed the presence of two minor DMT containing impurities of lower mobility (TLC silica gel dichloromethane/ ethanol (9:1) Rf(4)= 0.65; Rf(impurities)= 0.32 and 0.4). We assign these compounds to products of dimethoxytritylation of the uncyclized compound 2 (DMT-OCH_2-CH_2-CHOH-CHOH-CH_2OH and CH_2OH-CH_2-CHOH-CHOH-CH_2O-DMT). The desired product can be easily separated from these impurities by silica gel chromatography eluted with dichloromethane.

5-O-(4,4'-dimethoxytrityl)-1,4-anhydro-2-deoxy-D-ribitol 3-(2-cyanoethyl 2-chlorophenyl phosphate) (5).

Compound 4 (0.3 g, 0.70 mmol) was phosphorylated with cyclohexylammonium 2-chlorophenyl 2-cyanoethyl phosphate (0.51 g, 1.4mmol), 2,4,6-triisopropylbenzenesulfonyl chloride (0.86 g, 2.8 mmol) and 1-methylimidazole (0.45 mL,

5.6 mmol) following the procedure described by K. Yamada and R. Dohmori (8). Yield 0.44 g (95%).

5-O-(4,4'-dimethoxytrityl)-1,4-anhydro-2-deoxy-D-ribitol-3-(O-methyl)-N,N-diisopropylphosphoramidite (6).

Compound 4 (0.82 g, 2 mmol) was treated with 1.4 mL (8 mmol) of ethyldiisopropylamine and 0.6 mL (3 mmol) of chloro-N,N-diisopropylamino-methoxy phosphine in 4 mL of chloroform. After 1 hour, 0.2 mL of methanol were added and the reaction mixture was transferred to a separation funnel with a solution of ethyl acetate (50 mL) containing triethylamine (2 mL). The solution was washed with a cold solution of sodium bicarbonate (2x40 mL) and a cold saturated solution of sodium chloride (2x40 mL). The organic phase was dried and the solvent evaporated to an oil. Yield 1.0 g (1.8 mmol, 89%). TLC : one spot, Rf 0.8, ethyl acetate/ triethylamine (9:1). ^{31}P-NMR (Cl_3CD) : 144.6, 144.5 ppm (two diastereoisomers).

Oligonucleotide synthesis

Oligonucleotides were synthesized using solid-phase procedures. The synthesis of the heptamer was carried out, manually, on a 87 μmol scale using phosphotriester methodology (9) and poly(styrene-co-1%-divinylbenzene) as a solid support. After removal of all protecting groups, the product was purified by chromatography on a DEAE-Sephadex A-25 column (2x10 cm) eluted with a linear gradient of triethylammonium bicarbonate (pH 7.5) from 0.0015 M to 1 M. The overall yield (synthesis, deprotection and purification) was 52% (1800 O.D. units (260nm), 45 μmols).

The rest of the oligonucleotides were synthesized on controlled-pore glass (1 μmol scale) by successive additions of the appropriate phosphoramidites in an automated synthesizer. No difference on the incorporation yields were observed when compound 6 was employed. Overall yield: 5-25%.

At the end of the syntheses, the DMT-oligonucleotidyl-resins were deprotected (9) and the resulting DMT-

oligonucleotides purified by reversed-phase HPLC (10), treated with 80% acetic acid and rechromatographed (10).The homogeneity of the purified oligomers was checked by polyacrylamide gel electrophoresis (20% acrylamide, 7M urea).

5' End-Labeling

A reaction mixture (97 μL) for kination of the 15 bp primer contained 850 μM oligonucleotide, 7.3 mM $MgCl_2$, 0.4 M [γ-^{32}P]ATP (>4000 Ci/mmol), 12.9 mM DTT, and 5 units of T4 polynucleotide kinase in 56 mM Tris.HCl (pH 7.7). The mixture was incubated at 37 ^{o}C for 1.5 hrs and terminated by heating at 100 ^{o}C for 5 min.

Insertion analysis

Reaction mixtures consisted of the following components for primer extension by DNA Polymerase I-large fragment (Klenow) : 29 mM Tris.HCl pH 7.5, 7.6 mM $MgCl_2$, 26 mM NaCl, 1 mM DTT, 24 μg/mL BSA, 20 μM dTTP, 20 μM dCTP, 157 ng/mL of annealed primer template, and 20 μM of dATP and dGTP, respectively, in final volume of 5.7 μL. Reaction assays were run for one, five, and ten minutes at 25 ^{o}C with 1.35×10^{-2} polymerase units. Reactions were terminated by 10 mM EDTA followed by 100 ^{o}C for 5 min. Reaction samples were run on 16% denaturing polyacrylamide gel for 4 hrs at 2000 volts.

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