Insertion of Nucleotides Opposite Apurinic/Apyrimidinic Sites in Deoxyribonucleic Acid during in Vitro Synthesis: Uniqueness of Adenine Nucleotides†

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ABSTRACT: M13 DNA containing 20–30 apurinic/apyrimidinic (AP) sites per intact circular molecule was prepared by growing phage on an *ung⁻ dut⁻ Escherichia coli* mutant and treating the DNA with uracil *N*-glycosylase. AP sites obstruct in vitro DNA synthesis catalyzed by *E. coli* pol I. The position at which termination of synthesis occurs was determined for four enzymes. T4 DNA polymerase terminates one nucleotide before putative AP sites. DNA pol I, AMV reverse transcriptase, and DNA polymerase α terminate synthesis either before or at the site of an AP lesion depending on the particular sequence. We determined the identity of the nucleotide inserted opposite an AP site by synthesizing up to the lesion in a first-stage reaction using T4 DNA polymerase and then determining elongation in a second stage. Purines are inserted opposite AP sites more readily than pyrimidines, and dATP is more efficient than dGTP in promoting such elongation. The DNA-dependent conversion of dNTP to dNMP was determined in mixtures of all four dNTP's by using AP DNA. The production of dAMP from dATP occurs most readily. We conclude that there is an inherent specificity for the incorporation of adenine nucleotides opposite AP sites in this in vitro system. Insofar as the model system reflects in vivo mutational events, our data suggest that depurination should produce transversions and depyrimidination should produce transitions.

Apurinic/apyrimidinic (AP) sites in DNA are continually generated, both as the result of spontaneous depurination (Lindahl & Nyberg, 1972) and as a result of the protective action of DNA *N*-glycosylases in removing damaged or unusual bases from the DNA (Lindahl, 1982). Although active excision–repair mechanisms exist which restore the normal base sequence (Lindahl & Andersson, 1972; Lindahl, 1982), it is possible that an AP site could be encountered by the replicating mechanisms during DNA synthesis. We would expect such AP site(s) to be noninstructive, that is, to be devoid of information for Watson–Crick base pairing. AP sites have been reported to be blocks to DNA synthesis (Schaaper et al., 1983; Lockhart et al., 1982) as would be expected if such sites were noninstructive. On the other hand, there is evidence that AP sites are mutagenic (Kunkel et al., 1981). Since the establishment of a mutant clone requires a viable organism, mutagenesis implies that mechanisms do exist which permit bypass of the block to replication. This interpretation is supported by the finding that the mutagenic effect of AP sites in *Escherichia coli* is only expressed in cells induced for SOS functions (Schaaper & Loeb, 1981). This sequence of reactions permits low levels of synthesis past damaged sites in DNA (Moore et al., 1982a), even at the cost of mutation; the pathway as deduced from biological data (Radman et al., 1977) has been termed "error-prone" synthesis.

We have been engaged in the construction of in vitro models of the error-prone pathway (Strauss et al., 1982). Because of its noninstructive nature, the AP site seemed an important lesion for us to study. In this investigation, we have inquired as to the biochemical factors involved in the insertion of nucleotides by DNA polymerase(s) opposite AP sites in DNA, and we utilized a protocol which permits us to determine the specificity of nucleotide insertion (Moore et al., 1982b; Rabkin et al., 1983). We conclude that there is an overwhelming and unexpected specificity for the insertion of adenine nucleotides (A's) at such sites. This specificity, observed in our in vitro system, correlates well with the specificity of mutation observed in systems which behave as though AP sites were intermediates (Foster et al., 1983).

Materials and Methods

Enzymes and Chemicals. *E. coli* DNA polymerase Klenow fragment was obtained from Boehringer Mannheim (Indianapolis, IN): We designate this enzyme pol I. Bacteriophage T4 DNA polymerase was obtained from BRL (Bethesda Research Laboratories, Inc., Gaithersburg, MD). AMV reverse transcriptase was a preparation obtained from Life Sciences, Inc. (St. Petersburg, FL). DNA polymerase α, prepared from the human lymphoblastoid line Daudi, was supplied to us by Dr. K. Bose (Bose et al., 1978). Uracil *N*-glycosylase was prepared from frozen K12 cells according to Lindahl (1980) and assayed with [^{3}H]poly(dU) prepared as described by Cone & Friedberg (1981). One unit of enzyme renders 1 pmol of uracil acid soluble in 1 min at 37 °C. Nonradioactive deoxynucleoside triphosphates were obtained from Sigma and purified by diethylaminoethyl (DEAE) chromatography before use (Englund, 1972). ^{32}P-Labeled triphosphates were obtained from Amersham (Arlington, Heights, IL), and [^{3}H]methyl-labeled dTTP was from New England Nuclear. Poly(ethylenimine) (PEI) thin-layer plates were a product of Macherey-Nagel & Co. (Brinkmann Instruments, Westbury, NY). The following buffers were used routinely: low Tris buffer, 20 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.5, 20 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA); DNA polymerase reaction buffer, 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, and 8 mM $MgCl_2$ (0.5 mM $MnCl_2$ when substituted for Mg^{2+}). Phage M13, *E. coli* strains HfrC71 and BW313 (*ung⁻ dut⁻*) for cultivation of wild-type and uracil-containing M13, respectively, were kindly provided by Dr. T. Bonura, Stanford University.

Growth of Cells and Preparation of Phage. BW313 cells were grown at 37 °C with vigorous shaking on YT medium (5 g/L yeast extract, 8 g/L tryptone, and 5 g/L NaCl) containing 20 μg/mL thymidine and 100 μg/mL deoxyadenosine. At a cell density of 4×10^8/mL, the cells were centrifuged (5 min at 1000*g*), washed with YT medium, gently suspended

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in fresh warm medium containing 0.25 μg/mL uridine, and shaken for 5 min. M13 (wild type) was then added at a multiplicity of infection of 5. Both phage and double-stranded replicative form could be prepared from the same batch. *E. coli* HfrC71 was grown and infected in a similar manner, except that the YT medium contained 40 μg/mL thymine and was not replaced. At the end of 8-h incubation, the cells were collected by centrifugation and immediately resuspended and lysed for preparation of replicative form I (RF I, see below). The phage extruded into the medium (10^{12}/mL) was precipitated in the presence of 0.5 M NaCl and 5% poly(ethylene glycol) 6000 (Forsheit & Ray, 1971), purified on a CsCl gradient, and dialyzed against low Tris buffer.

Single-Stranded DNA and RF I. Single-stranded DNA was extracted from the purified virions with distilled phenol saturated with low Tris buffer and was then ethanol precipitated. RF I preparation was essentially as described by J. Messing (Messing et al., 1977), with the following modifications: after low-speed centrifugation, the cleared lysate was extracted 3 times with equal volumes of distilled–low Tris saturated phenol, and the DNA was precipitated with 2 volumes of ethanol and 0.1 volume of 2.5 M sodium acetate. The ethanol precipitate was resuspended in a small volume of low Tris buffer, 5 M NaCl was added to a final concentration of 1 M, and after 90 min at 4 °C, the precipitate was collected and discarded. Further purification of the RF from cellular DNA was accomplished by passing the supernatant (from 2 L of original culture) through a Sepharose 4B column (1.5 × 75 cm) equilibrated with 50 mM Tris, pH 7.5, 100 mM NaCl, and 2 mM EDTA. RF is eluted from the column first. The pooled RF fractions were diluted to 25.5 mL in low Tris buffer, mixed with CsCl (24.5 g) and ethidium bromide (0.48 mL of 10 mg/mL), and centrifuged at 40 000 rpm in a Beckman Ti50 rotor for 48 h. The tube was illuminated with UV light, and the lower major band containing RF I was collected by puncturing the side of the tube. Ethidium bromide was removed by repeated isoamyl alcohol extractions; the colorless DNA solution was dialyzed against low Tris buffer and precipitated with ethanol and sodium acetate.

Base Composition of Phage DNA. The base composition of both M13 wild type and M13 grown on *ung⁻ dut⁻* BW313 was determined by $HClO_4$ hydrolysis of the purified DNA. To 35–50 μg (20 μL) of DNA was added 0.1 mL of 72% $HClO_4$, the mixture was heated for 75 min at 100 °C, cooled on ice, and diluted with 0.8 mL of H_2O, and 4.0 N KOH was added to pH 4–5. For complete precipitation of $KClO_4$, the tube was kept on ice overnight and centrifuged, and the supernatant was lyophilized to dryness. The residue was redissolved in 50 μL of 1 N HCl and applied to MN 300 cellulose-coated plates (Analtech). The bases were separated by ascending chromatography in 2-propanol–HCl–H_2O (170:41:39) (Wyatt, 1955). The plates were prerun in the same solvent system used for the separation of the sample. UV-absorbing spots were scraped and eluted in 1 mL of 1 N HCl, and the concentration of each spot was determined by measurement of absorbancy (Table I). It can be seen that about 10% of the thymine residues have been substituted by uracils.

Preparation of AP DNA. A 100-μg sample of uracil-containing DNA was incubated with 50 units of uracil *N*-glycosylase in a 250-μL reaction mixture containing 70 mM (Hepes)–KOH (pH 8.0)–1 mM EDTA at 37 °C for 20 min. AP sites were stabilized by reduction in $NaBH_4$ as described by Bose et al. (1980), and the reduced DNA was purified by dialysis, phenol extraction, and ethanol precipitation.

Table I: Uracil Content of M13 Grown on an *ung⁻ dut⁻* Mutant[a]

reported (mol %) (Salivar et al., 1964) (wild type)		found (mol %) HfrC71 grown (wild type)	found (mol %) BW313 *ung⁻ dut⁻* grown
23	A	22	22
21	G	21	20
20	C	22	21
36	T	35	33
	U		4

[a] A trace of uracil was also found in the wild type, probably due to the deamination of cytosine. The percent of uracil in the mutant was corrected for this value.

Analysis of AP Sites. The number of AP sites formed was determined from the amount of uracil released by uracil *N*-glycosylase from M13 DNA labeled with [^{3}H]uridine and by the analysis of AP DNA on alkaline 1% agarose gels. Samples were mixed before being loaded with 0.5 volume of 0.3 N NaOH, 15 mM EDTA, and 50% glycerol. The running buffer was 30 mM NaOH plus 2 mM EDTA (McDonnel et al., 1977). Gels were electrophoresed for 2 h at 70 V, stained with ethidium bromide, and scanned on a Gilford spectrophotometer. The relative area under each portion of the gel was determined and used for the calculation of the number-average molecular weight (M_n) by using the relationship:

$$M_n = \frac{\sum C_i}{\sum C_i/M_i}$$

where C_i is the absorbancy in the ith section of the gel and M_i is the median molecular weight for this section, determined from the molecular weight markers. The average number of breaks for a circular molecule was calculated as M_n(control)/M_n(treated).

Preparation of Restriction Fragments. M13 RF I (200 μg) was digested at 65 °C with 200 units of *Taq*I (Biolab). After 1 h, an additional 200 units was added for a total of 2-h incubation. Fragments were separated on 1.2% agarose preparative gels at 60 V for 16 h in Tris–acetate buffer (40 mM Tris-HCl, pH 8.0, 5 mM sodium acetate, and 1 mM Na_2EDTA) to which ethidium bromide (0.1 μg/mL) was added. Fluorescent bands containing the fragments were excised and electroeluted into separate dialysis bags containing 2 mL of the Tris–acetate buffer, then precipitated with salt and ethanol, redissolved in a small volume of low Tris buffer, and stored at 4 °C.

DNA polymerase reactions and polyacrylamide gel electrophoresis were carried out as previously described (Moore et al., 1981).

First-Stage Reactions. Usually reactions of 10 μL contained 0.1 μg of DNA template with either 0.2 unit of pol I, 2 units of T4 DNA polymerase, or 8 units of AMV reverse transcriptase in polymerase buffer. A polymerase α preparation from the human lymphoid line Daudi was used, 0.6 μg of which catalyzed incorporation on a control DNA template equal to 20% of that of pol I. Incubation was at 37 °C (22 °C for pol I) for 30 min with mixtures of three deoxynucleotide triphosphates at 50 μM. The fourth which was α-^{32}P labeled was added at a concentration of 1.2–2.4 μM (5–10 μCi) for the first 15 min of the reaction. The concentration of the labeled dNTP was then adjusted to 50 μM with cold triphosphate for the second half of the incubation (Moore & Strauss, 1979). At the end of the incubation, samples were digested with 0.5 unit of *Taq*I at 65 °C and analyzed on sequencing gels. Numbering of the sequence is from the 3′-OH end of the primer.

Second-Stage Reactions. In some cases (see below), a second-stage reaction was performed (Moore et al., 1982b; Rabkin et al., 1983). The first-stage synthesis described above was scaled up with T4 DNA polymerase, and all four deoxynucleoside triphosphates were used for labeling as described above. The product was then isolated and purified from free dNTP's by phenol extraction, ethanol precipitation, and gel filtration on Sephadex G-50 Superfine columns. In the second stage, the ^{32}P-labeled, digested material from the first stage was incubated with either Mg^{2+} or Mn^{2+} in reaction mixtures similar to those used for the first stage. Deoxynucleotides were added to the individual tubes and incubated with the indicated polymerase for 10 min (amounts of enzyme and temperature of incubation were as in stage I). The samples were then denatured and applied to sequencing gels. Gels were autoradiographed with DuPont Cronex 4 film, and after development, the films were scanned at 600 nm by using a Gilford 250 spectrophotometer with a linear transport unit and the peaks recorded and integrated with a Varian CDS 401 computer. The area under the peaks is proportional to the amount of radioactivity.

Preparation of Primed Template. Primed templates were prepared by annealing a *Taq*I restriction fragment with single-stranded M13 DNA (50 μg/mL) at 65 °C for 90 min in 100 mM KCl as previously described (Moore et al., 1980). The molar ratio of fragment to single-stranded DNA was 1:1. The ratio should result in priming of approximately 50% of the single-stranded molecules. Most experiments were done by using either the 791 nucleotide fragment C or the 579 nucleotide fragment E (Konings & Schoenmakers, 1978).

Measurement of dNMP Release. Primed AP M13 DNA template was used to synthesize up to the lesion with T4 DNA polymerase and all four dNTP's (50 μM) in the first stage. The product was isolated on a Sephadex G50 column as described above and used to measure the release of free dNMP upon incubation with pol I and all four dNTP's in a DNA-dependent reaction. Values for each of the four dNTP's were determined independently by using a single α-^{32}P-labeled deoxynucleoside triphosphate in each mix. Incubation was carried out at room temperature, in 10 μL containing 0.125 μg of DNA, 0.4 unit of pol I, and 10 μM each of dATP, dCTP, dGTP, and TTP (specific activity of the labeled nucleotide was 2.5×10^4 cpm/pmol), in DNA polymerase buffer. At the times indicated, 1.5-μL aliquots were removed and spotted on PEI plates which had been prespotted with EDTA–sodium pyrophosphate and a mixture of the corresponding dNMP and dNTP markers. The plates were developed by ascending chromatography (Hershfield & Nossal, 1972), using 1.2 M LiCl for the samples containing ^{32}P-labeled dATP or dGTP and 0.9 M LiCl for dCTP or TTP. The spots corresponding to the origin, dNTP, and dNMP as well as the regions between the markers were identified by both UV absorbancy and autoradiography and then cut out and counted with toluene-based scintillant. For comparison of the rate of incorporation with the rate of release of dNMP for each of the deoxynucleoside triphosphates by pol I, a primed control DNA sample was incubated and analyzed by PEI chromatography under the same conditions as the AP template, but without prior synthesis with T4 DNA polymerase. Incorporation was determined from the radioactivity remaining at the origin on PEI plates.

Results

Production of AP Sites. Since our methodology requires many AP sites per molecule, we decided to produce a substrate enzymatically rather than by the heat treatment which results inevitably in strand breakage along with AP sites (Lindahl & Andersson, 1972). For this purpose, M13 bacteriophage was grown on an *ung*$^-$ (uracil *N*-glycosylase deficient) *dut*$^-$ (deoxyuridine triphosphatase deficient) mutant (Tye et al., 1978). Growth on this strain as a host results in a virus in which a portion of the thymine residues is substituted by uracils. Treatment of the uracil-containing DNA with uracil *N*-glycosylase (Lindahl, 1982) produces an alkali-sensitive product (Figure 1, lane C). In contrast, the substrate after borohydride reduction migrated in an alkaline agarose gel in a manner similar to that of the control, uracil-containing phage DNA. Treatment with a preparation of apurinic endonuclease (Bose et al., 1980) resulted in degradation of the glycosylase-treated DNA, but not of the control DNA. In order to determine the number of AP sites, we removed an aliquot of the incubation mixture with uracil *N*-glycosylase prior to addition of $NaBH_4$, extracted with phenol and precipitated with ethanol. The AP DNA was then incubated in 0.2 N NaOH at 37 °C for 1 h prior to analysis on an alkaline agarose gel (see Materials and Methods). The number-average molecular weight was calculated as about 20 from the ratio of the M13 DNA molecular weight to the fragmented weight. Using a ^{3}H-labeled DNA sample and calculating the number of uracils removed by the glycosylase, we arrive at an estimate of 30 sites.

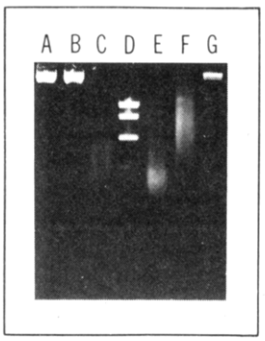

FIGURE 1: Alkaline agarose gel electrophoresis of AP DNA. All DNA samples were incubated for 1 h in 0.2 N NaOH at 37 °C and mixed before being labeled with 0.5 volume of 0.3 N NaOH, 15 mM EDTA, and 50% glycerol. The running buffer was 30 mM NaOH plus 2 mM EDTA. The channels are the following: (A) control, uracil-containing M13 DNA; (B) AP endonuclease (0.04 μg) treated control; (C) uracil *N*-glycosylase treated, nonreduced DNA (AP DNA), ethanol precipitated after the alkali treatment; (D) markers φX174 *Hae*II digest, 2314, 1565, 783, 269, and 185 nucleotides long; (E) $NaBH_4$-reduced AP DNA plus 0.04 μg of AP endonuclease; (F) $NaBH_4$-reduced AP DNA plus 0.01 μg of endonuclease; (G) AP DNA, no endonuclease, stabilized by $NaBH_4$ reduction.

An alternative way of measuring the number of AP sites is based on the finding that these lesions block the replication of DNA by DNA polymerase I (Schaaper et al., 1983; Lockhart et al., 1982). Single-stranded M13 AP DNA was used as a template primed with the *Taq*I restriction fragment C (Figure 2). Control DNA incorporated about 2200 nucleotides per molecule, and the AP template incorporated 0.11 as many. When there are many AP sites in the molecule, the fraction of residual synthesis is 1/(AP), where (AP) is the number of AP sites encountered in the region (Schaaper et al., 1983). When 1/(AP) is 0.11, (AP) = 9. Since only 2200/6400 = 0.34 of the M13 molecule was replicated by the control, the whole molecule contains 9/0.34 = 26 AP sites, a result about midway between the other two independent methods of determination.

Sites of Termination. Knowing that DNA synthesis halts or stutters at AP sites does not indicate the exact position of termination. We therefore carried out synthesis on restriction fragment primed templates and analyzed the products of

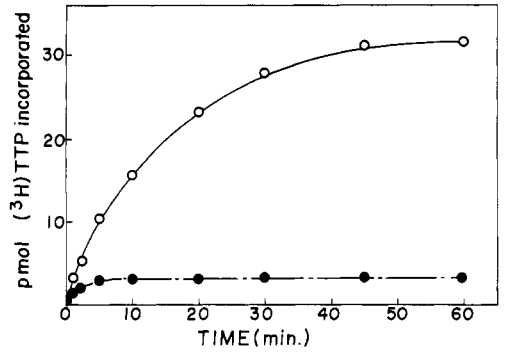

FIGURE 2: Time course and extent of DNA synthesis by pol I on control and AP M13 templates. AP and control M13 templates, primed with *TaqI* fragment C, were incubated with pol I (Klenow fragment) at 22 °C. Reaction mixtures contained polymerase reaction buffer (see Materials and Methods) and 50 μM each of dATP, dCTP, dGTP, and [^{3}H]TTP (930 dpm/pmol). The reaction was terminated by spotting aliquots on Whatman 3MM paper and washing with 5% trichloroacetic acid–1% sodium pyrophosphate and then ethanol. Each point represents the synthesis on 0.023 μg of DNA template with 0.14 unit of enzyme.

synthesis on sequencing gels. As previously described (Moore & Strauss, 1979), the length of the oligonucleotides produced depends on where the polymerase halts due to a block to synthesis. The exact positions of these stops can be determined from a sequencing ladder produced with untreated template and ddNTP's (Sanger et al., 1977) run alongside the experimental channels in the gel. The position of A's in the sequence ladder indicates the positions at which AP sites can be formed in the template due to the insertion of U instead of T and the removal of the U by glycosylase. Stops at (putative) AP sites appear as bands opposite A's in the ladder.

Synthesis on AP DNA and on control, uracil-containing templates was carried out by using either T4 DNA polymerase, *E. coli* pol I, AMV reverse transcriptase, or DNA polymerase α. AP DNA template produced a variety of strong termination bands (Figure 3). When control DNA was incubated with these enzymes, only polymerase α showed a pattern of strong termination bands, and these bands were not located at the positions seen with AP DNA. The termination bands with AP DNA lie mainly before, but in some cases at, the site of putative AP sites. A similar pattern of bands was obtained when AP DNA not reduced with $NaBH_4$ was used as a substrate.

So that the pattern of termination could be analyzed, the gels from several experiments were scanned, and the distribution of termination before and at putative AP sites was tabulated (Table II). Priming with different fragments provided us with the opportunity to examine a variety of sequences. For purposes of analysis, it is useful to consider first single-template T's and then termination at runs of two or three T's separately. The templates we use contain only 20–30 AP sites per molecule (see above), and it is unlikely that there will be two or three AP sites in sequence. Nonetheless, the analysis of sites with runs of T's is more complex.

Most of our experiments were done with *E. coli* pol I in the presence of Mg^{2+}, and we have given the average of the result of eight separate experiments in which the gels could be scanned (Table II). A rough measure of the reliability of the estimates of termination can be obtained from the standard deviation of the percent termination as calculated from all eight determinations. These values (of standard deviation) were 6 at positions 29–31, 9 at positions 36–37, 12 at positions 25–27, 11 at positions 18–19, 18 at positions 20–21, and 25 at positions 39–40 (fragment C). The high standard deviations arise from

Table II: Percent Termination of Synthesis at AP Sites by T4 DNA Polymerase, pol I, AMV Reverse Transcriptase, and Polymerase α[a]

(A) Primer: Fragment C

enzyme (cation)	3'...G	T	C	T	G	G	C	C	T	C	G	T	T	T	G	A	G	G	T	T	G	T	C	C	A	C	A	G	T	C	C	T	A	A	T	C	C	T	C...5'
		*		*					*	*		*	*	*					*	*		*							*				*	*				*
T4 DNA polymerase	100	0	100	0				47	49	4		23	31	46	0			54	45	2	99	1						92	8		100	0		100	0	0	100	0	0
pol I Mg^{2+}	79	21	89	11				18	64	17		7	55	36	3			42	56	2	15	85						47	53		10	90		78	22	92	8	100	0
pol I Mn^{2+}	66	34	83	17				9	51	40		2	41	51	6			3	71	27	3	97						24	76		11	89		71	29	0	100	0	100
AMV reverse transcriptase	100	0	61	39				27	54	20		11	31	45	14			60	41	0	95	5						95	5		24	77		100	0	100	0	42	58
polymerase α			41	59				8	45	48		0	27	50	23			0	49	51	0	100						0	100										

(B) Primer: Fragment E

enzyme (cation)	3'...A	T	T	C	A	T	A	T	C	G	G	G	G	C	C	T	T	T	A	T	C	C	A	C	A	T	A	G	T	G	G	C	A	T	G	A	G	T...5'
		*	*			*		*								*	*	*		*							*								*				*
T4 DNA polymerase	58	42	0		93	7	93	7								55	33	13	98	3						96	4		96	4				100	0				
pol I Mg^{2+}	31	69	0		28	72	81	19								24	57	20	80	20						61	39		66	34				60	40			47	53
pol I Mn^{2+}	26	61	13		16	84	60	40								21	61	19	62	38						51	49		6	94				23	77			0	100
AMV reverse transcriptase	18	72	9		49	51	95	6								41	14	46	68	32						47	53		54	46				54	46				
polymerase α																35	32	34	84	16						67	33		63	37				78	22			47	53

[a] DNA synthesis on AP templates primed with *TaqI* fragment C or E was carried out and analyzed as described in the legend for Figure 3. Gel autoradiographs were scanned as detailed under Materials and Methods. Distribution of radioactivity at and before a putative AP site was calculated as the percent of the total radioactivity at the site. This was done also for a run of two or three putative lesions. Values for polymerase α are corrected by subtracting values for termination bands in the control lane. * Positions of T's denote positions of putative AP sites in the template.

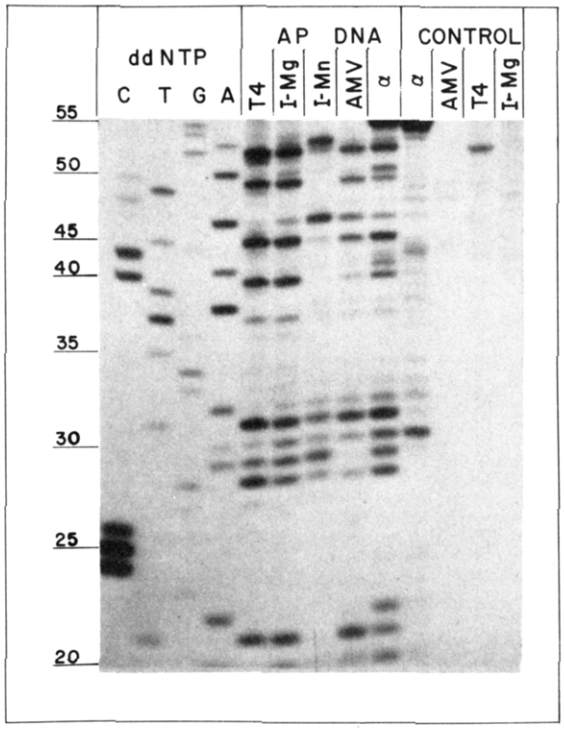

FIGURE 3: Polyacrylamide gel pattern of termination of DNA synthesis on an AP DNA template by pol I, T4 DNA polymerase, AMV reverse transcriptase, and polymerase α. Synthesis occurred on AP or control uracil-containing DNA templates (0.05 pmol) catalyzed by pol I (0.2 unit), AMV reverse transcriptase (8 units), T4 polymerase (2 units), or DNA polymerase α (0.6 μg). The DNA was primed with *Taq*I fragment E. Standard polymerase reactions were carried out in 10-μL mixtures as described under Materials and Methods. ddNTP channels (C, T, G, and A) are sequence standards synthesized with pol I and dideoxynucleotides on control DNA template. Abbreviations: T4, T4 DNA polymerase; I, pol I; AMV, AMV reverse transcriptase; α, DNA polymerase α (Bose et al., 1978). Mg^{2+} (8 mM) or Mn^{2+} (0.5 mM) was used, as specified.

one or two experiments which gave termination percentages quite different from the majority. The finding of "aberrant" termination percentage at one position was not necessarily associated with aberrant termination at other positions.

In the sequence of synthesis primed with fragment C, there are putative single AP sites at positions 19, 21, 40, 45, 48, 51, 53, and 55 (Table II). Such sites occur with fragment E primed synthesis at positions 20, 22, 32, 38, 41, 46, and 50. At all these sites, it is clear that T4 DNA polymerase catalyzed synthesis stops just before putative AP sites. The other enzymes tested show specific patterns of termination. *E. coli* pol I may stop mainly at the site of the lesion (C 40, 48; E 20), sometimes equally at or before the lesion (C 45; E 38, 41, 46, 50), and at some sites mostly one base before the AP site (C 19, 21, 51, 53, 55; E 22, 32), but termination before the AP site is not as complete as with the T4 DNA polymerase. Substitution of Mn^{2+} for Mg^{2+} results in increased procession to the position of the lesion (e.g., C 40, 45, 53, 55; E 41, 46, 50). AMV reverse transcriptase catalyzed synthesis stops before the lesion at some sites (C 40, 45, 51, 53; E 22) and before and at the lesion at other positions (C 21, 55; E 20, 32, 38, 41, 46), but at only one site (C 48) does synthesis proceed mainly to the site of the lesion. The data with DNA polymerase α are more limited but follow a pattern similar to that of pol I with synthesis terminating before or at the lesion at different positions in the sequence. For example, synthesis terminates mostly before the lesion at positions E 38, 41, and 46, mostly at the lesion at positions C 40 and 45, and equally before or at the AP site at positions C 21 and E 22 and 50.

We do not yet understand the structural features which give particular sequence positions their special properties. For example, at position 51 of fragment C, all of the enzymes appear to terminate mainly one nucleotide before the putative AP site. Positions C 48, E 38, and E 41 seem to favor termination at the site of the lesion (with the exception of the T4 DNA polymerase catalyzed reaction).

Multiple sites occur at positions C 26–27 (2), 30–32 (3), and 37–38 (2) and at E 16–17 (2) and 29–30 (2). It is possible to use these positions to test the assumption that AP sites are equally likely to be formed at any T position in the sequences. If T4 DNA polymerase stops one nucleotide before the site of a lesion, then there should be equal termination before and at the first putative AP site in a run of two T's and an equal amount of termination before the first and at the first two in a run of three template T's. This prediction seems to be supported by the data, for example, at C 26–27 and 37–38 and at E 16–17 (Table II). There are deviations from the expected at C 30–32 and E 29–30, but we do not know how significant these differences are. An enzyme that terminated equally well at, or before, a lesion would give a 1:2:1 distribution for the two possible AP sites in a doublet along with the proceding site and a 1:2:2:1 distribution at a triplet. Values approximating these distributions are obtained with pol I (Mg^{2+}) at position E 29–30 and with AMV reverse transcriptase at C 26–27 and 30–32.

Specificity of Insertion. The observation that at some sites a nucleotide is inserted opposite a putative AP site led us to ask which nucleotide(s) is (are) incorporated. In order to answer this question, we synthesize a substrate by using T4 DNA polymerase in a first-stage reaction with all four dNTP's (labeled) and with an AP template so that DNA synthesis terminated just before AP sites. This product was isolated and used in a second-stage reaction (Moore et al., 1982b; Rabkin et al., 1983) either with pol I (in the presence of Mg^{2+} or Mn^{2+}) or with the other enzymes. After reaction, the product was analyzed on a sequencing gel as described for the first-stage reaction.

Examination of such experiments (e.g., Figure 4) is complicated by the exonucleolytic activity of prokaryotic polymerases since it is necessary to consider whether a change in band pattern is due to elongation or to nucleolytic degradation. We have therefore adapted the following scheme for the analysis: the gels were scanned, and the total area under each peak was estimated. The areas under the peaks, corresponding to termination bands, were summed over the scanned region. The total area in the control lane was set as 100. Loss of radioactivity due to degradation in the experimental lanes should lead to values less than 100, as would bypass to give larger products, since such larger molecules would move up and beyond the gel region analyzed. Values of over 100 indicate degradation of larger material or elongation of smaller fragments so that they now appear in the scanned region. An analysis of a second-stage reaction carried out with pol I and Mg^{2+} (Figure 4) and a similar study carried out with pol I and Mn^{2+} are shown in Tables III and IV. In the presence of Mg^{2+}, the total radioactivity in the region scanned (positions 23–45) remains relatively constant (Table III), indicating no extensive migration of material into or out of the region. In contrast, in experiments in which all four dNTP's were added in the second stage (data not shown), only 45% of the radioactivity of the control was recovered in the same region, suggesting bypass.

We examined positions 23–28 and 36–45 of the sequence shown since the pattern at these sites seemed relatively simple.

Table III: Quantitative Analysis of Second-Stage Reactions: Mg^{2+} as the Divalent Cation[a]

position	template	control	[dATP] (μM)			[dGTP] (μM)			[dCTP] (μM)		50 μM TTP	H_2O
			5	10	50	5	10	50	10	50		
23	G	2	3	3	6	3	4	2	7	8	3	3
24	C	1	0	1	1	0	0	0	3	7	6	6
25	C	14	2	2	1	17	15	13	9	9	8	8
26	T*	14	2	3	2	11	14	14	11	11	10	11
27	T*	1	21	21	22	1	0	2	1	1	1	1
28	C	1	0	0	0	3	4	6	0	0	1	0
⋮												
36	G	12	3	4	3	6	8	8	18	18	12	7
37	T*	9	7	7	8	2	2	2	1	2	3	3
38	T*	0	4	4	4	0	0	0	0	0	0	0
39	G	15	3	3	0	6	5	4	12	10	10	9
40	T*	2	16	13	18	2	3	6	1	3	3	2
41	C	1	0	0	0	0	0	2	1	1	1	0
42	C	0	0	0	0	3	3	0	0	0	0	0
43	A	0	0	0	0	0	0	0	0	0	0	0
44	G	9	7	6	5	11	9	7	14	11	14	13
45	T*	2	8	13	11	0	2	2	0	0	0	0
total radioactivity in scan as % of control		100	106	102	100	109	108	114	100	111	109	96
% of radioactivity in analyzed sequence		83	76	80	81	65	69	68	78	81	72	63

[a] The gel scanned is shown in Figure 4. The numbers give the percent of radioactivity in the scan at that particular position to the nearest integer. * Positions of T's denote positions of putative AP sites in the template.

Table IV: Quantitative Analysis of Second-Stage Reactions: Mn^{2+} as the Divalent Cation[a]

position	template	control	[dATP] (μM)			[dGTP] (μM)			[dCTP] (μM)		50 μM TTP	H_2O
			5	10	50	5	10	50	10	50		
23	G	2	3	5	2	0	4	0	4	6	1	4
24	C	2	2	1	1	2	0	0	2	2	1	3
25	C	14	8	6	3	7	8	5	9	6	3	13
26	T*	15	7	5	3	15	20	17	11	12	13	12
27	T*	1	11	9	9	3	6	5	1	2	2	0
28	C	1	1	5	9	2	1	1	0	0	0	0
⋮												
36	G	12	4	4	3	10	4	7	10	8	9	12
37	T*	8	8	8	4	7	9	14	10	9	17	4
38	T*	0	6	9	13	3	0	2	0	8	0	0
39	G	14	5	4	0	3	2	0	12	7	6	13
40	T*	2	13	15	16	6	4	2	9	12	12	0
41	C	1	0	0	3	2	6	7	0	0	0	0
42	C	0	0	0	0	0	0	2	0	0	0	0
43	A	0	0	0	0	2	2	3	0	0	0	0
44	G	8	5	4	0	4	3	2	9	5	7	12
45	T*	2	11	11	13	7	8	8	7	10	8	2
total radioactivity in scan as % of control		100	90	89	81	80	64	63	96	99	98	79
% of radioactivity in analyzed sequence		82	84	86	79	73	77	75	84	87	79	75

[a] Reaction conditions and calculations as in the legends to Table III and Figure 4 except with Mn^{2+} (0.5 mM) as the divalent ion. * Positions of T's denote positions of putative AP sites in the template.

Some of the bands seen on the addition of single nucleotides, particularly in the intervening positions 29–35, are similar to bands appearing when stage I product is incubated with pol I in the absence of added nucleotides, suggesting that nucleolytic degradation occurs in addition to limited elongation. Table III shows the result of quantitation of the data in Figure 4. The addition of dATP results in elongation to position 27. We assume this to be the result of elongation to those AP sites present at position 27 as well as elongation to AP sites present at position 26, followed by bypass synthesis to position 27. The hypothesis that bypass occurs also accounts for the behavior of dGTP, which although not as efficient as dATP does appear to permit some elongation as seen by the gradual appearance of molecules at position 28. dCTP has no effect on elongation in this sequence nor does TTP; both seem to result in the same sort of degradation observed with the H_2O control where radioactivity accumulates at positions 23 and 24.

The events at positions 36–45 are particularly interesting because this section of the sequence contains possible single AP sites at positions 40 and 45. The addition of dATP promotes elongation to the site of the putative AP lesions at both sites. There is evidence for a concentration-dependent elon-

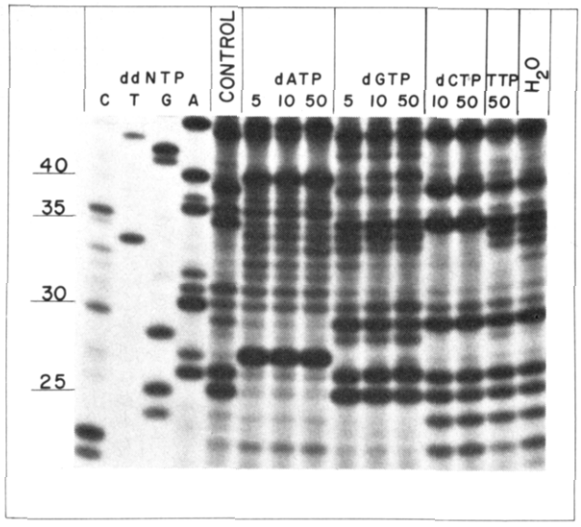

FIGURE 4: Polyacrylamide gel analysis of second-stage reaction products. DNA synthesis was carried out in two stages and analyzed as described under Materials and Methods. The template was primed with *Taq*I fragment C. Control channel: First-stage product with T4 DNA polymerase which was *Taq*I digested and used as substrate for the individual reactions with pol I. The concentrations of deoxynucleotides (in micromolar) are those used in the second stage and indicated on the figure. The numbers and lines at the side mark each fifth nucleotide in the sequence (template strand): 3′-GCC[25]TTCGT[30]TTGAG[35]GTTGT[40]CCAGT[45]CCTAA[50]-5′.

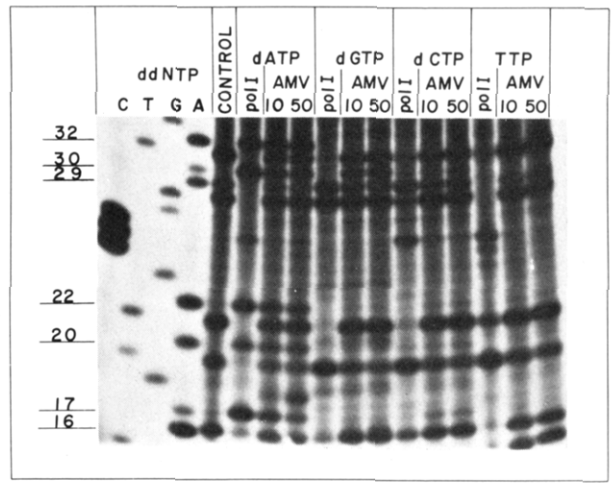

FIGURE 5: Polyacrylamide gel analysis of second-stage reaction products. Details of the reaction are as in the legend to Figure 4. DNA was primed with *Taq*I fragment E. Details of the protocol are as in the legend to Figure 4. Either pol I or AMV reverse transcriptase was used in the second stage, as indicated. The numbers at the side mark the positions of possible AP sites in the sequence (template strand): 3′-A[15]TTCAT[20]ATCGG[25]GCCTT[30]ATCCA[35]-5′.

gation produced by dGTP at position 40 along with some bypass to position 41 or 42, but there is clearly less radioactivity present in this portion of the gel after addition of dGTP. We think this pattern is the result of degradation to positions 29–35 (Figure 4). In contrast to the ability of dATP or dGTP (at one site) to promote elongation, both dCTP and TTP seem inefficient.

A relaxation of specificity in the presence of Mn^{2+} and pol I, previously reported (Rabkin et al., 1983), has also been observed (Table IV). In this experiment, elongation in the second stage was analyzed as in Table III, but with Mn^{2+} as the metal ion. Recovery of the original material (based on the scan of the corresponding region of the control channel) is lower than when Mg^{2+} is used, indicating either greater bypass or degradation, or possibly both. As with Mg^{2+}, elongation with dATP is observed at all possible AP sites. Elongation is observed with dGTP at positions 26–27, 40, and 45. A very significant elongation can be observed with either dCTP or TTP at position 40. Elongation with dCTP and TTP also may occur to position 26 as judged by the ratio of material at positions 25 and 26, but the data are not as clear as at position 40. These data support the view that as in the case of synthesis on a UV-damaged template (Rabkin et al., 1983) Mn^{2+} relaxes specificity.

We also examined the behavior of AMV reverse transcriptase in a second-stage reaction, using a different sequence (Figure 5). Although the addition of dATP does permit elongation to the site of (putative) AP lesions, with AMV reverse transcriptase there always remains a significant amount of material terminated one nucleotide before. Elongation with dGTP and dCTP occurs to a lesser extent than with dATP and at fewer sites (e.g., position 30). The pattern with TTP is like that of the control. The pattern obtained with this sequence and pol I contains special features. With dGTP added, there is only a trace of the control band at position 16. Control bands at positions 21 and 31 have disappeared while those at positions 19, 28, and 29 remain as in the control lane. We suppose that exonucleolytic degradation has occurred, transferring material from band 21 to position 19 and from position 31 to positions 28 and 29 with no elongation. The extra stop at position 26 with dCTP, TTP, and dATP does not seem related to any position of an AP site.

Production of Nucleoside Monophosphates. The observed preference for A insertion might be a result of reduced editing of the terminal A by the 3′→5′-nuclease activity of pol I. Although data in the literature suggest that the DNA-dependent production of dNMP from dNTP is greater for purine nucleotides than for pyrimidine nucleotides, the published measurements do not settle the question as to what happens at the site of AP lesions (Hershfield & Nossal, 1972). We therefore prepared AP DNA terminated one nucleotide before the first AP site by carrying out a first-stage reaction with nonradioactive nucleotides and T4 DNA polymerase. The DNA product was isolated and incubated in a reaction mixture containing Mg^{2+}, pol I, and a mixture of nucleoside triphosphates, one of which was α-^{32}P labeled, and the amount of dNMP was determined as described under Materials and Methods. We determined dNMP release in a stage one reaction with pol I and control DNA, as well as in a stage two reaction with control elongated DNA template isolated after completion of the stage one reaction with T4 DNA polymerase. No free dNMP was observed when either enzyme or DNA was omitted from the reaction mixture.

With both control and AP templates, the production of dAMP is observed to be greater than any of the other nucleotides (Figure 6). The production of dGMP is considerable though lower, but very little dCMP or TMP is observed. The order, and indeed the magnitude, of dNMP production is the same on the control template as on the AP substrate. Considerable incorporation into DNA is observed in the control as expected. We did not observe any radioactivity incorporated into the AP DNA substrate in this experiment. When release of dNMP is calculated per number of nucleotides incorporated into DNA, there is, of course, a tremendous difference between the activity of AP and control templates.

Discussion

AP sites are blocks to DNA synthesis, but the exact site of the block depends on the enzyme used and, at least in part, on the particular nucleotide sequence. Our data suggest that

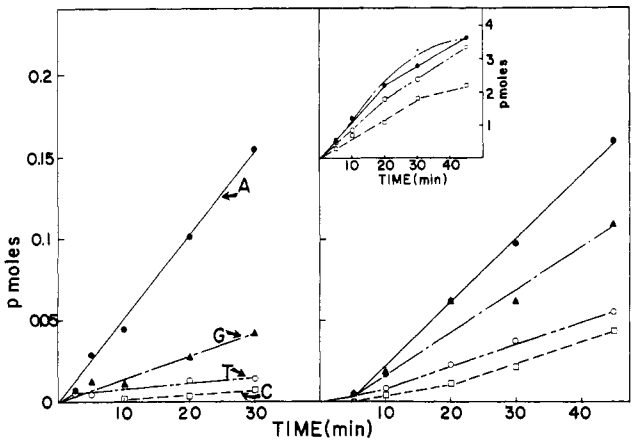

FIGURE 6: pol I catalyzed dNMP release in the presence of control and AP DNA templates. Incubation was carried out with DNA templates primed with the *Taq*I fragment C in reaction mixtures containing pol I and all four dNTP's, as detailed under Materials and Methods. (Left panel) Release of dNMP incubated with AP template in a second-stage reaction. (Right panel) Release of dNMP with control DNA template, incubated in a first-stage reaction. Insert: Incorporation into control DNA (calculated from radioactivity in the origin—one-stage reaction). No release of dNMP was detected in control reactions in which either DNA or enzyme was omitted. Background radioactivity of the ^{32}P-labeled deoxynucleoside triphosphates was determined and subtracted from all calculations.

the 3′→5′-exonucleolytic activity of prokaryotic polymerases is important since the T4 DNA polymerase exonuclease is particularly active (Hershfield & Nossal, 1972; Gillin & Nossal, 1976), and this polymerase is the most likely to stop completely one nucleotide before a putative AP site. However, this cannot be the whole explanation since at certain sites AMV reverse transcriptase, devoid of exonuclease activity, stops one nucleotide before an AP site and pol I, with 3′→5′-exonuclease activity, terminates opposite.

An alternative explanation for the observed oligonucleotide length is that synthesis is always blocked one nucleotide before an AP site but that in some cases slippage (Kornberg, 1974) occurs so that two identical bases are added. On denaturation and electrophoresis of the oligonucleotide, such slippage would appear as elongation to the site of the lesion. However, purines rather than pyrimidines are inserted when present one at a time. Insofar as this observation is relevant to what occurs when all four dNTP's are present, it suggests that at those sites in which a G precedes an AP site (e.g., position 40, sequence E, Table II) the elongation should be the result of the insertion of tandem C's. But dCTP is not effective in elongating stage one products, and therefore, the most likely explanation is that another base is inserted, i.e., that slippage is not an explanation.

Notwithstanding the obvious directing influence of enzyme and sequence, there is a special importance to the insertion of dA's at AP sites. dATP is the deoxynucleotide which is inserted at most sites with pol I and AMV reverse transcriptase. We also observe a preference for A in second-stage reactions with DNA polymerase α similar to that seen with AMV reverse transcriptase (data not shown). dGTP and TTP (with pol I) or dGTP and dCTP (with AMV reverse transcriptase) are incorporated at some sites. The supposition that A is in some way unique is supported by the finding that the rate of dATP to dAMP conversion is greater than that observed with any of the other nucleotides on an AP template. pol I of *E. coli* appears both to insert A and to excise it more readily than any other nucleotide when confronted with a noninstructive site.

AP sites are blocks to DNA synthesis (Schaaper et al., 1983; Lockhart et al., 1982) even though it is possible in some cases for synthesis to proceed to opposite the damaged site itself. Why should further synthesis be blocked? First, it is clear that bypass can occur once a base is added opposite the AP site. This is seen in Table II in which we show that addition of dGTP leads not only to elongation but also to bypass terminating one nucleotide past the AP site opposite a C in the template. Similarly, our quantitation of termination indicates that synthesis does proceed well past lesions when all four dNTP's are present in the second-stage reaction. The key problem in bypass would therefore seem to be insertion opposite the AP site itself followed by elongation before the added nucleotide can be removed (Fersht, 1979). It is not clear why synthesis in the first stage terminates before the AP site when it can be made to proceed to the site of the lesion when the experiment is done as a second-stage reaction with only one-tenth of the nucleotide concentration. One factor may be the infinite pool bias that exists in the second stage with only one nucleotide present. However, there still may be some difference between a reaction proceeding continuously along the molecule as opposed to starting synthesis from termination at an AP site.

These complications should not obscure the fact that dATP is specific for elongation to opposite AP sites when used with either *Escherichia coli* or avian myeloblastosis virus polymerases. Insofar as our in vitro model system has significance for in vivo conditions, these results make it possible to account for some recently published examples of mutagen specificity (Eisenstadt et al., 1982; Foster et al., 1983). If purines, particularly A, are inserted opposite AP sites, then one should be able to predict the type of mutation produced. Most depurination in vivo comes from the spontaneous loss of guanine (Lindahl & Nyberg, 1972), and one would therefore expect to see G–C to T–A transversions as both the major spontaneous mutation and the major change induced at artificially created AP sites. This is just what has been observed in experiments by Schaaper et al. (1983) in which AP DNA is used to infect bacteria. Aflatoxin B_1, which in its activated form reacts mainly at the N-7 position of guanine, thereby producing an unstable derivative prone to depurination, has been found to produce a high proportion of transversions in which an A has been inserted opposite the G (AP) site (Foster et al., 1983).

In these experiments, we assume that the AP site is completely noninstructive. If in some way the sequence had "remembered" that a T was originally present, the incorporation of A would be expected. Although this seems an unlikely explanation of the results and, indeed, pol I catalyzed A incorporation on an AP poly(dC) homopolymer template has recently been reported (Boiteux & Laval, 1982), we are proceeding to test the specificity of incorporation at AP sites which have been produced by the removal of other bases within the DNA.

Acknowledgments

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Registry No. dATP, 1927-31-7; dGTP, 2564-35-4; dCTP, 2056-98-6; TTP, 365-08-2; Mn^{2+}, 7439-96-5; Mg^{2+}, 7439-95-4; DNA polymerase, 9012-90-2; reverse transcriptase, 9068-38-6.

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