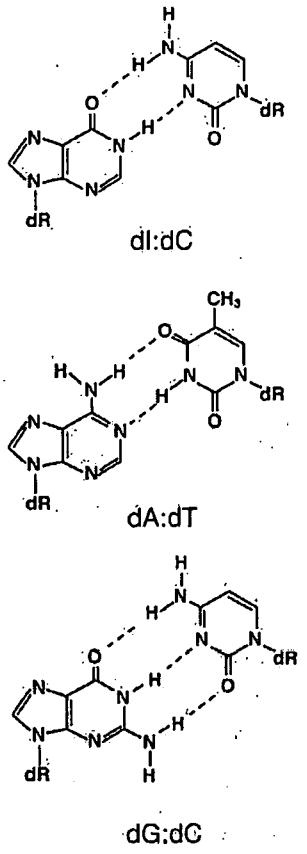

Fig. 8. Possible base pairing of the dI adduct with dC.

miscoding specificities and frequencies of the dI lesion catalyzed by Y-family human DNA pols. The dI adduct represents a highly miscoding lesion capable of generating A→G transitions, indicating that this $^{\bullet}NO$-induced lesion plays an important role in initiating inflammation-driven carcinogenesis.

Materials and Methods

General

Ultrapure dNTPs were from GE Healthcare. EcoRI restriction endonuclease (100 U/μL) was purchased from New England BioLabs. Blue Dextran (D5751) was obtained from Sigma. Human pol α was obtained from CHIMERx (Milwaukee, WI). Human pol η was purified as previously described.[19] Human pol κ (pol κΔC) was overexpressed in *E. coli* and purified as a C-terminally truncated form. The protein has 10× His tag at the N-terminal position and contains 559 amino acids from the N-terminus (N. Niimi and T. Nohmi *et al.*, unpublished results).

Preparation of oligodeoxynucleotides

All oligodeoxynucleotides, Alexa546 (Molecular Probes)-labeled primers, standard markers, and dI-modified template were obtained from Japan Bio Service Co. (Saitama, Japan). Alexa546 was conjugated at the 5'-terminus of primers and standard markers. A single dI was located at the 20th position from the 5'-termini in the modified 38-mer template (5'CATGCTGATGAATTCCTTCZCTTCTTTC-CTCTCCCTTT, where Z is dI). The oligomers were purified by using 20% denaturing PAGE before use.

Primer extension reactions

Primer extension reactions catalyzed by pol α, pol η, or pol κΔC were conducted at 25 °C for 30 min in a buffer (10 μL) containing all four dNTPs (100 μM each) using dI-modified and unmodified 38-mer templates (750 fmol) primed with an Alexa546-labeled 10-mer (500 fmol, 5' AGAGGAAAGA) (Fig. 3). The reaction buffer for pol α contains 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 60 mM KCl, 10 mM dithiothreitol, 250 μg/mL bovine serum albumin, and 2.5% glycerol. The reaction buffer for pol η and pol κΔC contains 40 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 10 mM dithiothreitol, 250 μg/mL bovine serum albumin, 60 mM KCl, and 2.5% glycerol. Reaction was stopped by addition of 2 μL formamide dye containing Blue Dextran (100 mg/mL) and ethylenediaminetetraacetic acid (50 mM) and incubation at 95 °C for 3 min. The whole amount of the reaction sample was subjected to 20% denaturing PAGE (30 × 40 × 0.05 cm). The positions of bands and homogeneities of oligodeoxynucleotides following PAGE were determined by using Molecular Imager FX Pro and Quantity One software (Bio-Rad). The linear range to quantitatively detect fluorescence-labeled oligomers was from 5 to 1500 fmol (Fig. 6).

Quantitation of miscoding specificity

Using dI-modified and unmodified 38-mer oligodeoxynucleotide (750 fmol) primed with an Alexa546-labeled 12-mer (500 fmol, 5'AGAGGAAAGAAG), we conducted primer extension reactions catalyzed by pol α (200 fmol), pol η (20 fmol), or pol κΔC (20 fmol) at 25 °C for 30 min in a buffer (10 μL) containing all four dNTPs (100 μM each) and subjected them to 20% denaturing PAGE (30 × 40 × 0.05 cm). Extended reaction products (>26 bases long) were extracted from the gel. The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer, cleaved with EcoRI, and subjected to two-phased PAGE (20 × 65 × 0.05 cm) containing 7 M urea in the upper phase and no urea in the middle and bottom phases (each phase contains 18%, 20%, and 24% polyacrylamide, respectively). The phase width is approximately 10, 37, and 18 cm from the upper phase. To quantify base substitutions and deletions, we compared the mobility of the reaction products with those of Alexa546-labeled 18-mer standards containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions[17,18] (Fig. 3).

Steady-state kinetic studies of nucleotide insertion and extension

Kinetic parameters associated with nucleotide insertion opposite the dI lesion and chain extension from the 3' primer terminus were determined at 25 °C, using varying amounts of single dNTPs. For insertion kinetics, reaction mixtures containing dNTP (0–250 μM) and either pol α (20–200 fmol), pol η (2–20 fmol), or pol κΔC (1–20 fmol) were incubated at 25 °C for 2 min in 10 μL of Tris–HCl buffer (pH 8.0) using a 38-mer template (750 fmol) primed with an Alexa546-labeled 12-mer (500 fmol; 5'AGAG-

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GAAAGAAG). Reaction mixtures containing a 38-mer template (750 fmol) primed with an Alexa546-labeled 13-mer (500 fmol; 5'AGAGGAAAGAAGN, where N is C, A, G, or T), with varying amounts of dGTP (0–250 μM) and either pol α (20–200 fmol), pol η (1–20 fmol), or pol κΔC (1–20 fmol), were used to measure chain extension. The reaction samples were subjected to 20% denaturing PAGE (30×40×0.05 cm). The Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were obtained from Hanes–Woolf plots. Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dT:dA base pair according to the following equation: $F=(V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dT:dA]}$.[24,25]

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