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Dual Roles for DNA Polymerase η in Homologous DNA Recombination and Translesion DNA Synthesis

Short Article

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Summary

Chicken B lymphocyte precursors and DT40 cells diversify their immunoglobulin-variable (IgV) genes through homologous recombination (HR)-mediated Ig gene conversion. To identify DNA polymerases that are involved in Ig gene conversion, we created DT40 clones deficient in DNA polymerase η (polη), which, in humans, is defective in the variant form of xeroderma pigmentosum (XP-V). Polη is an error-prone translesion DNA synthesis polymerase that can bypass UV damage-induced lesions and is involved in IgV hypermutation. Like XP-V cells, polη-disrupted (*polη*) clones exhibited hypersensitivity to UV. Remarkably, *polη* cells showed a significant decrease in the frequency of both Ig gene conversion and double-strand break-induced HR when compared to wild-type cells, and these defects were reversed by complementation with human polη. Our findings identify a DNA polymerase that carries out DNA synthesis for physiological HR and provides evidence that a single DNA polymerase can play multiple cellular roles.

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Introduction

HR is involved in the shuffling of allelic chromosomes during meiosis, the repair of DNA double-strand breaks (DSBs), and the release of DNA replication blocks. Furthermore, mating-type switching in yeast and IgV diversification of immunoglobulin (Ig) genes in some vertebrate species are made through HR-dependent gene conversion events (Haber, 1992; Reynaud et al., 1994). HR is a multistep process involving broken DNA and intact homologous sequences. During the early steps, DSB sites are processed, leading to formation of 3′ single-strand overhangs, which associate with Rad51. 3′ overhangs invade other homologous duplex DNA to form D loops. Finally, DNA synthesis from the invading strand results in gene conversion. Since D loop is unstable, efficient DNA synthesis may increase the rate of gene conversion (Paques et al., 1998).

Ig gene conversion is an excellent model for physiological HR in higher eukaryotes, because it is initiated by a well-characterized mechanism involving activation-induced deaminase (AID)-dependent deamination of dC to dU at the functional V(D)J joint (reviewed in Neuberger et al. [2005]). Subsequent HR reactions may involve single-strand break (SSB) formation associated with the elimination of dU, and invasion of the 3′ tail of the SSB onto the upstream intact duplex homologous sequences containing pseudo-V (ψV) segments, leading to loop formation (reviewed in Wyman et al. [2004]). Presumably, DNA synthesis from the 3′ invading tail using the ψV duplex as a template results in the transfer of several to a few hundred nucleotides from a ψV segment (donor of gene conversion) to the V(D)J joint (recipient). At the present time, it is unclear which DNA polymerase performs Ig gene conversion. Moreover, DNA polymerases acting in HR have not been clearly defined even in budding yeast, the best-characterized model organism for HR (Wang et al., 2004).

The release of DNA replication blocks is carried out not only by HR but also by error-prone translesion DNA synthesis (TLS). Specialized TLS polymerases take over from stalled replicative DNA polymerases at damaged template strands and flexibly undergo DNA synthesis past a variety of lesions including abasic sites (reviewed in Lehmann [2005]). A number of TLS polymerases have been identified in yeasts and mammals, and polη and polζ are conserved between species. A defect in polη is responsible for a variant form of XP-V (Cordonnier and Fuchs, 1999; Johnson et al., 1999; Masutani et al., 1999, 2000), which is characterized by a predisposition to skin cancer and elevated UV sensitivity (Lehmann, 2005).

To investigate the role of polη in IgV diversification and HR-mediated DSB repair in chicken B lymphocytes, we generated *POLη*$^{-/-}$ (hereafter called *polη*) clones in the DT40 line (Buerstedde and Takeda, 1991). The *polη* cells displayed 2- to 6-fold reduced frequency of Ig

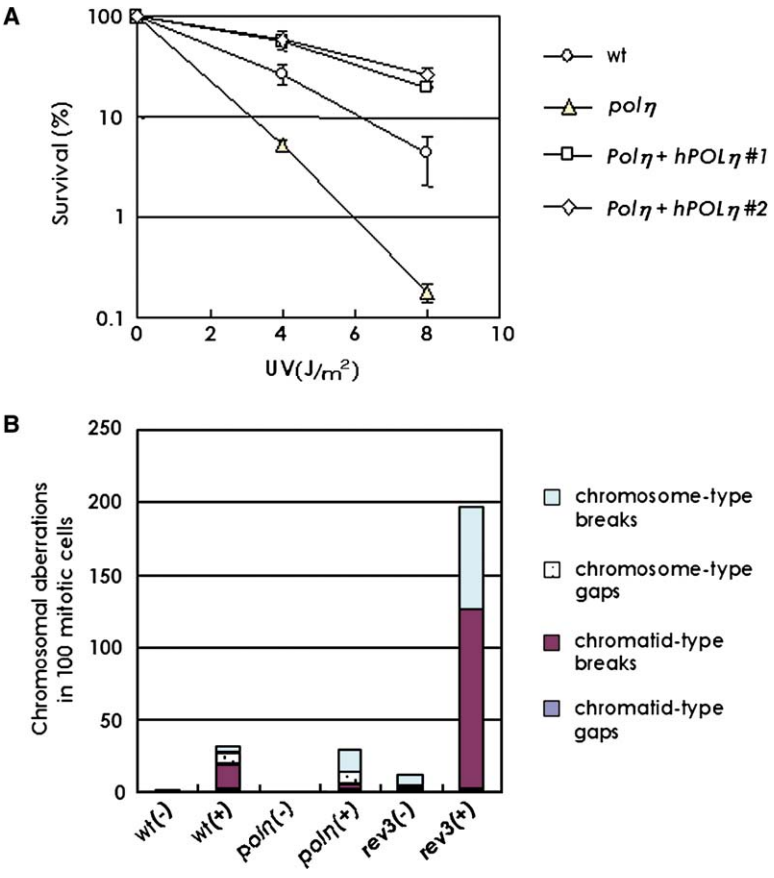

Figure 1. DNA Repair in polη-Deficient Cells in Response to UV and IR

(A) Sensitivity of *polη* cells to killing by UV. The fractions of surviving colonies after UV treatment compared with untreated controls of the same genotype are shown on the y axis on a logarithmic scale. The dose of UV is displayed on the x axis on a linear scale. "#1" and "#2" are two independent clones transfected with human *POLη*. The data shown were obtained from at least three experiments. Error bars indicate standard deviations.

(B) Proficient DSB repair during the G2 phase in *polη* cells. Chromosomal aberrations either during the cell cycle (−) or following exposure to 2 Gy γ-rays (+) of wt, *rev3* (*polζ*), and *polη* cells are shown. Cells were irradiated 3 hr before harvest. Mitotic cells were enriched by colcemid treatment for the last 3 hr before cell harvest.

gene conversion when compared with wild-type (wt) cells. Human *POLη*, but not its mutant gene defective in polymerase activity, reversed the rate of Ig gene conversion. These observations provide compelling evidence that polη can promote DNA synthesis during HR.

Results

Generation of polη-Deficient Cells

We isolated a chicken *POLη* cDNA and determined its amino acid sequence. The five (I–V) polymerase motifs show ~78.3% identity, and the overall identity to the human polη protein is 52.0%. Gene targeting constructs, which are expected to delete amino acids 185–316 containing two polymerase motifs (IV and V), were generated from the genomic DNA (see Figure S1A with the Supplemental Data available with this article online). Gene targeting of the *POLη* locus was confirmed by Southern blot analysis (Figure S1B). RNA protection assays confirmed that an exon located downstream of the deleted sequences in the *POLη* gene is not transcribed (Figure S1C). We examined the proliferative properties and DNA repair capacities of *polη* cells. Three independently isolated *polη* clones proliferated at a rate very similar to that of wt cells (data not shown). *Polη* cells exhibited higher sensitivity than wt cells to killing by UV (Figure 1A), but not to *cis*-diaminedichloroplatinum-II (cisplatin) or ionizing irradiation (IR) (our unpublished data). Primary human *polη*-defective XP-V cells are sensitive to UV only if they are treated with caffeine following irradiation (reviewed in Lehmann [2005]). However, SV40-transformed XP-V cells are sensitive even without caffeine, this sensitivity being attributed to loss of p53 (Limoli et al., 2002). The UV sensitivity of DT40 *polη* cells even without caffeine treatment may reflect the loss of functional p53 (reviewed in Hochegger et al. [2004]). Therefore, chicken DT40 *polη* cells have phenotype very similar to human XP-V cells.

Reduced Efficiency of HR-Mediated DSB Repair in *polη* Cells

To study the contribution of polη to HR, we examined gene targeting frequency, HR induced by megaendonuclease (I-SceI), and repair of IR-induced DSBs during the G2 phase. In all cases, wt and *polη* cells were directly compared. The gene targeting frequency was not significantly reduced in *polη* cells (Table S1). Next, HR-dependent DSB repair was evaluated using an artificial SCneo HR substrate DNA carrying the I-SceI recognition site, following insertion into the *OVALBUMIN* locus in DT40 cells (Fukushima et al., 2001). DSBs induced by transient expression of I-SceI are repaired by gene conversion from the upstream donor homologous sequences. Remarkably, using this assay, we found that the efficiency of HR-mediated DSB repair was reduced 10-fold in *polη* cells. Reconstitution of a human *POLη* transgene in *polη* cells restored the level of DSB repair to the wt level (Table 1). Thus, human polη can also participate in HR in DT40 cells. We previously reported that, following IR in G2 phase, HR is preferentially used for DSB repair in

Table 1. I-SceI-Induced Gene Conversion

Genotype	Percentage of G418-Resistant Colonies			
	Exp. 1	Exp. 2	Exp. 3	Average ±SD
Wt	2.4	1.2	1.3	1.6 ± 0.67
polη	0.1	0.1	0.2	0.1 ± 0.06
polη + *hPOLη*	1.9	1.0	1.3	1.4 ± 0.46

The frequency of HR-dependent DSB repair in the SCneo reporter construct in each genotype is shown as the percentage of G418-resistant colonies divided by the number of colonies obtained without G418 selection. SD is standard deviation.

DT40 cells, and, thus, the level of unrepaired chromosomal breaks at the subsequent M phase may reflect the capability of HR-dependent DSB repair (Sonoda et al., 2003). No significant differences were detectable in the level of IR-induced breaks between wt and *polη* cells following γ-ray irradiation in the G2 phase (Figure 1B). These observations are in marked contrast with the phenotype of *polζ* cells, which we described previously (Okada et al., 2005; Sonoda et al., 2003); polζ-deficient cells have a severe defect in repair of IR-induced DSBs during the G2 phase (Figure 1B) but show no significant defect in repair of I-SceI-induced DSBs and only a slight decrease in gene targeting efficiency. Thus, polη and polζ may be employed by different sets of HR reactions.

The Rate of Ig Gene Conversion Is Reduced in *polη* Cells

To analyze the contribution of polη to Ig gene conversion, its kinetics was measured. Our sIgM-negative cells carry a +G frameshift mutation in the rearranged VJ_λ segment at the same site as in the CL18 clone (Buerstedde et al., 1990). We monitored the gain of surface IgM (sIgM) expression resulting from the elimination of the frameshift mutation by gene conversion events (Figure 2A). We confirmed that three independently isolated *polη* cells indeed retained the same frameshift mutation as do the CL18 wt cells. We then performed sIgM-gain fluctuation analyses on 30–40 subclones derived from single sIgM-negative cells following a 3 week clonal expansion (Hatanaka et al., 2005). The three *polη* clones exhibited about a 2- to 6-fold decrease in the appearance of sIgM-positive revertant fractions in the subclones (Figure 2B). The reconstitution of human *POLη* transgene in *polη* cells reversed the level of sIgM gain to a wt level. The decreased rate of gain in *polη* cells was reproduced when the cells were treated with a histone deacetylase inhibitor, trichostatin A (TSA), which elevates the rate of Ig gene conversion by ~50 fold (Figure 2C) (Seo et al., 2005). To verify that the polymerase activity is required for Ig gene conversion, we expressed mutant human *POLη* cDNA carrying D115A/E116A mutations in the *polη* cells. These mutations abolish polymerase activity (data not shown), as previously reported with yeast polη (Johnson et al., 1999). The expression of the mutant polη did not reverse the sIgM gain (Figure 2B), indicating that polη is directly involved in Ig gene conversion by undergoing DNA synthesis in Ig gene conversion.

To assess the nature of Ig gene conversion events in *polη* cells, we determined the nucleotide sequence in the VJ in sIgM-positive revertants from the three

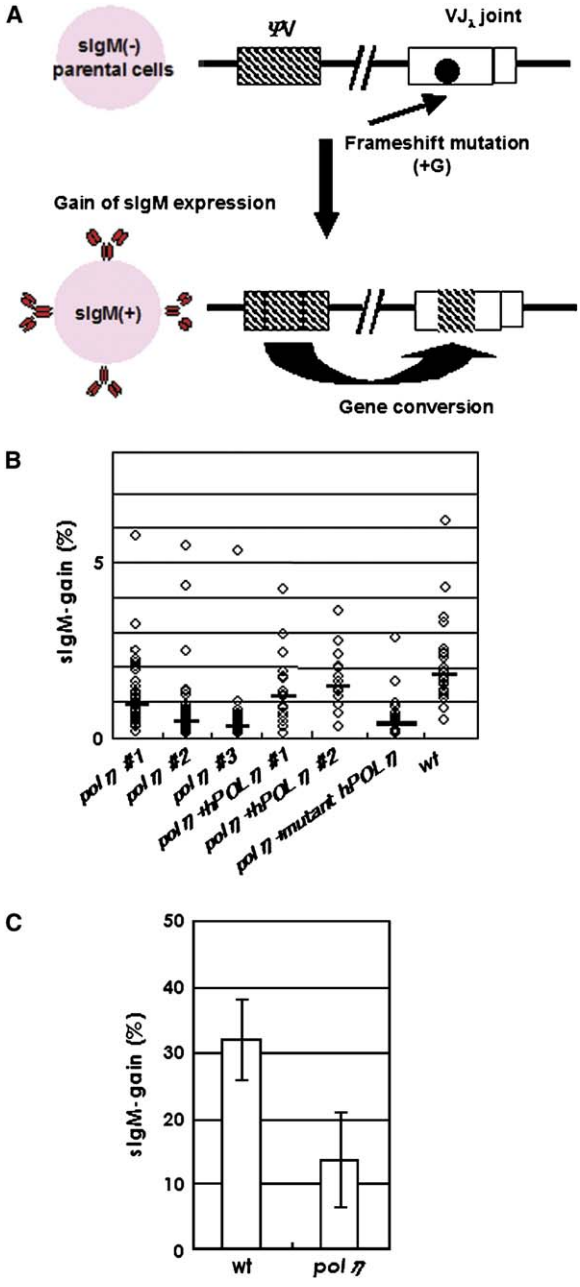

Figure 2. Reduced Ig Gene Conversion Rate in *polη* Cells

(A) The kinetics of Ig gene conversion is evaluated by measuring the frequency of sIgM gain. The frameshift mutation caused by insertion of G in the rearranged V_λ segment in the parental cells is shown by the closed circle. The mutation inhibits sIgM expression, whereas a gene conversion event using an upstream ψV as a donor can eliminate the frameshift mutation, leading to the expression of sIgM (left bottom). A ψV fragment is shown as a shaded box.

(B) The abundance of sIgM gain variants was determined in ~30 parallel cultures derived from sIgM-negative single cells after clonal expansion for 3 weeks. The horizontal bars in the panel indicate median percentages. Three independently generated *polη* clones and clones derived from #2 *polη* cells stably transfected with human intact or mutant *POLη* cDNA were analyzed in comparison with wt cells.

(C) The frequency of sIgM-gain revertants was determined in cultures at 14 days after addition of TSA. Cells were cultured in complete medium containing 1.25 ng/ml TSA. The percentage of sIgM-positive cells in wt and *polη* populations was ~1% when TSA was added. Error bars indicate standard deviations.

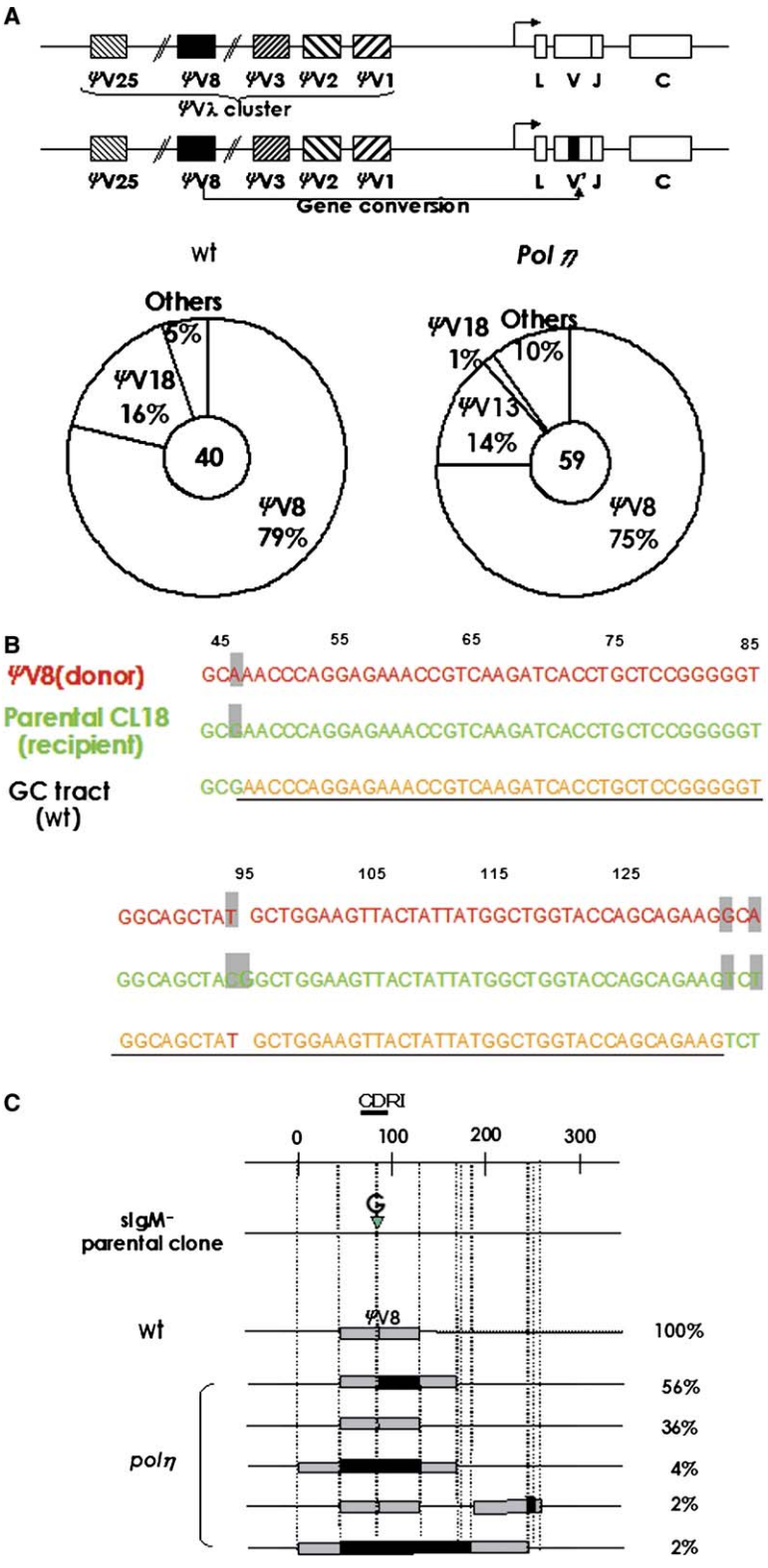

Figure 3. Increased Lengths of Ig Gene Conversion Tracts in *polη* Cells

(A) Preference of ψV gene usage as a donor for Ig gene conversion events in purified sIgM-gain populations. The primary structure of chicken Ig λ gene is shown in the upper panel. There are 25 ψV_λ segments upstream of the functional VJ_λ. The Ig gene conversion event using ψV_8 is illustrated in the upper panel as an example. The ψV_8, $_{13}$, and $_{18}$ are different ψV genes. The total numbers of VJ_λsequences analyzed in wt and *polη* cells are shown in the central circles.

(B) Base sequences around the frameshift (boldfaced **G** nucleotide) of the recipient VJ_λ gene. The maximum gene conversion tract in sIgM-positive wt cells is underlined. Shaded letters indicate mismatches between ψV_8 donor and recipient VJ_λ. The (1) donor ψV_8 and (2) recipient VJ are marked in red and green, respectively. In the converted sequences (3), donor- and recipient-derived nucleotides are marked in red and green, respectively, while the sequences that are derived from either the donor or recipient are marked in yellow. Thus, gene conversion is initiated and terminated somewhere within the yellow sequences flanking the red-colored T nucleotide. The number at the top indicates the nucleotide numbers in the second exon.

(C) Gene conversion tract spectra in the VJ_λ segment rearranged with ψV_8 in sIgM-positive revertants. The number at the top indicates the nucleotide numbers in the second exon. As discussed in the text, the conversion tract length can be estimated from the particular nucleotides at sites of mismatches between donor and recipient DNA. The minimum gene conversion length (black box) is the stretch between nucleotides that originate from the donor. The maximum conversion tract is the stretch between nucleotides that are in sequences shared by the donor and recipient. The identical sequence shared by the donor ψV_8 and recipient gene is indicated by lightly shaded boxes. The crossover sites must be within these lightly shaded boxes. Dotted vertical lines indicate the positions of mismatches between the donor ψV_8 and recipient VJ_λ (the positions of mismatches are −3, 46, 94, 95, 132, 134, 171, 172, 176, 186, 253, 256, and 260 from the first nucleotide of the second VJ_λ exon). The reversed green triangle indicates the position of the frameshift (corresponds to boldfaced letter **G** in Figure 3B) of the recipient VJ segment. The total numbers of the analyzed VJ_λ segments that were rearranged with the ψV_8 donor in wt and *polη*-deficient cells were 31 and 53, respectively.

independent *polη* clones. The sequences around the parental frameshift mutation were replaced by ψV sequences in most sequences analyzed (59 out of 60). The accuracy of Ig gene conversion is not compromised in the absence of polη, and the frequency of point mutation was low (~0.03%) in wt and polη-deficient DT40 cells. In both wt and *polη* cells, a majority of the gene conversion events utilized the ψV_8 segment as donor (Figure 3A), as this segment has the highest sequence similarity to the VJ_λ segment (Buerstedde et al., 1990).

The length of gene conversion tracts involving ψV_8 was estimated in the following manner. Sequence

comparison between (1) ψV_8 (gene conversion donor), (2) VJ_λ segment containing the frameshift mutation (gene conversion recipient), and (3) its converted sequence (Figure 3B) enables us to determine which sequences in the recipient (2) were replaced by the donor (1) in each product (3). For example, sequence analysis of tracts in wt cells indicates that TAC**G**GCT sequences in the recipient (2) were replaced by ψV_8 (1)-derived TATGCT sequences in all analyzed products (3) (Figure 3B; note that the boldfaced **G** causes the frameshift and corresponds to the green triangle in Figure 3C). Thus, C**G** of the recipient are replaced by "T" of the donor through gene conversion, and it restores the reading frame of the IgM protein. These converted sequences were flanked by identical sequence shared by all the donor, recipient, and converted sequences (sequences shown in yellow in Figure 3B). Outside this region of approximately 80 nucleotides, there are occasional mismatches, indicated by shading in Figure 3B and vertical lines in Figure 3C. In wt cells, all convertants contained the recipient sequences at the mismatches at nucleotide 46 and 132. This defines the maximum length of the conversion tract in wt cells; i.e., the gene conversion tract length was between 47 and 131 nucleotides. However, this was not always the case for the *polη* cells. By similar analysis of the origin of the mismatched bases in the conversion tracts in *polη* cells, we are able to define a minimum tract length (distance of the furthest mismatched bases between gene conversion product and recipient) and a maximum tract length (distance of the closest mismatches between gene conversion product and donor) (Figure 3C).

Interestingly, the average length of the gene conversion tract involving ψV_8 was dramatically increased in *polη* cells compared to wt cells (Figure 3C). The average length of maximum gene conversion tracts involving ψV_8 was increased by ~35% in *polη* cells compared to wt cells. Moreover, the minimum lengths of some gene conversion events in *polη* cells (86 and 145) were longer than even the maximum length in all gene conversion events in wt cells (84) (Figure 3C). In summary, polη increases the rate of gene conversion but does not affect the choice of donor ψV segments. The present data also reveal that, in the absence of polη, the length of conversion tracts was significantly increased, while the accuracy of gene conversion was not compromised.

Discussion

The present study demonstrates that polη plays a role in Ig gene conversion. The data identify the DNA polymerase that can promote DNA synthesis in physiological HR in higher eukaryotes. Moreover, polη significantly increases the efficiency of HR-dependent repair of I-SceI-induced DSBs. Taking the role for mammalian polη in generating single base substitutions in the IgV (Delbos et al., 2005; Martomo et al., 2005) into account, the data reveal multiple roles of polη in DNA metabolism, namely, HR- and single-base-substitution-dependent IgV diversification, HR-dependent DSB repair, and TLS past UV damage.

Chromosomal breaks following IR in the G2 phase also likely reflect the efficiency of HR-mediated DSB repair, because HR is preferentially used over NHEJ in the G2 phase in DT40 cells (reviewed in Hochegger et al. [2004]). We previously reported that cells deficient in Rev3, the catalytic subunit of polζ, display dramatic increases in the level of IR-induced chromosomal breaks, whereas Ig gene conversion is normal and there is no significant decrease in HR-mediated repair of I-*Sce*-I-induced DSBs (Sonoda et al., 2003) (E.S. and S.T., unpublished data). Thus, polη and polζ appear to have distinct roles in HR. Conceivably, since polζ is indifferent to the abnormal structure of template/primer strands (Lawrence and Maher, 2001), polζ can extend from the 3′ end of a DSB that is chemically modified by IR. Alternatively, the recruitment of each DNA polymerase might be strictly regulated depending on the type of DNA damage. For example, the ubiquitination of clamp protein (PCNA) appears to specifically facilitate the association of polη with a stalled replication fork (Kannouche et al., 2004; Stelter and Ulrich, 2003; Uto et al., 2004). Another question is whether polη can carry out DNA synthesis over the whole gene conversion tract or polη initiates DNA synthesis followed by extension by another DNA polymerase that has higher processivity. Recent biochemical work in the accompanying manuscript using fractionated cell-free extracts and purified recombinant proteins shows that polη vigorously extends DNA synthesis from D loop recombination intermediates (McIlwraith et al., 2005). These biochemical data suggest that polη could promote gene conversion of up to ~80 nucleotides, as observed in gene conversion tracts in wt cells (Figure 3C). Moreover, the vigorous extension from D loop by purified polη agrees with our genetic observation that polη significantly increases the rate of HR (Table 1 and Figure 2).

A number of DNA polymerases, including replicative polymerases, have functional redundancy in different types of HR. Thus, a comprehensive genetic study, such as phenotypic analysis of multiple gene-disrupted clones as well as conditional inactivation of replicative polymerases, may be required to enable us to understand the whole picture of DNA synthesis associated with various HR reactions.

Experimental Procedures

Isolation of the Chicken *POLη* Gene

To isolate the chicken *POLη* gene, we designed oligonucleotide PCR primers: CGGGGCCGCGATGTCGCGGGGCGGGAGC and CGCCCT TTTAAGATTCCCTGCTGGAAGCCCTGGGCCC, based on conserved sequences between the human and murine *POLη* orthologs (McDonald et al., 1999). By using these PCR primers, chicken cDNA fragments were isolated.

Plasmid Construction

Two *POLη* disruption constructs, *POLη-bsr/loxP* and *POLη-puro/loxP*, were generated from genomic PCR products combined with a bsr or puro selection marker cassette flanked by the loxP signals at both sides. To make a human polη expression plasmid, fragment of human *POLη* cDNA was inserted into the NotI site of the expression vector pMK10. A polymerase activity negative human *POLη* cDNA was generated by inserting D115A/E116A mutations. The resulting cDNA was PCR amplified with the primers aaggagatatacatATGGCTA CTGGACAGGAT containing an NdeI site and TTTAAGCCATTAACAC ATctcgagcaccaccac containing an XhoI site (note that the initiation and termination codons are underlined).

Cell Culture and DNA Transfection

Methods of cell culture, DNA transfection, and genotoxic treatments have been described previously (Hatanaka et al., 2005).

RNA Protection Assay

Nonisotopic RNA probes for chicken *POLη* were generated using an in vitro transcription kit (MAXIscript; Ambion, Texas). In brief, PCR-amplified cDNAs amplified by using primers (5′-GAGCTGCTGCCCA CCACCTTCGTGCAGGG-3′ and 5′-GCTGACAGGCATCTGGCTGAA GAGCTGCGGG-3′ for probe A and 5′-CGCAGCCAGCTCCATGGAG CACCCAGAGGG-3′ and 5′-GAGACCCAGAAGCTGCAGCCTTGGCG ATGGG-3′ for probe B in Figure S1C) were cloned into PCR2.1 vector, which contains a T7 promoter (Invitrogen, California). The ribonuclease protection assay kit (Ambion, Texas) was used according to the manufacturer's instruction.

Measurement of Targeted Integration Frequencies

Measurement of targeted integration frequencies was carried out as described previously (Buerstedde and Takeda, 1991; Fukagawa et al., 1999).

Measurement of Recombination Frequencies Using an I-SceI-Induced DSB Repair System

Measurement of recombination frequencies by an I-SceI-induced DSB repair system was performed as described previously with minor changes (Fukushima et al., 2001). Modified SCneo was inserted into the previously described *OVALBUMIN* gene construct and then targeted into the *OVALBUMIN* locus in wt and *polη* DT40 cells. In transient transfections, cells suspended in 0.5 ml of phosphate-buffered saline were mixed with the following plasmid DNAs without linearization: either 15 μg of pBluescript SK plus 15 μg of the I-SceI expression vector (pCBASce) or 15 μg of human *POLη* expression vector plus 15 μg of pCBASce. We obtained and counted more than 15 colonies in each sample to calculate a mean value and standard error. The total number of neomycin-resistant colonies, i.e., HR-dependent DSB repair events, is 141 for wild-type, 673 for *POLη*$^{-/-}$, and 119 for *POLη*$^{-/-}$ reconstituted with *POLη* cDNA.

Analysis of the Rate of Surface IgM Gain

The frequency of generation of sIgM-gain revertants was monitored by flow cytometric analysis of cells, which had been expanded for 3 weeks after subcloning and then stained with FITC-conjugated goat anti-chicken IgM (Bethyl, Montgomery, Texas). To enhance Ig gene conversion, trichostatin A (TSA) was added to sIgM-negative cell populations (concentration of TSA is 1.25 ng/ml), and the fraction of sIgM^{+} revertants was monitored with time, as described before (Seo et al., 2005).

Nucleotide Sequence Analysis of the Rearranged VJ_{λ} Segments Derived from sIgM-Gain Fractions

The frequencies of nontemplated single-base substitution and gene conversion were determined as previously described (Hatanaka et al., 2005).

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/20/5/793/DC1/.

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Accession Numbers

The base sequence of chicken *POLη* cDNA is deposited in the DNA Data Bank of Japan (DDBJ) database with accession number AB097589.