Translesion DNA synthesis (TLS) of damaged DNA templates is catalyzed by specialized DNA polymerases. To probe the cellular TLS mechanism, a host-vector system consisting of mouse fibroblasts and a replicating plasmid bearing a single DNA adduct was developed. This system was used to explore the TLS mechanism of a heptanone-etheno-dC (H- edC) adduct, an endogenous lesion produced by lipid peroxidation. In wild-type cells, H-edC almost exclusively directed incorporation of dT and da. Whereas knockout of the Y family TLS polymerase genes, Polh, Polk, or Poli, did not qualitatively affect these TLS events, inactivation of the Rev3 gene coding for a subunit of polymerase ζ or of the Rev1 gene abolished TLS associated with da, but not dT, insertion. The analysis of results of the cellular studies and in vitro TLS studies using purified polymerases has revealed that the insertion of da and dT was catalyzed by different polymerases in cells. While insertion of dT can be catalyzed by polymerase η, κ, and ι, insertion of da is catalyzed by an unidentified polymerase that cannot catalyze extension from the resulting da terminus. Therefore, the extension from this terminus requires the activity of polymerase ζ-REV1. These results provide new insight into how cells use different TLS pathways to overcome a synthesis block.

Endogenous reactive chemicals such as reactive oxygen species and certain lipid peroxidation products are thought to contribute significantly to aging, age-related degenerative diseases, and cancer (1–4). Celluar DNA is one of their targets, and replication of un-repaired DNA damage introduces mutations into the genome, thereby contributing to the aforementioned biological effects. Heptanone-etheno-dC (H-edC)2 (Fig. 1) is one of the substituted etheno-base DNA adducts generated by 4-oxo-2(E)-nonenal, a bifunctional electrophilic lipid peroxidation product derived from both arachidonic acid and linoleic acid (5–8). This adduct, as well as H-edG, serves as a biomarker of lipid peroxidation-mediated DNA damage (5). H-edC and H-edG were both found in the DNA of polyps from a cyclooxygenase-2 up-regulated Min mouse, a colorectal cancer mouse model (9). Our previous study has shown that H-edC blocks DNA synthesis and highly miscodes in human cells (10), raising the possibility that cyclooxygenase-2-mediated lipid peroxidation contributes to colorectal carcinogenesis in Min mice through the formation of DNA adducts. Due to its strong genotoxicity and physiological significance, H-edC is an attractive DNA lesion for the mechanistic study of mammalian translesion DNA synthesis (TLS).

Recently, it was revealed that various specialized DNA polymerases are actively engaged in DNA synthesis across a DNA lesion (11, 12). The catalytic sites of these polymerases are much more spacious than those of replicative polymerases so that they can accommodate a modified template base and an incoming nucleotide (13–15). Accordingly, their fidelity of DNA synthesis is compromised on undamaged template DNA (16–19). These specialized polymerases overcome the blocking effect of a DNA lesion by sacrificing the fidelity of DNA synthesis. They are widely distributed in various living organisms (20), and hence TLS is considered a general response to un-repaired DNA damage. In mammalian cells, polymerase η, polymerase κ, polymerase ι, and REV1 are found. They are structurally related and form a new family of DNA polymerases named the Y family (20). Polymerase η is known to be important for preventing sunlight-induced skin cancer (21), but the physiological roles of polymerase κ and polymerase ι have not yet been clarified. Among the four Y-family polymerases, REV1 is unique in its ability to act as a 2’-deoxyctydyl transferase (22). Although this transferase activity is suspected to play a role in TLS of abasic residues during somatic hypermutation of immunoglobulin genes (23, 24), it does not appear to be important for TLS of other types of DNA damage. This is because (i) the insertion of dC rarely occurs opposite UV-induced lesions, although REV1 is vital in UV mutagenesis, (ii) a catalytically inactive mutant can still support TLS (25), and (iii) a catalytically active mutant cannot always support UV mutagenesis (26). Although the function of REV1 in TLS has not yet been revealed clearly, it is known to cooperate with polymerase ζ (27). Polymerase ζ (28) is another translesion-specialized polymerase, which belongs to the B family (27), and consists of two subunits of REV3 (catalytic subunit) and REV7 (accessory subunit). This polymerase is thought to play a role in extending a primer opposite a lesion rather than in inserting a nucleotide opposite a lesion (29, 30).
A large number of in vitro TLS studies on various DNA lesions have reported the abilities of specialized polymerases to catalyze TLS with varying efficiencies and fidelities. However, it is not clear whether these activities have any significance to TLS across a given lesion in cellular DNA. One of a few examples known in mammalian cells is the critical role of polymerase η to conduct accurate TLS across UV-induced cis-syn cyclobutane pyrimidine dimers (21). Polymerase κ appears to play a critical role in TLS across benzo[a]pyrene DNA adducts (31, 32).

Here, we report the development of a host-vector system that can be used to study cellular roles of specialized polymerases in TLS across a defined DNA adduct. This system was applied to a mechanistic analysis of TLS across an H-ethyl-dC adduct. This study has revealed that two distinct erroneous TLS pathways operate on this adduct in cells: induction of H-ethyl-dC → dT transitions is dependent on polymerase ζ and REV1, whereas that of H-ethyl-dC → dA transversions is not. Possible mechanisms for these two TLS events are proposed based on the results of the cellular and in vitro TLS experiments.

**EXPERIMENTAL PROCEDURES**

**Construction of a Vector**

The replicating vector used for experiments conducted in the mouse embryonic fibroblast (MEF) lines was constructed by replacing the replication cassette consisting of the replication origin and the T antigen gene of BK virus in pBTEX3 (33) with a replication cassette from the mouse polyoma (Py) virus (Fig. 2A). To accomplish this replacement, the AatII-NotI fragment (3.3k bp) of pBTEX3. The NotI and EcoNI ends were made into blunt ends by filling-in reaction catalyzed by the Klenow enzyme, which were then ligated. A BsmBI site overlapping the unique AatII site was destroyed by digesting with AatII followed by trimming of 3′ overhangs by T4 DNA polymerase and subsequent ligation. The new plasmid, pMTEX4, contains blasticidin S- and ampicillin-resistant genes, Py T antigen gene, and four origins (Fl, ColE1, SV40, and Py) of replication.

**Construction of Site-specifically Modified Plasmid**

The synthesis, purification, and characterization of oligonucleotides containing H-ethyl-dC has been described previously (10). A modified 17-mer, 5′-CCATCTCCTCHTACCTTACCT, where H represents H-ethyl-dC, and its complementary 17-mer, 5′-TTC-CAGGTACGTAGGAG, were annealed to form 4-nucleotide overhangs on both ends and mismatches on both sides of and opposite the adduct. The strategy for incorporating this duplex oligonucleotide is shown in Fig. 2B. In step 1, pMTEX4 was digested with AflIII and BsmBI, and a large fragment was purified using a QIAquick PCR purification kit (Qiagen). The 5′-phosphorylated duplex oligonucleotide was ligated to the digested vector at the BsmBI-cleaved site at 4°C overnight (step 1) followed by digestion with Bsal (step 2). Following purification of a large fragment, DNA was subjected to self-ligation to form closed circular DNA (step 3). The ligation mixture was re-digested with AflIII to remove any residual parental plasmid. Closed circular constructs containing a site-specific, single H-ethyl-dC residue were purified by ultracentrifugation in a CsCl-ethidium bromide solution. The amount of modified construct was quantified by a UV spectrophotometer. This resulted in modified DNA, in which the H-ethyl-dC adduct was located 19 nucleotides downstream of a stop codon (TAA in Fig. 2B) of the blasticidin S-resistant gene.

To confirm that ligation of oligonucleotides had been accomplished successfully, the purified construct was digested with BpiI and PspOMI (step 4) and then labeled with [α-32P]dGTP and Klenow enzyme (step 5). Labeled fragments were separated in a denaturing 20% polyacrylamide gel and detected by using a Storm PhosphorImager (Amersham Biosciences). This procedure provided a labeled 35-mer (Fig. 2B, inset).

**Translesion Experiments in Gene Knockout MEFs**

MEFs—The immortalized MEFs used in this study were kind gifts from F. Hanaoka (Osaka University, Polk−/−) (35), H. Ohmori (Kyoto University, Polk−/−) (31), R. Woodgate (National Institutes of Health, Poli−/−) (36), and R. Wood (University of Pittsburgh, Rev3−/−) (37). The development of Rev1−/− mice has been described previously (23). Two independent immortalized Rev1−/− MEF lines (SCDE2 and SCDE4) were established from embryos of 14.5 gestation day by spontaneous immortalization.

**Introduction of Modified Plasmid into MEFs and Recovery of Progeny Plasmids**—Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml) under 5% CO2 at 37°C. Cells (1 × 106) were plated in a 25-cm2 flask and cultured overnight, after which they were transfected overnight with 500 ng of a modified construct by the FuGENE6 (Roche Applied Science) method according to the manufacturer’s instruction. The next day, cells were detached by treating with trypsin-EDTA, seeded in a 150-cm2 flask, and cultured for 4 days. Progeny plasmids were recovered by the method of Hirt (38) and analyzed for translesional events as described below.

**Analysis of Progeny Plasmids for Translesion Events**—To recovered plasmids, 5 ng of pVgRXR (Invitrogen), which coded for Zeocin resistance, was added. This plasmid served as an internal control for DpnI digestion. The mixture was treated with DpnI (1 unit) for 1 h to remove nonreplicated input DNA and then used to transform Escherichia coli DH10BMax elec-
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trocompetent cells [F–, mcrA, Δ(mrr-hsdRMS-mcrBC), δ80lacZΔM15, ΔacX74, deoR, recA1, endA1, araΔ139, Δ(ara, leu)7697, galU, galK, λ–, rpsL, nupG, tonA)] (Invitrogen) by an E. coli Pulser (Bio-Rad). Varying portions of a transformation mixture were plated on YT [1/11003] agar plates with ampicillin (100 g/ml medium) and blasticidin S (50 g/ml medium) or with Zeocin (25 g/ml medium). Because the adduct was located close to the blasticidin S-resistance gene, transformants containing progeny plasmid with large deletions around the adduct site should not grow on a blasticidin-containing plate and were excluded from analysis. A marked reduction in the number of colonies on a Zeocin-containing plate assured efficient digestion of nonreplicated plasmid by DpnI.

E. coli transformants were picked individually and subjected to oligonucleotide hybridization as described in detail previously (39). Oligonucleotide probes shown in Fig. 2 were used to determine the DNA sequence at the adduct site. L14 and R15 probes were used to confirm the presence of oligonucleotide inserts. U17 probe detects progeny derived from an unmodified strand. A17, T16, G16, and C16 probes identify targeted TLS events. Plasmids that did not hybridize to both L14 and R15 probes were omitted from a further analysis. U17 detected progeny derived from the unmodified complementary strand. A17, T16, G16, and C16 detected H- dC → dA, H- dC → dT, H- dC → dG, and H- dC → dC base substitutions, respectively. When plasmids did not hybridize to any of these four probes, DNA sequencing was conducted. More than 30 plasmids among those derived from TLS events were randomly selected for DNA sequencing to confirm the results of hybridization analyses. Thus, this strategy detected all types of events, including base substitutions, frameshifts, deletions, and insertions without any bias.

**Complementation of REV1 Deficiency by the Expression of Human REV1 cDNA**

A cDNA of the human REV1 (cREV1) gene, obtained from Origene, was cloned into pIREsneo2 (Clontech) using standard molecular biology techniques, including restriction enzyme digestion, PCR, and ligation, which created pIREsneo2(cREV1). The DNA sequence of a region amplified by PCR was verified by sequencing. SCDE4 cells were trans-
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TABLE 1
Translesional events in various translesion-specialized DNA polymerase-deficient MEFs

| Knockout gene         | No. of progeny derived from | Nucleotide inserted opposite H-edC | Other events |
|-----------------------|-----------------------------|------------------------------------|--------------|
|                       | UMS*                        | MS*                               | T | A | C | G     |                    |
| None (wild type)      | 208 (78)*                   | 59 (22)                            | 42 (71) | 13 (22) | 0  | 4 (7) | 0              |
| Polh (Expt 1)         | 134 (75)                    | 44 (25)                            | 31 (70) | 11 (25) | 1  (2) | 1 (2) | 0              |
| Polh (Expt 2)         | 187 (71)                    | 78 (29)                            | 47 (60) | 20 (26) | 2  (2) | 9 (12) | 0              |
| Polk                  | 244 (85)                    | 43 (15)                            | 26 (60) | 12 (28) | 3  (7) | 2 (5) | 0              |
| Pol                   | 140 (75)                    | 47 (25)                            | 21 (46) | 20 (43) | 0  | 5 (11) | 0              |
| Rev1 (Polζ)          | 158 (84)                    | 29 (16)                            | 25 (86) | 0  | 0  | 4 (14) | 2              |
| Rev1 (SCDE4)          | 191 (84)                    | 36 (16)                            | 34 (94) | 0  | 0  | 2 (6) | 0              |
| Rev1 (SCDE2)          | 74 (78)                     | 21 (22)                            | 20 (95) | 1 (5) | 0  | 0  | 0              |
| Rev1 (SCDE4) + cREV1  | 224 (84)                    | 44 (16)                            | 29 (66) | 14 (32) | 0  | 0  | 1*             |

* UMS, unmodified strand; MS, modified strand.
+ The numbers in parentheses represent percentages.
- TCHATA → TCAATT (H represents H-edC and an underline indicates a sequence change).
- TCHATA → TCAATA and TACTA.
+ TCHATA → TACATA.

RESULTS

To study the mechanism of mammalian TLS across an endogenous H-edC adduct, a single H-edC was site-specifically inserted into a plasmid vector. The modified vector was allowed to replicate in various MEF lines, in which one of the genes for TLS-specialized polymerases had been inactivated by gene targeting. This approach made it possible to evaluate the significance of each specialized polymerase in the TLS events observed in wild-type MEFs. Furthermore, in vitro TLS experiments using purified polymerases were conducted to complement cellular experiments.

DNA Synthesis Block by H-edC—We placed the lesion in the middle of three consecutive base mismatches. This made it possible to determine the number of progeny plasmid derived from modified and unmodified strands; the ratio of progeny reflects the degree of DNA synthesis blocking. When there is no blocking, the ratio should be 50:50 as revealed with a control construct that had three base mismatches without a lesion (41). DNA repair (removal of a DNA lesion and the two flanking mismatches followed by a gap-filling synthesis) would convert the three nucleotide sequence of the modified strand to the sequence complementary to the unmodified strand, thus losing the strand tag. Because a DNA repair mechanism active on H-edC is not known, the ratio obtained here in MEFs may not reflect a real blocking effect. However, the determined values are still useful in comparing the relative TLS efficiency among various MEFs. In fact, the detection of progeny derived from the H-edC-containing strand at a substantial ratio implies that the single H-edC lesion was not efficiently removed from our construct before TLS took place. The fraction (%) of progeny derived from the modified strand did not markedly decrease in any of gene knockout MEFs when compared with the wild-type MEFs (Table 1). The results indicate that none of the specialized polymerases dominates the entire TLS events.

Coding Specificity of H-edC in MEFs—When L14 and R15 probes were used to confirm the presence of the oligonucleotide inserts, the number of E. coli transformants that did not

20% polyacrylamide gel at 2000 V for 2 h. Gel images were captured by a Storm scanner and analyzed by the ImageQuanNT software package (Amersham Biosciences).
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give positive hybridization signals was small (<4%). These hybridization-negative plasmids most likely contained small deletions or untargeted point mutations in the vicinity of the adduct site. These plasmids were removed from further analysis. All plasmids included in Table 1 were positive in hybridization to both L14 and R15 probes. In wild-type MEFs, the insertion of dT opposite H-edC was dominant (71%) followed by the insertion of dA (22%) and dG (7%). Thus, the overall miscoding frequency was 93% (Table 1). Similar results were obtained with polymerase η- and polymerase κ-deficient MEFs. In polymerase ι-deficient MEFs, the frequency (43%) of dA insertion was somewhat higher than in wild-type, polymerase η-, and polymerase κ-deficient MEFs, reaching close to the level (46%) of dT insertion. This suggests that polymerase ι plays a more important role than do polymerase η and polymerase κ in dT insertion. However, these results, together with the effects on TLS efficiencies (previous section), suggest that polymerase ι, polymerase η, and polymerase κ individually are not essential for TLS across the H-edC lesion. In contrast, the coding events in MEFs deficient in REV1 or REV3 (polymerase ζ) were very different from those in the other MEFs. In these two MEF lines, TLS events accompanied by the insertion of dA opposite H-edC disappeared, and most translesion events were associated with dT insertion (Table 1). The same phenomena were observed in two independent REV1-deficient cell lines, SCDE2 and SCDE4. TLS accompanied by dA insertion was restored by expressing hREV1 in SCDE4 (Table 1). These results indicate that REV1 and polymerase ζ play a critical role in the TLS associated with dA, but not dT, insertion, pointing out that these two TLS events are conducted by different mechanisms.

TLS Studies with Purified Polymerases—The above cellular studies have revealed a vital role of REV1 and polymerase ζ (REV3) in TLS associated with the insertion of dA opposite H-edC. This result clearly indicates that the 2′-deoxyctydyl transferase activity of REV1 does not play any role and that REV1 exerts an unknown second function during this TLS event. Regarding the role of polymerase ζ, two possibilities are envisioned. First, polymerase ζ might catalyze the insertion of dA opposite H-edC, and second, the polymerase might be essential to the extension from a dA terminus that is generated by another polymerase. Since mammalian polymerase ζ has not yet been purified, yeast polymerase ζ was employed to address these possibilities. We examined in vitro the ability of polymerase ζ, as well as human polymerase η, polymerase κ, and polymerase ι, to insert a nucleotide opposite H-edC. H-edC was inserted into the same sequence context as that used in the cellular study. As shown in Fig. 3, polymerase ζ did not incorporate any of four nucleotides opposite H-edC when 1 μl (71 ng, 352 fmol) of the original enzyme solution, as well as diluted solutions (data not shown), was used. The addition of yeast proliferating cell nuclear antigen (214 nM) to a reaction mixture had no effect on the nucleotide incorporation (data not shown). No incorporation of a nucleotide by polymerase ζ opposite this adduct was observed at dNTP concentrations of 10, 50, 100, 500, or 1000 μM (data not shown). These results suggest that polymerase ζ cannot start DNA synthesis across from H-edC. This is consistent with the generally accepted concept that polymerase ζ is poor at inserting a nucleotide opposite a lesion in DNA (29, 30). On the other hand, polymerase κ and polymerase ι inserted mostly dT, which was the major coding event in MEFs. Remarkably, polymerase ζ inserted any of four nucleotides and extended this insertion product with multiple dA or dG homopolymeric stretches.

Because polymerase ζ cannot insert any nucleotide opposite H-edC, this polymerase may specifically be involved in catalyzing the extension from a dA terminus generated by another polymerase. A current model (30) predicts that polymerase ζ-REV1 is recruited to a stalled site to take over a role in extension when an inserter polymerase cannot perform extension following nucleotide insertion. Given that the insertion of dA and dT opposite H-edC is catalyzed by one polymerase, it is reasonable to assume that the dA terminus is more resistant to extension than is the dT terminus; consequently, the extension from the dA terminus requires polymerase ζ-REV1. We addressed this question by comparing the extension from the dT and dA termini, using pol η and pol κ, because they could catalyze extension from these termini in vitro (Fig. 4, A and B). A qualitative gel analysis with polymerase κ unexpectedly showed that extension from the dA terminus was more efficient than that from the dT terminus (Fig. 4B). To confirm this result, kinetic parameters of the extension reaction were determined using polymerase κ and polymerase η (Fig. 5 and Table 2). The extension, as determined by $K_{\text{eff}}$, was twice as efficient with the dA terminus when compared with the dT terminus in experiments using polymerase κ. This resulted mainly from the dif-
ference in $K_{\text{cat}}$ values. Extension from the dA terminus was also more facile when polymerase $\eta$ was used. In this case, $K_{\text{cat}}$ and $K_m$ values both contributed to cause a 2-fold difference. Thus, we did not obtain any evidence indicating that the dA terminus was more resistant to extension by these polymerases than was the dT terminus. Therefore, the requirement of the dA terminus for polymerase $\kappa$-REV1 does not seem to be due to a greater blocking effect on the subsequent extension compared with the dT terminus. This result suggests that the insertion of dA and dT is not catalyzed by the same polymerase. Rather, the two insertion events are catalyzed by two different polymerases: one polymerase (such as polymerase $\kappa$ and polymerase $\delta$) that inserts dT and can catalyze subsequent extension from this dT terminus to complete TLS, and another polymerase that inserts dA, but cannot catalyze the extension, and hence is replaced by polymerase $\zeta$-REV1. Indeed, polymerase $\kappa$ appears to catalyze a complete TLS (Fig. 4C) with dT insertion (Fig. 3).

To obtain support for this concept, we studied the extension from the dA and dT termini, using yeast polymerase $\kappa$ and yeast REV1 (Fig. 6). In this experiment, we observed that majority of extension terminated following the incorporation of one nucleotide (Fig. 6A), which was the correct dG (Fig. 6B), opposite the next template dC. Very little full extension of the two primers to the end of the 28-mer template was observed. Because REV1 was reported to enhance the catalytic activity of polymerase $\zeta$ (42), we also conducted the reaction in the presence of REV1. However, there was no enhancing effect on the extension. Further addition of proliferating cell nuclear antigen (214 nM) to the reaction mixture did not result in any enhancing effect. Again, this one nucleotide extension was found to be more efficient with a dA terminus than with a dT terminus, as was observed in the experiments using polymerase $\kappa$ and polymerase $\eta$.

**TABLE 2**

Extension catalyzed by polymerase $\kappa$ and polymerase $\eta$ from dA and dT termini pairing to H-dC template.

| Enzyme | Terminus | $K_m$ (μM) | $V_{\text{max}}$ (nM/min) | $K_{\text{cat}}$ | $K_{\text{eff}}$ ($K_{\text{cat}}/K_m$) |
|--------|----------|------------|---------------------------|-----------------|---------------------------------|
| Polymerase $\kappa$ | A | 2.5 ± 0.71 | 2.6 ± 0.18 | 0.59 | 0.24 |
| | T | 2.3 ± 0.55 | 1.2 ± 0.06 | 0.26 | 0.11 |
| Polymerase $\eta$ | A | 1.2 ± 0.36 | 0.70 ± 0.04 | 1.9 | 1.7 |
| | T | 1.9 ± 0.77 | 0.50 ± 0.04 | 1.4 | 0.73 |

**FIGURE 5.** Steady-state kinetics of extension from a dA and a dT terminus opposite H-dC catalyzed by polymerase $\eta$ and polymerase $\kappa$. 

**FIGURE 6.** Extension catalyzed by polymerase $\kappa$ and polymerase $\eta$ from dA and dT termini pairing to H-dC template.
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FIGURE 7. TLS model for H-edC (H) adduct. Refer to text for explanation.

insertion could be a replicative polymerase (lower minor pathway of Fig. 7). However, the polymerase cannot extend a primer from this dA terminus and so it disengages from TLS. Under this situation, pol ζ-REV1, which is very adept at elongating DNA from a terminus opposite a DNA lesion and a mismatch (25, 29, 30), is recruited to the dA terminus to complete TLS. However, this TLS is a minor event when a replicative polymerase encounters H-edC. A replicative polymerase largely disengages from DNA synthesis without inserting a nucleotide (dA) (upper major pathway). Upon this blockade, the Y-family polymerases (pol η, pol κ, and pol λ) are recruited to the site to catalyze nucleotide insertion. These three Y-family polymerases insert dT, which is almost exclusive with pol κ and pol λ (Fig. 3). Following the insertion of dT, possibly the same polymerase will catalyze extension from this dT terminus to complete TLS (Figs. 4 and 5). Inactivation of any one of these three polymerases caused no remarkable effect on the frequency of dT insertion (Table 1), which suggests that they complement this task each other. pol λ appears to contribute to this task more significantly than do the other two polymerases, because the frequency of dT insertion is lower in pol λ-deficient MEFs (Table 1). The possible contribution of the three polymerases to this TLS event will be clarified in future experiments using double and triple gene knockout MEFs.

The idea that the incorporation of dA and dT is catalyzed by different polymerases is also supported by qualitative (Fig. 4) and quantitative (kinetic) (Fig. 5 and Table 2) in vitro studies. If the insertion is catalyzed by the same polymerase, extension from a dA:H-edC pair should be more difficult than from a dT:H-edC pair and hence the specialized extension ability of pol ζ-REV1 would be required to complete TLS. The experiments using pol η and pol κ have shown that extension from a dA:H-edC pair is actually more facile than from a dT:H-edC pair (Figs. 4 and 5 and Table 2). These findings suggest that the two incorporations are catalyzed by different polymerases.

Our results have also shown that polymerase κ and polymerase η cannot substitute for polymerase ζ-REV1 in the task of extension from a dA:H-edC pair in cells (Table 1), although they can efficiently catalyze the extension in vitro (Fig. 4). This implies the existence of a cellular mechanism for the exclusive selection of pol ζ-REV1 for extension from a dA:H-edC pair. Although a complete understanding of the mechanism of TLS across the H-edC adduct needs further studies, we have clearly shown the existence of two distinct pathways acting upon the same DNA adduct. Our results illustrate how cells can use different TLS pathways to overcome a synthesis block. This new strategy, in conjunction with the use of single and multiple gene knockout MEFs, will be informative for elucidating the
mammalian TLS mechanisms operating on various DNA adducts.

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