

## Research Article

# Rev1, Rev3, or Rev7 siRNA Abolishes Ultraviolet Light-Induced Translesion Replication in HeLa Cells: A Comprehensive Study Using Alkaline Sucrose Density Gradient Sedimentation

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When a replicative DNA polymerase stalls upon encountering a lesion on the template strand, it is relieved by other low-processivity polymerase(s), which insert nucleotide(s) opposite the lesion, extend by a few nucleotides, and dissociate from the 3'-OH. The replicative polymerase then resumes DNA synthesis. This process, termed translesion replication (TLS) or replicative bypass, may involve at least five different polymerases in mammals, although the participating polymerases and their roles have not been entirely characterized. Using siRNAs originally designed and an alkaline sucrose density gradient sedimentation technique, we verified the involvement of several polymerases in ultraviolet (UV) light-induced TLS in HeLa cells. First, siRNAs to Rev3 or Rev7 largely abolished UV-TLS, suggesting that these 2 gene products, which comprise Pol $\zeta$ , play a main role in mutagenic TLS. Second, Rev1-targeted siRNA also abrogated UV-TLS, indicating that Rev1 is also indispensable to mutagenic TLS. Third, Pol $\eta$ -targeted siRNA also prevented TLS to a greater extent than our expectations. Fourth, although siRNA to Pol $\iota$  had no detectable effect, that to Pol $\kappa$  delayed UV-TLS. To our knowledge, this is the first study reporting apparent evidence for the participation of Pol $\kappa$  in UV-TLS.

## 1. Introduction

Multiple systems have evolved to manage the genomic photoproducts generated by harmful UV light. One such system is nucleotide excision repair (NER), which eliminates photoproducts from DNA strands by dual incision on both sides of a damaged base. The NER system cannot, however, remove all UV-damaged bases. When a replicative DNA polymerase stalls upon encountering a residual photoproduct on the template strand, it is relieved by other low-processivity polymerase(s), which incorporate nucleotide(s) opposite the lesion, extend by a few nucleotides and dissociate from the 3'-OH. The replicative polymerase then resumes DNA synthesis. This process, termed translesion replication (TLS) or replicative bypass (reviewed in [1]), is also one of the subtle systems that have evolved for the management of genomic photoproducts.

UV-C (100–290 nm wavelength) induces 2 main photoproducts [2]: the more frequent cyclobutane pyrimi-

dine dimer (CPD) and the several-fold lower pyrimidine-pyrimidone (6-4) photoproduct ((6-4)PP). *cis-syn* CPD, a predominant form of the multiple configurations, contains 2 adjacent pyrimidines that are covalently linked in parallel. Although the frequency of CPD varies with nucleotide composition, a ratio of T-T to C-T to T-C to C-C of 68 : 13 : 16 : 3 is obtained from UV-irradiated plasmid DNA. Cytosines within CPD are unstable, and are deaminated to uracil or 5-methylcytosine, and further deaminated to thymine [3]. The helical distortion caused by CPD is so inconspicuous that almost half of the lesions remain unrepaired by NER, even 6 hours after UV irradiation in the case of CHO cells [2].

The (6-4)PPs from T-C, C-C, and, less frequently, T-T sequences are detected in UV-irradiated DNA whereas that of C-T are not. In (6-4)PP, linkage between C-6 of one pyrimidine and C-4 of the adjacent pyrimidine cause the 2 bases to be in nearly perpendicular position. Consequently, formation of this lesion causes a major distortion in the double helix. NER preferentially removes (6-4)PP more

rapidly than it removes CPD from the genome in human and rodent cells [4].

At least 5 mammalian DNA polymerases are suggested to be implicated in UV-induced TLS: Pols  $\eta$ ,  $\iota$ ,  $\zeta$ ,  $\kappa$ , and Rev1, all of which belong to the Y family except for Pol $\zeta$  (B family) (reviewed in [1, 5, 6]). However, the participating polymerases and their roles have not been entirely characterized.

Patients with the autosomal recessive disorder, xeroderma pigmentosum variant (XP-V), have a predisposition to skin cancer, and XP-V cells demonstrate hypermutability after UV irradiation (reviewed in [7]). The defective gene in XP-V encodes Pol $\eta$ , which was first purified from a HeLa cell extract as an activity that complements TLS defect in XP-V cell extract [8]. Human Pol $\eta$  catalysed DNA synthesis past TT-CPD very efficiently and in a relatively accurate manner, as demonstrated by the lesion-bypass assay [7, 9]. When template DNA contained a (6-4)TT-PP, Pol $\eta$  incorporated one (random) nucleotide opposite the first thymine and another nucleotide opposite the second thymine of the lesion, but rarely continued across the lesion [7, 9].

Human Pol $\eta$  was also identified via a search for the homolog of yeast *Saccharomyces cerevisiae* *Rad30* gene, which encodes an error-free bypass protein [10]. Various XP-V causative mutations have been found in the Pol $\eta$  gene, *hRAD30A*, of XP-V patients [10, 11].

Pol $\iota$  (*RAD30B*) is the other mammalian homolog of yeast Pol $\eta$ , isolated by a similar approach [12]. In contrast to Pol $\eta$ , Pol $\iota$  is less efficient and less accurate [13].

Pol $\kappa$  was obtained by cloning of a human homolog of the *E. coli* *dinB* gene, encoding DNA Pol IV [14]. Pol $\kappa$  was reported to be unable to bypass either CPD or (6-4)PP [15, 16].

Originally, Rev1, 3, and 7 were cloned from *S. cerevisiae* isolates, in which the frequency of UV-induced reversion from *cyc1* mutations was reduced [17]. Human and mouse homologs (*Rev1*, 3, 7 gene) were later isolated [18–20]. Pol $\zeta$  is a complex of the *Rev3* and *Rev7* gene products, which act as the catalytic and regulatory subunit, respectively. Yeast Pol $\zeta$  is shown to be responsible for 98% of UV-induced base substitutions and 90% of frameshift mutations, in addition to spontaneous mutations [21]. Nonetheless, yeast Pol $\zeta$  itself was revealed to be too faithful to incorporate opposite CPD. Instead, it can efficiently extend from a matched or mismatched 3'-end (reviewed in [5, 6]). Human or mouse Pol $\zeta$  is assumed to have similar enzymatic properties to that of yeast, because several lines of antisense RNA expression or siRNA knockdown experiments in human or mouse cells have proven that Pol $\zeta$  is involved in mutagenic TLS [22–24].

Yeast and human Rev1 encode highly specialized DNA polymerases that preferentially insert a C residue opposite an abasic site in the template. This deoxycytidyl (dC) transferase activity is, however, unlikely to be required for UV-TLS, judging by observation in a yeast *rev1-1* mutant strain [25], which retains much of its dC transferase activity, but has a missense mutation (G193R) in the N-terminal BRCT domain. Rev1 protein also contains ubiquitin-binding motifs (UBMs) that interact with monoubiquitinated PCNA (a DNA polymerase sliding clamp) [26]. In the downstream C-terminal region, Rev1 interacts not only with Rev7 but also

with other bypass-polymerases [27], suggesting that Rev1 acts as a mediator and physical bridges between PCNA and Pol $\zeta$ .

Following DNA damage, such as that caused by UV and MMS, monoubiquitins are conjugated to PCNA arrested at the lesion-site by RAD6/RAD18 and recruit bypass-polymerases [28, 29]. In addition to Rev1, Pols  $\eta$ ,  $\iota$ , and  $\kappa$  possess UBMs and physically interact with PCNA [1, 5, 6]. Stalled replicative Pol $\delta$  is replaced, in turn, with one of these bypass polymerases bound on the PCNA by yet unknown “polymerase switching” mechanisms (reviewed in [1, 30]).

Translesion replication is typically detected with an alkaline sucrose density gradient centrifugation (ASDG) technique. Pulse-labelled replication products are smaller in UV-irradiated XP-V cells than in unirradiated cells; however, on prolonged incubation, the replication products in the irradiated cells eventually attain a high molecular weight similar to that in unirradiated cells. This conversion is interpreted that DNA synthesis is temporarily retarded by UV photoproducts, and then continues beyond the lesion, leaving a gap that is subsequently sealed [31]. The initial size of the newly synthesized DNA is approximately equal to the average distance between lesions in the template strands [32]. This means the gaps in the newly synthesized DNA are opposite the photoproducts [33]. Therefore, sealing of the gaps, by translesion or other postreplication repair mechanisms, can be observed by monitoring the molecular weight of labelled DNA.

Using a modified ASDG technique [34], we precisely detected the elongation of pulse-labelled replication products in the irradiated XP-V cells, showing that UV-TLS is delayed in the cells, but not completely abolished [35]. The marginal TLS is markedly prevented by caffeine at millimolar concentrations, as Lehmann et al. pointed out [31], and by proteasome inhibitors as well (unpublished results). In contrast, these agents do not retard UV-TLS in normal diploid cells. To know more about the inefficient polymerase(s) *in vivo*, we added specific DNA polymerase inhibitors. Butylphenyldeoxyguanosine (BuPGdR) inhibited TLS in XP-V cells [35], suggesting that Pol $\zeta$  may be involved in this Pol $\eta$ -independent bypass.

We recently reported that caffeine or proteasome inhibitors inhibit UV-TLS also in human cancer cells [36], and that, similar to XP-V cells, UV-TLS was much slower than in normal cells. These results suggested that UV bypass in cancer cells is predominantly of the Pol $\eta$ -independent type. Therefore, we expected that Pol $\zeta$  plays a major part in UV-TLS in cancer cells. Although Pol $\eta$  exists in normal quantity in these cells, it was supposed to be inactivated by some reasons. We explored these hypotheses here.

## 2. Materials and Methods

**2.1. Design of siRNAs.** All siRNAs duplexes were synthesized by JBioS (Japan) (Table 1). We designed the sequences for siRNA according to the JBioS guide (<http://www.JBioS.co.jp/RNAselect.htm>). Selection of target sequence for each siRNA proceeds as follows: first, an AAG or AAC sequence is found at least 75 nucleotides (nt) downstream from the start codon; the AAG (or AAC) and the following 18 nt sequence

TABLE 1: siRNAs used.

siRNA	Sense strand <sup>(a),(b)</sup>	Antisense strand <sup>(a),(b)</sup>	Prevention of UV-TLS (result)
siRev3-A	gcuuuacaugagauacaaaTT	uuuguaucauguaaagcTT	significant
siRev3-B	gacaguuuucagucaagauTT	aucuugacugaaaacugucTT	significant
siRev3-C	gagguaugauccugauuuTT	aauaucaggaucauaccucTT	significant
siRev3-D	guauugacuuaugucggauTT	auccgacauaagucaauacTT	significant
siRev3cont-A (6nt mismatches)	<b>gg</b> uuugag <u>ua</u> a <u>auguac</u> gauTT	auc <b>gu</b> acau <u>uuacu</u> caaa <b>acc</b> TT	no effect
siRev3cont-B (4nt mismatches)	<b>gg</b> auugag <u>uu</u> a <u>auguac</u> gauTT	auc <b>gu</b> acauaa <b>acu</b> caau <b>acc</b> TT	no effect
siRev3cont-C (2nt mismatches)	guauugag <u>uu</u> a <u>auguc</u> cgauTT	auc <b>gg</b> acauaa <b>acu</b> caauacTT	no effect
siRev7-A	gauccaggucaucaaggauTT	auccuugaugaccuggaucTT	partial
siRev7-B	gaugcagcuuuacguggaaTT	uuccaccguaaagcugcaucTT	significant
siRev7-C	cacugucugucuaaaauacTT	guauuugagacagacagugTT	significant
siRev7cont-A	gaugcag <u>uu</u> uacgucgaaTT	uuc <b>gac</b> guaa <b>acc</b> ugcaucTT	no effect
siRev1-A	cacauuuuuugccacaaaTT	uuuguggcauuuuuugugTT	significant
siRev1-B	gaagauugaaacggaaaaTT	auuuuuccguuucaauucTT	significant
siRev1-C	ccuucagacugcauuuuuaTT	uaaaauugcagucugaaggTT	significant
siRev1-D	gugugaauugacugaguuuTT	aaacucagucauuacacTT	significant
siRev1cont-E	ccuucacaccgcaac <u>cg</u> uuuTT	uaac <u>cg</u> uugcggugugaaggTT	little to no effect
siPol $\eta$ -4	uaaaccuugugcaguuuaTT	uacaacugcacaaguuuaTT	partial
siPol $\eta$ -A	gaaguuauguccagauuuTT	aagaucuggacauaaucucTT	significant
siPol $\eta$ -B	gcuucgcuuucaucucuuaTT	uaagagauagaaagcgaagcTT	significant
siPol $\eta$ cont-A	uaaaccu <b>gg</b> ugcagucgaaTT	uacgacugcaccagguuuTT	no effect
siPol $\iota$ -A	gccucauacagugaguuuaTT	uaaucucacuguaugaggcTT	no effect
siPol $\iota$ -5	aaguguccacaguugguuTT	auaccaacuguggacacuuTT	no effect
siPolk-A	gagaaaauuacaaaauuTT	uaauuuuguuuuuuucucTT	partial
siPolk-B	gaauaaacaaauggacaaTT	uuguccauuuugguuuuucTT	partial
siPolk-C	cuguuaccuuuaguuuuaTT	uucaacuuaaugguuacagTT	considerable
siPolkcont-B	gagaaa <u>g</u> uaaca <u>g</u> cuuaTT	uaa <u>g</u> cuuguuacuuuuucTT	little to no effect

<sup>(a)</sup>Small letters mean ribonucleotides, and large letters mean deoxyribonucleotides.

<sup>(b)</sup>Mismatched bases are illustrated in bold and italic letters.

(N18) are picked up. Ideally, the 2 nt following the N18 are TT, TN, NT, or AA. GC content of N18 is recommended to be about 40% to 50%. If GC content is less than 30%, or greater than 70%, avoid the sequence. The N18 had better be AT-rich in the 3' half and especially at the 3' end (2 nt).

**2.2. HeLa Cell Culture and siRNA Treatment.** HeLa cells were maintained in monolayers in Dulbecco's modified Eagle's medium (D'MEM) supplemented with 10% fetal calf serum (FCS) ("normal" medium), trypsinized and seeded into culture dishes ( $2 \times 10^5$  cells/ $\phi$  60 mm dish). About 6–8 hours later, the cells were treated with micelles of siRNA and OligofectAMINE (Invitrogen), formed according to the manufacturer's protocol, except Opti-MEM was replaced with D'MEM. The siRNA concentration used in RT-PCR analysis and western blot analysis was 5 nM.

**2.3. UV Irradiation and Translesion Replication.** Forty hours after siRNA addition, HeLa cells were exposed to UV light ( $10 \text{ J/m}^2$ ) from a germicidal lamp (Toshiba GL15) at

$0.6 \text{ J/m}^2$  per s. After 30 minutes in culture, the medium was changed to labeling medium consisting of D'MEM supplemented with 10% FCS and  $10 \mu\text{Ci/mL}$  of [ $U$ - $^{14}\text{C}$ ]thymidine (Moravek MC267,  $470 \text{ mCi/mmol}$ ). UV-irradiated cells were pulse-labelled for 1 hour, while nonirradiated cells were labelled for 30 minutes. The medium was changed to normal medium, and the cells were chased for 5 hours. These cells were harvested by trypsinisation and examined by ASDG [34].

**2.4. Alkaline Sucrose Density Gradient Centrifugation (ASDG).** Cells (about  $1 \times 10^5$  in  $50 \mu\text{L}$  PBS) were gently layered onto  $50 \mu\text{L}$  of 1% sucrose in PBS, which was overlaid on  $100 \mu\text{L}$  of lysis solution (0.6 M KOH, 2.0 M KCl, 10 mM EDTA, and 1% *N*-lauroylsarcosine), which was placed on top of a 4.35 mL alkaline 5–20% sucrose gradient (0.3 M KOH, 2.0 M KCl, 1 mM EDTA, and 0.1% *N*-lauroylsarcosine) with 0.4 mL of alkaline 80% sucrose as a cushion at the bottom. The gradient was centrifuged at 6,000 rpm ( $4,320 \times g$ ) for 15.6 hours at  $15^\circ\text{C}$  in a Beckman SW 50.1 rotor. The gradient was fractionated onto 30 circles of no. 17 paper

(Whatman). The paper circles were dried, immersed in cold 5% trichloroacetic acid for 10 minutes, washed 3 times with ethanol and once with acetone, and dried; radioactivity was then measured. As a molecular weight marker, [ $^{14}\text{C}$ ]-labelled T4 DNA phage particles were placed on the lysis layer and sedimented in a parallel run. The approximate fragment length of each fraction was estimated on the basis of the position of the T4 DNA marker and that of *E. coli* DNA and adjusted by sucrose density curve [34]. Average fragment length (in megabase(Mb)) of each profile is shown in Figures as fragment length of the median fraction [35]. (Median fraction is the middle fraction that separates the higher half of the profile from the lower half.)

**2.5. RT-PCR.** Total RNA was isolated using an RNeasy spin column (QIAGEN). One  $\mu\text{g}$  of total RNA was treated with DNase I (Invitrogen), reverse-transcribed using SuperScript II (Invitrogen) with random hexamers or PrimeScript II (TaKaRa) with oligo(dT) primers, followed by treatment with *E. coli* RNase H (Invitrogen). The PCR mixture contained cDNA templates, dNTPs, rTaq Pol (TOYOBO), anti-Taq (TOYOBO), and appropriate primers. Primers to assess knockdown of each gene were as follows: Rev3, 5'-GGAACGTCAACAGGAGCAAC-3' and 5'-GGAGCAATCCAACACCTGC-3'; Rev7, 5'-TGCTGTCCATCAGCTCAGAC-3' and 5'-AGAGCACTTGGAAATCAGGGC-3'; Rev1, 5'-CTCCTGCAGAGAAACCCCTG-3' and 5'-ACAAGCACTTATGGCACAGCT-3'; Pol $\eta$ , 5'-CCCAGGCAACTACCCAAAAC-3' and 5'-GGGCTCAGTTCCTGTACTTTG-3'; Pol $\iota$ , 5'-ATGATCAAGTGTGCCCCACAC-3' and 5'-ACATGACCCGACACAGTCAC-3'; Pol $\kappa$ , 5'-AGACAAGAATACCGCCAGCC-3' and 5'-AGGAAGGATTATTGCACTTGCC-3'; GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. All PCR reactions were carried out for 27 cycles, with the exception of GAPDH (25 cycles). PCR products were subjected to a MultiNA microtip electrophoresis DNA/RNA analyzer (Shimadzu Biotech).

**2.6. Western Blotting.** Cells were rinsed with PBS, lysed with  $1 \times$  SDS-PAGE loading buffer and collected by scraping. Cell lysate was boiled for 10 minutes, sonicated for 30 seconds and centrifuged at  $20,000 \times g$  for 10 minutes. The supernatant was used for western blotting after measuring protein content by Bradford method. The supernatant (60  $\mu\text{g}$  protein) was separated on 5–20% (Rev7, Rev1 and Pol $\iota$ ) or 3–10% (the rest) SDS-polyacrylamide gels (Wako) and transferred onto Immobilon-P<sup>SQ</sup> membranes (Millipore). The membranes were incubated with antibodies to bypass-polymerases (Rev1, Santa Cruz Biotechnology sc-48806; Rev7, BD Transduction Laboratories 612266; Pol $\eta$ , Santa Cruz Biotechnology sc-17770; Pol $\iota$ , Abnova H00011201-M01; Pol $\kappa$ , a kind gift from Dr. Tomoo Ogi, Nagasaki University), and then with horseradish peroxidase-conjugated secondary antibodies (anti mouse IgG, DAKO PO-0447; anti-rabbit IgG, DAKO PO-0448). Antibodies were diluted with CanGetSignal (TOYOBO) to enhance immunoreactivity. The signals were developed with ECL-Plus (GE Healthcare),

and blots were stripped for reprobing with anti  $\alpha$ -tubulin antibody (Sigma T5168).

### 3. Results

**3.1. Effect of siRNAs to Rev3 or Rev7 on HeLa UV-TLS.** We selected 4 target sites from the human Rev3 mRNA sequence (NCBI locus: NM\_002912), according to the JBioS guide. All the Rev3 siRNAs (Table 1) effectively knocked down expression of Rev3 (Figure 1(a)) and, at 5 nM, abolished UV-TLS in HeLa cells (Figure 1(b)). Replication products immediately after UV irradiation were sedimented as a sharp peak, illustrated by a thin line in Figure 1(b), slightly larger in size than the T4 phage DNA marker. When only Oligofectamine-treated cells were chased in normal medium for 5 hours, the products joined to form larger DNA with lengths in the order of megabases (Mb), illustrated by a thick line. In Rev3 siRNA-transfected cells, the products remain in smaller size, as depicted by a thin line with open marks, demonstrating these siRNAs prevent UV-TLS (Figure 1(b)).

We assessed how many mismatched nucleotides (nt) are necessary at minimum for the negative control siRNA (Figure 1(c)) and found that siRev3cont-C, designed from siRev3-D with 2 nt mismatches, did not prevent UV-TLS, indicating that these Rev3 siRNAs degrade Rev3 mRNA with high specificity. The dose-response profile of siRev3-D, shows that only 1 nM siRNA sufficiently inhibited UV-TLS (Figure 1(d)). The siRev3-D siRNA had no effect on normal replication (Figure 1(e)).

We selected 3 target sites from the human Rev7 mRNA sequence (NM\_006341) (Table 1), and found that all siRNAs effectively reduced Rev7 expression (Figure 2(a)); 5 nM siRev7-B or siRev7-C completely abolished UV-TLS (siRev7-A elicited only partial prevention) (Figure 2(b)). The siRev7cont-A, designed from siRev7-B with 2 nt mismatches, had no effect (ibid). The dose-response profile of siRev7-B shows that 1 nM siRNA was sufficient to inhibit UV-TLS (Figure 2(c)); Rev7 siRNAs had no effect on normal replication (Figure 2(d)).

**3.2. SiRNAs to Rev1 Significantly Abrogated UV-TLS.** For knockdown of Rev1 expression, we selected 4 sites from the human Rev1 mRNA sequence (NM\_016316) (Table 1) (Figure 3(a)). The siRNAs (5 nM) targeted to these sites abolished UV-TLS in HeLa cells (Figure 3(b)). The siRev1cont-E, designed from siRev1-C with 4 nt mismatches, had little to no effect. The dose-response profile of siRev1-C shows that 1 nM of the siRNA was enough to inhibit UV-TLS (Figure 3(c)); siRev1-C or siRev1-D siRNA had no effect on normal replication (Figure 3(d)).

**3.3. SiRNAs to Pol $\eta$  Prevented UV-TLS to a Great Extent.** First, we tested the siRNA described by Choi and Pfeifer [37], siPol $\eta$ -4, which partially inhibited UV-TLS (Figure 4(a)). The negative control, siPol $\eta$ cont-A, was designed from siPol $\eta$ -4 with 2 nt mismatches, had no effect (ibid). We added 2 Pol $\eta$  siRNAs of new design (human Pol $\eta$  mRNA sequence: NM\_006502) (Table 1), and these siRNAs also effectively

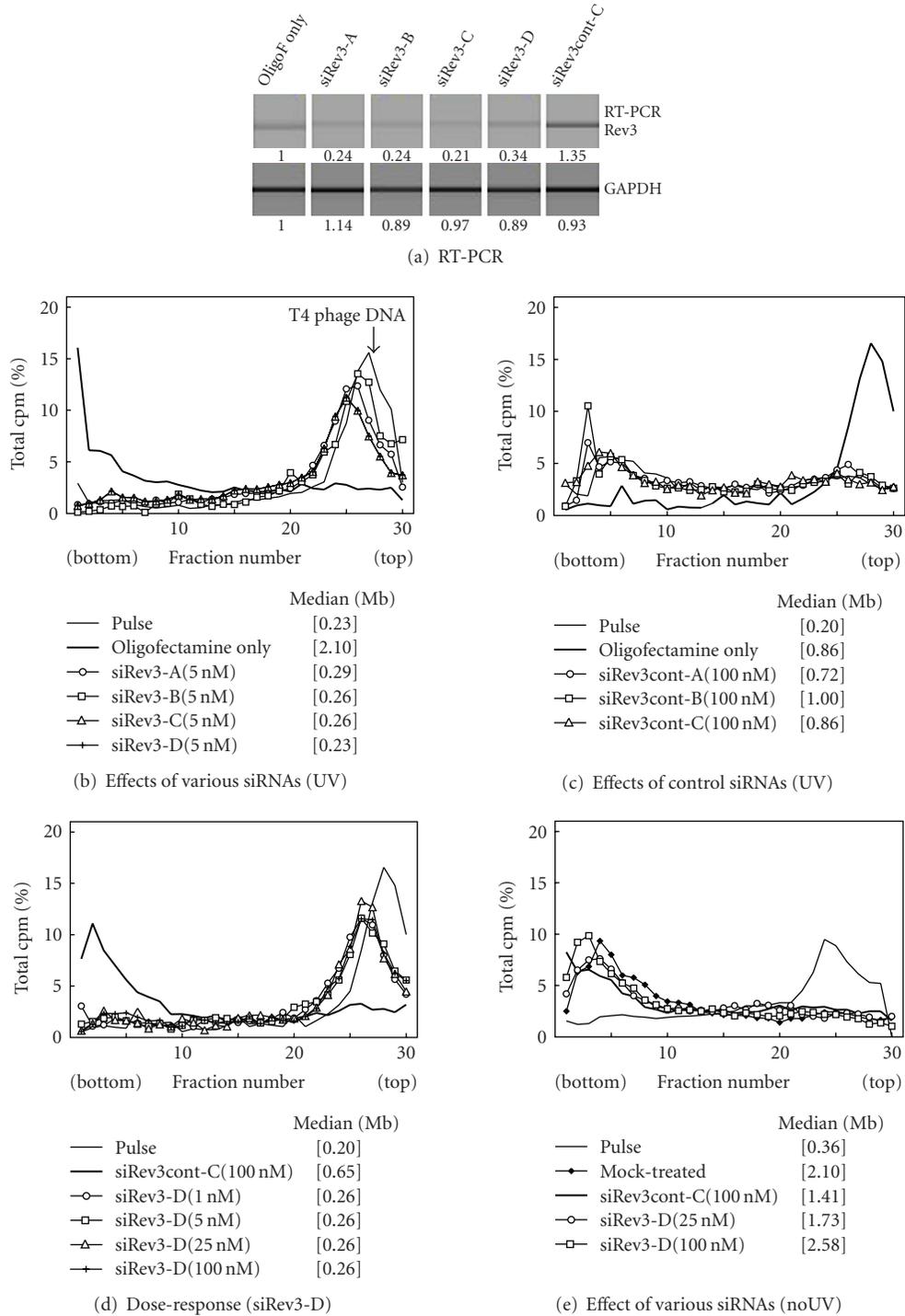


FIGURE 1: Efficient knockdown by Rev3 siRNAs and their effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Rev3 expression (RT-PCR analysis); (b) Effects of various Rev3 siRNAs; (c) Effects of Rev3 control siRNAs; (d) Dose-response of siRev3-D; (e) Effect of various Rev3 siRNAs (no UV control). Twenty-four hours after Rev3 siRNA transfection, total RNA was isolated and Rev3 RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Rev3 siRNA transfection, cells were UV-irradiated ( $10 \text{ J/m}^2$ ), incubated in normal medium for 30 minutes, pulse-labelled with  $10 \mu\text{Ci/mL}$  of [ $^{14}\text{C}$ ]thymidine for 1 hour, washed twice with PBS, and incubated for 5 hours at  $37^\circ\text{C}$  in normal medium (b, c, d). siRev3cont-A, 6 nt mismatches; siRev3cont-B, 4 nt mismatches; siRev3cont-C, 2 nt mismatches (c). Forty hours after Rev3 siRNA transfection, cells were not UV-irradiated, pulse-labelled with  $10 \mu\text{Ci/mL}$  of [ $^{14}\text{C}$ ]thymidine for 30 minutes, washed twice with PBS, and incubated at  $37^\circ\text{C}$  in normal medium for 1 hour (e). Some of these profiles overlap (c, d, e). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately  $5.5 \times 10^7 \text{ Da}$ /single strand). Labelled *E.coli* DNA (approximately 4 Mb) sedimented near the bottom (fractions 3–6) (4). Average fragment length (in Mb) of each profile is shown in square brackets as fragment length of the median fraction.

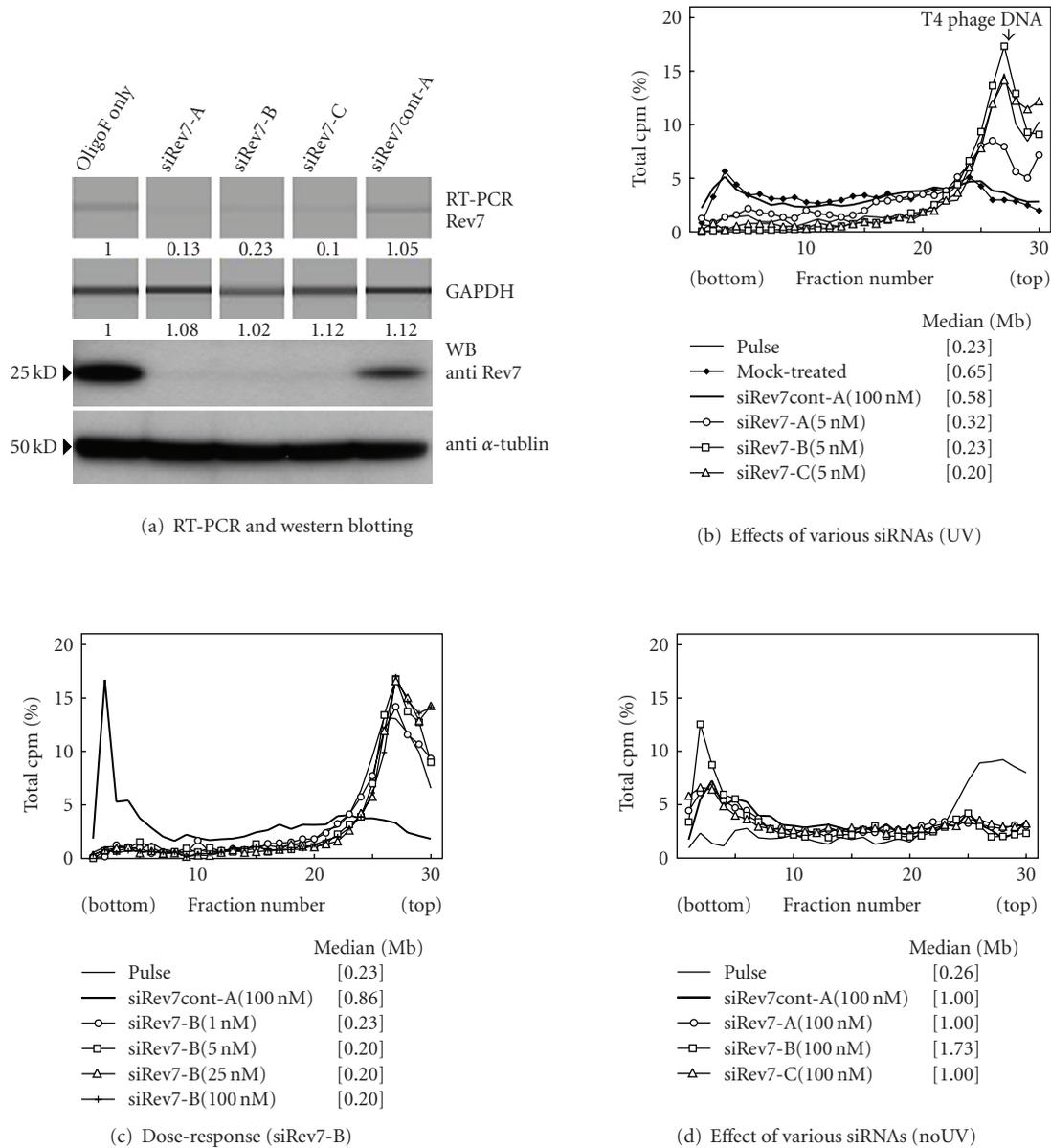


FIGURE 2: Efficient knockdown by Rev7 siRNAs and their effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Rev7 expression (RT-PCR analysis and western blot analysis); (b) Effects of various Rev7 siRNAs; (c) Dose-response of siRev7-B; (d) Effect of various Rev7 siRNAs (no UV control). Twenty-four hours after Rev7 siRNA transfection, total RNA was isolated and Rev7 RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Rev7 siRNA transfection, whole cell extracts were prepared and Rev7 protein was quantified by western blot analysis (a). Forty hours after Rev7 siRNA transfection, cells were UV-irradiated ( $10 \text{ J/m}^2$ ), incubated in normal medium for 30 minutes, pulse-labelled with  $10 \mu\text{Ci/mL}$  of  $[^{14}\text{C}]$ thymidine for 1 hour, washed twice with PBS, and incubated at  $37^\circ\text{C}$  in normal medium (b, c). Forty hours after Rev7 siRNA transfection, cells were not UV-irradiated, pulse-labelled with  $10 \mu\text{Ci/mL}$  of  $[^{14}\text{C}]$ thymidine for 30 minutes, washed twice with PBS, and incubated at  $37^\circ\text{C}$  in normal medium for 1 hour (d). Some of these profiles overlap (c, d). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately  $5.5 \times 10^7$  Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

knocked down Pol $\eta$  expression (Figure 4(b)). These latter siRNAs at 5 nM abolished UV-TLS to a greater extent than siPol $\eta$ -4 (Figure 4(c)). The Pol $\eta$  siRNAs had no effect on normal replication (Figure 4(d)).

3.4. Polk siRNAs Delayed UV-TLS, While siRNAs to Pol $\iota$  Did Not. Although Pol $\iota$  siRNAs efficiently prevented Pol $\iota$  expression (Figure 5(a)), we could not detect these effects on ASDG profiles (Figure 5(b)). The siPol $\iota$ -5 was reported by

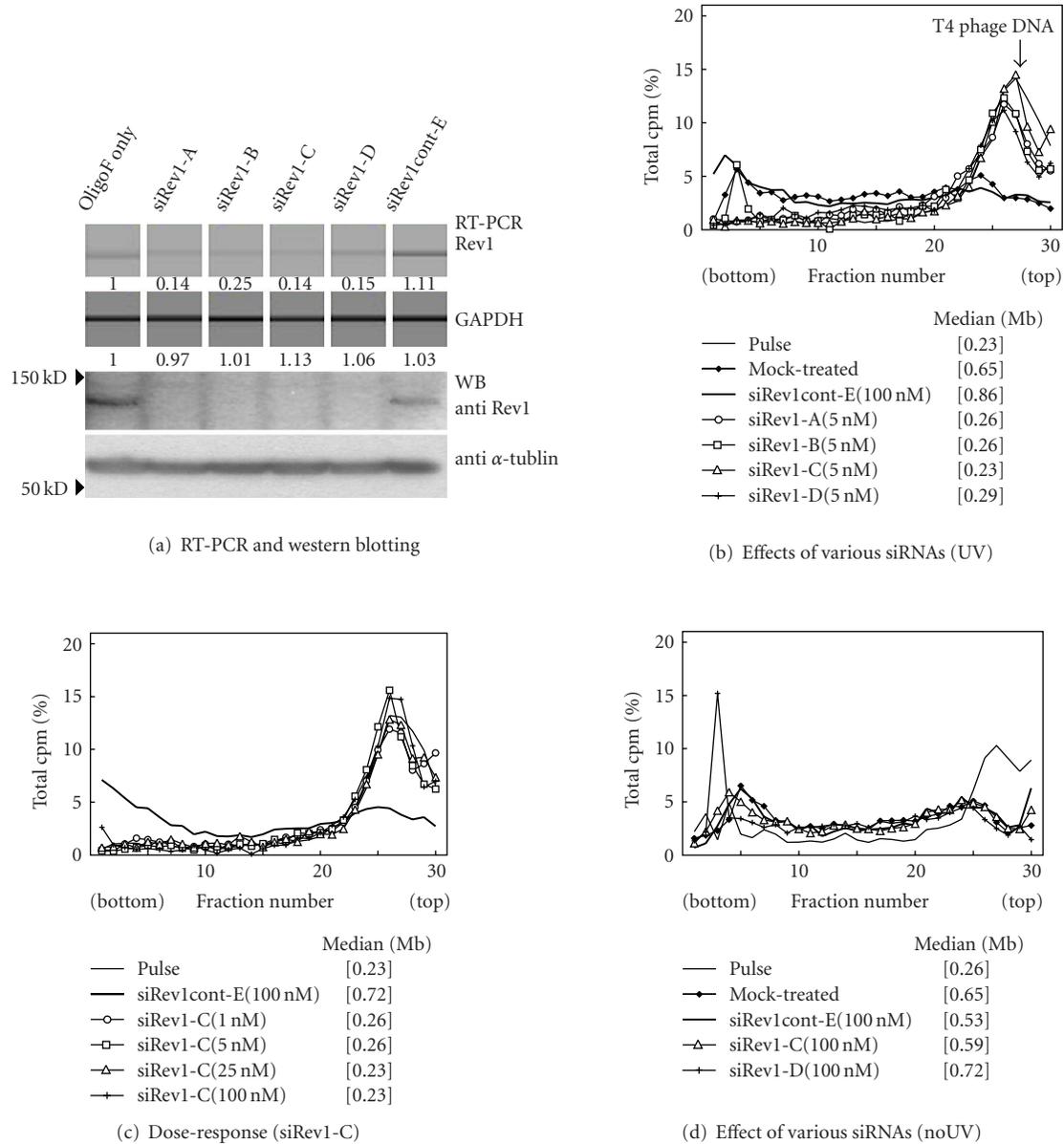


FIGURE 3: Efficient knockdown by Rev1 siRNAs and their effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Rev1 expression (RT-PCR analysis and western blot analysis); (b) Effects of various Rev1 siRNAs on UV-TLS; (c) Dose-response of siRev1-C on UV-TLS; (d) Effect of various siRNAs (no UV control). Twenty-four hours after Rev1 siRNA transfection, total RNA was isolated and Rev1 RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Rev1 siRNA transfection, whole cell extracts were prepared and Rev1 protein was quantified by western blot analysis (a). Forty hours after Rev1 siRNA transfection, cells were UV-irradiated ( $10 \text{ J/m}^2$ ), incubated in normal medium for 30 minutes, pulse-labelled with  $10 \mu\text{Ci/mL}$  of [ $^{14}\text{C}$ ]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at  $37^\circ\text{C}$  in normal medium (b, c). Forty hours after Rev1 siRNA transfection, cells were not UV-irradiated, pulse-labelled with  $10 \mu\text{Ci/mL}$  of [ $^{14}\text{C}$ ]thymidine for 30 minutes, washed twice with PBS, and incubated at  $37^\circ\text{C}$  in normal medium for 1 hour (d). Some of these profiles overlap (b, c). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately  $5.5 \times 10^7 \text{ Da}$ /single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

Choi et al. [38], and the target sequence of siPol $\kappa$ -A was 4 nt downstream from the one reported by Machida et al. [39] (human Pol $\kappa$  mRNA sequence: NM.007195).

We prepared 3 siRNAs for knockdown of Pol $\kappa$  mRNA (NM.016218) (Figure 6(a)). In contrast to siPol $\kappa$ , these Pol $\kappa$  siRNAs (5 nM) delayed UV-TLS in HeLa cells

(Figure 6(b)). The siPol $\kappa$ cont-B, from siPol $\kappa$ -A with 3 nt mismatches, had little to no effect. The dose-response profile of siPol $\kappa$ -A shows that the molecule sufficiently inhibited UV-TLS at 1 nM (Figure 6(c)). The Pol $\kappa$  siRNAs had no effect on normal replication (Figure 6(d)).

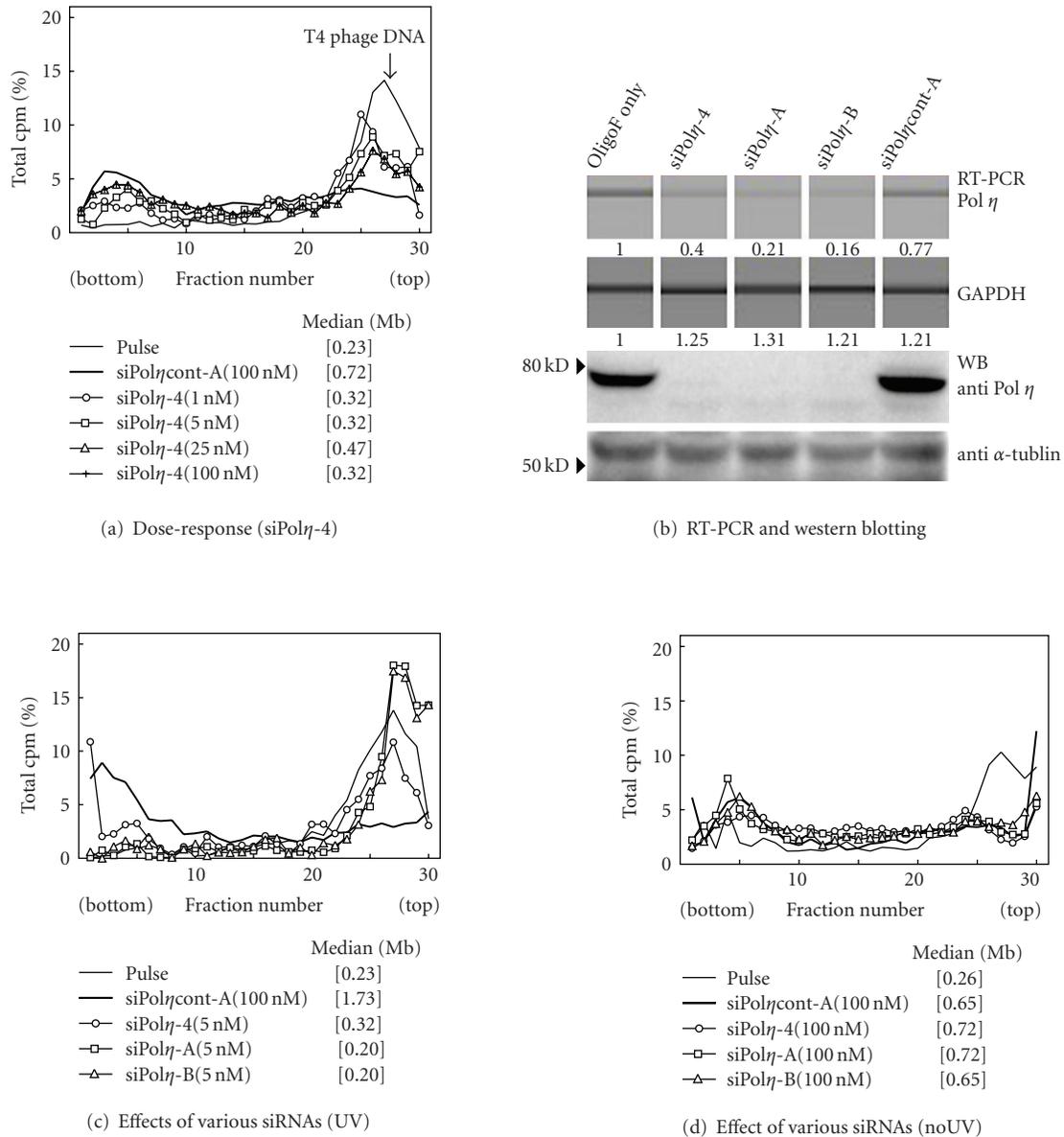


FIGURE 4: Efficient knockdown by Pol $\eta$  siRNAs and the effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Dose-response of siPol $\eta$ -4 on UV-TLS, Efficiency of knockdown on Pol $\eta$  expression; (b) RT-PCR analysis and western blot analysis, (c) Effects of various Pol $\eta$  siRNAs on UV-TLS, (d) Effect of various Pol $\eta$  siRNAs (no UV control). Twenty-four hours after Pol $\eta$  siRNA transfection, total RNA was isolated and Pol $\eta$  RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (b). Forty hours after Pol $\eta$  siRNA transfection, whole cell extracts were prepared and Pol $\eta$  protein was quantified by western blot analysis (b). Forty hours after Pol $\eta$  siRNA transfection, cells were UV-irradiated (10 J/m<sup>2</sup>), incubated in normal medium for 30 minutes, pulse-labelled with 10  $\mu$ Ci/mL of [<sup>14</sup>C]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (a, c). Forty hours after Pol $\eta$  siRNA transfection, cells were not UV-irradiated, pulse-labelled with 10  $\mu$ Ci/mL of [<sup>14</sup>C]thymidine for 30 minutes, washed twice with PBS, and incubated at 37°C in normal medium for 1 hour (d). Some of these profiles overlap (d). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately  $5.5 \times 10^7$  Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

#### 4. Discussion

We verified the involvement of multiple bypass polymerases in UV-TLS in HeLa cells using original siRNAs and ASDG

technique, which is consistent with the recent model of 2 polymerase mechanisms [40, 41]. Rev3 and Rev7, which comprise Pol $\zeta$ , were confirmed to participate in mutagenic UV-TLS. Also, Rev1 was suggested to play an important role

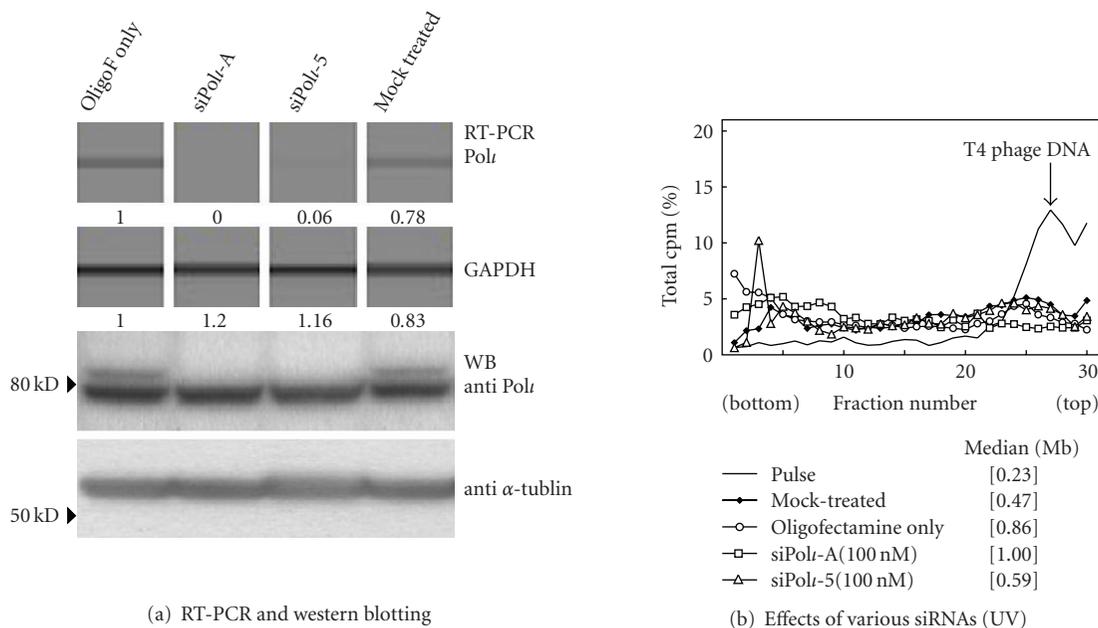


FIGURE 5: Efficient knockdown by *Polι* siRNAs and the effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on *Polι* expression (RT-PCR analysis and western blot analysis); (b) Effects of two *Polι* siRNAs on UV-TLS. Twenty-four hours after *Polι* siRNA transfection, total RNA was isolated and *Polι* RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after *Polι* siRNA transfection, whole cell extracts were prepared and *Polι* protein was quantified by western blot analysis (a). Forty hours after *Polι* siRNA transfection, cells were UV-irradiated ( $10\text{ J/m}^2$ ), incubated in normal medium for 30 minutes, pulse-labelled with  $10\ \mu\text{Ci/mL}$  of [ $^{14}\text{C}$ ]thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at  $37^\circ\text{C}$  in normal medium (b). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately  $5.5 \times 10^7$  Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

in human TLS, although in avian DT40 cells, Rev1 may have a distinct role [42]. We were surprised to find that siRNAs against *Polη* prevented TLS to a great extent. TLS was delayed in *Polκ* siRNA-transfected cells, but not in si*Polι*-transfected cells.

We anticipated a limited participation of *Polη*, because UV-TLS in HeLa cells is very slow (i.e., inefficient) and caffeine-sensitive [35]. However, siRNAs against *Polη*, particularly si*Polη*-A and si*Polη*-B, prevented TLS to a great extent. Since both Rev3 and Rev7 siRNAs also significantly abolished UV-TLS, these results suggest that the *Polζ*-dependent TLS pathway and the *Polη*-dependent process are not mutually exclusive but overlapped.

Enzymology of yeast *Polζ* revealed that this polymerase is too faithful to insert nucleotides opposite a CPD, although it efficiently extends from a matched or mismatched 3' end [5, 6]. Therefore, we assumed that mutagenic (error-prone) TLS proceeded through the insertion by *Polι* or *Polκ* of mismatched nucleotides opposite UV photoproducts, followed by extension by *Polζ*. Our data showed, however, that si*Polι* had no effect, and si*Polκ* partially prevented TLS. These results suggest that in some cases, *Polη*, and to a lesser extent *Polκ*, may insert nucleotide(s) opposite UV photoproducts, followed by extension by *Polζ*.

*Polη* is capable of bypassing a CPD without aid of other TLS polymerases. Both yeast and human *Polη*, however, incorporate wrong nucleotide at a fairly high rate and can

extend these mismatched primer termini with only a frequency of  $\sim 10^{-2}$  to  $10^{-3}$  relative to extension from matched primer termini [6, 43]. Plausibly, *Polη* dissociates from there and the proof-reading exonuclease of *Polδ* removes the wrong nucleotide [44]. To the primer termini, *Polη* is recruited again and incorporates a new nucleotide. This cycle is repeated until *Polη* incorporates a correct nucleotide. We suppose that disruption or malfunction of this cooperation renders mismatched primer termini accessible to *Polζ*.

Recently, Yoon et al. published 2 papers describing the effects of siRNA knockdown on the efficiency of TLS at TT-CPD [45] or (6-4)TT PP [46] on duplex plasmids in human cells. They also reported the effects of siRNA knockdown on mutation frequencies in the  $\lambda$  phage *cII* gene lysogenized in mouse cells expressing a (6-4)PP photolyase [45] or CPD photolyase [46]. The results of this tremendous and detailed study demonstrated that *Pols η, κ, and ζ* contribute to CPD bypass, wherein *Pols κ* and  $\zeta$  promote mutagenic TLS and *Polη* executes error-free bypasses (*Polι* siRNA had no effect) [45]. As for (6-4)PP bypass, *Pols η* and  $\iota$  provide alternate pathways for mutagenic TLS, and *Polζ* acts in a predominantly error-free manner (*Polκ* siRNA had no effect) [46].

The participation of *Pols κ* or  $\iota$  in CPD bypass was similarly demonstrated by our results and those of Yoon et al. [45]. Because (6-4)PP is a minor photoproduct, which is removed predominantly by NER, and because HeLa cells

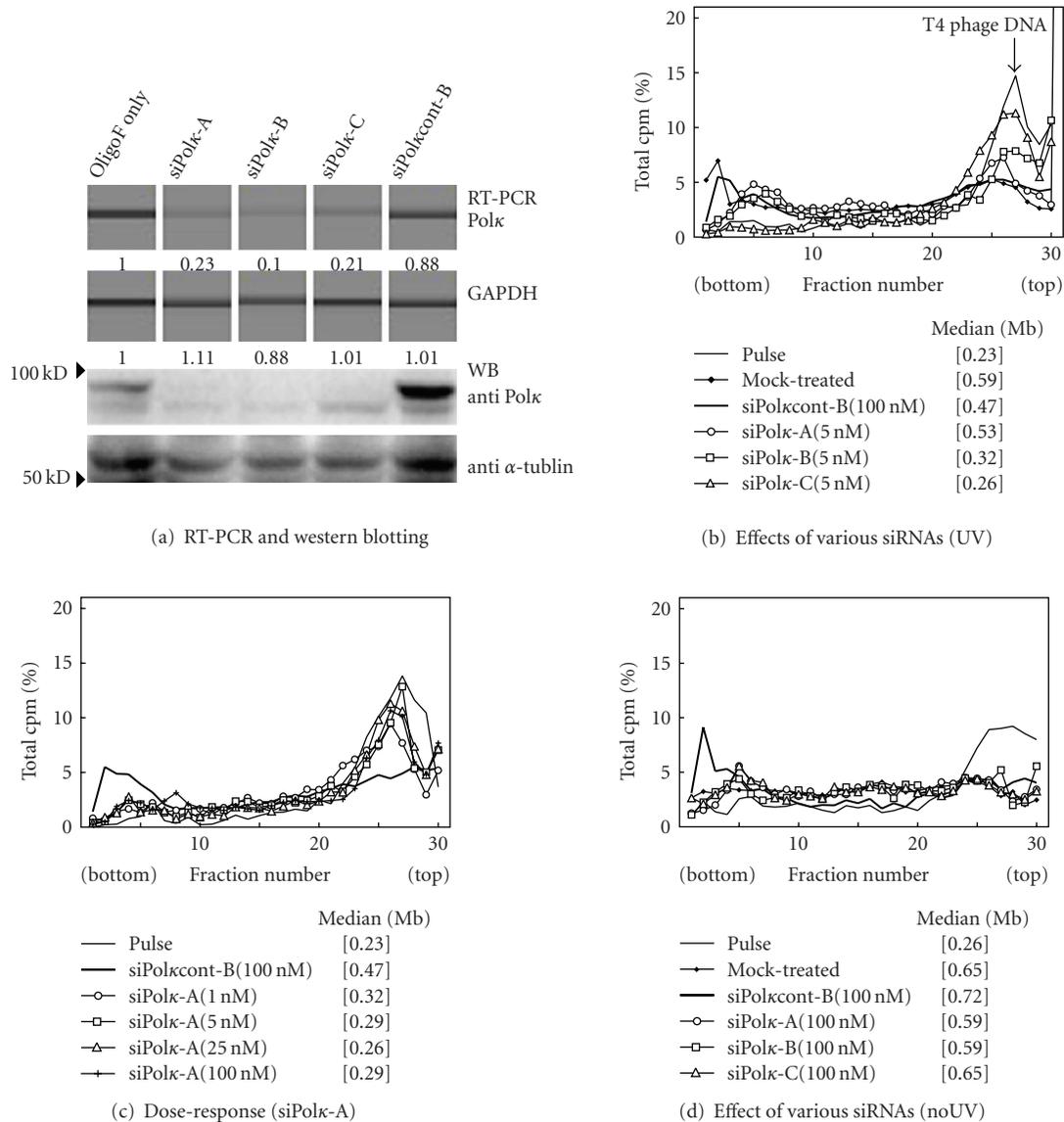


FIGURE 6: Efficient knockdown by *Polk* siRNAs and the effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on *Polk* expression (RT-PCR analysis and western blot analysis), (b) Effects of various *Polk* siRNAs on UV-TLS, (c) Dose-response of siPolk-A on UV-TLS, (d) Effect of various *Polk* siRNAs (no UV control). Twenty-four hours after *Polk* siRNA transfection, total RNA was isolated and *Polk* RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after *Polk* siRNA transfection, whole cell extracts were prepared, and *Polk* protein was quantified by western blot analysis (a). Forty hours after *Polk* siRNA transfection, cells were UV-irradiated ( $10 \text{ J/m}^2$ ), incubated in normal medium for 30 minutes, pulse-labelled with  $10 \mu\text{Ci/mL}$  of  $^{14}\text{C}$ thymidine for 1 hour, then washed twice with PBS, and incubated for 5 hours at  $37^\circ\text{C}$  in normal medium (b, c). Forty hours after *Polk* siRNA transfection, cells were not UV-irradiated, pulse-labelled with  $10 \mu\text{Ci/mL}$  of  $^{14}\text{C}$ thymidine for 30 minutes, washed twice with PBS, and incubated at  $37^\circ\text{C}$  in normal medium for 1 hour (d). Some of these profiles overlap (c, d). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately  $5.5 \times 10^7 \text{ Da}$ /single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

possess high NER activity (unpublished observation), it is reasonable to conclude that our phenomena observed in HeLa cells by ASDG are largely attributable to CPD, although we have not yet determined the extent of remaining (6-4)PP.

We may also conclude that Rev1 is indispensable for TLS across CPD. Thus far, it is unknown if Rev1 is equally involved in TLS across CPD and (6-4)PP, or if it exhibits

some preference. Nelson et al. [25] demonstrated that Rev1p participates in UV-TLS across (6-4)PP, based on yeast transfected with a (6-4)PP-carrying plasmid; only slight differences were observed with a CPD-carrying plasmid.

*In vitro* lesion-bypass assay showed that Pol $\eta$  alone accomplishes bypass across TT-CPD as above (i.e., both insertion and extension) [8, 9]. However, Yoon et al.

presented complex results showing involvement of multiple bypass polymerases. They used SV-untransformed XP-A and XP-V cells, but did not include SV-untransformed normal fibroblasts, wherein we detected quick and caffeine-insensitive UV-TLS [35, 36]. It is possible that the kind of damage, as well as cell status (normal, transformed, or cancerous) may determine the participation of bypass polymerase(s).

We have presented the first apparent evidence that Pol $\kappa$  participates in UV-TLS. Pol $\kappa$  knockout mouse embryonic cells are known to be UV sensitive [47], but the mechanism had not yet been determined. Pol $\kappa$  is also thought to play a part in the repair-synthesis step of NER [48, 49]. From the results of lesion-bypass assays, human Pol $\kappa$  was suggested to be unable to bypass CPD or (6-4)PP. Because the outcomes of such *in vitro* assays depend on the assay conditions [12], these results must be validated *in vivo*, such as by ASDG analysis.

## 5. Conclusions

Using siRNAs originally designed and ASDG technique, we verified the participation of multiple bypass polymerases in UV-induced TLS in HeLa cells, which is consistent with recent model of 2 polymerase mechanisms. UV-TLS was largely abolished by siRNAs to Rev3 or Rev7, suggesting that these 2 proteins, which constitute Pol $\zeta$ , play a primary role in mutagenic TLS. Rev1-targeted siRNAs also significantly abolished UV-TLS, consistent with prior suggestions that Rev1 is indispensable in mammalian mutagenic TLS. Unexpectedly, siRNAs to Pol $\eta$  prevented TLS to a great extent, implying that the Pol $\eta$ - and Pol $\zeta$ -dependent processes do not alternate but overlap. Pol $\kappa$  siRNAs, but not siRNAs to Pol $\iota$ , delayed TLS; this is the first apparent evidence for the participation of Pol $\kappa$  in UV-TLS.

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