MOLECULAR AND CELLULAR BIOLOGY, July 2005, p. 6103–6111 Vol. 25, No. 14
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.14.6103–6111.2005

Multiple Roles of Vertebrate *REV* Genes in DNA Repair and Recombination†

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Received 8 September 2004/Returned for modification 24 November 2004/Accepted 5 April 2005

In yeast, Rev1, Rev3, and Rev7 are involved in translesion synthesis over various kinds of DNA damage and spontaneous and UV-induced mutagenesis. Here, we disrupted Rev1, Rev3, and Rev7 in the chicken B-lymphocyte line DT40. *REV1*$^{-/-}$ *REV3*$^{-/-}$ *REV7*$^{-/-}$ cells showed spontaneous cell death, chromosomal instability/fragility, and hypersensitivity to various genotoxic treatments as observed in each of the single mutants. Surprisingly, the triple-knockout cells showed a suppressed level of sister chromatid exchanges (SCEs), which may reflect postreplication repair events mediated by homologous recombination, while each single mutant showed an elevated SCE level. Furthermore, *REV1*$^{-/-}$ cells as well as triple mutants showed a decreased level of immunoglobulin gene conversion, suggesting participation of Rev1 in a recombination-based pathway. The present study gives us a new insight into cooperative function of three Rev molecules and the Polζ (Rev3-Rev7)-independent role of Rev1 in vertebrate cells.

Homologous DNA recombination (HR) is associated with various cell functions. It is involved in the repair of DNA double-strand breaks (DSBs), which may be caused by ionizing radiation (IR) or DNA interstrand-cross-linking agents, and which may lead to cell death if left unrepaired. HR also functions as a part of postreplication repair (PRR), avoiding DNA replication blocks induced by various endogenous or environmental genotoxic stresses. Mating-type gene switching in yeast and diversification of the immunoglobulin gene (*Ig*) in the immune cells of some vertebrate species are partly made through gene conversion events mediated by HR (15, 37). In germ cells, HR is crucial to meiotic recombination, the process that increases genetic variation within a species (18).

In yeast, the major components of PRR are divided roughly into two groups: translesion DNA synthesis (TLS) involving the Rad6-Rad18 epistasis group and HR composed of the Rad52 epistasis group (reviewed in references 4 and 10). TLS allows tolerance of DNA damage, employing a number of specialized DNA polymerases that are able to synthesize directly across template DNA lesions (reviewed in references 13 and 17). Vertebrate TLS polymerases have been reported to have biochemical functions basically similar to those of their yeast homologs. However, the biological role of the TLS polymerases is complicated and poorly characterized in vertebrate cells. Similarly, DNA polymerases involved in HR are only poorly understood even in yeast (reviewed in reference 16).

REV genes were originally identified as responsible genes for reversible mutation of UV light-induced mutagenesis in the yeast *Saccharomyces cerevisiae* (21, 23). Longstanding work in yeast and recent studies in vertebrates have revealed that Rev1 is one of the Y-family DNA polymerases (34), having deoxycytidyl transferase activity (31), and that Rev3 and Rev7 are the catalytic and regulatory subunits of DNA polymerase ζ (Polζ) capable of bypassing a UV-induced thymidine dimer (32; reviewed in references 20 and 30). The three yeast *rev* mutants and their triple mutant show very similar sensitivity to various genotoxic treatments. However, there is also a distinct phenotypic difference between the yeast *rev1* mutant and the other *rev* mutants; for example, UV-induced frameshift mutagenesis depends much more on Rev3 and Rev7 than on Rev1 in yeast (22).

The vertebrate Rev1 and Rev3 molecules are significantly larger than their yeast counterparts, suggesting that the vertebrate enzymes may have additional functions despite similarities in their basic biochemical behavior (12, 25). Indeed, biochemical studies indicate that mammalian Rev1 can physically interact with Rev7 (26, 29) or with other Y-family polymerases, such as Polκ, Polι, and Polη (14) via a C-terminal region that is lacking in yeast Rev1. Another difference between vertebrate and yeast Rev functions is that the contribution of Polζ to tolerance of DNA damage appears to be significantly greater in vertebrates than in budding yeast. Although mutation of yeast Rev3 has comparatively little impact on viability or sensitivity to genotoxic agents, disruption of Rev3 in mice causes embryonic lethality (2, 9, 46) and significant genome instability and extreme hypersensitivity to killing by a variety of genotoxic agents, particularly, cisplatin in chicken DT40 (42). Vertebrate

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† Supplemental material for this article may be found at http://mcb.asm.org/.

Rev7 has homology with vertebrate Mad2 to a similar extent with yeast Rev7 (7, 28), also suggesting that it might act as a spindle checkpoint protein (8, 36).

The chicken B-lymphocyte line DT40 is characterized by a high efficiency of gene targeting and phenotypic stability (6, 41), providing us with a unique opportunity of dissecting the mechanism of HR by comparing the phenotypes of a variety of HR mutants (3, 40, 45). As suggested from yeast and vertebrate studies mentioned above, the vertebrate Rev molecules are supposed to have distinct as well as collaborative function. To understand the genetic interrelationship among the three Rev molecules in the higher eukaryote, we generated *REV7* gene-disrupted cells (*rev7* cells) and triple mutants of the three *REV* genes (*rev1 rev3 rev7*) in DT40. In this study, we reveal that the three Rev molecules may act as a functional unit in cellular tolerance to a variety of genotoxic stresses, including IR, but that Rev1 acts independently of Rev3-Rev7 in HR-dependent diversification of the *Ig* gene variable (*Ig V*) region: i.e., *Ig* gene conversion. We also present the nonredundant, overlapping role of these Rev molecules in the HR-mediated PRR: i.e., sister chromatid exchange (SCE), in vertebrate cells.

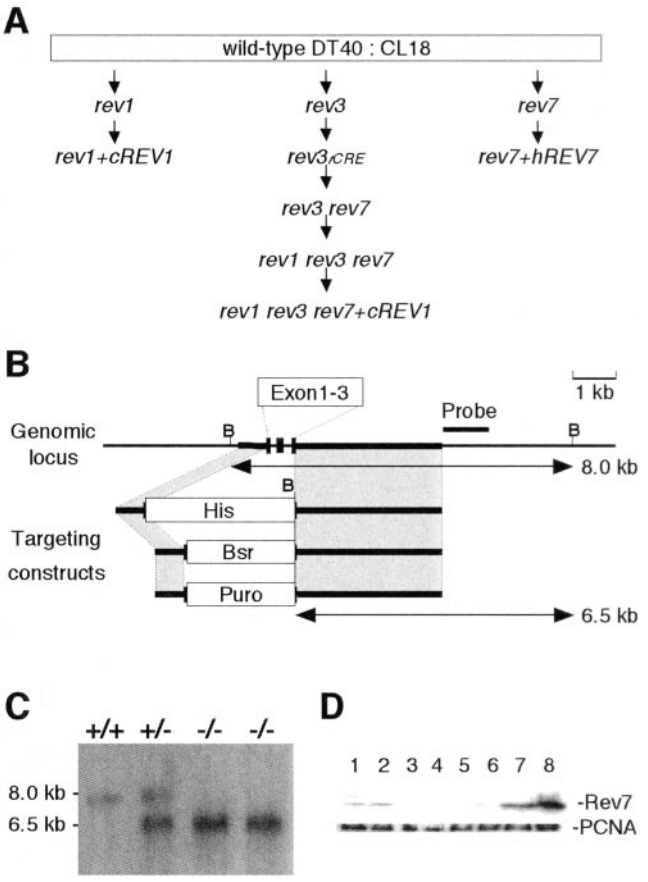


FIG. 1. Experimental strategy and gene targeting of *REV7* locus. (A) Functional analysis of *REV1*, *REV3*, and *REV7* by comparing the wild type and *REV*-disrupted and *REV*-reconstituted mutants. *rev3/CRE* indicates a *rev3* mutant that expresses Cre recombinase-estrogen receptor fusion protein (CreER) to remove drug selection markers. (B) Configuration of the chicken *REV7* genomic locus and the gene disruption constructs. Solid boxes indicate the positions of exons that were disrupted. B indicates relevant BamHI restriction sites. BamHI digestion causes 8.0-kb and 6.5-kb fragments in wild-type and targeted alleles, respectively. (C) Southern blot of genomic DNA from wild-type (+/+), $REV7^{+/-}$ (+/−), and *rev7* (−/−) clones digested with BamHI and hybridized with the probe indicated in panel B. (D) Western blot of whole-cell lysate from cells of each genotype treated with anti-human Rev7 rabbit antibody. Lane 1, $REV7^{+/-}$; 2, wild type; 3, *rev7*#1; 4, *rev7*#2; 5, *rev1 rev3 rev7*#1; 6, *rev1 rev3 rev7*#2; 7, *rev7+hREV7*; 8, Ramos (human B-cell line).

MATERIALS AND METHODS

Cloning of the chicken *REV1* and *REV7* genes. Chicken *REV1* partial cDNA sequences were obtained by chicken BLAST search with human *REV1* sequence. For determination of the unknown rest of the sequences, 5′-cDNA fragments were amplified from our rapid amplification of cDNA ends (RACE) cDNA library derived from chicken testis total RNA with the Marathon cDNA amplification kit (Clonetech) by reverse transcription-PCR with Pyrobest polymerase (Takara, Kyoto, Japan) using AP1 primer and the primer 5′-GCTGGGGGTT TGCACCAGGGCG-3′ designed from the database-obtained sequences. Chicken *REV1* complete cDNAs were amplified with Pyrobest using the primers 5′-ggacgcggcggaagaagcttcagtATGAGG-3′ and 5′-caagtTCAGATAACTTTTA ATGTGCTTCCG-3′ (coding sequences are denoted in uppercase) and cloned into pCR-BluntII vectors (Invitrogen).

Chicken *REV7* partial cDNA sequences were also obtained by chicken BLAST search with human *REV7* sequence. cDNA fragments containing undetermined 5′ sequences were amplified from LambdaZAPII chicken testis cDNA library by reverse transcription-PCR with XL polymerase (Perkin-Elmer) using the M13R primer and the primer 5′-GGGTGATTTCGAAGACAAATC-3′ designed from the obtained sequences. Eight amplified PCR products were cloned into the pGEM-T Easy vector (Promega), and the complete sequences were determined.

Chicken *REV7* genomic DNAs were amplified from the EMBL3 chicken genomic DNA library (Clontech) with LA-Taq (Takara) using the primers designed from the cDNA sequence. The positions of exons and introns were determined by base sequencing.

Plasmid construction. Three *REV7* disruption constructs, *REV7-hisD*, *REV7-bsr*, and *REV7-puro*, were generated from genomic PCR products combined with *hisD*, *bsr*, and *puro* selection marker cassettes. Genomic DNA sequences were amplified from DT40 genomic DNA and inserted unique restriction sites near the 5′ and 3′ ends using the following primers: for the left arm (0.6 kb), 5′-AG CTTCTAGACTGGAGGCGTTTAAGGACAT-3′ (denoted XbaI site with underlined) and 5′-AGCTGGATCCTTAAGGTCCTGTCGTGTGAG-3′ (BamHI site); and for the right arm (3.5 kb), 5′-AGCTGGATCCTGCACTGTGTAAA GCCACTG-3′ (BamHI site) and 5′-AGCTGGTACCTACCTCAGGATTGTG ACCCG-3′ (KpnI site). Amplified PCR products for each arm were cloned into pGEM-T Easy vector (Promega), and SalI-BamHI fragment containing the left arm was inserted into the right arm vector, and then each selection marker cassette flanked by BamHI site was inserted. *REV1* disruption constructs *REV1-neo* and *REV1-puro* and *REV3* disruption constructs *REV3-his/loxP* and *REV3-bsr/loxP* were generated as described previously (39, 42). Modified *REV1* disruption constructs *REV1-hisD*, *REV1-bsr*, and *REV1-hygro* were made from *REV1-neo* by replacing the 1-kb short arm with a new 3.5-kb genomic fragment in order to improve targeting efficiency and by alternating each selection marker cassette. The new 3.5-kb fragments were obtained by PCR with primers 5′-GGGGGAT CCAAAGCAGGCAGGTCTGCACTTAAAACTG-3′ (BamHI site) and 5′-GG GCTCGAGTCTGGTAATATTGCCATTCAAGCTGGG-3′ (XhoI site). The human *REV7* expression construct, *p345*, was generated from pEGFP-N2 (Clontech), in the EcoRI site of which a human *REV7* cDNA (containing stop codon) was inserted. The chicken *REV1* expression construct, pCR3-*loxP-cREV1*/IRES-*EGFP-loxP* (named *p667* in short), in which chicken *REV1* and eukaryotic green fluorescent protein (*EGFP*) genes are flanked by the *loxP* sequences, was made by inserting a chicken *REV1* cDNA into the BamHI-XhoI site of pCR3-*loxP*-MCS/IRES-*EGFP-loxP* (11, 47). The plasmids were linearized prior to transfection into DT40 cells; *REV7-hisD*, *REV7-bsr*, *REV7-puro*, *p345*, and *p667* were digested with KpnI, ScaI, KpnI, NotI, and PvuI, respectively. *REV1-hisD*, *REV1-bsr*, and *REV1-hygro* were all linearized with NotI.

Cell culture and DNA transfection. The conditions for cell culture, selection, and DNA transfections have been described previously (42, 44).

Generation of mutant cells. CL18 is a wild-type (WT) DT40 clone which is negative in surface IgM (sIgM) and is characterized its sequence at the *IgL* locus (5). CL18 cells were sequentially transfected with *REV1-hisD* and *REV1-bsr* targeting constructs to obtain new *rev1* clones (Fig. 1A). Also CL18 cells and *rev3/CRE* cells without a drug-resistant cassette (42) were sequentially trans-

fected with *REV7-hisD* and *REV7-bsr* constructs to generate *rev7* and *rev3 rev7* cells, respectively. *rev7+hREV7* cells were obtained from *rev7* cells by transfection with the *p345* expression vector. *rev1 rev3 rev7* cells were derived from *rev3 rev7* cells by successive transfection with *REV1-puro* and *REV1-neo* constructs (39). *rev1* and *rev1 rev3 rev7* cells were transfected with *p667*, resulting in *rev1+cREV1* and *rev1 rev3 rev7+cREV1* cells, respectively (Fig. 1A). *ATM*$^{-/-}$ (*atm*) and *rad18* cells were described previously (27, 47). All the mutant DT40 clones described in this paper are deposited in the RIKEN Cell Bank (RIKEN, Wako, Japan).

Flow cytometric analyses of cell cycle progression and mitotic index. The experimental methods for cell count, cell cycle analysis, and green fluorescent protein (GFP) expression were described previously (44). Analyses of G_2/M DNA damage checkpoint and the spindle assembly checkpoint using mitotic index measurement were also described previously (42). Briefly, to analyze the mitotic DT40 cells, a subconfluent culture (2×10^5 to 5×10^5/ml) was subjected to genotoxic treatment and was incubated with 0.1 μg/ml Colcemid (Gibco-BRL, Grand Island, NY) and then harvested each hour for 6 h and fixed with 70% ethanol. Fixed cells were treated with rabbit anti-phospho-histone H3 (Ser10) monoclonal antibody (Upstate) and subsequently with fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (Southern Biotech.). The cells were resuspended in phosphate-buffered saline (PBS) containing propidium iodide (PI) at 5 μg/ml for subsequent analysis with FACScaliber (Becton Dickinson).

Colony formation assay. The colony formation assay was performed as described previously (35), with the following modification in exposure to UV and hydrogen peroxide. UV (wavelength = 254 nm) irradiation was performed without medium exchange for PBS since the treatment of *rev* mutants with PBS causes the significant reduction of cell viability. UV dose applied to the cells within complete medium was estimated and calibrated accordingly. Hydrogen peroxide (H_2O_2; 30% [wt/vol]; Santoku, Japan) was appropriately diluted with distilled water just before use. For treatment of cells with H_2O_2, 1×10^5 cells were incubated at 39.5°C in 1 ml of complete medium containing a pertinent concentration of H_2O_2 for 1 h. Then cells were serially diluted and plated in triplicate onto six-well plates with 5 ml/well of 1.5% (wt/vol) methylcellulose (Aldrich) containing Dulbecco's modified Eagle's medium–F-12 (Gibco-BRL), 15% fetal calf serum (Equitech-Bio), 1.5% chicken serum (Sigma), and 10 μM β-mercaptoethanol and were incubated at 39.5°C for 6 to 7 days (wild-type cells) or 10 to 14 days (mutant cells) for the estimation of surviving colonies.

Chromosomal aberration analysis. Preparation of chromosome spreads and karyotype analysis were performed as described previously (35, 40). For the enrichment of mitotic cells, Colcemid was added to the last 3 h of incubation.

Measurement of SCE level. Measurement of SCE level was carried out as described previously (35, 47). Cells were treated with Colcemid for the last 2 h of incubation to enrich mitotic cells. To compare the SCE results, nonparametric statistical analysis (Mann-Whitney U test) was performed using StatView5.0 software.

Measurement of gene targeting frequency. Targeted gene integration frequency was measured as described previously (44).

Measurement of *Ig* gene conversion rate. Measurement of the *Ig* gene conversion rate using surface IgM detection was performed as described previously (3, 5, 38).

Antibody. Anti-Rev7 antibody was raised against glutathione *S*-transferase (GST)–human Rev7 and purified by a maltose birding protein (MBP)-human Rev7 column.

Nucleotide sequence accession numbers. The chicken *REV1* and *REV7* cDNA sequences have been submitted to the GenBank database under accession no. AY675169 and AY675170, respectively.

RESULTS

***rev1*, *rev3*, and *rev7* single-gene-disrupted clones show similar defective growth properties.** The chicken Rev7 protein consists of 211 amino acids, showing a high degree of amino acid identity to its human (96%), mouse (96%), and frog (94%) orthologs. In contrast, relatively lower identity is found when compared with budding yeast Rev7 (21%) and human Mad2 (24%) (see Fig. S1 in the supplemental material). We generated gene targeting constructs which would delete the first three coding exons of the *REV7* gene corresponding to amino acids 1 to 72 (Fig. 1B). Targeting events were verified by the appearance of a 6.5-kb band and the disappearance of an 8.0-kb band in Southern blot analysis of BamHI-digested genomic DNA (Fig. 1C). We isolated two independent *REV7*$^{-/-}$ (*rev7*) clones from two *REV7*$^{+/-}$ clones and reconstituted the *rev7* clones with a human *REV7* cDNA (*rev7+hREV7*). The *REV7*

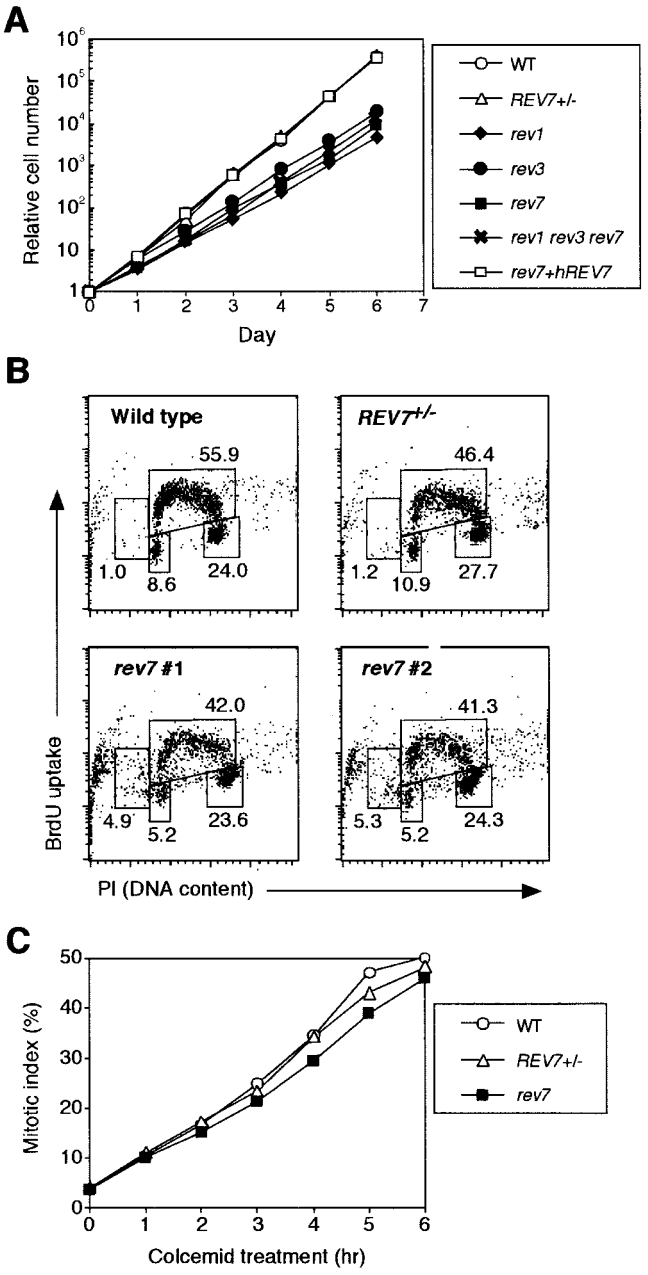


FIG. 2. Growth and cell cycle properties of wild-type and *rev* mutant DT40 cells. (A) Growth curves of WT and mutant DT40 cells. Each value represents the mean of the results from two independent clones for each genotype. (B) Representative cell cycle distribution of the indicated cell cultures as measured by bromodeoxyuridine (BrdU) incorporation and DNA content in flow cytometric analysis. Each of the gates at the upper half, lower left, and lower right and the leftmost gate correspond to cells incorporating BrdU (~S phase), G_1 cells, G_2/M cells, and sub-G_1 cells, respectively. Numbers show the percentage of cells falling in each gate. (C) Normal spindle assembly checkpoint in *rev7* cells. Cells in mitosis were identified by costaining with PI and antibody to phosphorylated histone H3, and the percentages of the mitotic cells are shown as the mitotic index. The period of chase indicates time after addition of 0.1 μg/ml of Colcemid.

TABLE 1. Level of spontaneous chromosomal aberrations in wild-type and *rev* mutant DT40 cells

Cells (clone name)	No. of cells analyzed	No. of:					Total (per cell)
		Chromatid type		Chromosome type		Chromatid exchanges	
		Gaps	Breaks	Gaps	Breaks		
Wild type (CL18)	150	3	3	1	0	0	7 (0.05)
rev1[a]	100	0	2	2	7	0	11 (0.11)
rev3	150	0	4	1	8	1	14 (0.09)
rev7#1	100	0	2	0	1	4	7 (0.07)
rev7#2	100	0	6	1	5	0	12 (0.12)
rev1 rev3 rev7	200	0	2	3	15	0	20 (0.10)

[a] Clone from reference 39.

gene disruption and reconstitution were confirmed in Western blot analysis (Fig. 1D). We modified original *REV1* gene disruption constructs (39; see also Materials and Methods) and made *rev1* cells from wild-type DT40 cells that carried the same frameshift mutation in the VJλ joint (CL18) (5), in order to accurately measure the rate of *Ig* gene conversion.

The growth properties of these two *rev7* clones were monitored using growth curves and cell cycle analysis. The *rev7* clones exhibited growth properties very similar to those of *rev1* and *rev3* clones, including retarded growth kinetics (Fig. 2A) and higher fractions of dead cells (Fig. 2B). Complementation of the *rev7* cells with human Rev7 resulted in growth kinetics indistinguishable from those of WT cells (Fig. 2A). The significant homology between vertebrate Mad2 and Rev7 (see Fig. S1 in the supplemental material) suggested that Rev7 might be involved in the spindle assembly checkpoint similarly to Mad2 (24). To address this question, we measured the mitotic index with time after addition of Colcemid, an inhibitor of microtubule formation. *rev7* cells were normally arrested before entering metaphase in the presence of Colcemid, indicating that disruption of Rev7 has no effect on the spindle checkpoint (Fig. 2C). Similarly, suppression of DNA synthesis after IR irradiation and a delay in G_2/M transition after exposure to IR or UV were observed in Rev7-deficient cells as well as in wild-type cells, also suggesting that disruption of Rev7 has no effect on DNA damage checkpoints (see Fig. S2 in the supplemental material).

We have previously shown that spontaneous chromosomal breaks in *rev3* cells may stimulate a DNA damage checkpoint, leading to retarded cell growth and cell death. We therefore compared spontaneous chromosome aberrations in each *rev* single mutant and wild-type cells. All the disrupted clones exhibited a similar level of increase in spontaneous chromosomal breaks, while wild-type and reconstituted *rev7+hREV7* clones exhibited only few aberrations (Table 1). These results suggest that the three Rev molecules act broadly cooperatively in maintaining chromosomal DNA in cycling cells.

The three *rev* mutants and the *rev1 rev3 rev7* triple mutant exhibit the same level of elevated sensitivity to a variety of DNA-damaging agents. Our previous studies revealed *rev1* and *rev3* cells are sensitive to killing by a variety of genotoxic stresses (39, 42). In the present study, we compared the sensitivities of the three *rev* mutants to a variety of genotoxic treatments using colony survival assays. We examined at least two independent clones of each genotype to assess the extent of variation of their phenotypes because of the genome instability associated with the *rev* defects. Figure 3A shows the UV sensitivity of the representative wild-type, *rev* mutant, and *REV7*-reconstituted cells. Interestingly, the data show that the sensitivities of the three *rev* mutants to UV (wavelength = 254 nm) are similar. *rev7* cells also exhibit elevated sensitivity to IR (γ rays; ^{137}Cs), cisplatin [*cis*-diaminedichloroplatinum (II); CDDP], H_2O_2, and methyl methanesulfonate (MMS), as do *rev1* and *rev3* cells (Fig. 3B to E). The sensitivity of each *rev* single mutant to these agents is also similar.

To confirm the epistatic relationship of the three Rev molecules, we generated two independent *rev1 rev3 rev7* triple mutants (Fig. 1A). Briefly, two *rev3 rev7* clones were first made from a *rev3* clone. The phenotypes of the *rev3* and *rev3 rev7* clones were essentially the same (data not shown), in agreement with biochemical data that yeast and vertebrate polymerase ζ appears to be composed of the Rev3 and Rev7 subunits (28, 32). Subsequently, we generated two *rev1 rev3 rev7* clones from one *rev3 rev7* clone. *rev1 rev3 rev7* cells showed a phenotype similar to that of single *rev3* and *rev7* mutants, which includes proliferation rate (Fig. 2A), the level of spontaneous chromosomal aberrations (Table 1), and sensitivity to killing by various genotoxic stresses (Fig. 3). The overall similarity in the phenotypes of the *rev* single mutants and the *rev1 rev3 rev7* triple-mutant cells supports the notion that the three Rev molecules might operate as a single functional entity in the repair or tolerance of DNA damage.

The three *rev* mutants have indistinguishable phenotypes in HR-mediated DSB repair while exhibiting variant phenotypes in sister chromatid exchange. We previously showed that the significant increase in IR sensitivity in *rev3* cells is presumably caused by a defect in HR-mediated DSB repair as well as in TLS (42). The observed hypersensitivity of *rev1* and *rev7* mutants to IR and cisplatin led us to address further assessment of role for Rev molecules in HR. To investigate the role for Rev1 and Rev7 in HR, we studied HR-dependent DSB repair, gene targeting frequency, SCE, and *Ig* gene conversion in the *rev* single and triple mutants.

To specifically evaluate the contribution of HR-dependent DSB repair in *rev1* and *rev7* cells, asynchronous populations of cells were exposed to 2 Gy of IR and induced chromosome breaks were measured at 3 h after the irradiation. The majority of the cells that enter mitosis within 3 h after irradiation should have been irradiated in the G_2 phase but not in the late S phase (42). Thus, the number of induced chromosomal breaks in metaphase spreads should reflect DSB repair capability during the G_2 phase, mainly mediated by HR (44). Following this

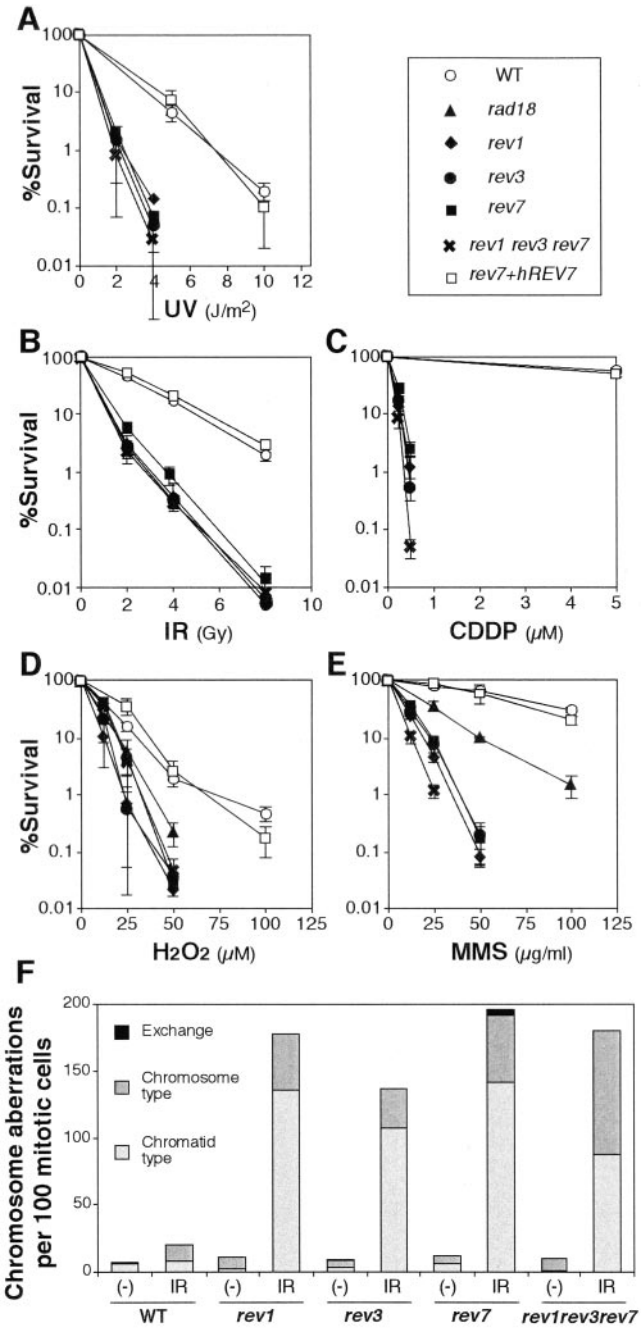


FIG. 3. Sensitivities of *rev* single mutants and *rev1 rev3 rev7* cells to killing by various DNA-damaging agents. Results of the colony survival assay after treatment with (A) UV light (254 nm), (B) γ rays (^{137}Cs), (C) cisplatin (CDDP), (D) H_2O_2, and (E) methyl methanesulfonate (MMS) are indicated. The symbols indicating each genotype, shown at the right top of panels, represent the mean values of at least three independent experiments. Each error bar shows the standard deviation of the mean. The doses of genotoxic agents are displayed on the *x* axis on a linear scale, and the percentile fractions of surviving colonies are displayed on the *y* axis on a logarithmic scale. Plating efficiencies of wild-type and mutant cells were ~80% in the UV, IR, CDDP, and H_2O_2 assays and were reduced to ~50% in the MMS assay because of 1 h of incubation in serum-free medium. (F) Level of chromosomal aberrations induced by IR. For each preparation, 100 mitotic cells were analyzed at 3 h after 2-Gy γ-ray (^{137}Cs) irradiation. (−) indicates spontaneous aberrations.

protocol, *rev1* and *rev7* cells also showed a marked increase in the level of chromosomal aberrations (Fig. 3F). A similar increase in IR-induced aberrations was seen in the triple mutants. This observation suggests that Rev1 and Rev7, as well as Rev3 (42), are essential for efficient DSB repair following DNA damage, most likely during the DNA synthesis step of the HR reaction.

On the contrary, the levels of SCEs, which are thought to reflect HR-mediated PRR, tended to be different among the *rev* mutants (Fig. 4). We assessed SCE levels of two clones at least for each single and triple *rev* mutant and confirmed the tendency that the levels of SCEs are the same among the identical genotypic clones (data not shown). Since the distribution of the number of SCEs per cell in each mutant does not follow the normal distribution, we employed nonparametric statistical analysis, the Mann-Whitney U test. According to this test, we obtained statistical orders that WT < *rev1 rev3 rev7* < *rev1* = *rev7* = *rev3* < *rev1 rev3 rev7+cREV1* in spontaneous SCE level, and that WT = *rev1 rev3 rev7* < *rev3* = *rev1* = *rev7* = *rev1 rev3 rev7+cREV1* in 4-nitroquinolone oxide (4-NQO)-induced SCE level (=, not significantly different; <, significantly lower [$P < 0.002$]). As expected, *rev7* cells showed the same level of increase in SCE events, as did *rev3* cells, both with and without 4-NQO treatment. *rev1* cells also showed a significant increase in the level of SCE, which is similar to those of *rev3* and *rev7* cells. This observation does not differ much from Sale's original work, as the level of spontaneous SCE in their *rev1* mutant appeared to be indeed higher than that of wild-type cells (39). Remarkably, *rev1 rev3 rev7* triple mutants show nearly a wild-type level of SCE even in the induced condition. Expression of chicken *REV1* cDNA in *rev1 rev3 rev7* cells increased SCE events to a level similar to that of *rev3* and *rev7* cells (Fig. 4).

We also measured gene targeting efficiency using an *OVALBUMIN* locus construct (6) with the *Eco-gpt* selection marker. The ratio of targeted to random integration was a few fold lower in *rev3* and *rev7* cells, while this ratio of *rev1* and *rev1 rev3 rev7* cells was not reduced when compared with that of wild-type cells (Table 2). These observations imply distinct as well as redundant roles for Rev1 and the other two Rev molecules in some of the HR reactions.

Reduced Ig gene conversion rate in *rev1* and *rev1 rev3 rev7* cells but not in *rev3* or *rev7* cells. To analyze in more detail the role of the Rev molecules in HR, we analyzed *Ig* gene conversion in the *rev* mutants. In the B cells of some vertebrates, an *Ig* gene conversion event is supposed to arise through intragenic HR between two diverged homologous sequences: i.e., pseudo-V and VJλ segments following completion of V(D)J rearrangement. This DT40 assay allows for the determination of gene conversion events as well as the identification of aberrant events. To assess the rate of *Ig* gene conversion, we monitored the gain of sIgM expression from sIgM-negative cells that carried the same frameshift mutation in the rearranged VJλ joint (CL18) (5). The gain of sIgM reflects the repair of the frameshift mutation through superimposed gene conversion events. We previously showed that a defect in Rev3 has no impact on the *Ig* gene conversion in chicken DT40 (42).

In the current study, we newly made three *rev1* clones from CL18 (Fig. 1A; see Materials and Methods) and analyzed *Ig* gene conversion in the cells together with *rev1 rev3 rev7* cells

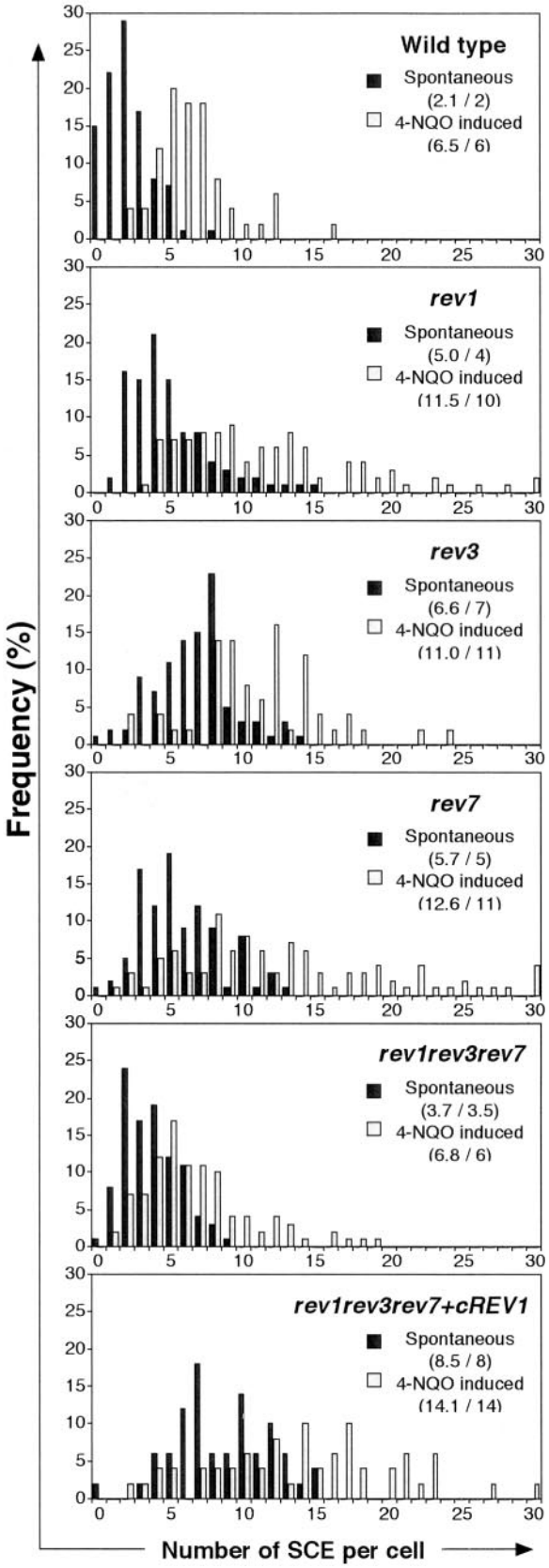


FIG. 4. Level of SCE in asynchronous populations of *rev* mutant cells. Distribution of spontaneous and 4-NQO-induced SCEs from indicated cells. Solid bars, spontaneous SCEs; shaded bars, SCEs induced by 0.2 ng/ml of 4-NQO. Mean/median numbers of SCEs are shown in parentheses at the top right in each panel. Cells were incubated with bromodeoxyuridine (BrdU) for two cell cycle periods and treated with Colcemid for the last 2 h to enrich mitotic cells. For each preparation, 100 mitotic cells were analyzed. Note that the *rev1* mutant was from reference 39. The Mann-Whitney U test indicated WT < *rev1rev3rev7* < *rev1* = *rev7* = *rev3* < *rev1 rev3 rev7+cREV1* in spontaneous SCE level and WT = *rev1 rev3 rev7* < *rev3* = *rev1* = *rev7* = *rev1 rev3 rev7+cREV1* in 4-NQO-induced SCE level (=, not significantly different; <, significantly lower [$P < 0.002$]).

(Fig. 5). Analyses of fluctuations in sIgM gain were performed on 30 to 40 subclones expanded from single sIgM-negative clones following a 3-week culture. Remarkably, *rev1* and *rev1 rev3 rev7* cells exhibited about a 3.5-fold decrease in appearance of sIgM-positive revertant subclones, while *rev3* cells showed a wild-type level (Fig. 5A). The expression of chicken *REV1* transgene in *rev1* cells normalized the level of sIgM gain to a wild-type level. This observation suggests that Rev1, but neither Rev3 nor Rev7, is required for efficient Ig gene conversion.

To assess the nature of *Ig* gene conversion events in *rev1* cells, we determined nucleotide sequences in the VJλ segments in sIgM-positive revertants from the three independent *rev1* clones. On the whole, revertant subclones derived from *rev1* mutants showed rare somatic mutation events occurred independently of gene conversion events, as observed in wild-type cells (Fig. 5B). The sequences around the parental frameshift mutation were indeed replaced by pseudo-V sequences in sIgM-positive revertant subclones in *rev1* mutants, as observed in wild-type cells (see Fig. S3 in the supplemental material). In *rev1* cells, the average length of the gene conversion tract and the selection of putative pseudo-V donor were comparable to those of wild-type cells (Fig. 5B; average tract length is 92.8 bp in the wild type versus 98.2, 76.9, and 78.7 bp in three *rev1* mutants). These results suggest that Rev1, acting independently of Rev3 and Rev7, apparently influences the rate of *Ig* gene conversion but not the length or fidelity of conversion tract or the choice of donor segments.

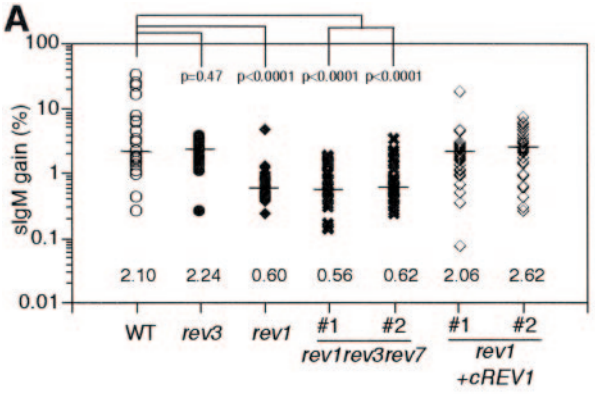
DISCUSSION

The present study reveals cooperative and multiple functional relationships of the three Rev molecules. *rev3* and *rev7* mutants showed similar phenotypes in every assay studied, indicating that Rev7 is required for Rev3, the catalytic subunit of Polζ. In marked contrast, Rev1 may act in two different manners, as an additional component of Polζ complex and in a role independent of Polζ. *rev1* cells and the other *rev* mutants exhibited similar phenotypes as follows: defective growth properties with spontaneous chromosomal aberrations, elevated sensitivity to a variety of DNA-damaging agents, and induced chromosomal breaks after IR in the G_2 phase; indicating that the similarity in phenotypes reflects the cooperative function of the three Rev molecules in these circumstances. On the other hand, the *rev1* and *rev1 rev3 rev7* clones, but not *rev3* or *rev7* clones, exhibited reduced *Ig* gene conversion, suggesting that Rev1 may be required for the intragenic HR independently of Polζ or presumably by associating with other unknown DNA polymerase(s). In summary, the current study demonstrates

TABLE 2. Target integration frequencies

Cells	No. of targeted clones/ no. analyzed (%)[a]
Wild type (CL18)	58/75 (77.7)
rev1[b]	24/25 (96.0)
rev1[c]	19/27 (70.4)
rev3	19/44 (43.2)
rev7	8/18 (44.4)
rev1 rev3 rev7	26/31 (83.8)

[a] Transfection with the targeting constructs for the *OVALBUMIN* locus was repeatedly performed. The data shown are the number of targeted clones/the number of drug-resistant clones analyzed (percent frequency of targeting).
[b] Clone from reference 39.
[c] Clone newly established in this study.



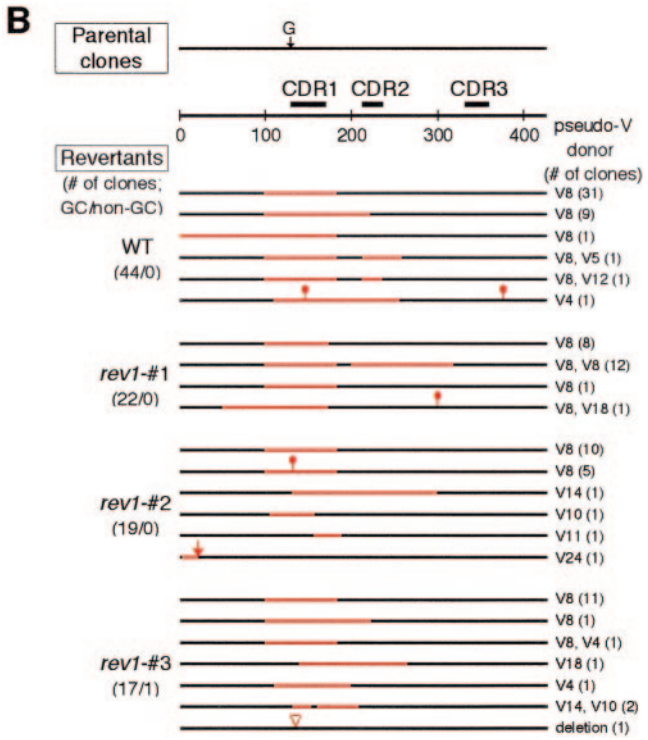


FIG. 5. Fluctuation analysis of the generation frequency of sIgM gain revertants. (A) The abundance of sIgM gain variants was determined in 30 parallel cultures derived from single sIgM-negative parental cells after clonal expansion (4 weeks); median percentages are noted below each data set and are indicated by the solid line. The spectra of sIgM gain are indicated on a logarithmic scale. *P* values calculated by nonparametric statistical analysis (Mann-Whitney U test) between wild-type and mutant DT40 cells are shown. (B) The representative gene conversion tract spectra from sIgM-positive revertants in the wild type and three *rev1* mutants are shown on the left as the number of sIgM revertants by gene conversion event/by non-gene conversion event. Each horizontal line represents the rearranged VJλ segment (427 bp) with gene conversion tracts (red lines) and mutations including insertion (arrow), deletion (inverted open triangle), and base substitution (lollipop shape). The parental clone of each genotype has the same frameshift mutation (arrow with G) within this segment. A putative pseudo-V donor is shown on the right of each spectrum with the number of subclones.

that Rev1 plays Rev3-Rev7-dependent and independent roles in the cellular tolerance of DNA-damaging agents and HR.

Cooperative function of the Rev1, Rev3, and Rev7 proteins in DNA damage response. The current study showed that the three *rev* single mutants displayed similar sensitivities to a variety of DNA damage. This phenotypic similarity can be interpreted in two different ways. First, all the Rev proteins may be equally required for efficient TLS past a variety of DNA damage. Alternatively, although Rev1 may have another contribution to cellular tolerance of DNA damage, as suggested from a biochemical study indicating that Rev1 interacts with another TLS polymerase (14), the contribution might be little in DT40 cells, resulting in *rev1* cells lacking a more prominent phenotype than *rev3* or *rev7* cells. We favor the former idea, because the triple mutant also exhibited the same level of sensitivities as did all the single mutants, and because an essential role for Rev1 in Polκ-dependent TLS is unlikely at least in DT40, from our observation that *rev3* and *polκ* double mutations caused an additive increase in UV sensitivity (manuscript in preparation). Thus, it is unlikely that some Rev molecules can act independently of the other Rev molecules in tolerance to exogenous DNA damage.

We previously demonstrated that Rev3 is involved in not only TLS but also HR-dependent DSB repair after IR (42). Since Rev3 is relatively indifferent to structural distortions at DSBs caused by IR, Rev3 may play an important role in DNA synthesis of HR-mediated DSB repair, particularly after IR. Interestingly, *rev1*, *rev7*, and *rev1 rev3 rev7* mutants as well as *rev3* cells all exhibited very similar levels of chromosomal breaks after IR irradiation (Fig. 3F). We, therefore, conclude that the three Rev molecules may participate in HR-mediated DSB repair as a functional unit, though their involvement in other unknown DSB repair pathways is not completely eliminated.

Rev1 and Rev3-Rev7 may contribute nonredundantly to SCE. It is thought that SCE is a result of PRR events mediated by HR resulting in crossover events in higher eukaryotic cells (19). We have shown that TLS mutant DT40 cells display elevated level of SCE, while HR mutant DT40s show reduced SCE level (16). The level of SCE should be determined by the balance of two major factors of PRR: TLS and HR. A defect in TLS may result an increase in the number of unfilled gap, which may activate HR-mediated PRR (i.e., SCE), whereas a defect in HR should directly reduce SCE events. To assess the role of each Rev molecule in HR-mediated PRR, we measured the levels of SCE comparing the three *rev* single mutants as well as the triple mutant. Remarkably, two clones of *rev1 rev3 rev7* cells exhibited suppressed levels of spontaneous or induced SCE events, although each *rev* single mutant consis-

tently exhibited a significant increase in spontaneous SCE (Fig. 4). Furthermore, reconstitution of the triple mutant with *REV1* cDNA increased in the level of SCE to those of the three *rev* single mutants.

It is unclear how SCE levels in *rev* triple mutants are suppressed, although those of *rev* single mutants are elevated. The suppressed SCE levels in *rev* triple mutants are reminiscent of HR-deficient DT40 mutants that demonstrate SCE is a PRR mediated by HR (41). It is likely that SCE in eukaryotic cells reflects only a proportion of total HR events which result in crossing over. Furthermore, it is difficult to know what is leading to the reduced level of SCEs in *rev* triple mutants, since a reduction in SCEs may reflect a decreased number of lesions being processed by recombination and/or a decreased number of crossover recombination events.

Rev1 may act independently of Rev3-Rev7 proteins in *Ig* gene conversion. In this study, we found *rev1* cells displayed a significant decrease in the kinetics of *Ig* gene conversion, although the previous study did not find such reduction in their *rev1* clone using a distinctly different assay system (39). They examine the gene conversion events that inactivate an *IgV* gene and thereby lead to loss of sIgM expression. This assay is suitable for detection of single-nucleotide substitutions, as they wished to examine, but does not necessarily provide reproducible data for counting the number of HRs, because the vast majority of gene conversion events do not inactivate an *IgV* gene and their rate should be substantially affected by the nucleotide sequences of the *IgV* gene. To solve these problems, we created for this study three additional *rev1*-deficient clones as well as the other *rev* mutants from the wild-type cells that carry the defined *IgV* sequences, including a specific frameshift mutation (5), and examined in all genotypes analyzed the number of *Ig* gene conversion events that occurred around identical sequences, including the frameshift mutation. Indeed, we have obtained consistent result from each genotype, including wild-type cells, two *rev1* clones, two clones reconstituted with Rev1 cDNA, and two clones of *rev1 rev3 rev7* triple mutants. We, therefore, conclude that a defect in Rev1 indeed had an impact on the *Ig* gene conversion rate, whereas neither Rev3 nor Rev7 appears to participate in this intragenic HR reaction. Thus, Rev1 may play a role in HR even in the absence of Polζ. There are two possible mechanisms of action of Rev1. First, the nontemplate nucleotide insertion activity of Rev1 might contribute to the initiation of DNA synthesis in gene conversion. However, this possibility may be unlikely, because yeast Rev1 does not require its conserved deoxycytidyl transferase activity for at least TLS (1, 33). Alternatively, Rev1 associates with other unknown DNA polymerase(s) and may play a regulatory role in DNA synthesis, as suggested from the biochemical study (14). Our observation that the defect in Rev1 significantly reduced the rate of *Ig* gene conversion but had no impact on the length of gene conversion tract suggests a certain regulatory role of Rev1 in *Ig* gene conversion, which agrees with the latter idea. This model is open to further testing, and we need to identify the DNA polymerases involved. We assume that this task should be performed by TLS polymerases that can extend from mismatched 3′ end, such as Polκ, as well as Polι and Polη, because substantial sequence divergence is present between donor pseudo-V and recipient VJλ segments in *Ig* gene conversion. Presumably, the DNA synthesis in gene conversion may be achieved by the combination of at least two DNA polymerases: One may require Rev1, whereas the other polymerase can act without Rev1 and might have extensive processivity.

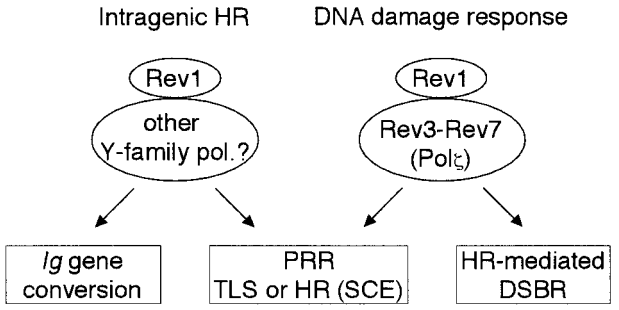


FIG. 6. Schematic model of the roles of vertebrate Rev molecules in DNA damage response and HR. Rev1 cooperates with a Rev3-Rev7 complex (i.e., Polζ) as a functional unit in translesion synthesis (TLS) and HR-mediated double-strand break repair (DSBR) for DNA damage response. On the other hand, Rev1 plays a role independent of Polζ in *Ig* gene conversion, an intragenic HR reaction. In this reaction, Rev1 might cooperate with other Y-family polymerases like Polκ, Polι, or Polη. Rev1 may also act independently of the Rev3-Rev7 complex in the formation of SCE. Thus, Rev1 may regulate two pathways: DNA damage response and intragenic HR.

In conclusion, the present data suggest two types of Rev1 action in HR; Rev1 is involved in HR-mediated repair of IR-induced DSBs as a component of the Polζ complex and also participates in *Ig* gene conversion without interacting with Polζ. From a view of DNA repair, the three Rev molecules may form a functional unit to undergo TLS past a wide range of DNA lesions and also achieve the repair of IR-induced DSBs. Furthermore, Rev1 and Rev3-Rev7 might act independently of each other and be required for HR-mediated PRR of DNA damage (Fig. 6). As reviewed in a recent report concerning genes involved in chromosome fragility syndromes (43), further study of the *REV* genes and other HR-related genes disrupted in DT40 clones would help in dissecting the complex and independent roles of each Rev molecule as well as revealing unknown genetic interaction with other molecules in a variety of DNA repair and HR with the related pathways.

ACKNOWLEDGMENTS

We thank Y. Nishimura, H. Onisawa, and Y. Sato for their technical assistance. We also acknowledge J. E. Sale (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) and K. Kamiya (Research Institute for Radiation Biology and Medicine, Hiroshima, Japan) for critical reading and suggestion. Anti-human Rev7 antibody was kindly provided by T. Hirota (Kumamoto University).

Financial support was provided in part by a Core Research for Evolutional Science and Technology grant from Japan Science and Technology Corporation; by the Center of Excellence (COE) grant for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government; and by grants from the Uehara Memorial Foundation and the Naito Foundation.

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