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Multiple roles of Rev3, the catalytic subunit of polζ in maintaining genome stability in vertebrates

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Translesion DNA synthesis (TLS) and homologous DNA recombination (HR) are two major postreplicational repair (PRR) pathways. The *REV3* gene of *Saccharomyces cerevisiae* encodes the catalytic subunit of DNA polymerase ζ, which is involved in mutagenic TLS. To investigate the role of *REV3* in vertebrates, we disruped the gene in chicken DT40 cells. *REV3$^{-/-}$* cells are sensitive to various DNA-damaging agents, including UV, methyl methanesulphonate (MMS), cisplatin and ionizing radiation (IR), consistent with its role in TLS. Interestingly, *REV3$^{-/-}$* cells showed reduced gene targeting efficiencies and significant increase in the level of chromosomal breaks in the subsequent M phase after IR in the G_2 phase, suggesting the involvement of Rev3 in HR-mediated double-strand break repair. *REV3$^{-/-}$* cells showed significant increase in sister chromatid exchange events and chromosomal breaks even in the absence of exogenous genotoxic stress. Furthermore, double mutants of *REV3* and *RAD54*, genes involved in HR, are synthetic lethal. In conclusion, Rev3 plays critical roles in PRR, which accounts for survival on naturally occurring endogenous as well as induced damages during replication.

Keywords: DNA polymerase ζ/genome instability/postreplication repair/REV3/translesion DNA synthesis

Introduction

Numerous and varied DNA lesions are generated continuously not only by environmental factors but also by endogenous damage. Unrepaired DNA damage can lead to replication fork arrest and the formation of gaps, and occasionally double-stranded DNA breaks (DSBs) in one of the daughter strands. To remove such DNA lesions arising during DNA replication, cells have evolved methods of postreplication DNA repair (PRR). PRR is carried out mainly by two pathways: translesion DNA synthesis (TLS) and homologous recombination (HR) repair (reviewed in Friedberg *et al*., 1995). TLS functions by filling gaps on a daughter strand using a number of specialized TLS polymerases, while HR repair promotes DNA synthesis from a damaged chromatid templated on the other intact sister chromatid. Compared with our understanding of HR in maintenance of chromosomal DNA (reviewed in Sonoda *et al*., 2001b), the role of TLS is only poorly characterized in vertebrate cells.

A number of TLS polymerases have been identified in *Saccharomyces cerevisiae* and mammals (reviewed in Ohmori *et al*., 2001; Wood *et al*., 2001). Three of TLS polymerases (Pol), Polη (McDonald *et al*., 1997; Johnson *et al*., 1999b; Masutani *et al*., 1999), Polζ (Morrison *et al*., 1989; Gibbs *et al*., 1998; Lin *et al*., 1999; Van Sloun *et al*., 1999; reviewed in Lawrence, 2002) and Rev1 (Nelson *et al*., 1996a; Gibbs *et al*., 2000; Simpson and Sale, 2003) are conserved between these species. Human Polη is mutated in a variant form of xeroderma pigmentosum (XP-V) (Johnson *et al*., 1999b; Masutani *et al*., 1999), which is characterized by predisposition to skin cancer and elevated UV sensitivity. Yeast and mammalian polζ is comprised of the Rev3 catalytic subunit and the Rev7 subunit (Nelson *et al*., 1996b; Murakumo *et al*., 2001). Yeast *rev3* mutant strains exhibit a moderate sensitivity to UV as well as reduced levels of mutagenesis induced by a very broad range of mutagens (Lemontt, 1972; Lawrence and Christensen, 1976). Although a defect in Rev3 does not affect the viability of yeast, *REV3$^{-/-}$* murine embryos died around midgestation, and *rev3*-deficient mammalian cell lines have not yet been established (Bemark *et al*., 2000; Esposito *et al*., 2000; Wittschieben *et al*., 2000). To analyze the essential function of Rev3 in higher eukaryotes, we generated Rev3-deficient cells from the chicken B lymphocyte line DT40 (Buerstedde and Takeda, 1991). Here, we report genetic evidence that Rev3 protein is involved in maintenance of chromosomal DNA as well as in tolerance of various DNA damages by TLS and HR.

Results

Slower growth kinetics of REV3-deficient cells

We isolated a chicken *REV3* cDNA and determined its sequence. The six polymerase motifs show ~95% identity, and the overall identity to the human Rev3 protein is ~75%. Gene targeting constructs, which are expected to disrupt four polymerase motifs (VI, III, I, V; Figure 1A) were generated from the amplified genomic DNA. Gene

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targeting of the *REV3* locus was confirmed by Southern blot analysis (Figure 1B). The proliferative properties of two independently isolated *REV3*$^{-/-}$ clones were monitored using growth curves and cell cycle analysis. *REV3*$^{-/-}$ cells proliferated in slower kinetics compared with wild-type cells, while *REV3*$^{+/-}$ cells proliferated with normal kinetics (Figure 1C). Pulse–chase bromodeoxyuridine (BrdU) labeling revealed that the length of cell cycle time was extended to 9 h (wild type, 8 h) in the absence of Rev3 (Figure 1E). Furthermore, a higher fraction of the *REV3*$^{-/-}$ cells accumulated in the G_2 phase as well as in a sub-G_1 fraction when compared with wild-type cells (Figure 1D). These observations indicate that the G_2 phase is elongated in *REV3*$^{-/-}$ cells. Accumulation of cells in the G_2 phase is also observed in HR-deficient cells, such as *RAD51*$^{-/-}$ DT40 cells (Sonoda *et al*., 1998), which show extensive chromosomal breaks probably leading to stimulation of the G_2 damage checkpoint. Likewise, *REV3*$^{-/-}$ cells exhibited a significant increase in the level of spontaneous chromosomal breaks (Table I) as well as elevated level of dying sub-G_1 cells (Figure 1D). These observations indicate that the slower proliferation rate of *REV3*$^{-/-}$ cells is explained by both an extended G_2 phase and increased cell death, presumably caused by spontaneous chromosomal breaks.

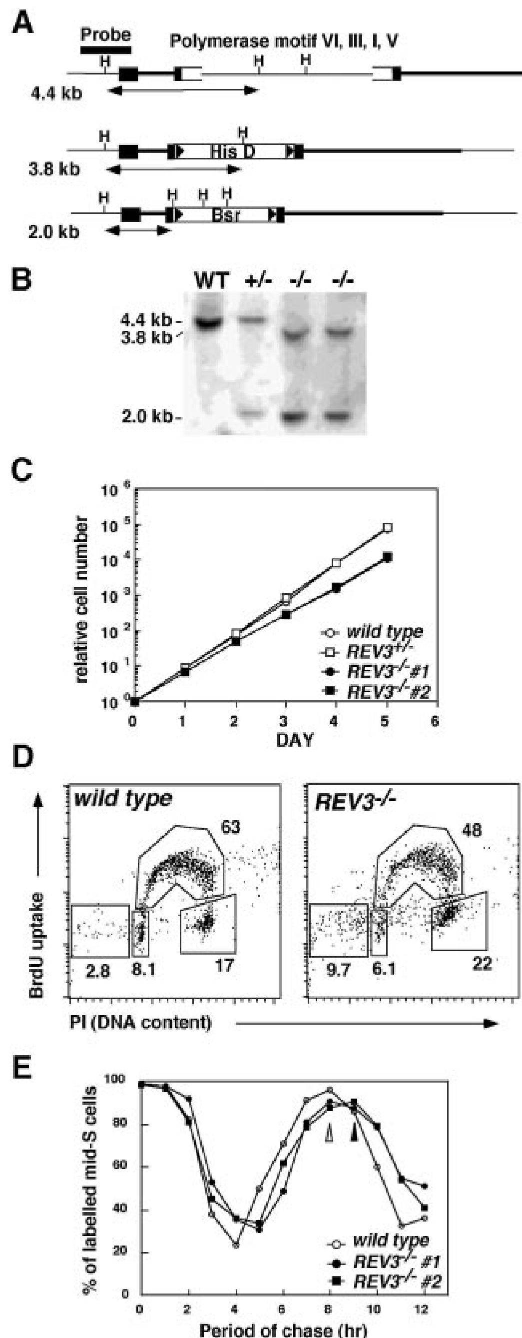

Fig. 1. Gene targeting of the *REV3* locus. (**A**) Schematic representation of part of the *REV3* locus, the gene disruption constructs, and the configuration of the targeted alleles. Solid boxes indicate the position of exons. Only disrupted exons are indicated. Relevant *Hin*dIII sites and the position of the probe used in Southern blot analysis are indicated. (**B**) Southern blot analysis of *Hin*dIII-digested genomic DNA from cells with the indicated genotypes of the *REV3* gene, using the probe shown in (A). The positions and sizes of hybridizing fragments of the wild-type and targeted loci are indicated. (**C**) Growth curves corresponding to the indicated cell cultures. Data shown are the average of the results from two separate clones for each genotype. Error bars show the standard deviation of the mean for three experiments. (**D**) Representative cell cycle distribution of the indicated cell cultures as measured by BrdU incorporation and DNA content in flow cytometry analysis. Cells were pulse–labeled for 10 min, and subsequently stained with FITC-anti-BrdU to detect BrdU incorporation (*y*-axis, log scale) and propidium iodide to detect total DNA (*x*-axis, linear scale). The upper gate identifies cells incorporating BrdU (~S phase), the lower-left gate identifies G_1 cells, and the lower-right gate displays G_2/M cells. Sub-G_1 cells reflect dead cells. Numbers show the percentages of cells falling in each gate. (**E**) The calculated relative percentages of cells in the indicated gate are plotted with time. The symbols for each sample are shown on the right. The progression of BrdU-labeled (i.e. S phase) cells out of S phase, and into first G_2/M (4n DNA content), then G_1 (2n DNA content), and back into S phase with time is indicated.

REV3$^{-/-}$ cells are sensitive to a variety of genotoxic stress

We examined viability of wild-type and *REV3*$^{-/-}$ cells after genotoxic treatment by colony survival assays. *REV3*$^{-/-}$ cells exhibited elevated sensitivity to UV, methyl methanesulphonate (MMS), cisplatin, and ionizing radiation (IR), when compared with wild-type and *REV3*$^{+/-}$ cells (Figure 2). Elevated sensitivity to a variety of DNA-damaging agents is reminiscent of the phenotype of yeast mutants of the Rad6/Rad18 pathway (McKee and Lawrence, 1980; Keszenman *et al*., 1992; Simon *et al*., 2000).

To investigate the cause of cell death following UV irradiation, we measured UV-induced chromosomal breaks, which reflect DSBs in the chromosomal DNA. It is known that DSBs are not induced by UV alone, but can be induced by DNA replication over UV-damaged template DNA (van Zeeland *et al*., 1980). Chromosome breaks were measured at 3, 6, 9 or 12 h after UV irradiation (Figure 2E). The data show that a defect in Rev3 dramatically increased UV-induced chromosomal breaks. In particular, cells that were exposed to UV when they were at early S phase showed a maximum amount of chromosome breaks (9 h after irradiation). This suggests that Rev3 may process DNA replication block caused by UV damage.

In yeast, all the components of the TLS pathways including Rev3 are epistatic to the Rad6–Rad18 molecules (reviewed in Lawrence, 1994; Broomfield *et al*., 2001). It

is known that Rad6 is a ubiquitin-conjugating enzyme (E2), forming a tight complex with the Rad18 protein (Bailly *et al.*, 1994, 1997). We have previously shown that *RAD18*$^{-/-}$ cells are sensitive to IR, UV and crosslinking agents (Yamashita *et al.*, 2002). This phenotype is similar to that observed in yeast *rad18* mutant (Lawrence and Christensen, 1976; Prakash, 1981; Fabre *et al.*, 1989). Interestingly, *REV3*$^{-/-}$ cells exhibited significantly higher sensitivity to cisplatin, MMS and IR when compared with *RAD18*$^{-/-}$ cells (Figure 2). Thus, unlike in yeast, Rev3 may not be fully regulated by the Rad18 protein in vertebrate cells.

Defective DSB repair after completion of DNA replication in REV3$^{-/-}$ cells

REV3$^{-/-}$ as well as *RAD18*$^{-/-}$ cells exhibited elevated IR sensitivity when compared with wild-type cells (Figure 2D). This could be explained by a defect in any of the following four systems: a damage checkpoint, TLS, and two major DSB repair pathways (reviewed in Kanaar *et al.*, 1998; van Gent *et al.*, 2001)—nonhomologous end-joining (NHEJ) and HR. Two Gray γ-irradiation of wild-type and *REV3*$^{-/-}$ cells suppressed DNA replication and stimulated the G_2 checkpoint (Supplementary figure 1, available at *The EMBO Journal* Online), indicating that Rev3 deficiency does not affect damage checkpoints. No difference between *REV3*$^{-/-}$ and wild-type cells was seen in recircularization of transfected linearized plasmid (Supplementary figure 2) indicating that NHEJ also appears to work normally (Verkaik *et al.*, 2002).

To dissect the contribution of the TLS and HR pathways to IR tolerance, we analyzed IR sensitivity of synchronised populations of *REV3*$^{-/-}$ and wild-type cells. Defective TLS would increase IR sensitivity particularly when the cells were exposed to IR prior to DNA replication. In contrast, defective HR would increase IR sensitivity in late S to G_2 phases, because HR between sister chromatids, but not HR between homologous chromosomes, is responsible for DSB repair in higher eukaryotes (Takata *et al.*, 1998). We synchronized cells using either nocodazole–mimosine treatments (Sonoda *et al.*, 2001a; Figure 3A) or elutriation (Takata *et al.*, 1998; data not shown), and obtained consistent data. As expected, *REV3*$^{-/-}$ cells exhibited elevated IR sensitivity in the G_1 and early S phases more prominently when compared with late S to G_2 phases (Figure 3B), suggesting that defective TLS accounts for their IR sensitivity at least partially as well as for the MMS sensitivity (Figure 2C).

Interestingly, *REV3*$^{-/-}$ cells exhibited significant increase in IR sensitivity also in late S to G_2 phases, as observed in HR deficient *RAD54*$^{-/-}$ cells (Bezzubova *et al.*, 1997; Figure 3B). The increased IR sensitivity in the G_2 phase in *REV3*$^{-/-}$ cells can be explained by the following two mechanisms. First, IR induces base damages, which interfere with DNA, leading to formation of DSBs. Secondly, IR-induced DSBs are not repaired efficiently in *REV3*$^{-/-}$ cells. To investigate the role of Rev3 in DSB repair, asynchronous populations of cells were exposed to IR, and chromosome breaks were measured at 3, 6, 9 or 12 h after IR (Figure 3C; Takata *et al.*, 1998; Takao *et al.*, 1999). The majority of the cells that entered mitosis between 0 and 3 h after 2 Gy IR should have been irradiated in the G_2 phase, but not in the late S phase, for the following two reasons. First, the irradiated *REV3*$^{-/-}$ cells were partially arrested up to 4 h in the G_2 phase (Supplementary figure 1). Secondly, no BrdU-labeled mitotic chromosomes were detected at 3 h after IR when the irradiated cells were exposed to both BrdU and colcemid between 0 and 3 h (Supplementary table 1). Strikingly, like *RAD54*$^{-/-}$ cells (Takata *et al.*, 1998), *REV3*$^{-/-}$ cells exhibited marked increase in the levels of chromosomal breaks at 3 h after IR, while these levels decreased at later time points (Figure 3C). This pattern of IR-induced chromosomal breaks indicates that Rev3 plays a direct role in IR-induced DSB repair even after completion of S phase. Since the absence of Rev3 does not appear to affect NHEJ, we favor the possibility that Rev3 is involved in DSB repair by participating in the HR pathway. In conclusion, Rev3 deficiency may cause defects in both TLS- and HR-mediated DSB repair, resulting in hypersensitivity to IR.

To evaluate HR capability of *REV3*$^{-/-}$ cells, we measured the rate of the immunoglobulin (Ig) gene conversion and gene targeting efficiency. Chicken B lymphocyte precursors as well as DT40 cells diversify the variable region of the Ig genes through HR between V(D)J joints and their upstream pseudo V segments (reviewed in Reynaud *et al.*, 1994). We examined the rate of gene conversion at the Ig λ locus in *REV3*$^{-/-}$ cells by measuring the gain and loss of surface Ig expression as described previously (Buerstedde *et al.*, 1990; Sale *et al.*, 2001). However, we were not able to detect any effect of the Rev3 deficiency on Ig gene conversion frequency (Supplementary figure 3) or nontemplated base substitution (two mutations out of 18 and one mutation out of eight gain of surface Ig expression events in wild-type and *REV3*$^{-/-}$ cells, respectively). In contrast, the efficiency of gene targeting onto three loci was consistently reduced in the absence of Rev3 (Table II), indicating the involvement of Rev3 in some types of HR. In summary, Rev3 may play a direct role in HR, particularly in repair of IR induced DSBs.

Table I. Frequency of spontaneous chromosomal aberrations in wild-type and Rev3-deficient DT40 cells

Cells	No. of cells analyzed	Chromatid-type		Chromosome-type		Chromatid exchange	Total (per cell)
		Gaps	Breaks	Gaps	Breaks		
Wild type	100	3	2	1	0	0	6 (0.06)
REV3$^{-/-}$	100	2	3	7	4	0	18 (0.18)

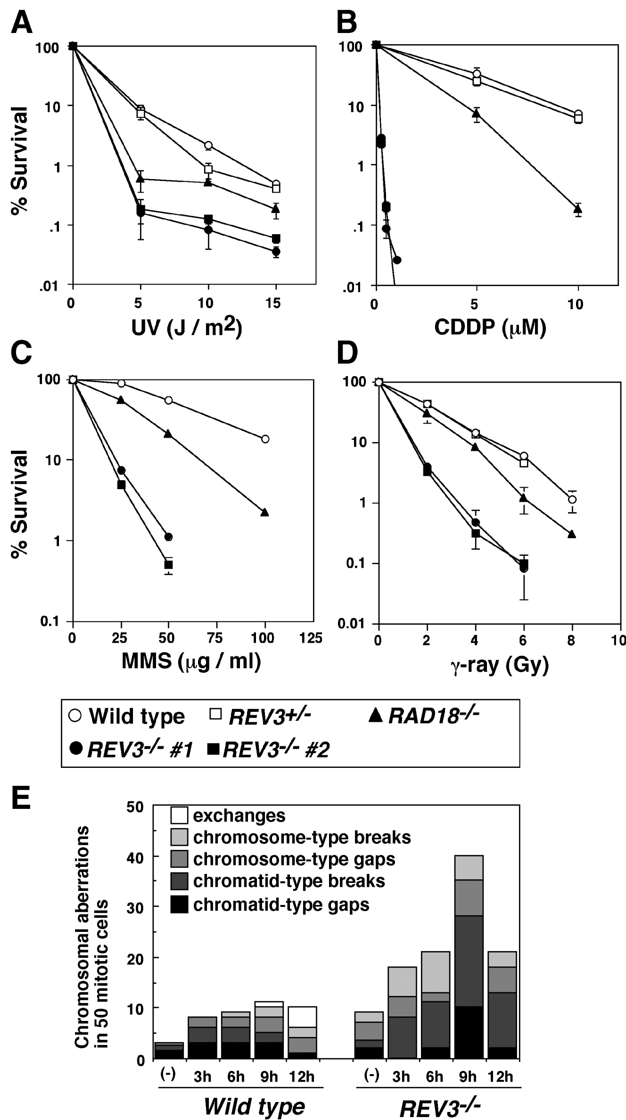

Fig. 2. Sensitivity of wild-type and *REV3*$^{-/-}$ cells to DNA-damaging agents, and UV-induced chromosomal aberrations. (A–D) The fractions of surviving colonies after the indicated treatment of cells compared with untreated controls of the same genotype are shown on the *y*-axis on a logarithmic scale. (**A**) UV; (**B**) cisplatin (CDDP); (**C**) MMS; (**D**) γ-rays. The dose of ^{137}Cs γ-rays and UV, and concentrations of cisplatin and MMS are displayed on the *x*-axis on a linear scale in each graph. The data shown are the representative results from three separate experiments. (**E**) UV-induced chromosome aberrations of wild-type and *REV3*$^{-/-}$ cells. Cells were exposed to 5 J/m^2 UV.

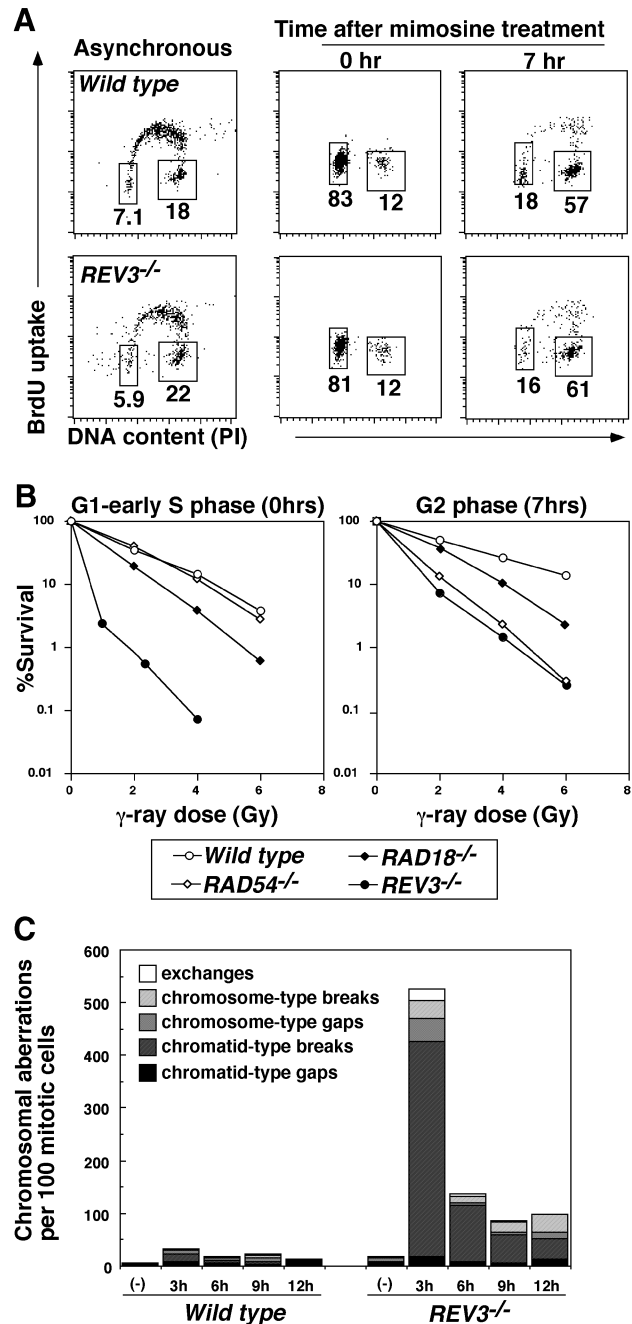

Fig. 3. Increased radiosensitivity in G_2 as well as G_1/S boundary in the absence of Rev3 (**A**) Cells of the indicated genotypes were synchronized at the G_1/S boundary with nocodazole–mimosine treatment for 16 h, and released into culture at 0 h. The vast majority of the cells were in G_1/S boundary at 0 h and in late S–G_2 phases at 7 h after the release. Live cells were gated according to their FSC and SSC profiles. (**B**) IR sensitivity of these synchronized populations is shown as in Figure 2D. (**C**) IR-induced chromosome aberrations. Results from wild-type cells and *REV3*$^{-/-}$ are shown. Cells were exposed to 2 Gy γ-rays.

Postreplication repair in REV3$^{-/-}$ *cells*

To assess the capability of PRR of *REV3*$^{-/-}$ cells, we measured the size of newly replicated DNA before and after UV-irradiation by velocity sedimentation analyses in alkaline sucrose gradients (Prakash, 1981; Tateishi *et al.*, 2000; Yamashita *et al.*, 2002). There was no obvious difference in the strand growth after UV irradiation between wild-type and *REV3*$^{-/-}$ cells (Figure 4, bottom panels), in agreement with the yeast *rev3* mutation, which has no effect on PRR of UV-damaged DNA (Prakash, 1981).

Since TLS and HR are thought to constitute major PRR pathways in yeast (Prakash, 1981; Broomfield *et al.*, 2001) as well as in *Escherichia coli* (Friedberg *et al.*, 1995; Berdichevsky *et al.*, 2002), we wanted to know which pathway is mainly affected in *REV3*$^{-/-}$ cells. To evaluate HR-mediated PRR events, we measured the level of microscopically visible sister chromatid exchange (SCE) events (Sonoda *et al.*, 1999; Dronkert *et al.*, 2000; Wang *et al.*, 2000). *REV3*$^{-/-}$ cells showed a few-fold higher spontaneous SCE level than wild-type cells (Figure 5A). This result suggests that Rev3 plays an important role

Table II. Targeted integration frequencies[a]

Genotype	Targeted locus		
	OVALBUMIN	*Ig LAMBDA*	*RAD54*
Wild type	43/47 (91.5%)	28/59 (47.4%)	53/77 (68.8%)
REV3$^{-/-}$	32/48 (66.7%)[b]	15/75 (20.0%)[c]	6/34 (17.6%)[d]
RAD18$^{-/-}$	22/54 (40.7%)	ND	3/38 (7.9%)
RAD54$^{-/-}$	0/44 (0%)	3/48 (6.3%)	ND

Wild-type and *REV3*$^{-/-}$ cells were transfected with targeting constructs of the indicated loci.
[a]The data shown are the number of targeted clones at each locus divided by the number of drug-resistant clones analyzed. The percent frequency is in parentheses.
[b–d]Significantly different from wild-type levels ([b]χ^2 = 8.8037, $P < 0.003$; [c]χ^2 = 11.4244, $P < 0.0007$; [d]χ^2 = 24.8146, $P < 0.0001$). ND, not determined.

during DNA replication even in the absence of exogenous genotoxic stress. We next measured the level of SCE following exposure of cells to UV and 4-nitroquinoline 1-oxide (4NQO), which damages base residues in a manner similar to UV irradiation (reviewed in Friedberg *et al*., 1995). The levels of UV- and 4NQO-induced SCE were 5.2 and 4.1 SCEs/cell, respectively, for wild-type cells, and 5.0 and 4.4 SCEs/cell, respectively, for *REV3*$^{-/-}$ cells (Figure 5). Thus, a defect in Rev3 elevates the level of spontaneous SCE but not UV- or 4NQO-induced SCE. It is surprising that a defect in Rev3 does not significantly affect DNA ζ synthesis on UV-damaged templates (Figure 4B), although the same defect significantly reduced colony survival (Figure 2A) and increased induced chromosomal breaks (Figure 2E) in response to UV. Presumably, a subtle defect in TLS past each UV damage may not be detectable by measuring DNA synthesis on UV-damaged templates for the following reason. Thirty seven per cent survival dose of UV irradiation induces as many as 40 000 UV damages per human cell (BEIRV, 1990), and a half-life of cyclobutane pyrimidine dimer (CPD), the major UV damage, is nearly one day (Setlow, 1985). Thus, even a slight decrease in TLS past each UV damage may substantially increase the number of gaps in each cell, leading to significant increase in the level of chromosomal breaks and reduction of colony survival.

Double mutants of REV3 and RAD54 are not able to proliferate

Increased SCE in *REV3*$^{-/-}$ cells led us to investigate the functional overlap between Rev3-dependent PRR and HR in processing gaps caused by replication block. To this end, we generated cells deficient in both Rev3 and Rad54. We showed previously that Rad54-deficient DT40 cells exhibit hypersensitivity to IR, but are able to proliferate with nearly normal kinetics (Bezzubova *et al*., 1997; Takata *et al*., 1998). Since we have failed to generate cells deficient in both Rev3 and Rad54, we generated conditional *REV3*$^{-/-}$*RAD54*$^{-/-}$ double mutant cells using the tamoxifen-inducible Cre–loxP system (Zhang *et al*., 1998; Fujimori *et al*., 2001). Upon the addition of tamoxifen to the culture media, virtually all cells lost their *RAD54* transgene (designated as *loxP-RAD54* hereafter) through Cre-recombinase (the transgene designated as *CRE* hereafter) action within 24 h (data not shown), generating mutant cells that had completely lost functional *REV3* and *RAD54* genes.

Continuous exposure of *RAD54*$^{-/-}$ or *REV3*$^{-/-}$ cells to tamoxifen did not affect their exponential proliferation (Figure 6A). In marked contrast, the *REV3*$^{-/-}$*RAD54*$^{-/-}$ (*w/loxP-RAD54, CRE*) cells exhibited dramatic reduction in growth rate in the presence of tamoxifen and eventually died. To investigate the cause of cell death, we analyzed chromosomal breaks in *REV3*$^{-/-}$*RAD54*$^{-/-}$cells. The *REV3*$^{-/-}$*RAD54*$^{+/-}$ cells showed only few chromosomal aberrations even after tamoxifen treatment (Figure 6B). In contrast with these control populations, the *REV3*$^{-/-}$*RAD54*$^{-/-}$ cells had 1.25 aberrations/cell at day 3 after adding tamoxifen (Figure 6B). We showed previously that the level of spontaneous chromosomal breaks of various HR-deficient DT40 clones is closely correlated with the rate of cell death during the cell cycle (reviewed in Morrison and Takeda, 2000; Sonoda *et al*., 2001b). These observations indicate that the increased chromosomal breaks may account for the massive cell death of *REV3*$^{-/-}$*RAD54*$^{-/-}$ cells.

Discussion

Requirement of Rev3 for PRR in DT40 cells

The present study reveals multiple roles of chicken Rev3 in PRR. First, the data show that a defect in Rev3 caused increase in sensitivities to several different types of DNA lesions. Secondly, we present genetic evidence that Rev3 is involved in targeted integration and HR-mediated DSB repair particularly following IR. Thirdly, we show that *REV3*$^{-/-}$ cells exhibited increased levels of spontaneous SCE events and chromosomal breaks. These observations as well as synthetic lethality of the *rev3* and *rad54* mutations suggests that Rev3 plays an important role in maintaining chromosomal DNA during the cell cycle. Fourthly, *REV3*$^{-/-}$ cells show a more prominent phenotype compared with *RAD18*$^{-/-}$ cells in terms of the level of spontaneous chromosomal breaks as well as sensitivity to genotoxic treatments. Thus, Rad18 does not fully regulate TLS DNA polymerases in higher eukaryotes, which is in marked contrast with yeast Rad18. Like Rad18-deficient DT40 cells, the *REV3*$^{-/-}$ DT40 cells exhibited high sensitivity to UV, IR, MMS and cisplatin, which is consistent with their role in PRR. These observations support the notion that chicken Rev3 is involved in TLS, as has been demonstrated for yeast Rev3. However, the phenotype of the DT40 *rev3* mutant is not necessarily the same as that of the yeast mutant. The yeast *rev3* mutant shows only mild sensitivity to UV and marginal sensitivity to IR and MMS (Lemontt, 1972; McKee and Lawrence, 1979; Johnson *et al*., 1998; Xiao *et al*., 1999). Thus, yeast Rev3 appears to account for only a small component of PRR.

The present study highlights Rev3 as a key factor for determining cellular response to cisplatin. There are a few explanations for the extremely high sensitivity of the *rev3* mutant to cisplatin. First, after other TLS polymerases insert deoxynucleotides opposite cisplatin damages, such as intrastrand crosslinks, Rev3 may be essential for extending from such deoxynucleotides. Another

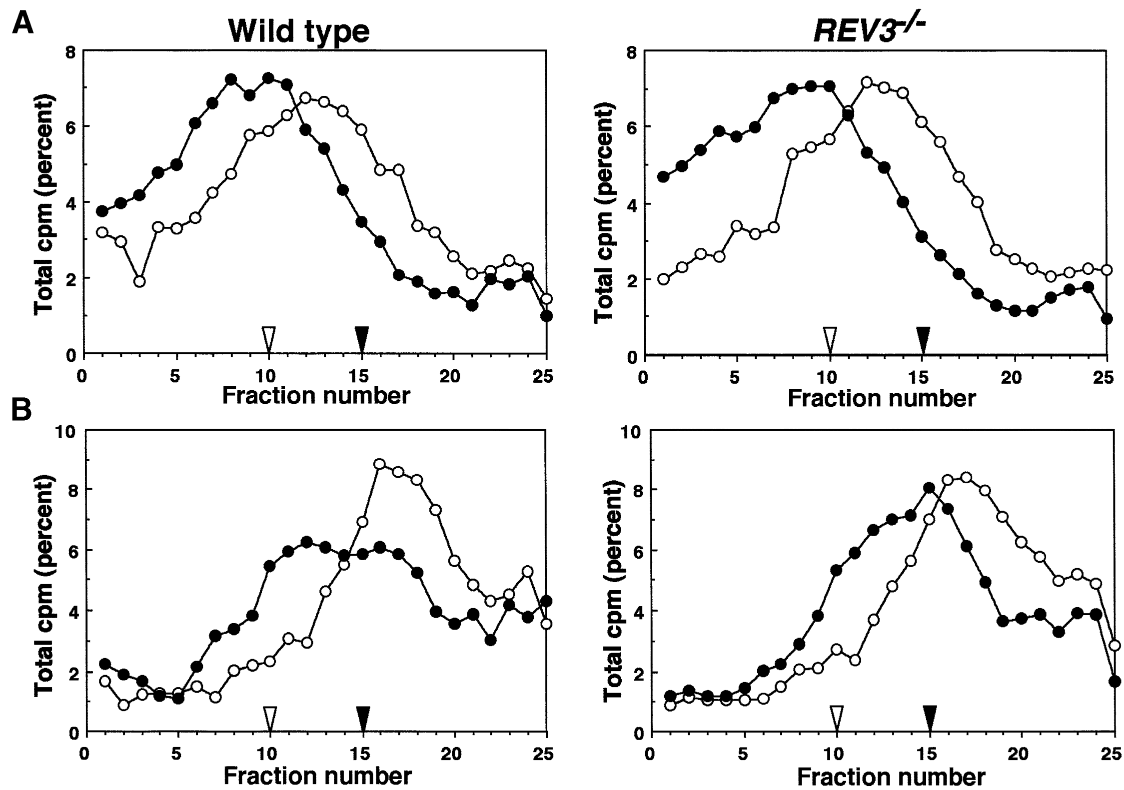

Fig. 4. Postreplication repair of Rev3-deficient DT40. Alkaline sucrose-gradient analysis of DNA from wild-type and *REV3*$^{-/-}$ cells. (**A**) Wild-type (left) and Rev3-deficient (right) DT40 cells were pulse-labeled with [^{3}H]thymidine (0.93 MBq/ml) for 15 min without UV irradiation (open circles). In a pulse–chase experiment, the pulse-labeled cells were further incubated for 30 min (closed circles) in fresh medium containing 10 μM unlabeled thymidine and uridine. Samples were sedimented on 5–20% alkaline sucrose gradients from right to left. (**B**) Wild-type DT40 cells and Rev3-deficient DT40 cells were irradiated with UV (8 J/m^2), incubated for 10 min, and then pulse-labeled with [^{3}H]thymidine (0.93 MBq/ml) for 15 min (open circles). In a pulse–chase experiment, the pulse-labeled cells were further incubated for 90 min (closed circles) in the chase medium containing 10 μM unlabeled thymidine and uridine. The samples were analyzed by the same method as (A). Closed and open arrowheads indicate the positions of bacteriophage λ DNA (42 kb) and T4GT7 DNA (165.6 kb), respectively.

explanation is synergistic effect of defective TLS and HR on processing of cisplatin damage in the *rev3* mutant. The present study suggests that analysis of the expression of polζ would be useful for predicting cisplatin sensitivity of tumor cells.

Critical roles of Rev3 in repair of IR-induced DNA damage

We have shown that a defect in Rev3 as well as Rad18 causes an elevation of IR sensitivity. This phenotype appears to be explained by defects in both TLS and probably another repair pathway, which accounts for the increased levels of chromosomal aberrations observed in cells that are irradiated in the G_2 phase of the cell cycle. The obvious first candidate repair pathways are the two major DSB repair pathways NHEJ and HR (Kanaar *et al*., 1998; van Gent *et al*., 2001). We presume that the major DSB repair pathway in G_2 cells is HR, as we showed previously (Takata *et al*., 1998). Furthermore, involvement of the Rev3 polymerase in the NHEJ pathway has not been shown in yeast genetic studies, while Rev3 is implicated in mutagenesis associated with homology-dependent DSB repair (Holbeck and Strathern, 1997). Indeed, we have failed to detect a defect in NHEJ in *REV3*$^{-/-}$ cells, using an assay to measure the relative usage of accurate recircularization of transfected linear plasmid (Tauchi *et al*., 2002; Verkaik *et al*., 2002; Supplementary figure 2). However, we did find indications of reduced HR, most notably a reduced efficiency of gene targeting in three different loci in the absence of Rev3 (Table II). The reduced HR capability can be explained by the following two explanations. First, since the HR pathway in *REV3*$^{-/-}$ cells has to repair a large number of endogenous DNA damage caused by defective TLS in the S phase, it could not efficiently repair additional IR-induced DSBs. This explanation is, however, unlikely, because cells that were exposed to γ-rays in the S phase (3–6 and 6–9 h in Figure 3C) exhibited a significant reduction in the levels of induced chromosomal breaks when compared with cells irradiated in the G_2 phase (0–3 in Figure 3C). Secondly, Rev3 may play a critical role in HR-mediated DSB repair of IR-induced DSBs, because IR-induced DSBs may be associated with chemical modification, which may make the 3′ ends unsuitable as primers for most DNA polymerases. Rev3, however, is relatively indifferent to structural distortions in the primer and template strands, including a mismatch near the 3′ end of a primer (Johnson *et al*., 2000; Lawrence *et al*., 2000; reviewed in Lawrence and Maher, 2001; Woodgate, 2001). Therefore, Rev3, but not other DNA polymerases, may be critical for initiation of DNA synthesis from the 3′ end of IR-induced DSBs in HR-mediated repair.

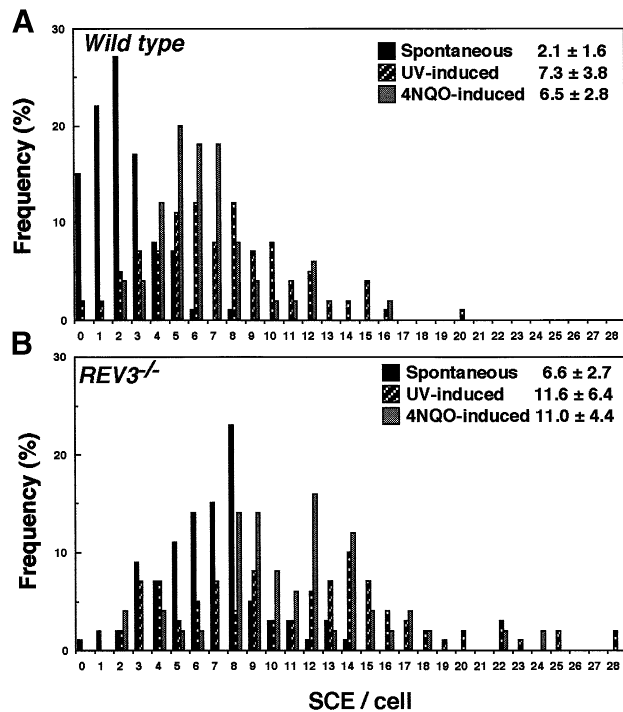

Fig. 5. Spontaneous sister chromatid exchange is elevated in *REV3*$^{-/-}$ cells. Levels of SCE per cell are shown in wild-type and *REV3*$^{-/-}$ cells. Mean ± standard error is shown at the top of each panel. Solid box, distribution of SCE/cell without treatment; shaded and dotted box, distribution of SCE/cell with 0.25 J/m^2 UV light and 0.2 ng/ml 4NQO treatment, respectively.

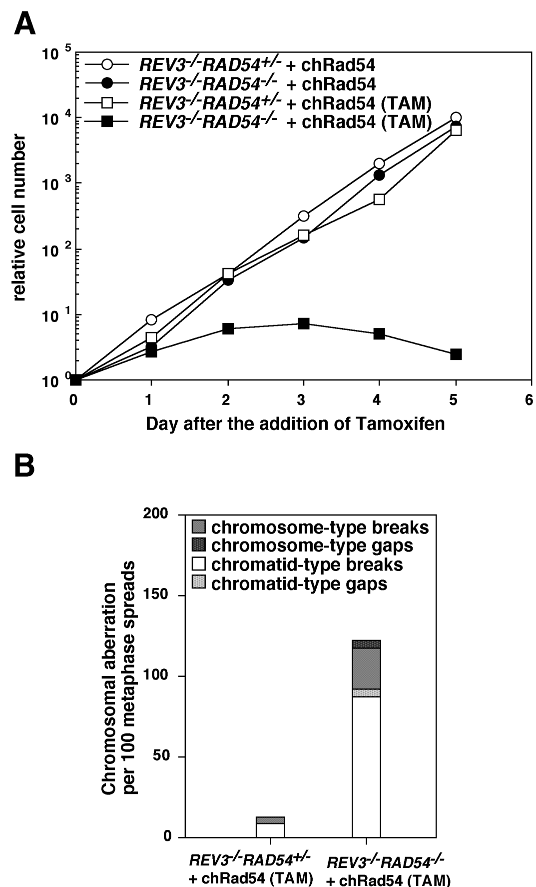

Fig. 6. *REV3*$^{-/-}$*RAD54*$^{-/-}$ double mutant cells are not viable. (**A**) Growth curves of the indicated cell cultures in the absence and presence of tamoxifen (TAM). (**B**) Chromosomal breaks are accumulated in dying *REV3*$^{-/-}$*RAD54*$^{-/-}$ double mutant cells. Cells were exposed to tamoxifen for 3 days. One hundred mitotic cells were analyzed in each case.

Although the data discussed above suggest that Rev3 is involved in HR, it should be noted that Rev3 does not play an important role in some types of HR, such as the Ig gene conversion. Likewise, a defect in Rev3 appears to result in only mild impairment of HR-mediated PRR of spontaneously arising DNA damage, since the level of spontaneous SCE was rather increased in the absence of Rev3. On the other hand, a defect in Rev3 appears to impair HR as well as TLS in repairing gaps at UV damage. Indeed, it is surprising that a defect in Rev3 did not cause an increase in the level of UV-induced SCE, although the number of UV-induced gaps should be dramatically increased in the absence of Rev3. Presumably, Rev3-dependent HR might also play a role in repairing gaps at UV damage. Alternatively, since 37% survival dose of UV irradiation induces as many as 40 000 UV damages per human cell (BEIRV, 1990), the capability of HR-mediated PRR might be saturated particularly in *REV3*$^{-/-}$ cells. Taken together, we favor the hypothesis that HR is directly affected by the Rev3 mutation.

Rev3-dependent PRR contributes to the maintenance of chromosomal DNA

Accumulating evidence has suggested that Rev3 plays an important role in maintaining chromosomal integrity in cycling cells. First, loss of Rev3 leads to embryonic lethality in the mouse (Bemark *et al*., 2000; Esposito *et al*., 2000; Wittschieben *et al*., 2000). Furthermore, a significant increase in DSBs as well as chromatid and chromosome aberrations was observed in primary cells from *REV3*$^{-/-}$ murine embryos (Van Sloun *et al*., 2002).

In this study, we show that *REV3*$^{-/-}$ DT40 cells exhibited increase in spontaneous chromosomal breaks (Table I). These observations indicate frequent employment of Rev3 in PRR during the normal cell cycle. We hypothesize that the main DNA lesions that trigger PRR in vertebrate cells may be abasic sites, as they are among the most frequently formed DNA lesions, which block the replicative DNA machinery. Since biochemical studies have shown that polζ efficiently bypass AP sites in combination with other polymerases such as polδ and Rev1 (Nelson *et al*., 1996a, 2000; Haracska *et al*., 2001), polζ may make a significant contribution to the tolerance to AP sites *in vivo*. Indeed, even if yeast cells simultaneously lack Ntg1, Ntg2 and Apn1, all of which are responsible for the processing of AP sites following base damages, they can survive because polζ readily bypasses the AP sites (Swanson *et al*., 1999). Since yeast polζ is implicated in spontaneous mutation (Quah *et al*., 1980; Roche *et al*., 1994; Harfe and Jinks-Robertson, 2000), vertebrate polζ also may have a significant impact on accumulation of base substitution and tumorigenesis by participating in PRR during physiological cell cycle.

We also found that *REV3*$^{-/-}$ cells exhibited a significant increase in the level of spontaneous SCE (Figure 5). We

interpret this increase as more frequent usage of HR-mediated PRR pathway due to a defect in the TLS-mediated PRR pathway. Alternatively, it is possible that the HR pathway is hyperactivated in *REV3*$^{-/-}$ cells. This is unlikely because the frequency of targeted integration is reduced in *REV3*$^{-/-}$ cells compared with wild-type cells (Table II). Thus, we conclude that a defect in TLS in *REV3*$^{-/-}$ cells increases the number of gaps, leading to more frequent usage of HR-mediated PRR in the mutant cells than in wild-type cells. This conclusion supports the notion that a substantial fraction of the DNA lesions that occur during DNA replication can be processed by both HR and Rev3-dependent PRR pathways. To investigate functional redundancy between these two pathways, we generated *REV3*$^{-/-}$ cells that were conditionally deficient for Rad54. The Rad54 appears to have a minor role in HR when compared with Rad51 in vertebrates, as manifested by normal embryogenesis in Rad54-deficient mice (Essers *et al*., 1997), which is in marked contrast with lethality of Rad51 depletion to the cells (Sonoda *et al*., 1998). We previously found that *rad54* and *rad18* mutations are synthetic lethal (Yamashita *et al*., 2002). Likewise, *rad54* and *rev3* mutations were synthetic lethal to the cells exhibiting extensive chromosomal breaks, although either type of the single mutants is able to proliferate. Presumably, since a large numbers of gaps, some of which could then be processed into DSBs, are generated in the absence of Rev3, even a minor defect in HR, i.e. a defect in *RAD54*, may be lethal to the cells. In conclusion, synthetic lethality of *rev3* and *rad54* and that of *rad18* and *rad54* suggest that two major PRR pathways, TLS and HR, cooperatively contribute to the maintenance of chromosomal DNA in higher eukaryotes.

Materials and methods

Plasmid construction

Two *REV3* disruption constructs, *REV3-hisD* and *REV3-bsr*, were generated from genomic PCR products combined with hisD- and bsr-selection marker cassettes. Genomic DNA sequences were amplified using the primers 5′-TCCCGCTTCTTCAGCAACGCTGG-3′and 5′-TCGTGAAATACTGTGAGATGGTGCC-3′ (for the left arm of the KO construct); and 5′-ACAGAGGTAGCTCTGCTTACAAGCC-3′ and 5′-ACGGGATCTGTCGGTCAAAGCAGCC-3′ (for the right arm of the KO construct). Amplified PCR products were cloned into pCRII-TOPO vecotor (Invitrogen). The 1.1 kb *Eco*RV fragment from the left arm were cloned into *Xho*I (blunt ended)–*Eco*RV site of pCRII containing the 4 kb right-arm sequence. The *Eco*RV site was used to clone marker gene cassettes. The 0.8 kb *Hin*dIII fragment from the genomic DNA amplified using the primers 5′-ATTACGTTAGCCGGGTCCATGGG-3′ and 5′-AGAACAGCGTTGCTGTAGAAGCGGG-3′was used as a probe for Southern blot analysis. *RAD54-puro* and *RAD54-bsr* disruption constructs were described previously (Takata *et al*., 1998). We constructed an expression vector pCR3-*loxP-RAD54*/IRES-*EGFP-loxP* (named *loxP-RAD54* in short), in which *RAD54* and green fluorescent protein (*EGFP*) genes are flanked by the *loxP* sequences, by inserting a *RAD54 Bam*HI fragment into the *Bam*HI site of pCR3-*loxP*-MCS-*loxP* (Fujimori *et al*., 2001; Yamashita *et al*., 2002).

Cell culture and DNA transfection

Cells were cultured in RPMI1640 supplemented with 10^{-5} M β-mercaptethanol, 10% fetal calf serum, and 1% chicken serum (Sigma, St Louis, MO) at 39.5°C. Methods of DNA transfection and genotoxic treatments are as described previously (Takata *et al*., 1998). Cell synchronization at the G_1/S phase transition was achieved as described previously, either by sequential nocodazole and mimosine blocks (Sonoda *et al*., 2001a) or by elutriation (Takata *et al*., 1998).

Generation of *REV3*$^{-/-}$ and *REV3*$^{-/-}$*RAD54*$^{-/-}$ (w/loxP-RAD54, CRE) cells

The wild-type DT40 cells were sequentially transfected with *REV3-bsr*- and *REV3-hisD*-targeting constructs to obtain *REV3*$^{-/-}$ cells. Then, the plasmid containing CreER chimeric recombinase (pANMerCreMer; Zhang *et al*., 1998) was transfected to delete the drug-resistant cassettes. The *REV3*$^{-/-}$ *w/CRE* cells without drug-resistant cassettes were transfected with *RAD54-puro*-targeting construct. The resulting *REV3*$^{-/-}$*RAD54*$^{+/-}$ clone was transfected with *loxP-RAD54*, which can be selected by neo-selection marker (Fujimori *et al*., 2001). *REV3*$^{-/-}$*RAD54*$^{+/-}$ (*w/loxP-RAD54*) cells were subsequently transfected with *RAD54-bsr*-targeting construct to obtain *REV3*$^{-/-}$*RAD54*$^{-/-}$ (*w/loxP-RAD54*) cells. We also transfected the plasmid containing the CreER chimeric recombinase into *RAD54*$^{-/-}$ cells, and obtained *Rad54*$^{-/-}$ *w/CRE* cells. These cells were treated by 100 nM tamoxifen as described previously (Yamashita *et al*., 2002).

Measurement of the length of cell cycle time using pulse–chase labeling of cells

The method of flowcytometric analysis of cell cycle is as described previously (Takata *et al*., 1998).

Colony formation assay following genotoxic treatments

Colony formation assay was performed as described previously (Okada *et al*., 2002).

Measurement of the size of newly synthesized DNA strands following UV irradiation

Postreplication repair was analyzed by the sedimentation velocity method as described previously (Tateishi *et al*., 2000; Yamashita *et al*., 2002).

Analysis of irradiation-induced damage checkpoints

To monitor the S phase checkpoint, cells were either untreated or irradiated with 4 Gy γ-rays, then incubated for 1 h. [^{3}H]thymidine (20 μCi/ml) was pulsed for the last 15 min. Inhibition of DNA synthesis, which indicates the activation of S phase checkpoint, was monitored by the [^{3}H]thymidine incorporation. To monitor the G_2/M checkpoint, cells were either untreated or irradiated with 2 Gy γ-rays, then incubated with colcemid and aliquots of the culture were taken every 1 h. Cells in mitosis were identified by co-staining with PI and antibody to anti-phospho-histone H3 (Upstate, New York).

Chromosome aberration analysis

Karyotype analysis was performed as described previously (Sonoda *et al*., 1998). For the morphological analysis of chromosome aberrations, cells were treated with colcemid for 3 h to enrich mitotic cells.

Measurement of SCE levels

Measurement of SCE levels was performed as described previously (Okada *et al*., 2002; Yamashita *et al*., 2002).

Measurement of targeted integration frequencies.

To analyze targeted integration events at the *Ovalbumin Igλ* (Buerstedde and Takeda, 1991) and *RAD54* (Bezzubova *et al*., 1997) loci, disruption construct DNAs were transfected into cells, and Southern blot analysis was performed following selection of clones resistant to appropriate antibiotics.

Analysis of Ig gene conversion

CL18 is a surface IgM$^-$ (sIgM$^-$) subclone of DT40 (Buerstedde *et al*., 1990) and is the parental clone for the Rev3 mutants described here. We confirmed Rev3-deficient clones retained the same frameshift mutation in the Vλ sequence as do wild-type CL18 cells. As described previously (Buerstedde *et al*., 1990; Sale *et al*., 2001), we assessed the Ig gene conversion by measuring the gain and loss of sIgM expression during a 3 week period.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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