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A novel role for the Bcl-2 protein family: specific suppression of the *RAD51* recombination pathway

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The oncogenic role of Bcl-2 is generally attributed to its protective effect against apoptosis. Here, we show a novel role for Bcl-2: the specific inhibition of the conservative *RAD51* recombination pathway. Bcl-2 or Bcl-x_L overexpression inhibits UV-C-, γ-ray- or mutant p53-induced homologous recombination (HR). Moreover, Bcl-2 recombination inhibition is independent of the role of p53 in G_1 arrest. At an acute double-strand break in the recombination substrate, Bcl-2 specifically inhibits *RAD51*-dependent gene conversion without affecting non-conservative recombination. Bcl-2 consistently thwarts recombination stimulated by *RAD51* overexpression and alters Rad51 protein by post-translation modification. Moreover, a mutant ^{G145A}Bcl-2, which is defective in Bax interaction and in apoptosis repression, also inhibits recombination, showing that the death and recombination repression functions of Bcl-2 are separable. Inhibition of error-free repair pathways by Bcl-2 results in elevated frequencies of mutagenesis. The *Bcl-2* gene therefore combines two separable cancer-prone phenotypes: apoptosis repression and a genetic instability/mutator phenotype. This dual phenotype could represent a mammalian version of the bacterial SOS repair system.

Keywords: apoptosis/DNA repair/homologous recombination/mutagenesis/*RAD51*

Introduction

Genome integrity and cell proliferation/viability are commonly regulated by a network of pathways including cell cycle checkpoints, DNA repair/recombination and programmed cell death. In response to genotoxic attacks, proliferating cells temporarily stall in their cycle, allowing the repair of the injured DNA (Hartwell, 1992, Hartwell *et al*., 1994). Alternatively, cells activate their programmed cell death (Rich *et al*., 1999; Wyllie *et al*., 1999).

p53 is the gene most frequently found to be mutated in human tumours (Hollstein *et al*., 1991; Levine *et al*., 1991). The p53 protein mediates cell cycle checkpoints and apoptosis (for review see Donehower and Bradley, 1993; Hainaut, 1995; Smith and Fornace, 1995; Ko and Prives, 1996). In addition, the status of p53 can affect homologous recombination frequency (Wiesmuller *et al*., 1996; Bertrand *et al*., 1997; Mekeel *et al*., 1997; Dudenhoffer *et al*., 1999; Saintigny *et al*., 1999). However, the effect of p53 on homologous recombination can be independent of G_1 checkpoint alteration, suggesting that p53 acts on recombination via a pathway other than the G_1 checkpoint control (Dudenhoffer *et al*., 1999; Saintigny *et al*., 1999). Since p53 protein also controls apoptosis, this raises the question, what effect does apoptosis regulation have on homologous recombination. In line with this, the mammalian recombination protein *RAD51* is a target for caspase degradation during programmed cell death (Flygare *et al*., 1998; Huang *et al*., 1999). However, the relationship between apoptosis and regulation of homologous recombination is poorly understood.

One efficient and classic way to repress apoptosis is to overexpress Bcl-2 oncogene family members. Indeed, DNA damage can induce two pathways to apoptosis, one p53 dependent and one p53 independent, and both pathways can be inhibited by Bcl-2 (Strasser *et al*., 1994). Bcl-2 becomes oncogenic when overexpressed, such as in follicular B-cell lymphomas resulting from a t(14:18) translocation (Bakhshi *et al*., 1985; Cleary and Sklar, 1985; Tsujimoto *et al*., 1985). Bcl-2, as well as other members of its family such as Bcl-X_L, has anti-apoptotic activity (for review see Adams and Cory, 1998). Since cells become more resistant to genotoxic agents, this raised the question of how DNA repair pathways are regulated when protecting the cells against apoptosis. Here we address the question of whether the expression of Bcl-2 affects homologous recombination efficiency. Using a combination of different substrates and strategies to monitor recombination, the present paper focuses on the detailed characterization of the recombination pathways affected by Bcl-2 (or Bcl-X_L) overexpression. We overexpressed Bcl-2 in mammalian cell lines containing the tandem repeat recombination substrates depicted in Figure 1. These substrates allow the monitoring of different intrachromosomal recombination pathways. Using a substrate containing a cleavage site for the rare-cutting endonuclease I-*Sce*I (Figure 1B), it is possible to target a unique double-strand break (DSB) in the recombination substrate (Liang *et al*., 1998). This strategy has permitted the definition of two homology-directed DSB repair pathways: (i) a non-conservative recombination mechanism arising by single-strand annealing (SSA) and leading to deletion of the intervening sequence (Neo^R/Hyg^S); (ii) a conservative recombination mechanism initiated by strand invasion and leading to gene conversion with or without associated crossing over (double-resistant Neo^R/Hyg^R). In contrast to SSA, conservative recombination is *RAD51* dependent in both yeast (Ivanov *et al*., 1996) and mammalian cells (Lambert and Lopez, 2000).

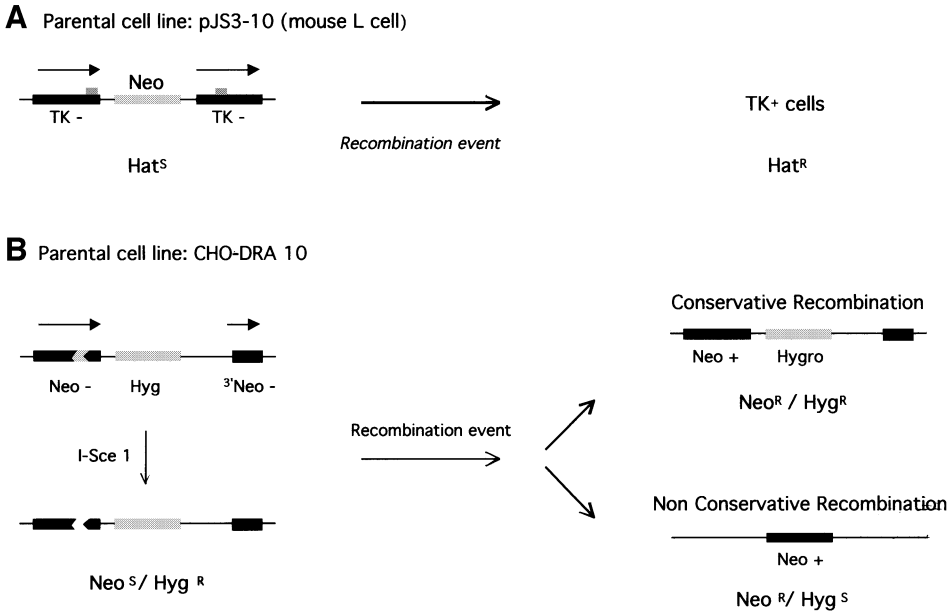


Fig. 1. Recombination substrates. (**A**) Parental pJS3-10 is a mouse L*tk*⁻ cell line containing a tandem repeat of two inactive *TK* genes (black boxes) from herpes simplex virus type I (the grey square corresponds to the inactivating mutations). The cells are deficient in thymidine kinase activity (*tk*⁻) and, thus, sensitive to the HAT selective medium. Recombination between the two *TK* sequences restores a functional *TK* gene and resistance to HAT. The frequency of recombination is estimated by the frequency of HAT-resistant clones (Liskay *et al*., 1984). (**B**) Parental CHO-DRA10 is a hamster CHO K1 cell line containing a tandem repeat of two inactive neomycin (Neo) resistance genes (black boxes). In between the two Neo sequences is the hygromycin-resistant (Hyg) sequence (grey box). The parental lines are sensitive to G418 (Neo^S) and resistant to hygromycin (Hyg^R). All the recombinants become G418 resistant (Neo^R). One Neo cassette contains a cleavage site (grey arrow) for the yeast rare-cutting enzyme I-*Sce*I. Expression of I-*Sce*I results in a DSB targeted in the recombination substrate. Conservative recombination leads to Neo^R/Hyg^R double-resistant clones. Non-conservative recombination leads to Neo^R single-resistant clones (Liang *et al*., 1998). Non-conservative recombination mainly corresponds to single-strand annealing (SSA), an *RAD51*-independent process. Conservative recombination corresponds to gene conversion or intrachromatid crossing over (followed by re-integration of the excised product), two *RAD51*-dependent processes (Ivanov *et al*., 1996; Lambert and Lopez, 2000).

Here we show that Bcl-2 expression specifically inhibits the *RAD51*-dependent conservative recombination pathway, independently of programmed cell death repression. We also show that the expression of Bcl-2 results in modification of post-translation regulation of Rad51 protein. Taken together, the results suggest that *RAD51*-dependent conservative recombination and apoptosis are controlled by two separable functions of Bcl-2 (or Bcl-X_L). In addition to its anti-apoptotic phenotype, Bcl-2 also shows a mutator phenotype, both of which could contribute to a pre-cancerous predisposition.

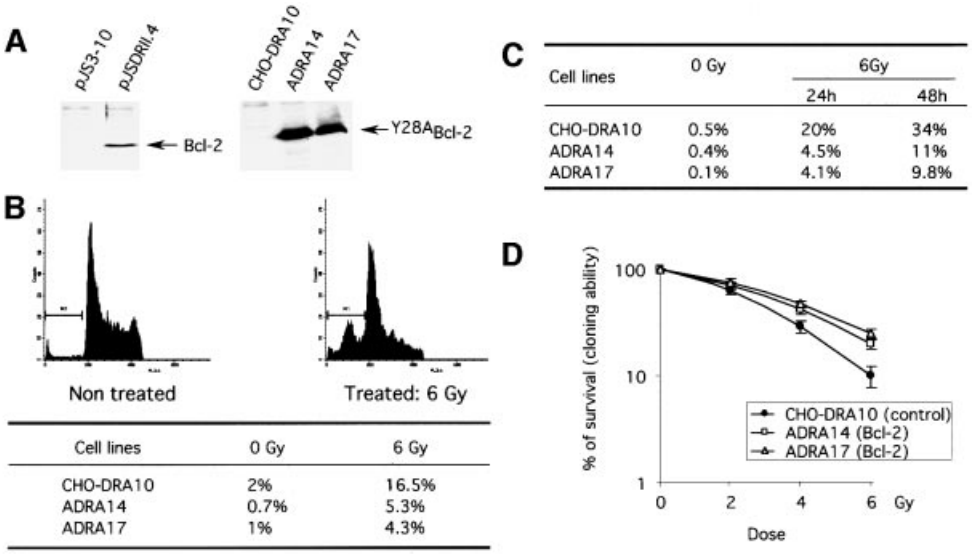
Results

Expression of Bcl-2 in cell lines carrying intrachromosomal recombination substrates

We used the recipient lines and strategies described in Figure 1. The pJS3-10 line derives from mouse L*tk*⁻ cells, which are sensitive to HAT medium, and contains a copy of a duplication of inactive herpes simplex type I *TK* gene. Recombination between the two *TK* sequences restores a functional *TK* gene and thus HAT resistance. The frequency of recombination can be calculated from the number of HAT-resistant clones relative to the total number of viable plated cells. CHO-DRA10 contains a direct repeat of two inactive neomycin (*NEO*) resistance genes, separated by a hygromycin-resistant gene. Recombination restores a functional *NEO* gene and thus resistance to G418 (Neo^R). One NEO cassette contains one I-*Sce*I cleavage site. Expression of the I-*Sce*I rare-cutting endonuclease produces a DSB targeted into the recombination NEO cassette (Liang *et al*., 1998). Repair of this DSB via a conservative gene conversion event produces a double-resistant Neo^R/Hyg^R clone, and a non-conservative event produces a Neo^R but hygromycin-sensitive (Hyg^S) clone. A few double-resistant Neo^R/Hyg^R (2 out of 11 double-resistant clones analysed) recombinant clones result from intrachromatid crossing over then random re-integration of the excised product (Lambert and Lopez, 2000). These events are also initiated by strand exchange and are *RAD51* dependent.

The different lines were transfected with mammalian expression vectors, to obtain derivative lines stably expressing Bcl-2 or mutant Bcl-2 proteins (Figure 2A). The wild-type Bcl-2 protein inhibits apoptosis and affects G_0–G_1 cell cycle entry. These two functions of Bcl-2 can be separated and the mutant ^{Y28A}Bcl-2 has been shown to maintain its anti-apoptosis function but to be deficient in its role in G_0–G_1 cell cycle entry (Huang *et al*., 1997). Mutant ^{G145A}Bcl-2 has been shown to be deficient in its interaction with Bax and in apoptosis repression (Yin *et al*., 1994). The different derivative lines are listed in Table I. Analysis by immunofluorescence shows that overexpression of Bcl-2 in our lines does not seem to modify its classical localization (data not shown).

We first verified the apoptotic cell death repression activity resulting from ^{Y28A}Bcl-2 expression. The protective effect of ^{Y28A}Bcl-2 in the CHO-DRA10 line, after treatment with ionizing radiation, was checked by different methods: (i) the frequency of cells with a sub-G_1 DNA



Cell lines	0 Gy	6 Gy
CHO-DRA10	2%	16.5%
ADRA14	0.7%	5.3%
ADRA17	1%	4.3%

Cell lines	0 Gy	6Gy	
		24h	48h
CHO-DRA10	0.5%	20%	34%
ADRA14	0.4%	4.5%	11%
ADRA17	0.1%	4.1%	9.8%

Fig. 2. Bcl-2 expression and apoptosis repression. (**A**) Detection by western blotting of the expression of Bcl-2. pJS3-10 is the mouse parental cell line; pJSDRII.4 is a pJS3-10 line overexpressing Bcl-2. CHO-DRA10 is the hamster parental line; ADRA14 and ADRA17 are two independent clones overexpressing ^{Y28A}Bcl-2. (**B**) Apoptotic sub-G_1 population, measured by FACS, 48 h after 6 Gy irradiation. Upper panel, an example of a typical histogram (M1 corresponds to the sub-G_1 population); lower panel, frequency of sub-G_1 cells 48 h after irradiation. (**C**) Measurement of apoptosis by means of Hoechst fluorescence; the percentages correspond to the percentage of cells with fragmented nuclei. ADRA14 and ADRA17 correspond to two independent clones expressing ^{Y28A}Bcl-2. (**D**) Effect of Bcl-2 expression on survival of CHO-DRA10, after γ-rays.

Table I. Cell lines

Cell lines	Origin	Expression of an exogenous human protein	
		p53 mutant protein	Bcl-2 protein
pJS 3.10	mouse L cell	none[a]	none
pJS DR II.4	*pJS 3.10*	none	Bcl-2
H175 DR 211	*pJS 3.10*	175 (Arg→His)	none
H175 DR II.3	*pJS 3.10*	175 (Arg→His)	Bcl-2
H175 DR II.5	*pJS 3.10*	175 (Arg→His)	Bcl-2
H273 DR11	*pJS 3.10*	273 (Arg→His)	
P273 DR 4	*pJS 3.10*	273 (Arg→Pro)	
CHO-DRA10	hamster CHO-K1	none[b]	none
Cm3[c]	*CHO-DRA10*	none	none
A3[c]	*CHO-DRA10*	none	none
ADRA 8	*CHO-DRA10*	none	^{Y28A}Bcl-2
ADRA14	*CHO-DRA10*	none	^{Y28A}Bcl-2
ADRA17	*CHO-DRA10*	none	^{Y28A}Bcl-2
BDRA1	*CHO-DRA10*	none	^{G145A}Bcl-2
BDRA2	*CHO-DRA10*	none	^{G145A}Bcl-2

[a]The pJS 3.10 parental cell line expresses an endogenous wild-type p53 protein (see Bertrand *et al*., 1997; Saintigny *et al*., 1999).
[b]The CHO-DRA10 parental cell line expresses an endogenous mutant p53 protein.
[c]The Cm3 and A3 cell lines are independent clones from the CHO-DRA10 parental cell line transfected with the empty expression vector.

content, measured by fluorescence-activated cell sorting (FACS); and (ii) the nuclear fragmentation, visualized by means of Hoechst 33342, a fluorescent DNA intercalating agent. Forty-eight hours after irradiation (6 Gy), the percentage of apoptotic cells (sub-G_1 cell population) was 16.5% in the control cells and 5.3 and 4.3% in two independent clones expressing ^{Y28A}Bcl-2 (Figure 2B). The frequency of fragmented nuclei (monitored by Hoechst fluorescence) consistently decreased 3- and 3.5-fold in two independent irradiated lines expressing ^{Y28A}Bcl-2 (Figure 2C). Thus, both methods confirmed in our cell line the well-established cell death repression activity of ^{Y28A}Bcl-2. These results are in agreement with a 2- to 3-fold increased viability after γ-rays (6 Gy) of both mouse fibroblasts (pJS3-10) and CHO-DRA10 lines expressing Bcl-2 or ^{Y28A}Bcl-2 (Figure 2D).

Bcl-2 expression suppresses the induction of recombination by γ-rays or UV-C

Both γ-rays and UV-C stimulated homologous recombination in the parental CHO-DRA10 lines (Figure 3). ^{Y28A}Bcl-2 expression inhibited induction of recombination by γ-rays (Figure 3A) as well as by UV-C (Figure 3B). Since these two different genotoxic stresses produce different types of DNA damage, Bcl-2 recombination inhibition (BRI) is not specific to the type of genotoxic stress.

Anti-recombination and anti-apoptosis are separable functions of Bcl-2

One important question is whether BRI requires the interaction between Bcl-2 and Bax proteins, and whether it is associated with or separable from the apoptosis repression activity. More generally, BH3-only members of the Bcl-2 family are critical initiators of apoptosis that can be repressed by Bcl-2. However, mutants in the BH1 domain of Bcl-2 are unable to inhibit the pro-apoptotic activity of such BH3-only proteins (O'Connor *et al*., 1998).

To address this question, we expressed the mutant ^{G145A}Bcl-2 (mutated in the BH1 domain of Bcl-2) in the CHO-DRA10 cell line. ^{G145A}Bcl-2 is defective in the Bax interaction and the protection against apoptosis (Yin *et al*., 1994). We first verified the effect on apoptosis by

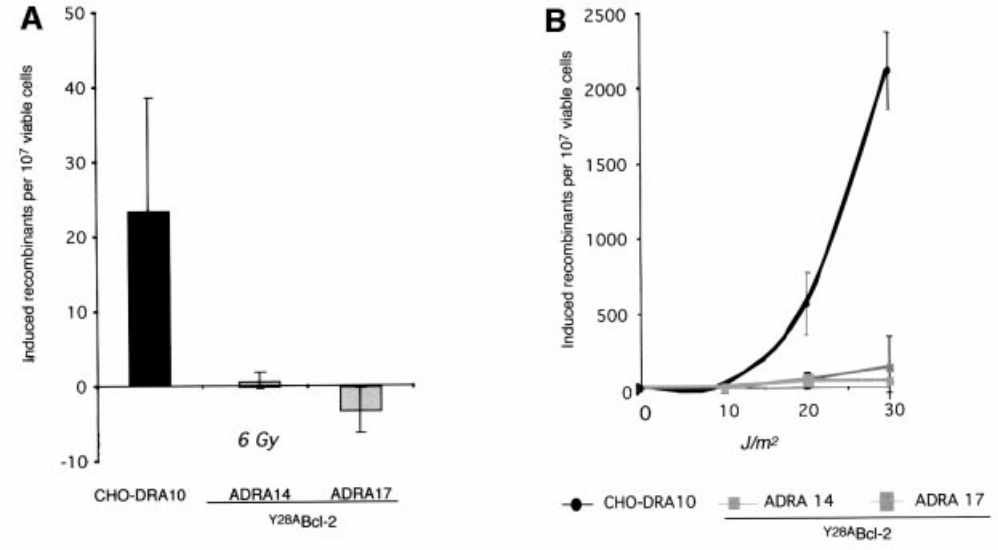


Fig. 3. Bcl-2 expression inhibits radiation-induced recombination. (**A**) γ-rays (6 Gy). (**B**) UV-C; the doses are indicated on the figure. Black bar, parental CHO-DRA10 line (control line); grey bar (ADRA14, ADRA17), two independent clones expressing ^{Y28A}Bcl-2.

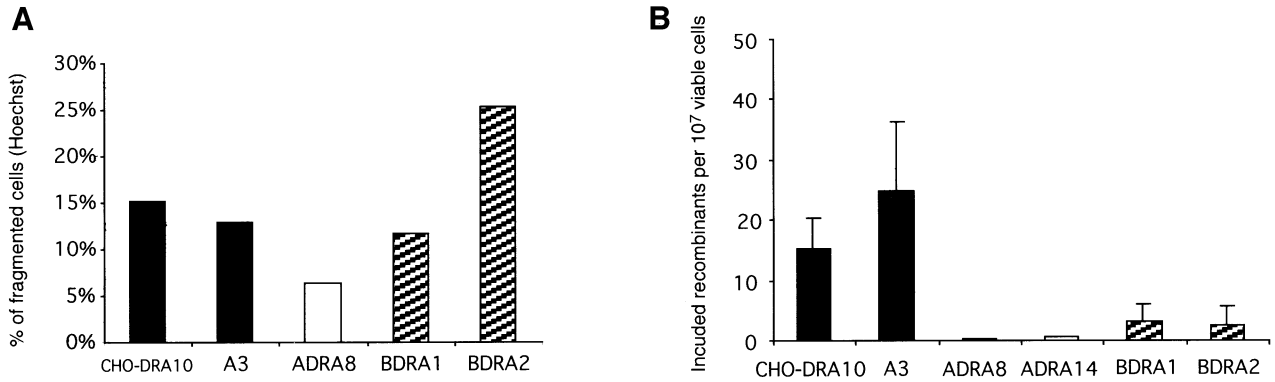


Fig. 4. Effect of the ^{G145A}Bcl-2 mutant on apoptosis (A) and radiation-induced recombination (B), 48 h after irradiation (6 Gy). (**A**) Frequency of fragmented nuclei measured by Hoechst fluorescence. (**B**) Induction of recombination by γ-rays. CHO-DRA10, parental line (control); A3, CHO-DRA10 transfected with an empty expression vector (control); ADRA8, CHO-DRA10 expressing ^{Y28A}Bcl-2; BDRA1 and BDRA2, two independent clones expressing ^{G145A}Bcl-2.

measuring the frequency of fragmented nuclei after exposure to radiation. ^{Y28A}Bcl-2 exhibited a protective effect, whereas ^{G145A}Bcl-2 showed no anti-apoptotic phenotype in two independent clones (Figure 4A). We then measured the effect of ^{G145A}Bcl-2 expression on radiation-induced recombination. Expression of ^{G145A}Bcl-2 as well as of ^{Y28A}Bcl-2 strongly impaired the induction of recombination by ionizing radiation (Figure 4B). This result shows that recombination inhibition and apoptosis repression are separable activities of Bcl-2. In addition, this result suggests that the Bcl-2–Bax interaction is not required for BRI.

Transient expression of Bcl-2 or Bcl-X_L inhibits radiation-induced recombination

Bcl-2 belongs to a family of anti-apoptosis genes (Adams and Cory, 1998). In order to determine whether BRI is specific to Bcl-2, we repeated these tests in cells expressing another member of the Bcl-2 family: Bcl-X_L (Adams and Cory, 1998). We measured the impact of transient overexpression of Bcl-2, ^{Y28A}Bcl-2 or Bcl-X_L on radiation-induced recombination, tested 24 h after transfection in the pJS3-10 cell line and its derivatives (Figure 5).

Transient expression of Bcl-2 as well as of Bcl-X_L completely impaired radiation-induced recombination (Figure 5A). This result shows that BRI is not specific to Bcl-2 since another member of the family, such as Bcl-X_L, can also suppress the induction of recombination.

Furthermore, the fact that transient expression of Bcl-2 or Bcl-X_L suppresses the recombination induction shows that BRI results directly from Bcl-2 or Bcl-X_L expression and not from a secondary associated phenotype selected during the long-term isolation of stable transfectants (2–3 weeks of selection).

Bcl-2 and Bcl-X_L inhibit recombination independent of p53 status

To investigate whether BRI is affected by p53 status, we used either the parental pJS3-10 (wild-type p53) or pJS3-10 expressing different mutant p53 proteins with

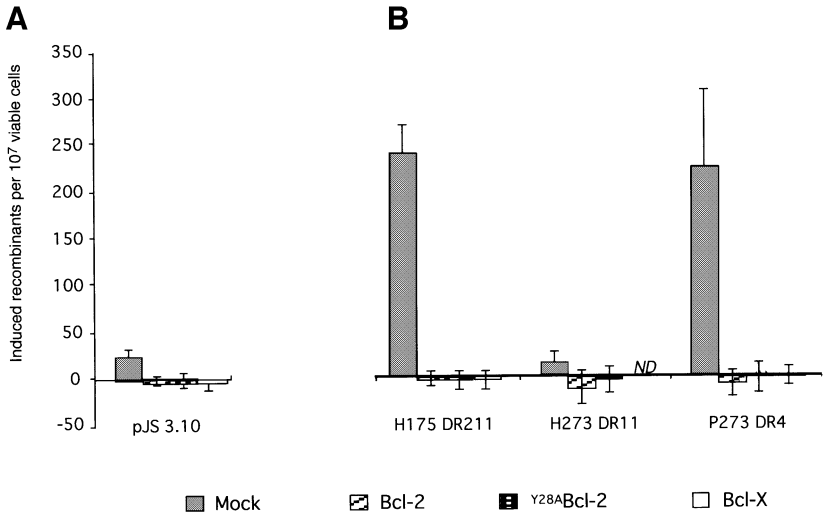


Fig. 5. Transient expression of Bcl-2 family members inhibits radiation-induced recombination, independently of p53 status. Cells were irradiated at a dose of 6 Gy. The expressed transgenes and their respective symbols are indicated on the figure. (**A**) Parental (wild-type p53) pJS3-10. (**B**) pJS3-10 derivative cell lines expressing different mutant p53 with various effects on recombination (Saintigny *et al.*, 1999). The expressed mutant p53 are reported in Table I.

Table II. Spontaneous homologous recombination between direct repeat sequences

Cell lines	Expression of exogenous Bcl-2 or Y28ABcl-2 protein	Number of independent cultures	Recombination rate ($\times 10^{-6}$/cell/generation)	
			Luria and Delbrück	Lea and Coulson
pJS 3.10	none	18	1.5 ± 0.8	1.9
pJS DR II.4	Bcl-2	12	2 ± 0.7	2.3
H175 DR 211	none	18	9.7 ± 0.7	11.7
H175 DR II.3	Bcl-2	6	1.8 ± 0.6	1.7
H175 DR II.5	Bcl-2	12	2.7 ± 0.6 ($\times 10^{-7}$/cell/generation)	3.4
CHO-DRA10	none	6	5.5 ± 0.4	6.6
ADRA14	^{Y28A}Bcl-2	6	1.3 ± 0.6	1.5
ADRA17	^{Y28A}Bcl-2	6	1.4 ± 0.6	1.6

various effects on cell cycle control and/or on recombination efficiency. His175 p53 affects the G_1–S checkpoint after radiation, whereas neither His273 nor Pro273 p53 modifies the G_1 block after irradiation. Moreover, His175 or Pro273 mutant p53 proteins stimulate radiation-induced recombination whereas, the His273 mutant p53 protein does not (Saintigny *et al.*, 1999).

Irradiation moderately stimulated recombination in the parental pJS3-10 and H273DR11 cell lines (expressing the His273 p53 protein) and strongly stimulated recombination in lines expressing either His175 or Pro273 p53 (Figure 5B), as previously described (Saintigny *et al.*, 1999). Transient expression of either Bcl-2, ^{Y28A}Bcl-2 or Bcl-X_L inhibited radiation-induced recombination in all cell lines tested (Figure 5B). Thus, the status of p53 (for recombination as well as for the G_1 checkpoint) did not affect BRI. Whatever the extent of recombination stimulation, Bcl-2 suppressed radiation-induced recombination.

Effect of Bcl-2 overexpression on spontaneous recombination

Expression of Bcl-2 inhibits recombination induced by profound genotoxic stresses such as UV or γ-radiation. We checked whether spontaneous recombination, i.e. without a drastic exogenous genotoxic stress, is also affected by Bcl-2 expression. Spontaneous recombination was measured by fluctuation analysis using two assays: the Luria and Delbrück or Lea and Coulson test (Luria and Delbrück, 1943; Lea and Coulson, 1948; Capizzi and Jameson, 1973).

In mouse L-cells, expression of Bcl-2 has no effect on the spontaneous recombination rate in the parental pJS3-10 line (Table II). Expression of the mutant His175 p53 protein led to a 6-fold increase in spontaneous recombination in the pJS3-10 line (Table II; Bertrand *et al.*, 1997; Saintigny *et al.*, 1999). In these lines, expression of Bcl-2 abolished the p53 stimulation of the spontaneous recombination rate to the level of the parental wild-type p53 pJS3-10 line (Table II). The hamster CHO-DRA10 line contains an endogenous mutant (Lys211) p53 protein (Hu *et al.*, 1999). In two independent CHO-DRA10 derivative lines, expression of ^{Y28A}Bcl-2 resulted in a 4-fold decrease in the spontaneous recombination rate (Table II).

Thus, Bcl-2 does not inhibit the basal level of spontaneous recombination, but suppresses the stimulation of

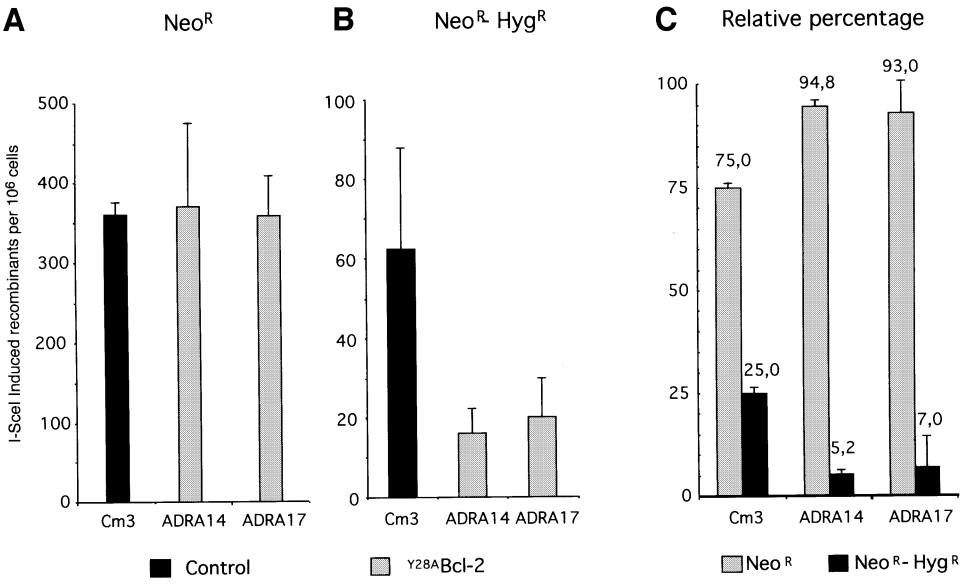


Fig. 6. Effect of Bcl-2 on recombination induced by I-*Sce*I. (**A**) Number of total recombinants (Neo^R). (**B**) Number of conservative events (Neo^R/Hyg^R). (**C**) Distribution (percentage) of class of events: non-conservative events (grey bars); conservative events (black bars). The numbers on top of the histograms indicate the exact value of the percentage. Cm3, control corresponding to the parental CHO-DRA10 cell line transfected with the empty expression vector. ADRA14 and ADRA17 correspond to two independent clones expressing ^{Y28A}Bcl-2.

recombination resulting here from the expression of mutant p53 proteins, even in the absence of profound exogenous genotoxic stress.

Bcl-2 specifically inhibits conservative recombination events induced by a unique DSB

Taken together, these results show that Bcl-2 expression inhibits recombination stimulation by UV-C and γ-rays as well as by mutant p53 proteins, independently of the toxicity of the treatment. In order to further the molecular characterization of the recombination pathway affected, we checked the effect of Bcl-2 expression on a unique and acute DSB, targeted to the recombination substrate. We used the I-*Sce*I strategy in the CHO-DRA10 lines (Figure 1). Expression of I-*Sce*I, which produces the DSB, is not toxic for the cells but strongly stimulates homology-directed recombination (Liang *et al.*, 1998). More importantly, this strategy distinguishes between two recombination pathways: conservative recombination (double-resistant Neo^R/Hyg^R) and non-conservative recombination (single Neo^R). Conservative events (double-resistant Neo^R/Hyg^R) are initiated by strand invasion and are thus *RAD51*-dependent, whereas non-conservative events (single Neo^R) mainly correspond to SSA, an *RAD51*-independent process (Ivanov *et al.*, 1996; Lambert and Lopez, 2000). SSA systematically results in the deletion of the sequence between the tandem recombination markers; it is thus an error-prone pathway.

As previously shown, transfection by I-*Sce*I stimulated the total number of recombinant (Neo^R) by 100- to 1000-fold (Liang *et al.*, 1998). These recombinants correspond to the sum of conservative and non-conservative events. The expression of Bcl-2 did not modify the overall frequency of Neo^R recombinants (Figure 6A). However, the expression of Bcl-2 did modify the relative proportion of the different classes of events. Bcl-2 expression resulted in a significant 3-fold decrease in the frequency of double-resistant (Neo^R/Hyg^R) clones (Figure 6B). This result shows that the expression of Bcl-2 does not affect the non-conservative recombination events, but specifically inhibits conservative recombination events (double-resistant Neo^R/Hyg^R). The percentage of conservative events (double-resistant Neo^R/Hyg^R colonies) relative to all the recombinant colonies (Neo^R alone) was examined (Figure 6C). Since this value is normalized to the frequency of Neo^R colonies (representing the whole recombinant population), the calculation is based on an internal standard and is independent of transfection and cleavage efficiencies. In the control cell line, conservative events (Neo^R/Hyg^R colonies) comprised 25% of the total recombinant colonies. In the two independent lines overexpressing Bcl-2, the percentage of double-resistant Neo^R/Hyg^R colonies fell to 5.2 and 7.6%, respectively (Figure 6C).

Taken together, these results show that global DSB healing is unaffected and that Bcl-2 does not act on all recombination pathways, but specifically on the conservative homologous recombination pathway. The fact that the frequency of Neo^R is unaffected suggests that non-homologous end-joining (NHEJ) and SSA pathways are not inhibited by Bcl-2 expression.

Finally, this result shows that Bcl-2 is able to exercise its effect on a unique DSB, in the absence of an extreme deleterious genotoxic stress.

Bcl-2 suppresses RAD51-induced recombination

Conservative recombination is *RAD51* dependent, whereas NHEJ and SSA are *RAD51* independent. To confirm further that Bcl-2 overexpression inhibits recombination events promoted by *RAD51*, we used a CHO-DRA10 derivative cell line overexpressing mammalian *RAD51*. We have previously shown that overexpression

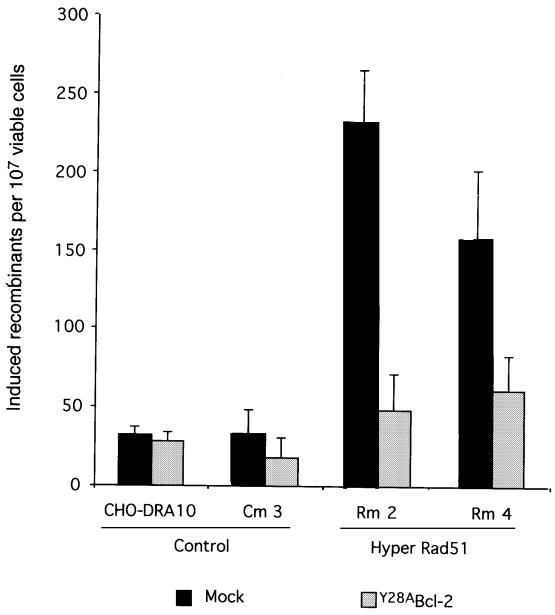


Fig. 7. Bcl-2 inhibits recombination stimulated by *RAD51*. Cells were irradiated at 6 Gy. Cm3, control cell line corresponding to the parental CHO-DRA10 transfected with the empty expression vector. Rm2 and Rm4 are two independent clones overexpressing mouse *MmRAD51*, leading to a stimulation of radiation-induced recombination (Lambert and Lopez, 2000). Black bars, transfection with an empty vector; grey bars, transfection with a ^{Y28A}Bcl-2 expression vector.

of the mouse *MmRAD51* cDNA strongly stimulates homologous recombination after ionizing radiation (Lambert and Lopez, 2000). We tested here whether Bcl-2 expression is able to suppress the stimulation of radiation-induced recombination resulting from the expression of *MmRAD51*.

At a dose of 6 Gy, overexpression of *MmRAD51* led to a 10-fold stimulation of radiation-induced recombination compared with the control lines. In the lines transfected with the Bcl-2 expression vector, *RAD51*-stimulated recombination was reduced to the level of the control lines (Figure 7). This result indicates that the recombination stimulation provoked by the overexpression of *MmRAD51* was thwarted by the expression of Bcl-2, and is consistent with the data showing a specific inhibition of conservative recombination events.

Bcl-2 expression affects post-translation modification of Rad51 protein

The data presented above show a specific inhibition of the *RAD51* recombination pathway. In order to gain some clues regarding the molecular mechanisms involved, we focused on the Rad51 protein. Rad51 protein acts in a huge protein complex, but we have previously shown that it plays a pivotal role in gene conversion regulation. Overexpression of only Rad51 protein or of a dominant-negative form is sufficient to stimulate or to inhibit gene conversion (Lambert and Lopez, 2000). These results indicate that acting on the regulation of Rad51 protein alone could be sufficient to regulate the whole gene conversion pathway. We thus checked the status of Rad51 protein in cell lines overexpressing Bcl-2. First, the amount of Rad51 protein was measured by western blotting (Figure 8A). The fact that the amount of Rad51 protein was identical in lines expressing Bcl-2 and in control lines showed that Bcl-2 did not affect transcription, RNA maturation or translation efficiencies of *RAD51*. Thus, if Rad51 is a target, the consequences of Bcl-2 expression should act post-translationally.

After genotoxic stress, Rad51 protein re-localizes in nuclear foci (Haaf *et al.*, 1995). Since Bcl-2 has been reported to inhibit the nuclear transport of proteins such as p53 (Ryan *et al.*, 1994), we checked whether Bcl-2 expression affects the formation of Rad51 nuclear foci (Figure 8B). No differences in the percentage or kinetics of Rad51 focus formation were observed between the Bcl-2-overexpressing and control cell lines. This result excludes the down-regulation of gene conversion via inhibition of Rad51 protein nuclear transport.

We analysed Rad51 protein by two-dimensional gel electrophoresis (TDGE). In the first dimension, proteins migrate according to their isoelectric point; the second dimension corresponds to a classical SDS–polyacrylamide gel and the proteins migrate essentially according to their molecular weight. A post-translation modification can affect either the molecular weight and/or the charge of the protein. Subtle modifications can thus be detected by TDGE. In the control line, Rad51 protein, detected with an anti-Rad51 antibody, corresponded to a faint spot plus two main spots, with the same molecular weight. This showed that Rad51 was present in three forms, which differ according to their charge. In the extract from cell lines expressing Bcl-2, the total amount of Rad51 protein is the same, but the distribution between the three spots changed. Indeed, most of the Rad51 protein is present in the central spot and the most basic spot almost disappeared. This result suggests that a large fraction of Rad51 protein became more acid.

Taken together, the western blot and TDGE results show that expression of Bcl-2 affects the post-translation modification of Rad51 protein.

Bcl-2 leads to a mutator phenotype

Although the *RAD51*-dependent repair pathway is affected, these cells have increased resistance to ionizing radiation, implying that other repair pathways should be proficient. Our results show that Bcl-2 specifically inhibits the error-free *RAD51* pathway, but that the alternate error-prone repair pathways are proficient. This hypothesis is attested to by the fact that SSA, an error-prone recombination process that systematically leads to sequence deletion, is not decreased by Bcl-2 expression (see above). Consequently, one prediction would be that mutagenesis should be increased in cells expressing the ectopic Bcl-2.

We first measured spontaneous mutagenesis in the *Na^+/K^+-ATPase* membrane pump gene. Mutation in this gene leads to ouabain resistance of the mutant cells. Spontaneous mutagenesis was calculated by fluctuation analysis using the Luria and Delbrück or the Lea and Coulson assay (Luria and Delbrück, 1943; Lea and Coulson, 1948; Capizzi and Jameson, 1973). In two independent clones, expression of Bcl-2 led to a 2.5-fold increase in the spontaneous rate of mutagenesis per cell per generation (Table III).

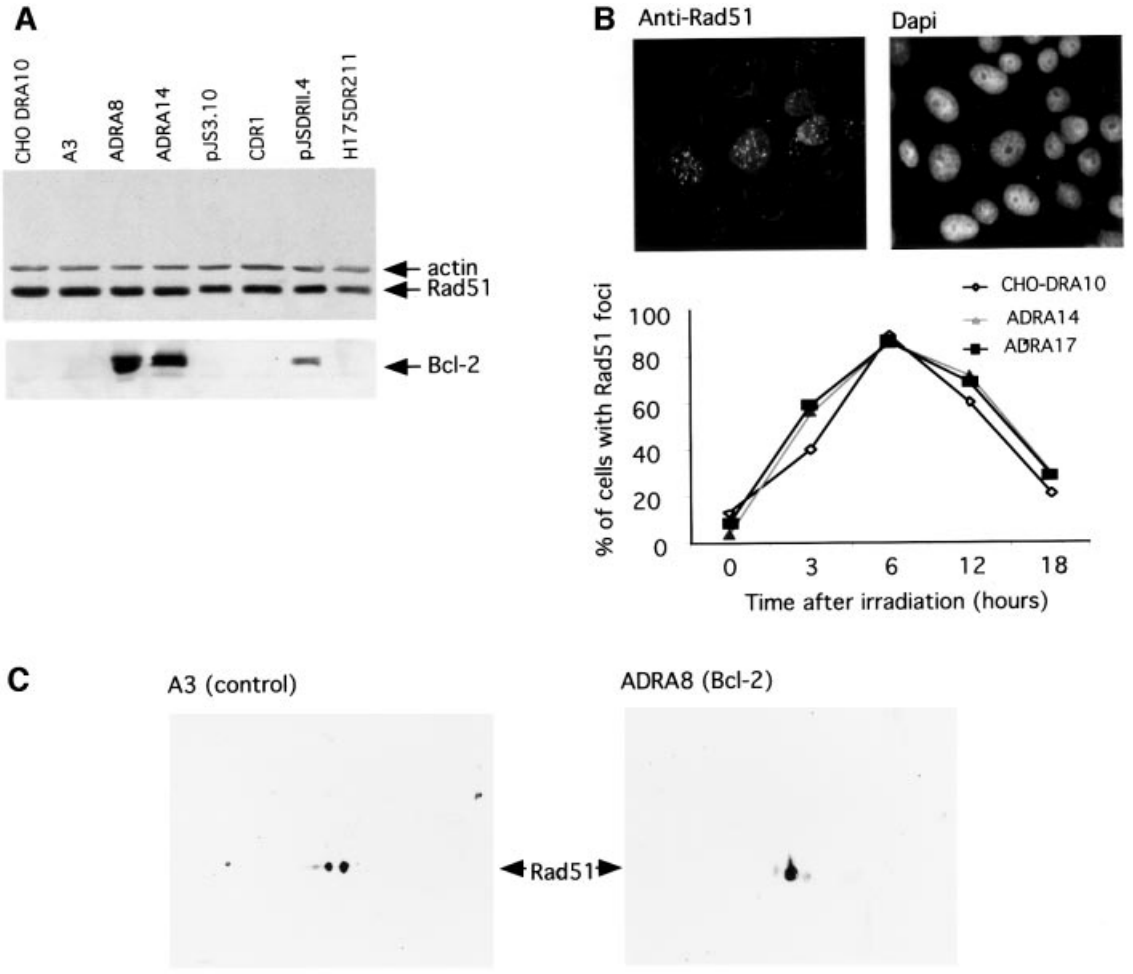


Fig. 8. Status of Rad51 protein in lines expressing Bcl-2. (**A**) Western blot. Arrows indicate Rad51 protein, actin (internal standard) and overexpression of the exogenous human Bcl-2. Cell lines are described in Table I. CDR1 is a clone from pJS3-10 transfected with an empty expression vector. (**B**) Rad51 foci measured by immunofluorescence. Top: an example of Rad51 foci (left panel) and nucleus coloration by DAPI (right panel). Bottom: the kinetics of Rad51 foci formation after irradiation (6 Gy). (**C**) TDGE revealed with an anti-Rad51 antibody. Rad51 is indicated by an arrow. Left panel, extract from the control cell line (A3); right panel, extract from a cell line expressing an ectopic Bcl-2 (ADRA8).

Table III. Spontaneous mutagenesis rate

Cell lines	Expression of exogenous ^{Y28A}Bcl-2 protein	Number of independent cultures	Mutagenesis rate ($\times 10^{-8}$/cell/generation)	
			Luria and Delbrück	Lea and Coulson
CHO-DRA10	none	6	6.1 ± 0.56	5.8
ADRA14	Bcl-2	6	14 ± 0.49	14.9
ADRA17	Bcl-2	6	16 ± 0.48	12.7

We then measured mutagenesis induced either by UV-C or by ionizing radiation. Expression of Bcl-2 led to a strong stimulation of UV-induced mutagenesis (Figure 9A). At a dose of 30 J/m^2, UV-induced mutagenesis of the *Na$^+$/K$^+$-ATPase* membrane gene locus was stimulated from 18- to 20-fold in two independent clones expressing Bcl-2, compared with the control cell line.

We also measured the γ-ray-induced mutagenesis of two different loci: the *Na$^+$/K$^+$-ATPase* membrane gene and the *HPRT* gene. Mutation of the latter gene leads to 6-thioguanine (6-TG) medium resistance. In two independent clones, Bcl-2 expression stimulated γ-ray-induced mutagenesis of both loci (Figure 9B). At a dose of 6 Gy, mutagenesis was stimulated 10-fold at the ouabain resistance locus and 80- to 100-fold at the *HPRT* locus.

Discussion

The oncogenic role of Bcl-2 is generally attributed to the inhibition of a variety of apoptotic deaths. Thus, Bcl-2 is

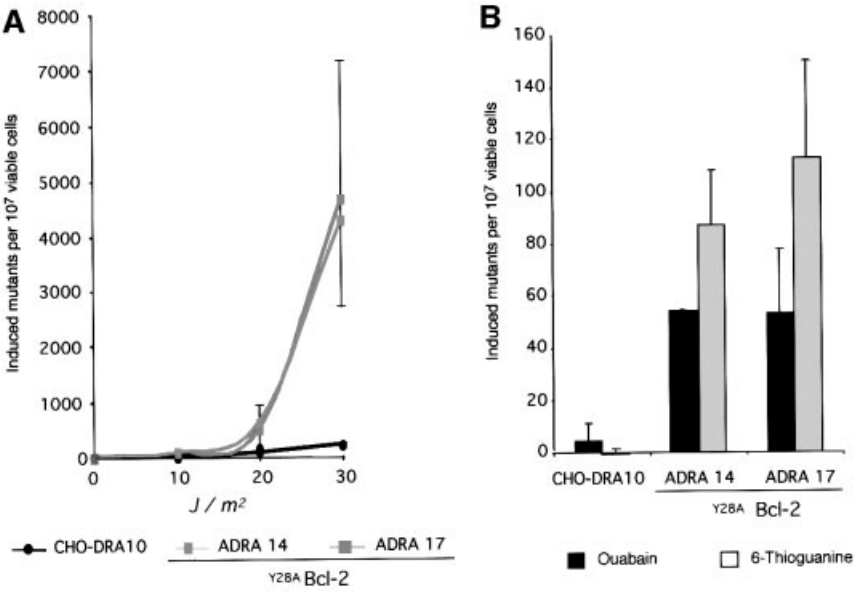


Fig. 9. Bcl-2 stimulates induced mutagenesis. In black is the control line CHO-DRA10. In gray are ADRA14 and ADRA17, two independent clones expressing ^{Y28A}Bcl-2. The values correspond to the number of induced mutants: the number of resistant clones (ouabain or 6-TG) for 10^7 viable treated cells minus the number of resistant clones (ouabain or 6-TG) in 10^7 non-treated cells. (**A**) UV-C-induced mutagenesis. Mutagenesis was measured at the ouabain locus. (**B**) γ-ray-induced mutagenesis at two loci: ouabain resistance (in black) and HPRT locus (6-TG resistance, in grey). Cells were irradiated at 6 Gy.

an oncogene when it is overexpressed, such as in follicular B-cell lymphomas, in which Bcl-2 overexpression results from a t(14;18) translocation (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985). Consequently, Bcl-2 studies classically use overexpression of Bcl-2 to mimic the pathological level that occurs in some cancers. In the present paper we show that overexpression of Bcl-2 protects the cells against apoptosis after genotoxic stress such as ionizing or UV radiation, but suppresses the associated stimulation of homologous recombination. This effect is not specific to Bcl-2 since Bcl-X_L shows the same behaviour. Moreover, it is not affected by the impact of p53 on the G_1 checkpoint and on recombination. One hypothesis could be that, after radiation, cells bearing improperly repaired DNA and accumulating mutations are protected from apoptosis by Bcl-2. This hypothesis may be true in part, but several lines of evidence do not totally fit with it. Indeed, for a unique and non-toxic DSB, we show here that Bcl-2 does not affect all DSB repair systems, but very specifically the conservative *RAD51*-dependent recombination pathway. Moreover, the effect on recombination does not require activation by a profound genotoxic stress, and Bcl-2 decreases the recombination frequency to the basal level of the non-treated control cell line independently of the extent of recombination stimulation by different stresses (γ, UV, mutant p53 protein expression). In addition, the functions of Bcl-2 in recombination and apoptosis can be separated in the mutant ^{G145A}Bcl-2. We show that Bcl-2 expression affects post-translational regulation of Rad51 protein, even in the absence of genotoxic stress. This suggests that Bcl-2 acts on the recombination pathway itself. This conclusion is consistent with the fact that the ^{G145A}Bcl-2 mutant exhibits separation of functions for the death repression activity and BRI.

The use of the rare-cutting endonuclease I-*Sce*I allowed us to determine the precise recombinational DSB repair pathway affected by Bcl-2. I-*Sce*I produces a DSB targeted in the recombination substrate with no detectable associated effect on cell survival. Bcl-2 expression does not significantly modify the total frequency of I-*Sce*I-induced recombination (Neo^R clones), but specifically decreases the frequency of conservative recombination events (double Neo^R/Hyg^R resistant clones). Since non-conservative recombination is *RAD51* independent and conservative recombination is *RAD51* dependent (Ivanov *et al.*, 1996; Lambert and Lopez, 2000), the present data show that Bcl-2 expression specifically affects the *RAD51* pathway even in the absence of stress able to induce apoptosis. These results were confirmed when using cell lines overexpressing the mouse *MmRAD51* protein. In the present paper we studied more specifically the effect of Bcl-2 expression on Rad51 protein. Overexpression of only Rad51 protein or of a dominant-negative form is sufficient to stimulate or to inhibit gene conversion (Lambert and Lopez, 2000). Thus, the regulation of Rad51 protein would be sufficient to control the whole gene conversion pathway. We have shown that the amount of Rad51 protein was not modified by Bcl-2 expression. This shows that Bcl-2 does not act on the efficiency of *RAD51* gene transcription or translation of Rad51 protein. We also show here that the expression of Bcl-2 does not affect the nuclear transport of Rad51 or foci formation. Nevertheless, since gene conversion is inhibited, if Rad51 foci actually represent repair foci, this result suggests that either the foci are formed but are inactive or that the late steps of the recombination process are inhibited. One hypothesis could be that Bcl-2 affects the post-translation regulation of Rad51 protein. This hypothesis is consistent with the fact that the amount of Rad51 protein measured

by western blotting remains unchanged by the expression of Bcl-2. We also show here that Bcl-2 affects the distribution of the different Rad51 protein forms, as measured by TDGE analysis. Indeed, the basic fraction of Rad51 became more acid in the Bcl-2-overexpressing cell line. Phosphorylation is a good candidate mechanism, compatible with our TDGE data. Phosphorylation modifies the charge of the protein, resulting in a change in the migration pattern in TDGE. Rad51 was shown to undergo phosphorylation. For instance, phosphorylation by cAbl inactivates Rad51 strand-exchange activity (Yuan *et al*., 1998), but favours the formation of Rad51 foci (Chen *et al*., 1999). Other kinases could act on Rad51 protein, and the characterization of the transduction signal pathway involved in BRI is under investigation.

It has recently been reported that targeted cytoplasmic radiation induces mutation of the nuclear genome, suggesting, as is the case here, that there is a tight connection between cytoplasm metabolism and the maintenance of genome stability (Wu *et al*., 1999).

Paradoxically, although expression of Bcl-2 inhibits one DSB repair pathway, these cells show increased resistance to irradiation. Among the different DSB repair pathways, Bcl-2 selectively affects the error-free DSB repair pathway (the *RAD51*-conservative recombination pathway), but is without effect on the mutagenic pathways (SSA, NHEJ). Since Bcl-2-overexpressing cells remain more resistant to genotoxic stress, despite its effects on error-free DNA repair systems, error-prone DNA repair systems must compensate. We show here that SSA remains proficient. Conservative recombination is not the only error-free DNA repair pathway affected by Bcl-2. Expression of Bcl-2 has also been shown to result in an attenuation of the nucleotide excision repair (NER) system in UV-irradiated cells (Liu *et al*., 1997). The fact that different repair pathways are affected suggests that distinct patterns of mutagenesis should occur. In line with this, Bcl-2 overexpression results in increased mutagenesis induced by different genotoxic stresses such as benzene metabolite-induced oxidative stress (Kuo *et al*., 1999), UV or γ-rays (this work), but also in increased spontaneous mutagenesis in the mutant p53 lines (this work). We have measured mutagenesis at two different loci, *HPRT* and the ouabain resistance gene, that do not exactly monitor the same kind of mutagenesis (Friedrich and Coffino, 1977). We have also measured mutagenesis induced by different types of genotoxic stress (UV-C, γ-rays), which produce different kinds of damage. We show that, in all cases, Bcl-2 overexpression results in an increase in mutagenesis. High levels of chromosome aberrations after ionizing radiation have also been noted in human lymphoblast lines expressing Bcl-2 (Cherbonnel-Lasserre *et al*., 1996). Taken together, these results suggest a wide mutagenesis spectrum. Since NER, base excision repair as well as conservative recombination are error-free repair pathways, this indicates that Bcl-2 (or Bcl-X_L) expression results in a concerted and specific inhibition of the error-free pathways, resulting in a strong mutator phenotype in response to a broad spectrum of genotoxic stresses.

The data presented here show that Bcl-2 does not affect all the DSB repair pathways, but acts on the regulation of the balance between conservative versus non-conservative recombination. Control of this pathway is of particular importance in cancer predisposition. It is generally assumed that the oncogenic role of Bcl-2 overexpression (as in some lymphomas) results from its death-repression activity. The present data reveal another separate oncogenic role of Bcl-2 overexpression: the mutator and genetic instability phenotypes due to the inhibition of error-free DNA repair pathways, such as conservative homologous recombination.

More generally, expression of Bcl-2 confers increased resistance to genotoxic stress associated with an increase in mutagenesis. This dual phenotype is reminiscent of the general consequences of the SOS system in bacteria, despite differences in the exact molecular control.

Materials and methods

DNA manipulations

All DNA manipulations were performed as described (Sambrook *et al*., 1989; Ausubel *et al*., 1999).

pSFFv-Bcl-2 expression vector was kindly provided by C.Cherbonnel-Lasserre and is described elsewhere (Cherbonnel-Lasserre *et al*., 1996). pEF-^{Y28A}Bcl-2 expression vector was kindly provided by Drs J.Adams and S.Cory, and is described elsewhere (Huang *et al*., 1997). The ^{G145A}Bcl-2 mutant expression vector was kindly provided by Dr Korsmeyer (Yin *et al*., 1994).

Cells and Rad51 foci

Mouse L, pJS3-10 (Liskay *et al*., 1984) and CHO-K1 DRA10 cells (Liang *et al*., 1998) and their derivative lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. TK^+ (recombinant) clones were selected in HAT medium (100 μM hypoxanthine, 2 μM aminopterin, 15 μM thymidine) as described (Liskay *et al*., 1984). Neo^+ (recombinant) clones were selected with 500 μg/ml G418 and hygromycin-resistant clones were selected with 500 μg/ml hygromycin. Puromycin selection was performed at 5 μg/ml. Single transfections were performed using Transfast (Promega, Madison, WI). Co-transfections were performed using the calcium phosphate precipitate technique (Sambrook *et al*., 1989). The Rad51 foci were analysed as described (Haaf *et al*., 1995) using an anti-Rad51 antibody (Oncogene Research Products, Cambridge, MA).

Western blot analysis

All extract preparation steps were performed at 4°C. After washing with phosphate-buffered saline (PBS), cells were suspended in lysis buffer A (25 mM Tris–HCl pH 7.5, 1 mM EDTA, 600 mM NaCl, 0.5% NP-40, 2 μg/ml leupeptin, 2 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride) and incubated for 40 min on ice. Extracts were centrifuged for 30 min at 15 000 *g*, supernatant was retrieved and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). A total of 40 μg/well of the boiled samples was loaded onto a 10% SDS–PAGE gel. After migration, the proteins were electrotransferred onto a nitrocellulose membrane and probed with specific antibodies: anti-human Bcl-2 (SC-509; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Rad51 (Ab-1; Oncogene Research Products). Standard procedures were used for the electrophoresis, transfer and western blotting. Antibodies were visualized using the ECL detection kit (Amersham Pharmacia Biotech, Orsay, France).

Measurement of apoptosis

Two different methods for the measurement of apoptosis were used (Celis, 1994).

Profile of DNA content (FACS analysis). For each point, 10^6 cells were plated in DMEM and incubated for 48 h at 37°C. Cells were then irradiated at 6 Gy using a ^{137}Cs irradiator (2 Gy/min). After 24 or 48 h incubation at 37°C in DMEM, cells were trypsinized, collected by centrifugation (5 min at 2000 g), resuspended in 500 μl of PBS and fixed by adding 1.5 ml of cold ethanol. The DNA content was estimated by propidium iodide fluorescence and DNA flow cytometry (Becton FACScalibur).

During the subsequent rinse, apoptotic cells permeabilized by ethanol fixation leak low-molecular-weight DNA into the cytoplasm. The lower

DNA content of these cells means that they contain less DNA stained by the fluorochrome. Thus, cells with lower DNA staining than that of G_1 cells (the so-called 'sub-G_1 peaks') are considered apoptotic.

Analysis of the morphological changes (Hoechst fluorescence). For each point, 5×10^4 cells were plated in DMEM and incubated for 48 h at 37°C. Cells were then irradiated at 6 Gy using a ^{137}Cs irradiator (2 Gy/min). Twenty-four or 48 h after irradiation, cells were washed and stained for 30 min at 37°C with Hoechst 33342 in culture medium. Hoechst 33342 penetrates the plasma membrane without permeabilization and intercalates into the DNA. The cells were visualized by fluorescence microscopy. In contrast to normal cells, the nuclei of apoptotic cells appear to be one or more featureless, bright spherical beads. For each point, >500 cells (normal and apoptotic cells) were counted.

Recombination and mutagenesis measurements

Fluctuation analysis was used to measure spontaneous recombination and spontaneous mutagenesis. For each line analysed, several independent cultures were plated and cultured to confluence. Cells were then trypsinized, counted, and one portion was used for plating efficiency estimation. The remaining cells were plated under selection medium. Recombinant (*TK*$^+$) L cells (pJS3-10 and pJS4-7-1 and derivatives) were selected on HAT selective medium. Recombinants (*Neo*$^+$) from the CHO-DRA10 cell line (and derivatives) were selected on G418 or G418 + hygromycin. The resulting number of *TK*$^+$ or *Neo*$^+$ clones allowed us to calculate the recombination frequency. The mutant colonies were selected on 2 mM ouabain or 20 µM 6-TG. The resulting number of ouabain- or 6-TG-resistant clones allowed us to calculate the mutagenesis frequency. The rate of recombination or mutagenesis per cell per generation was calculated by using the fluctuation analysis of Luria and Delbrück (Luria and Delbrück, 1943; Capizzi and Jameson, 1973) or Lea and Coulson (1948).

Recombination frequency after treatment with γ-rays (in PBS, using a ^{137}Cs irradiator 2 Gy/min) or UV-C (254 nm at 0.7 J/m^2/s) was measured at the dose indicated. After irradiation, the cells were incubated in DMEM at 37°C for 24 h. The cells were then trypsinized and divided into two fractions. The first fraction was used to calculate the viability by measuring the plating efficiency. The second fraction was plated under HAT or G418 selection to measure the frequency of recombinant clones.

Recombination after induction of a DSB was measured: 3×10^5 cells (for the control lines DRA10 and Cm3) or 1.8×10^6 cells (for ADRA14 and ADRA17) were plated and transfected with 2 and 12 µg, respectively, of an expression vector for the I-*Sce*I endonuclease (pCMV I-*Sce*I). Twenty-four hours after transfection, G418 or G418 + hygromycin selection was initiated. The Neo^R clone frequency was estimated by the ratio of (number of Neo$^+$ clones)/(total number of cells plated). The Neo^R/Hyg^R clone frequency was estimated by the ratio of (number of double-resistant Neo^R/Hyg^R clones)/(total number of cells plated). The percentage of SI recombination events was calculated from the ratio of (frequency of double-resistant Neo^R/Hyg^R clones)/(frequency of single-resistant Neo^R clones).

Two-dimensional gel electrophoresis

Extract preparation. CHO cells were pelleted at 1200 r.p.m. for 5 min and washed twice in PBS. The pellet was resuspended quickly in extraction buffer [8 M urea, 1 M thiourea, 0.5% CHAPS, 50 mM dithiothreitol (DTT), 24 mM spermine] and incubated for 1 h at room temperature. After ultracentrifugation at 200 000 *g* for 1 h at 20°C, the concentration of supernatant was measured using Bradford reagent (Bio-Rad).

Two-dimensional electrophoresis. IPG strips (pH 4–7) were rehydrated overnight with 150 µl of extraction buffer containing 1% IPG buffer and 200 µg protein sample and traces of bromophenol blue. Proteins were isoelectrofocused using a Multiphor II Electrophoresis Unit cooled at 20°C with successive 30 min steps at 200, 1000 and 2000 V, and a final step at 3500 V for 2.5 h.

Focused IPG strips were incubated for 15 min in equilibration solution (50 mM Tris–HCl pH 6.8, 6 M urea, 30% glycerol, 1% SDS, bromophenol blue) containing 50 mM DTT and then for 15 min in equilibration solution containing 200 mM iodoacetamide. For the second dimension, 10% SDS–PAGE was performed.

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