Targeted disruption of one allele of the *Y-box binding protein-1* (*YB-1*) gene in mouse embryonic stem cells and increased sensitivity to cisplatin and mitomycin C

Kotaro Shibahara,[1,2] Takeshi Uchiumi,[1,7] Takao Fukuda,[1] Shinobu Kura,[3] Yohei Tominaga,[4] Yoshihiko Maehara,[2] Kimitoshi Kohno,[5] Yusaku Nakabeppu,[4] Teruhisa Tsuzuki[3] and Michihiko Kuwano[6]

[1]Departments of Medical Biochemistry, [2]Surgery and Science, [3]Medical Biophysics & Radiation Biology, [4]Faculty of Medical Sciences, Division of Neurofunctional Genomics, Medical, Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, [5]Department of Molecular Biology, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu 807-8555; and [6]Research Center for Innovative Cancer Therapy, 21st Century COE Program for Medical Science, Kurume University, 67 Asahimachi, Kurume, Fukuoka 830-0011

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The eukaryotic Y-box binding protein-1 (YB-1) functions in various biological processes, including transcriptional and translational control, DNA repair, drug resistance, and cell proliferation. To elucidate the physiological role of the YB-1 protein, we disrupted one allele of mouse *YB-1* in embryonic stem (ES) cells. Northern blot analysis revealed that *YB-1*$^{+/-}$ ES cells with one intact allele contain approximately one-half the amount of mRNA detected in wild-type (*YB-1*$^{+/+}$) cells. We further found that the protein level of *YB-1*$^{+/-}$ cells was reduced to approximately 50–60% compared with that of *YB-1*$^{+/+}$ cells. However, no apparent growth difference was found between *YB-1*$^{+/-}$ and *YB-1*$^{+/+}$ cells. *YB-1*$^{+/-}$ cells showed increased sensitivity to cisplatin and mitomycin C, but not to etoposide, X-ray or UV irradiation, as compared to *YB-1*$^{+/+}$ cells. YB-1 may have the capacity to exert a protective role against cytotoxic effects of DNA damaging agents, and may be involved in certain aspects of drug resistance. (Cancer Sci 2004; 95: 348–353)

The Y-box protein family, which is widely distributed from bacteria to mammals, contains a cold-shock domain which is highly conserved from prokaryotic cold-shock proteins.[1)] The human Y-box binding protein, YB-1, which is located on chromosome 1p34, was initially identified as a transcription factor which associates with the Y-box sequence appearing in the major histocompatibility complex class II genes.[2–4)]

It has been hypothesized that YB-1 might play a role in promoting cell proliferation through the transcriptional regulation of various relevant genes, including proliferating cell nuclear antigen, epidermal growth factor receptor, DNA topoisomerase IIα, thymidine kinase, and DNA polymerase α.[5, 6)] In our laboratory, we have shown that YB-1 is involved in transcriptional activation of the human multidrug resistance 1 gene.[7–9)] and also the DNA topoisomerase IIα gene[10)] in response to various environmental stimuli. YB-1 appears to play a critical role in cell proliferation, DNA replication, and drug resistance. The biological roles of YB-1 include modification of chromatin, translational masking of mRNA, participation in the redox signaling pathway, RNA chaperoning, and stress response regulation.[11)] It has also been demonstrated that eukaryotic Y-box proteins regulate gene expression at the translational level by recognizing RNA.[12, 13)] The murine YB-1 protein (MSY1) is specifically expressed in testis rather than other tissues, and regulates the translation of germ cell RNA.[14)] The Y-box binding proteins thus appear to play critical roles in both mRNA turnover and translational control.

YB-1 also appears to protect mammalian cells from the cytotoxic effects induced by DNA damage. We have previously reported that human cancer cell lines overexpressing YB-1 showed resistance to cisplatin, while the reduction of YB-1 itself leads to increased drug sensitivity to cisplatin, other DNA-interacting drugs, and UV irradiation.[15)] We also demonstrated that YB-1 protein is localized mainly in the cytoplasm, but translocates to the nucleus when cells are irradiated with UV or treated with anticancer drugs.[16)] YB-1 specifically binds to cisplatin-modified DNA, apurinic DNA and also 8-oxo-guanine-containing RNA.[17–19)] We have further demonstrated that YB-1 binds directly to repair-associated proteins such as proliferating cell nuclear antigen and p53 protein.[18, 20)] YB-1 may thus be involved in the process of DNA repair and/or DNA damage response. In clinical studies on YB-1, the cellular level of YB-1 was found to be closely associated with tumor growth and prognosis in ovarian cancers, lung cancers, and breast cancers.[21–23)]

To gain more insight into how YB-1 proteins exert their multiple functions, we carried out a targeted disruption of the *mouse YB-1* gene (*MSY1*) in mouse embryonic stem (ES) cells. We have established ES cell lines with a heterozygously targeted disruption of the *YB-1* gene (*YB-1*$^{+/-}$), which we found to result in hypersensitivity to cytotoxic agents, such as cisplatin and mitomycin C.

Materials and Methods

Cell growth characteristics. CCE ES cells and YB-1 knockout cells were maintained on a feeder cell layer in DMEM supplemented with 20% heat-inactivated fetal bovine serum and 100 units/ml of recombinant leukemia inhibitory factor at 37°C in an atmosphere of 10% CO_2 in air.

Construction of the targeting vector. The mouse *YB-1* gene was isolated from a 129/Sv genomic library by the standard plaque hybridization method, using mouse *YB-1* cDNA as a probe. The targeting vector contained approximately 8.4 kb of genomic sequence interrupted by a *pol*II-*neo*-poly(A) cassette. Insertion of the neomycin (*neo*) cassette resulted in deletion of a 1.8-kb *Sal*I/*Bgl*II fragment of the *YB-1* gene, containing 43 nucleotides of exon 5 and 84 nucleotides of exon 6, as well as 240 nucleotides of intron 5 and 1.4 kb of intron 6. A pair of herpes simplex virus thymidine kinase (TK) cassettes (TK1 and TK2, both under control of the MC1 promoter) were placed flanking

[7]To whom correspondence should be addressed.
E-mail: uchiumi@biochem1.med.kyushu-u.ac.jp
Abbreviations: YB-1, Y-box binding protein-1; ES, embryonic stem; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PES, phenazine ethosulfate.

the *YB-1* genomic sequence in the targeting vector to allow for positive and negative selection of DNA when introduced into ES cells.[24] The targeting construct was linearized at a unique *Not*I site located on the plasmid vector.

Isolation of heterozygous mutant embryonic stem cell lines. The ES cell line CCE was cultured on a feeder cell layer and electroporated, using 5×10^7 cells and 50 μg of the linearized targeting vector DNA, as described.[25] The transfected cells were subjected to positive and negative selection, using G418 (250 μg/ml; Geneticin, GIBCO/BRL) and ganciclovir (GANC) (5 m*M*; a gift of Nihon Syntex) as selective agents. Colonies doubly resistant to G418 and GANC were grown on 24-well plates to expand them for Southern blot analysis. DNA was isolated from each cell line and analyzed by Southern blot hybridization.

Southern blot analysis. Genomic DNA (8 μg) was digested with *Eco*RV and *Bgl*II, then run on a 0.7% agarose gel and transferred to a nylon membrane filter (Hybond N1; Amersham). The filter was hybridized with a 0.3-kb *Eco*RV/*Eco*RI fragment (5′ internal probe A) and a 0.3-kb *Xho*I/*Hin*dIII fragment (3′ flanking probe B), labeled with [α-^{32}P]dCTP. The membrane was washed, applied to an imaging plate, and analyzed using a Bio-image analyzer BAS 2000 (Fuji Photo Film Co., Kanagawa).

RNA isolation and northern blot analysis. Cells in the exponential growth phase were transferred to a medium without feeder layer cells and further cultured on a gelatin-coated dish for 4 days to avoid contamination with *YB-1* mRNA derived from feeder cells. Total RNA was isolated using an RNeasy spin column (Qiagen, Hilden, Germany). RNA samples (10 μg/lane) were separated on a 1% formaldehyde-agarose gel and were transferred to a membrane. The membranes were hybridized with ^{32}P-labeled mouse YB-1 1.2-kb cDNA as a probe.[15] Radioactivity was visualized by autoradiography and was analyzed using a Fujix Bas 2000 bioimaging analyzer (Fuji Photo Film Co., Tokyo).

Immunoblotting of the YB-1 protein. The cells were lysed in TNE buffer (50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 0.5% NP-40, 1 m*M* PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin), and boiled in western sample buffer for 10.0% SDS-PAGE and western blot analysis. An antibody to YB-1 was generated as described previously.[15] PCNA-specific antibody (PC10; Santa Cruz) and p21 (sc-817; Santa Cruz) were used for western blotting.

Proliferation rates. To determine the proliferation rates of ES cells, 5×10^5 cells were plated in triplicate in 6-well plates and the cell numbers were determined at the indicated time points using a CASYR cell counter.

Clonogenic survival assay. ES cells were plated on gelatin-coated 6-well plates at a density of approximately 500 cells/well. Twenty-four hours after plating, ES cells were treated with various chemical agents, X-rays or UV irradiation. Plates were incubated for 7 days and the surviving ES cell colonies in each well were counted after staining with Giemsa. The plating efficiency was ~60–80%. The relative sensitivity of each clone of *YB-1*$^{+/-}$ ES cells was determined by dividing the IC_{90} value for each cell line by that of wild-type (*YB-1*$^{+/+}$) ES cells.

MTS survival assay. CellTiter 96 Aqueous One Solution cell proliferation Assay (Promega) was used to evaluate drug sensitivities. The 96-well plates were inoculated with 4000 cells/well in a volume of 100 μl of ES medium. Twenty-four hours later, drugs were added at various concentrations. Seventy-two hours later, 20 μl of MTS/PES was added, and incubation was continued for 2 h at 37°C. In the presence of the electron-coupling reagent PES, MTS is reduced by dehydrogenase enzymes found in metabolically active cells into a formazan product that is readily soluble in tissue culture medium. The quantity of formazan product was measured in terms of the absorbance at 490 nm. At least five different drug concentrations were used to determine the IC_{50} values, and each drug concentration was replicated in 4 wells for each individual experiment. The relative sensitivity of each *YB-1*$^{+/-}$ ES cell clone was determined by dividing the IC_{50} value for each cell line by the *YB-1*$^{+/+}$ ES cell line IC_{50} value.

Results

Targeted disruption of the *YB-1* gene. The mouse *YB-1* gene, which encodes a 49-kDa protein, is composed of 8 exons, spans more than 16 kb, and is 99% identical to human YB-1. A region of genomic DNA carrying part of two exons and the adjacent intron region, was replaced by a *neo* cassette (Fig. 1A). This region was chosen as the target since it encodes the C-terminal domain of the YB-1 protein, which is essential for protein interaction and nucleoside binding.[26] The resulting construct was electroporated into ES cells, and cells showing increased resistance to both G418 and GANC were selected. The DNAs of resistant clones were digested with restriction enzymes and hybridized with several different probes. Homologous recombinants were characterized by the appearance of a 5.5-kb *Eco*RV fragment with the 5′-internal probe A, and a 10.3-kb *Bgl*II fragment with the 3′-flanking probe B (Fig. 1, B and C). Additional Southern blot analysis using other restriction enzymes confirmed the targeted disruption of one allele of *mouse YB-1* (data not shown). Furthermore, hybridization with the *neo* probe showed that the predicted genomic DNA fragment size is similar in all of these clones. Approximately 4% of G418- and GANC-doubly resistant cells carried the expected structure for the mutated allele.

Decrease in mRNA expression and protein levels in heterozygous *YB-1*$^{+/-}$ ES cells. Northern blot analysis of YB-1 mRNA, using the full-length YB-1 cDNA as a probe, was performed in *YB-1*$^{+/+}$ cells and three different clones of *YB-1*$^{+/-}$ cells. We found that *YB-1*$^{+/-}$ cells contain approximately half the amount of mRNA detected in *YB-1*$^{+/+}$ cells (Fig. 2A). Consistent with this observation, western blot analysis of heterozygously disrupted mutant cells also showed a reduction in YB-1 protein levels. For semi-quantitative analysis, various amounts of cell lysate were applied to the same gel, and we estimated that the protein level of *YB-1*$^{+/-}$ cells was reduced to approximately 50–60% of the wild-type level (Fig. 2B). *YB-1*$^{+/-}$ cells thus established grew normally in ES medium, despite these characteristics. The doubling time of *YB-1*$^{+/+}$ and three clones (1, 2, and 5) of *YB-1*$^{+/-}$ cells were 10.2, 10.0, 9.0, and 10.2 h, respectively (Table 1). Thus, no apparent growth retardation or abnormal cell morphology was found in *YB-1*$^{+/-}$ cells, in spite of the reduced content of YB-1 protein.

Drug, X-ray, and UV irradiation sensitivity of targeted cells to DNA damaging agents. YB-1 has been proposed to be involved in the sensitivity of cells to DNA-damaging agents such as cisplatin and mitomycin C.[15] We therefore explored the role of YB-1 heterozygosity in sensitizing ES cells to a variety of cytotoxic agents using both MTS and clonogenic survival assays. As shown in Table 2, we found that *YB-1*$^{+/-}$ cells are more sensitive to cisplatin and mitomycin C, and moderately more sensitive to etoposide than *YB-1*$^{+/+}$ cells. This enhanced sensitivity was seen in three independently isolated *YB-1*$^{+/-}$ clones (1, 2, and 5), and the depletion of YB-1 was required for drug sensitization. Comparisons made at the IC_{50} dose for cisplatin revealed that *YB-1*$^{+/-}$ cells were approximately 3-fold more sensitive to cisplatin than *YB-1*$^{+/+}$ cells (IC_{50}=20.0 μ*M*). Similarly, comparisons made at the IC_{50} dose for mitomycin C revealed that *YB-1*$^{+/-}$ cells were approximately 5-fold more sensitive to mitomycin C than *YB-1*$^{+/+}$ cells (IC_{50}=9.0 μ*M*). Also, comparisons made at the IC_{50} dose for etoposide revealed that *YB-1*$^{+/-}$ cells showed a moderate level of sensitivity (1.4-

fold), compared with *YB-1*$^{+/+}$ cells (IC_{50}=0.6 μ*M*) (Table 2A). Taken together, the results described above suggest that the concentration of YB-1 appears to correlate inversely with cellular sensitivity to DNA-damaging agents. Clonogenic survival assays were also performed on *YB-1*$^{+/+}$ and *YB-1*$^{+/-}$ cells to determine whether differences observed in MTS survival assays would translate into differences in clonogenicity. We treated cells with a variety of chemotherapeutic drugs, X-ray or UV irradiation and then incubated them for 7 days before counting colonies with greater than 50 cells per colony. This assay provides a longer-term assessment of cell growth than the MTS assay and directly assesses the ability of individual cells to proliferate into viable colonies. We tested the cytotoxicity of cisplatin, mitomycin C, etoposide, X-rays, and UV irradiation against *YB-1*$^{+/+}$ and *YB-1*$^{+/-}$ cells. The dose of agent required to reduce colony formation to 10% (IC_{90}) of that by the control non-treated cells is shown in Table 2B. Consistent with data gained in MTS assays and previous observations in KB cells,[15] *YB-1*$^{+/-}$ cells exhibited greater sensitivity to cisplatin and mitomycin C than *YB-1*$^{+/+}$ cells. The dose-modifying factor for equivalent cell killing (IC_{90}) was approximately 2.0-fold for cisplatin and mitomycin C. In contrast, *YB-1*$^{+/-}$ cells were found to be as sensitive as *YB-1*$^{+/+}$ cells to etoposide, X-ray and UV irradiation (Table 2B, Fig. 3). Our results suggested that the reduction of YB-1 level in ES cells preferentially enhances their sensitivity to DNA cross-linking agents.

No change of p21 levels between YB-1$^{+/+}$ and YB-1$^{+/-}$ cells. Swamynathan *et al*. established DT40 cells, in which one allele of Chk-YB-1b is disrupted.[27] The DT40YB1b (+/−) cells showed multiple abnormalities, such as slow growth rate, increased cell size, increased genomic DNA content, and reduced p21 levels.[27] We found no apparent growth retardation or abnormal cell morphology in *YB-1*$^{+/-}$ cells, in spite of the reduced content of YB-1 protein. We examined whether p21 levels were changed in these wild-type and mutant ES cells. Western blot analysis of heterozygously disrupted mutant cells (YB-1$^{+/-}$) also showed a reduction in YB-1 protein levels, but no change of p21 levels was observed in these wild and mutant ES cells (Fig. 4).

Discussion

To clarify the biological role of YB-1 by modulating the amount of cellular YB-1, cell lines defective in the *YB-1* gene should be useful. In the present study, we have generated *YB-1*$^{+/-}$ cell lines using a gene-targeting techniques. The cell lines established have a sequence alteration in a defined region of one allele of the *YB-1* gene, and display a significant depletion of YB-1 mRNA (approximately 50%). Consistent with the reduction in mRNA transcripts, the protein level of the *YB-1*$^{+/-}$ cells was reduced to approximately 50–60%, compared with that of *YB-1*$^{+/+}$ cells. In this study, insertion of the neomycin (*neo*) cassette resulted in deletion of a 1.8-kb region of the *YB-1* gene, containing a part of exon 5 and all of exon 6. The mutant allele may produce a truncated protein and this protein may function in a dominant-negative manner. We could not detect

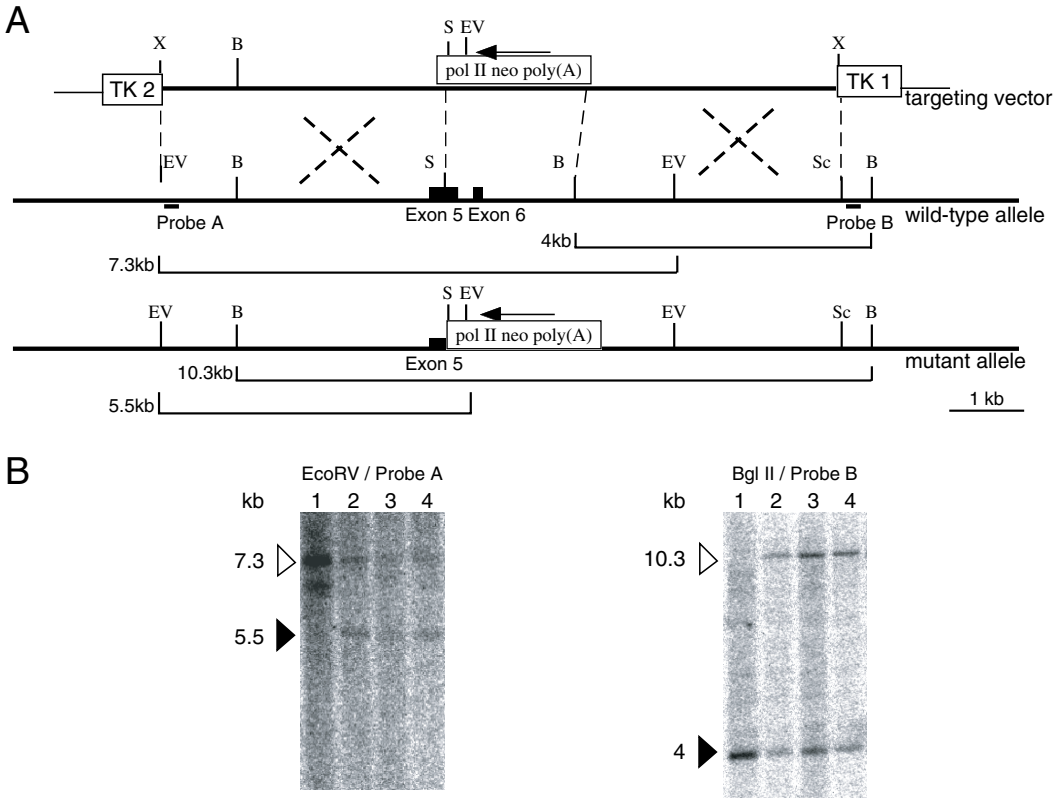

Fig. 1. Targeted disruption of the *mouse YB-1* gene. (A) Configurations of the intact and mutated alleles. The targeting vector carried approximately 8.4 kb of the genomic sequence, within which a part of exon 5 and all of exon 6 was replaced by the *pol*II-*neo*-poly(A) cassette. Restriction enzyme sites are shown; *Xho*I (X), *Bgl*II (B), *Sal*I (S), *Eco*RV (EV), and *Sac*I (Sc). Thick lines indicate the genomic sequence and thin lines represent the bacterial plasmid. The 5′ to 3′ orientation of the *mouse YB-1* gene is left to right, while the 5′ to 3′ orientation of the *pol*II *neo* pA cassette, HSV-1 thymidine kinase gene (TK1) and HSV-2 thymidine kinase gene (TK2) is right to left. Positions of the 5′- and 3′-probes, indicated as probes A and B, respectively, are also shown. (B and C) Southern blot analysis of the DNA isolated from the embryonic stem cell lines. An *Eco*RV digest hybridized with probe A (5′ internal), and a *Bgl*II digest hybridized with probe B (3′ flanking) yielded a wild-type band and a mutant band as indicated. In both cases, genotypes of cells are shown as follows: lane 1, *YB-1*$^{+/+}$; lane 2, clone 1 of *YB-1*$^{+/-}$; lane 3, clone 2 of *YB-1*$^{+/-}$; lane 4, clone 5 of *YB-1*$^{+/-}$.

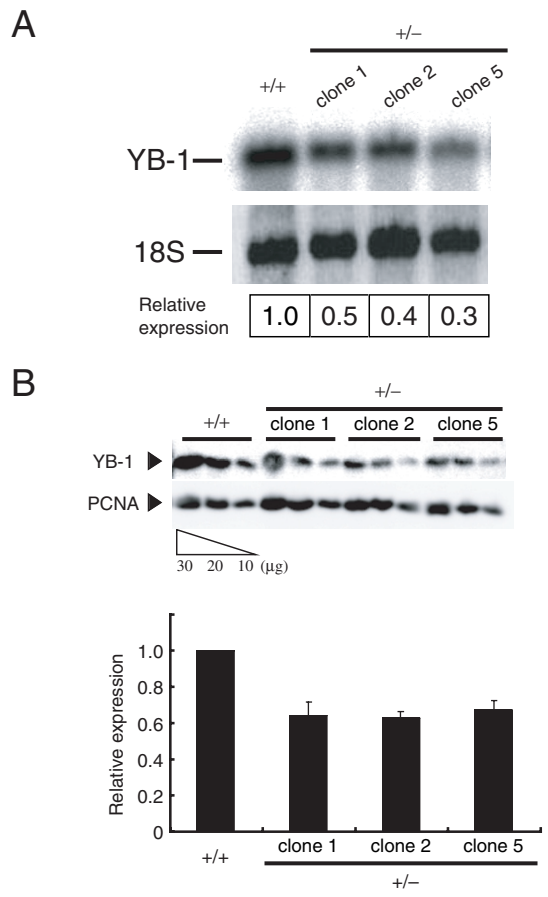

Fig. 2. (A) Northern blot analysis of the *YB-1* mRNA isolated from the germ-line-transmitted embryonic stem cell lines. Total RNA (10 μg) from each cell line was separated on a 1% agarose gel containing 2.2 *M* formaldehyde, transferred to a Hybond N^+ membrane, and hybridized with ^{32}P-labeled YB-1 cDNA (1060 bp). Relative expression levels of *YB-1* mRNA are presented following normalization to 18S ribosome RNA. (B) Immunoblot analysis of the YB-1 protein isolated from the germ-line-transmitted embryonic stem cell lines. To detect the protein level of YB-1 semi-quantitatively, 30, 20, and 10 μg of total cell lysate were applied to adjacent lanes. The amount of YB-1 in each cell line was quantitated by immunoblot analysis of the same membrane with anti-PCNA antibody and is expressed relative to the amount of PCNA. Relative expression of YB-1 is presented following normalization to PCNA levels.

Table 1. Doubling time of YB-$1^{+/+}$ and YB-$1^{+/-}$ ES cells

Cell lines	Doubling time (h)
YB-$1^{+/+}$	10.2
YB-$1^{+/-}$	
Clone 1	10.0
Clone 2	9.0
Clone 5	10.2

Each value is the mean of duplicate determinations.

the NH_2-terminally truncated form of the YB-1 protein by immunoblotting using an antibody against the NH_2-terminus of YB-1 (data not shown). Although we tried to establish double knockout ES cells (*YB-$1^{-/-}$*) by subsequent culture of heterozygous mutant cells in an elevated concentration of G418, we could not isolate homozygous null mutant ES cells, suggesting that a complete lack of YB-1 may be lethal in ES cells. We have previously shown that YB-1 is directly involved in multidrug-resistance 1 gene activation at the transcriptional level.

Table 2. A. Sensitivity of YB-$1^{+/+}$ and YB-$1^{+/-}$ ES cells to various drugs on MTS assay

Agent	IC_{50}[1] for *YB-$1^{+/+}$*	Relative sensitivity[2] *YB-$1^{+/-}$*		
		Clone 1	Clone 2	Clone 5
Cisplatin (μ*M*)	20.0	0.5	0.3	0.2
Mitomycin C (μ*M*)	9.0	0.3	0.2	0.1
Etoposide (μ*M*)	0.6	1.5	0.5	0.2

B. Sensitivity of YB-$1^{+/+}$ and YB-$1^{+/-}$ ES cells to various drugs on colonogenic assay

Agent	C_{90}[1] for *YB-$1^{+/+}$*	Relative sensitivity[2] *YB-$1^{+/-}$*		
		Clone 1	Clone 2	Clone 5
Cisplatin (μ*M*)	1.5	0.6	0.7	0.4
Mitomycin C (μ*M*)	0.16	0.6	0.6	0.5
Etoposide (μ*M*)	0.07	1.2	1.2	0.9
UV (J/m^2)	3.8	1.1	0.9	0.9
γ-Rays (Gy)	5.4	1.0	1.2	0.8

1) The IC_{50} of *YB-$1^{+/+}$* and *YB-$1^{+/-}$* cells for each agent was determined by MTS assay and the IC_{90} was determined by colony formation assays. *2*) The relative sensitivity of each clone of *YB-$1^{+/-}$* ES cells was determined by dividing the IC_{50} or IC_{90} value for each cell line by that of *YB-$1^{+/+}$* ES cell line. Values are means derived from two separate experiments.

YB-1 is directly required for basal promoter activation in response to genotoxic stresses, including carcinogens, anticancer agents, and UV irradiation.[7–9] Also, varying levels of expression of the YB-1 protein are associated with many biological phenomena, including cell proliferation and transformation.[5, 21, 22, 28, 29] Determining how YB-1 plays a role in biological processes in eukaryotic cells is therefore important. We have shown that YB-1 is located mainly in the cytoplasm, and then accumulates in the nucleus when cells are exposed to genotoxic stress.[16] We have observed that YB-1 is overexpressed in human cancer cells lines that are resistant to cisplatin, and that the amount of YB-1 correlates with the sensitivity of these cells to anti-cancer drugs, cisplatin, mitomycin C and UV irradiation.[15] However, the previously observed inverse correlation between YB-1 levels and drug sensitivity in human cancer cell lines may result from genetic and epigenetic differences unrelated to YB-1 among these cell lines. Single knockout ES cells of various genes, such as O^6-methylguanine-DNA methyltransferase (MGMT) and multiple drug related protein 1 (MRP1), which are associated with DNA repair and drug resistance, respectively, displayed a higher sensitivity to anticancer drugs as compared with their wild-type counterparts.[30, 31] In principle, these particular ES cell lines, having a defect in one allele of the *YB-1* gene, could facilitate studies on the biological role of the YB-1 protein.

Cisplatin is widely used in treating a variety of human malignancies. Resistance to this agent is mediated through pleiotropic mechanisms, including decreased drug accumulation, detoxification of the drug, and DNA repair.[32, 33] We have also shown that YB-1 levels correlate with sensitivity to cisplatin, suggesting that YB-1 is directly involved in both the cellular response to cisplatin and cisplatin resistance.[15] We therefore examined the sensitivity of the *YB-$1^{+/-}$* cells to various anticancer drugs, X-rays, and UV irradiation. YB-$1^{+/-}$ cells showed an increased sensitivity to cisplatin and mitomycin C; drugs which induce cross-linking of DNA. Conversely, no dramatic difference in sensitivity to etoposide compared to *YB-$1^{+/+}$* cells was noted in the MTS assay, or the colony formation assay. Essentially, these results are consistent with the sensitivity levels of YB-1 antisense transfectants, in terms of colony formation.[15]

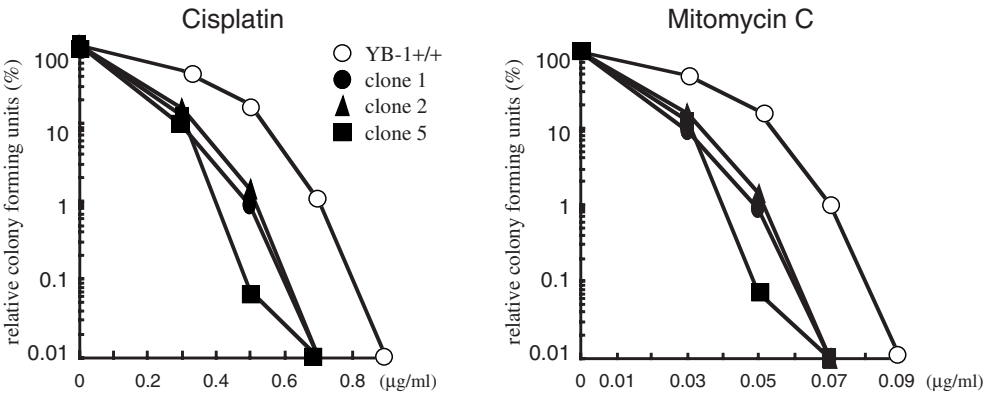

Fig. 3. Dose-response curve to anticancer drugs of *YB-1*$^{+/+}$ and *YB-1*$^{+/-}$ ES cells. Approximately 500 cells were plated on gelatin-coated 6-well plates and incubated in the absence of any drug for 24 h. The cells were then exposed to various concentrations of drugs for 7 days, and the remaining number of colonies was counted; 100% corresponds to the colony number of the same cell line in the absence of any drug. Data points: average values for triplicate dishes.

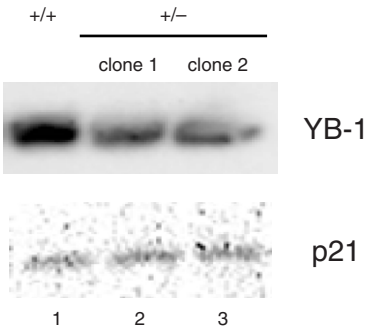

Fig. 4. Immunoblot analysis of the YB-1 and p21 proteins isolated from the germ-line-transmitted embryonic stem cell lines. Total cell lysate (20 μg) was applied to adjacent lanes. In both cases, genotypes of cells are shown as follows: lane 1, *YB-1*$^{+/+}$ ES; lane 2, clone 1 of *YB-1*$^{+/-}$ ES; lane 3, clone 2 of *YB-1*$^{+/-}$ ES.

We consider that the MTS assay can be markedly affected by the rate of cell death, rather than clonogenic survival, and that in turn, can be dependent on the proportion of cells undergoing apoptosis. This may be the reason why we still observed a sensitivity to etoposide in the MTS assay of *YB-1*$^{+/-}$ cells. ES cells with the *YB-1*$^{+/-}$ background had a 60% expression level of YB-1 protein compared to *YB-1*$^{+/+}$ cells. This reduced protein level in *YB-1*$^{+/-}$ cells clearly reflects the sensitivity level of the heterozygous mutant cells, as expected from the findings that stoichiometric amounts of YB-1 protein are needed for sensitivity to cisplatin and mytomicin C. These results indicate that YB-1 is involved in the cellular response to DNA-damaging agents, especially DNA-cross-linking agents. However, in this study no apparent difference in sensitivity to UV irradiation was found between *YB-1*$^{+/-}$ and *YB-1*$^{+/+}$ cells, as compared with the previous study.[15] The IC_{50} dose for UV irradiation of wild-type ES cell was 3.8 (J/m^2), compared to 7.0 (J/m^2) in KB cells. This finding may be a result of the genetic and epigenetic differences between human cancer cell lines and ES cells.

YB-1 has been shown to bind preferentially to cisplatin-modified DNA, apurinic DNA, and RNA containing 8-oxoguanine,[18, 19, 26] suggesting that this protein may bind preferentially to structurally altered DNA.[17] Several nuclear proteins that recognize cisplatin-DNA adducts have been characterized.[32, 34] Among the HMG protein family, HMG1 and HMG2 have been shown to bind specifically to DNA that contains cisplatin-induced intrastrand cross-links[35] and to sensitize cancer cells to cisplatin.[36, 37] Also, IXRI, a yeast protein containing an HMG box, confers sensitivity to cisplatin, though a correlation between the cellular levels of HMG proteins and the repair of damaged DNA has not been demonstrated.[38] It has been established that various repair processes, mainly nucleotide excision repair and mismatch repair, are involved in repair of the cisplatin-induced DNA damage.[39] Cells deficient in DNA repair have been found to be particularly sensitive to cisplatin. Mammalian cells defective in XPF- and ERCC-1, among the proteins involved in nucleotide excision repair, are extremely sensitive to cisplatin.[40] In fact, DNA repair activity has been implicated as a main cause of the resistance of many cell lines to cisplatin. Thus, the cellular sensitivity to cisplatin may be determined by a dynamic interaction between DNA damage recognition processes and DNA repair proteins. We have demonstrated that YB-1 interacts directly with proliferating cell nuclear antigen and p53, which are essential proteins in DNA replication and repair.[18, 20] We have also shown that overexpression of the p53-associated protein p73, involved in DNA repair and apoptosis, increases cellular levels of YB-1,[41] and that YB-1 possesses 3′-5′ DNA exonuclease activity. YB-1 may recognize DNA damaged by a DNA-cross-linking agent, such as cisplatin, and be involved in the process of DNA repair, possibly through interaction with other repair-related proteins.

Recent studies using heterozygous DT40 YB1b (+/−) cells with one copy of the wild-type Chk-YB-1b allele showed a slower rate of growth, abnormal cell morphology, increased cell size, and increased genomic DNA content, compared to wild-type DT40 cells, and it was concluded that YB-1 plays an important role in cell proliferation.[27] In contrast, we found no such apparent growth retardation or abnormal cell morphology of *YB-1*$^{+/-}$ ES cells compared with the wild type. We also found no difference of p21 levels between the two (Fig. 4). This may be due to the following reasons: 1) The level of YB-1 protein in *YB-1*$^{+/-}$ cells was approximately 60% of that of *YB-1*$^{+/+}$ cells and this level of YB-1 protein may be sufficient for the proliferation of *YB-1*$^{+/-}$ cells. 2) DT40 cells were derived from chicken B lymphocytes and do not express p53. Therefore, mouse ES cells may differ greatly from DT40 cells with respect to genetic background, especially regarding the cell cycle and apoptosis control processes. Additional studies are required to determine the differences in phenotype between mouse ES and chicken DT40 cells. Also, the cellular level of YB-1 is associated with tumor growth in ovarian cancers, lung cancers, and breast cancers.[21–23] At present, we have not been able to establish a homozygous null mutant in ES cells. Taken together, these findings suggest that the YB-1 function may be essential for cell viability and proliferation.

In conclusion, we have established YB-1 single knockout cell lines, using gene targeting techniques, and shown that the extent of YB-1 expression correlates with cellular sensitivity to the cytotoxic effects of cisplatin and mitomycin C. YB-1 appears to protect cells or DNA integrity from the toxic insults associated with exposure to DNA-damaging agents. YB-1 is therefore expected to be involved in variety of biological roles, including transcription, cell proliferation, drug resistance, and

DNA repair. To elucidate these physiological roles of the YB-1 protein, establishment of mouse lines defective in *YB-1* genes is in progress. For more definitive studies, designed to evaluate the precise role of the YB-1 protein *in vivo*, the generation of conditional YB-1 mutants may be necessary.

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1. Wolffe AP, Tafuri S, Ranjan M, Familari M. The Y-box factors: a family of nucleic acid binding proteins conserved from *Escherichia coli* to man. *New Biol* 1992; **4**: 290–8.
2. Makino Y, Ohga T, Toh S, Koike K, Okumura K, Wada M, Kuwano M, Kohno K. Structural and functional analysis of the human Y-box binding protein (YB-1) gene promoter. *Nucleic Acids Res* 1996: **24**: 1873–8.
3. Toh S, Nakamura T, Ohga T, Koike K, Uchiumi T, Wada M, Kuwano M, Kohno K. Genomic organization of the human Y-box protein (YB-1) gene. *Gene* 1998; **206**: 93–7.
4. Didier DK, Schiffenbauer J, Woulfe SL, Zacheis M, Schwartz BD. Characterization of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc Natl Acad Sci USA* 1988; **85**: 7322–6.
5. Ladomery M, Sommerville J. A role for Y-box proteins in cell proliferation. *BioEssays* 1995; **17**: 9–11.
6. Wolffe AP. Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *BioEssays* 1994; **16**: 245–51.
7. Uchiumi T, Kohno K, Tanimura H, Matsuo K, Sato S, Uchida Y, Kuwano M. Enhanced expression of the human multidrug resistance 1 gene in response to UV light irradiation. *Cell Growth Differ* 1993; **4**: 147–57.
8. Asakuno K, Kohno K, Uchiumi T, Kubo T, Sato S, Isono M, Kuwano M. Involvement of a DNA binding protein, MDR-NF1/YB-1, in human MDR1 gene expression by actinomycin D. *Biochem Biophys Res Commun* 1994; **199**: 1428–35.
9. Ohga T, Uchiumi T, Makino Y, Koike K, Wada M, Kuwano M, Kohno K. Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. *J Biol Chem* 1998; **273**: 5997–6000.
10. Furukawa M, Uchiumi T, Nomoto M, Takano H, Morimoto RI, Naito S, Kuwano M, Kohno K. *J Biol Chem* 1998; **273**: 10550–5.
11. Swamynathan SK, Nambiar A, Guntaka RV. Role of single-stranded DNA regions and Y-box proteins in transcriptional regulation of viral and cellular genes. *FASEB J* 1998; **2**: 515–22.
12. Matsumoto K, Wolffe AP. Gene regulation by Y-box proteins: coupling control of transcription and translation. *Trends Cell Biol* 1998; **8**: 318–23.
13. Sommerville J. Activities of cold-shock domain proteins in translation control. *BioEssays* 1999; **21**: 319–25.
14. Tafuri SR, Familari M, Wolffe AP. A mouse Y box protein, MSY1, is associated with paternal mRNA in spermatocytes. *J Biol Chem* 1993; **268**: 12213–20.
15. Ohga T, Koike K, No M, Makino Y, Itagaki Y, Tanimoto M, Kuwano M, Kohno, K. Role of the human Y box-binding protein YB-1 in cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and ultraviolet light. *Cancer Res* 1996; **56**: 4224–8.
16. Koike K, Uchiumi T, Ohga T, Toh S, Wada M, Kohno K, Kuwano M. Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Lett* 1997; **417**: 390–4.
17. Hasegawa SL, Doetsch PW, Hamilton KK, Martin AM, Okenquist SA, Lenz J, Boss JM. DNA binding properties of YB-1 and dbpA: binding to double-stranded, single-stranded, and abasic site containing DNAs. *Nucleic Acids Res* 1991; **19**: 4915–20.
18. Ise T, Nagatani G, Imamura T, Kato K, Takano H, Nomoto M, Izumi H, Ohmori H, Okamoto T, Ohga T, Uchiumi T, Kuwano M, Kohno K. Transcription factor Y-box binding protein 1 binds preferentially to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen. *Cancer Res* 1999; **59**: 342–6.
19. Hayakawa H, Uchiumi T, Fukuda T, Ashizuka M, Kohno K, Kuwano M, Sekiguchi M. Binding capacity of human YB-1 protein for RNA containing 8-oxoguanine. *Biochemistry* 2002; **41**: 12739–44.
20. Okamoto T, Izumi H, Imamura T, Takano H, Ise T, Uchiumi T, Kuwano M, Kohno K. Direct interaction of p53 with the Y-box binding protein, YB-1: a mechanism for regulation of human gene expression. *Oncogene* 2000; **19**: 6194–202.
21. Kamura T, Yahata H, Amada S, Ogawa S, Sonoda T, Kobayashi H, Mitsumoto M, Kohno K, Kuwano M, Nakano H. Is nuclear expression of Y box-binding protein-1 a new prognostic factor in ovarian serous adenocarcinoma? *Cancer* 1999; **85**: 2450–4.
22. Shibahara K, Sugio K, Osaki T, Uchiumi T, Maehara Y, Kohno K, Yasumoto K, Sugimachi K, Kuwano M. Nuclear expression of the Y-box binding protein, YB-1, as a novel marker of disease progression in non-small cell lung cancer. *Clin Cancer Res* 2001; **7**: 3151–5.
23. Janz M, Harbeck N, Dettmar P, Berger U, Schmidt A, Jurchott K, Schmitt M, Royer HD. Y-box factor YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biologic factors HER2, uPA and PAI-1. *Int J Cancer* 2001; **97**: 278–82.
24. Mansour SL, Thomas KR, Capecchi MR. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* 1988; **336**: 348–52.
25. Tsuzuki T, Fujii Y, Sakumi K, Tominaga Y, Nakao K, Sekiguchi M, Matsushiro A, Yoshimura Y, Morita T. Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. *Proc Natl Acad Sci USA* 1996; **93**: 6236–40.
26. Izumi H, Imamura T, Nagatani G, Ise T, Murakami T, Uramoto H, Torigoe T, Ishiguchi H, Yoshida Y, Nomoto M, Okamoto T, Uchiumi T, Kuwano M, Funa K, Kohno K. Y box-binding protein-1 binds preferentially to single-stranded nucleic acids and exhibits 3′→5′ exonuclease activity. *Nucleic Acids Res* 2001; **29**: 1200–7.
27. Swamynathan S, Varma B, Weber K, Guntaka R. Targeted disruption of one allele of the Y-box protein gene, Chk-YB-1b, in DT40 cells results in major defects in cell cycle. *Biochem Biophys Res Commun* 2002; **296**: 451–7.
28. Oda Y, Sakamoto A, Shinohara N, Ohga T, Uchiumi T, Kohno K, Tsuneyoshi M, Kuwano M, Iwamoto Y. Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human osteosarcoma. *Clin Cancer Res* 1998; **4**: 2273–7.
29. Shibao K, Takano H, Nakayama Y, Okazaki K, Nagata N, Izumi H, Uchiumi T, Kuwano M, Kohno K, Itoh H. Enhanced coexpression of YB-1 and DNA topoisomerase II alpha genes in human colorectal carcinomas. *Int J Cancer* 1999; **83**: 732–7.
30. Lorico A, Rappa G, Flavell RA, Sartorelli AC. Double knockout of the MRP gene leads to increased drug sensitivity *in vitro*. *Cancer Res* 1996; **56**: 5351–5.
31. Tominaga Y, Tsuzuki T, Shiraishi A, Kawate H, Sekiguchi M. Alkylation-induced apoptosis of embryonic stem cells in which the gene for DNA-repair, methyltransferase, had been disrupted by gene targeting. *Carcinogenesis* 1997; **18**: 889–96.
32. Chu G. Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair. *J Biol Chem* 1994; **269**: 787–90.
33. Stavrovskaya A. A cellular mechanisms of multidrug resistance of tumor cells. *Biochemistry (Mosc)* 2000; **65**: 95–106.
34. Zamble DB, Lippard SJ. Cisplatin and DNA repair in cancer chemotherapy. *Trends Biochem Sci* 1995; **20**: 435–9.
35. Timmer-Bosscha H, Mulder NH, de Vries EG. Modulation of *cis*-diamminedichloroplatinum(II) resistance: a review. *Br J Cancer* 1992; **66**: 227–38.
36. Arioka H, Nishio K, Ishida T, Fukumoto H, Fukuoka K, Nomoto T, Kurokawa H, Yokote H, Abe S, Saijo N. Enhancement of cisplatin sensitivity in high mobility group 2 cDNA-transfected human lung cancer cells. *Jpn J Cancer Res* 1999; **90**: 108–15.
37. He Q, Liang CH, Lippard SJ. Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. *Proc Natl Acad Sci USA* 2000; **97**: 5768–72.
38. Brown SJ, Kellett PJ, Lippard SJ. Ixr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin. *Science* 1993; **261**: 603–5.
39. Damia G, Imperatori L, Stefanini M, D'Incalci M. Sensitivity of CHO mutant cell lines with specific defects in nucleotide excision repair to different anti-cancer agents. *Int J Cancer* 1996; **66**: 779–83.
40. De Silva IU, McHugh PJ, Clingen PH, Hartley JA. Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. *Nucleic Acids Res* 2002; **30**: 3848–56.
41. Uramoto H, Izumi H, Ise T, Tada M, Uchiumi T, Kuwano M, Yasumoto K, Funa K, Kohno K. p73 interacts with c-Myc to regulate Y-box-binding protein-1 expression. *J Biol Chem* 2002; **277**: 31694–72.