Effects of Estrogenic Agents 17β-Estradiol (E2) and Bisphenol A on the Expression of RecQ DNA Helicases in Mammary Tumor MCF-7 Cells

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17β-Estradiol (E2) and bisphenol A (BPA) damage DNA in estrogen receptor (ER)-positive human mammary tumor MCF-7 cells. The effect of E2 and BPA is associated with increased expression of Bloom helicase (BLM) and formation of nuclear foci consisting of BLM and γH2AX at double stranded break regions. BLM is one of five human RecQ helicases that participate in the maintenance of genomic stability. This study investigated if the expressions of RecQ helicases Werner syndrome helicase (WRN), Rothmund-Thomson syndrome helicase (RTS), RecQ5 helicase (RecQ5), RecQ protein-like 1 helicase (RecQL1, also known as RecQ1) and BLM helicase are affected in MCF-7 cells by treatment with estrogenic agents. The expression of RTS mRNA significantly increased although the expression in RTS protein only slightly increased. The expressions of WRN, RecQ5 and RecQL1 were almost unaffected both at mRNA and protein levels. These data suggest that the expression of human RecQ helicases is differentially regulated depending on the tissues and cells. We showed that some helicase expressions are regulated depending on the DNA damage.

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INTRODUCTION

17β-Estradiol (E2) is believed to cause carcinogenesis by genotoxicity[1)] combined with a stimulatory effect on cell proliferation.[2)] Various studies using laboratory animals and cultured cells have confirmed that E2 is carcinogenic.[3,4)] Experiments with DNA microarray technology characterized the profiles of estrogen-responsive genes.[5,6)] E2 upregulates cell cycle-associated genes, especially associated with DNA synthesis, and downregulates the expression of antiproliferative and proapoptotic genes,[5)] resulting collectively in the formation of mammary tumor cells and sustained proliferation. A similar effect is also observed for bisphenol A (BPA),[7)] an endocrine-disrupting chemical, widely used in material for polycarbonate plastic and epoxy resin.[8)] BPA has a weak estrogenic activity to stimulate cell proliferation of estrogen receptor (ER)-positive mammary tumor cells, and so is attracting considerable attention.[9,10)] E2 and BPA damage DNA in mammary tumor MCF-7 cells via ER, and Bloom helicase (BLM) foci colocalize at sites of DNA damage, suggesting that BLM has a role in the repair of damaged DNA.[11)]

Genome-maintaining enzymes, such as RecQ DNA helicases, participate in replication of DNA during cell proliferation and prevent the DNA structure from various types of damage. BLM is a member of RecQ helicase family having ATPase and 3′–5′ DNA unwinding activity.[12)] Other human RecQ helicases in the family are: Werner syndrome helicase (WRN),[13)] Rothmund-Thomson syndrome helicase (RTS),[14)] RecQ5 helicase (RecQ5),[15)] and RecQ1 helicase (RecQL1).[16,17)] RecQ helicases share a part of the amino acid sequence encoding seven motifs that may be relevant to DNA unwinding activity and other specified features of each helicase.[15)] Biochemical studies have shown that BLM, WRN and RecQL1 have a 3′–5′ DNA unwinding activity and DNA-dependent ATPase activity.[18)] BLM and WRN helicases appear to have differential functions in DNA surveillance, but can coordinately work for particular types of DNA damage associating with a group of proteins including RPA, Rad51, Rad51D and MLH1.[19–21)] RecQL1 helicase catalyzes branch migration of Holliday junc-

tion structures suggesting a role of homologous recombination repair after DNA damage.[22] Little is known about the function of RTS and RecQ5. In this study, we addressed the question of whether the expression of all five RecQ helicases are similarly or differentially affected by estrogenic agents, and we found that they are differentially regulated.

MATERIALS AND METHODS

Chemicals —— E2 and BPA were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan).

Cells, Cell Culture and Chemical Treatment —— Mammary tumor cells MCF-7 cells were obtained from the American Type Cell Culture (Bethesda, MA, U.S.A.). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin (both from Sigma-Aldrich, St. Louis, MO, U.S.A.) in a humidified atmosphere under 5% CO_2 at 37°C. For all chemical treatments, the cells were transferred to phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped FBS (Hyclone, Logan, UT, U.S.A.) for 48 hr before use to eliminate steroid hormones, including estrogen. The cells were treated with E2 or BPA for 48 hr at the concentrations indicated in the figures. In a time-course study, cells were treated with 10^{-10} M E2 or 10^{-5} M BPA, E2 and BPA for the 48 hr treatment at various concentrations and the time-course study were solubilized in ethanol and ethanol was adjusted to 0.1% in the culture of 0.1%. Control culture cells were exposed to a culture medium containing 0.1% ethanol.

Gene Expression —— BLM, WRN, RTS, RecQ5 and RecQL1 mRNA were quantified by using reverse transcriptase-polymerase chain reaction (RT-PCR) with the TaqMan method and an ABI prism 7000 (Applied Biosystems, Foster City, CA, U.S.A.). One-Step RT-PCR Master Mix Reagents Kit and TaqMan MGB probes specific for *BLM*, *WRN*, *RTS*, *RecQ5* and *RecQL1* were purchased from Applied Biosystems. Total RNA was extracted by using an RNeasy Mini kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's protocol. The experiments were repeated three times and RNA samples were assayed in duplicate. The percentage of changes in expression was calculated by using a comparative C_T method (ABI PRISM user bulletin #2) with human β-glucuronidase expression as an internal control.

Antibodies and Immunoblotting —— Immunoblot analysis of RecQ helicases was done as described previously.[23] Briefly, after treatment with E2 or BPA, cells were solubilized in radio immunoprecipitation assay buffer containing 20 mM Tris-HCl (pH 7.4), 0.1% sodium dodecyl sulfate, 1% TritonX-100 and 1% sodium deoxycholate. The amounts of protein were determined by using the Bradford protein assay kit (BioRad, Hercules, CA, U.S.A.). Solubilized proteins (50 μg) were electrophoresed on 2–15% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan) at 200 V for 1 hr and were electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, U.S.A.) for 90 min at 6 V. Goat polyclonal antibodies against human BLM (C-18) were purchased from Santa Cruz, CA, U.S.A. Mouse monoclonal antibody 4H12 against the C-terminal end of WRN was reported previously.[23] These mouse monoclonal antibodies against human RTS and RecQ5β were prepared by immunizing mice with a purified recombinant C-terminal fragment of RTS (amino acid residues 907–1208) or of RecQ5β (amino acid residues 848–991), respectively.[24] Rabbit polyclonal antibodies against human RecQL1 were kindly provided by Dr. Enomoto.[18] RecQ helicase proteins were detected by using specific monoclonal or polyclonal antibodies and then by using rabbit anti-goat immunoglobulin, rabbit anti-mouse immunoglobulin or goat anti-rabbit immunoglobulin that were conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark). The membrane was developed by using increased chemiluminescence with ECL Plus Western Blotting Detection Reagents (Amersham Life Science, Buckinghamshire, U.K.). Anti α-actin (ICN, Costa Mesa, CA, U.S.A.) was used to normalize the sample loading.

Statistical Analysis —— Dunnett's test was used.

RESULTS AND DISCUSSION

Estrogenic agents E2 and BPA stimulated proliferation of E2-responsive MCF-7 cells.[8] We showed that transcription of not only *BLM*, but also cell-cycle-associated genes, such as *MCM5*, *MCM2*, *Myt1*, *PCNA* and *AuroraA*, were stimulated under the same conditions.[7]

In this study, we examined mRNA expression

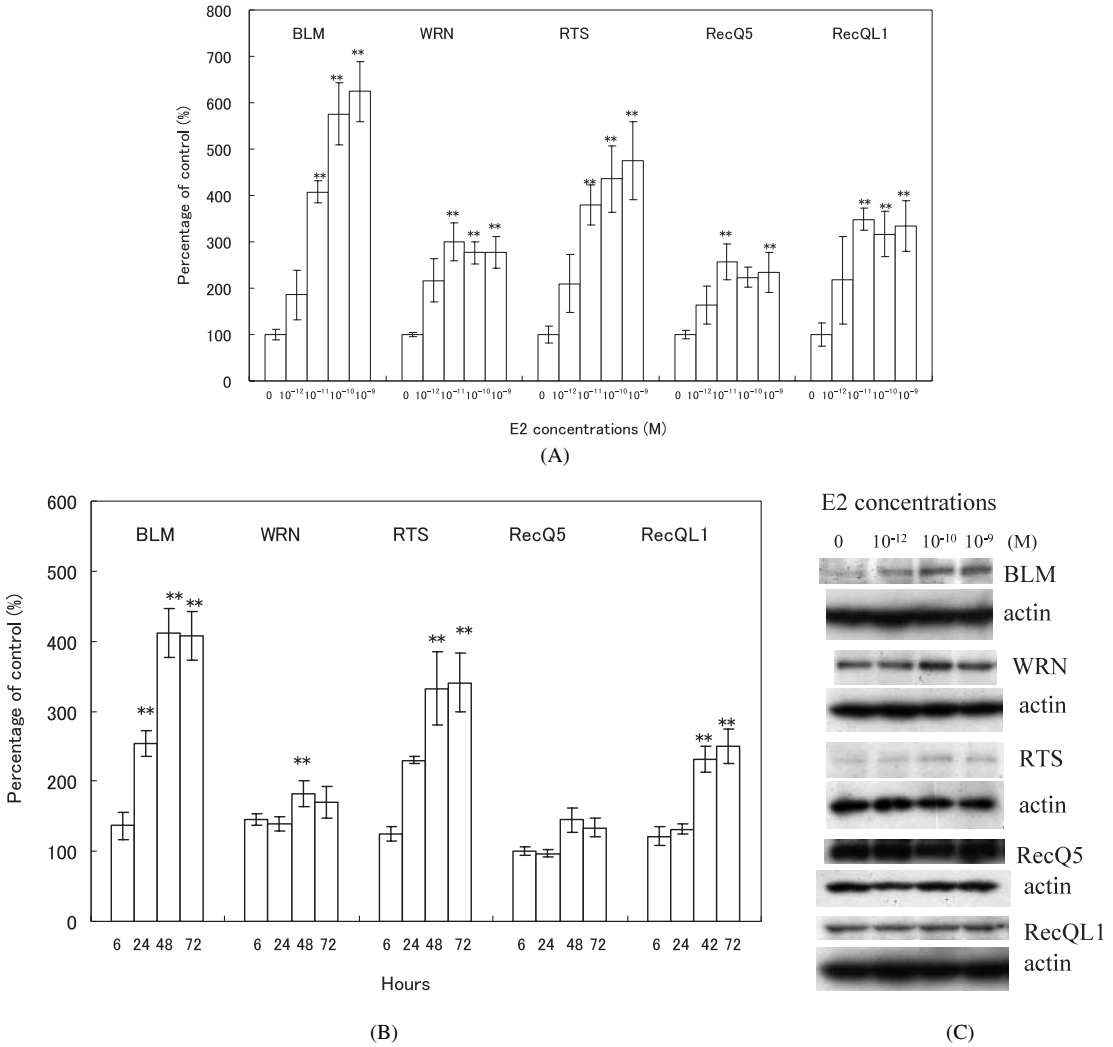

Fig. 1. Expression of mRNA and Its Product of RecQ Helicases in MCF-7 Cells after Treatment with E2

(A) Concentration-dependent effect of E2 for 48 hr on MCF-7 cells. Each point represents the mean ± standard deviation of triplicate samples. The vertical axis indicates the percentage of expression of control (treated cells/untreated control cells × 100%). ** Significantly different from control cells, $p < 0.01$. (B) Time course of the effect of 10^{-10} M E2 in MCF-7 cells. See also (A). (C) Expression of RecQ helicase proteins in MCF-7 cells as assessed by immunoblotting. MCF-7 cells were treated with E2 at the indicated concentrations for 72 hr.

of *BLM*, *WRN*, *RTS*, *RecQ5* and *RecQL1* (Fig. 1A). Figure 1A shows that the amount of mRNA of BLM and RTS increased dose-dependently from 10^{-12} M to 10^{-9} M E2. The three other mRNAs increased moderately and reached similar levels above 10^{-11} M E2 concentration. Figure 1B shows the time course of mRNA levels of these RecQ helicases after treatment with 10^{-10} M E2. BLM and RTS mRNA levels increased significantly shortly after 6 hr treatment with 10^{-10} M E2 and the increase continued until reaching similar levels at 48 hr and 72 hr. E2 and BPA stimulates the rate of MCF-7 cell proliferation and increases the cell number after 144 hr.[8] In our study, E2 at 10^{-12} and 10^{-9} M or BPA at 10^{-8} and 10^{-5} M accelerated cell proliferation for 96 hr (data not shown). The cell number was 1.4 times higher at 10^{-9} M E2 or 10^{-5} M BPA than non-treated control cells after 96 hr treatment. Comparison of mRNA expression of five helicases assessed because of slightly increased cell numbers showed that the mRNA levels of BLM and RTS were specifically upregulated by estrogenic agents. The mRNA levels of BLM and RTS reached the highest level after 72 hr of treatment, and thus we determined the protein levels of BLM and RTS at around 72 hr (Fig. 1B). Figure 1C shows the expression of the products of these genes in MCF-7 cells as assessed by immunoblotting at 72 hr after E2 treatment at the indicated doses. As expected, the BLM protein expression was stimu-

lated dose-dependently, but the RTS protein level was did not increase as distinctly as the BLM helicase protein. The protein levels of WRN, RecQ5 and RecQL1 were not affected, even though their mRNA synthesis significantly increased by E2 treatment.

We investigated the effect of BPA treatment on mRNA synthesis of the five RecQ helicases. BPA needed a 100–1000 times higher concentration than E2 to induce the same levels of BLM mRNA expression. This differential effect is correlated with the intrinsic binding affinity of E2 and BPA to the estrogen receptor.[25–27] Figure 2A shows the results with BLM, WRN, RTS, RecQ5 and RecQL1, in relation to their mRNA levels in MCF-7 cells after 48 hr treatment with 10^{-9} to 10^{-5} M BPA. BLM and RTS mRNAs specifically increased by BPA treatment dose-dependently, but WRN, RecQ5 and RecQL1 mRNAs increased slightly due perhaps to slightly increased cell numbers. Figure 2B shows the time course of mRNA expression after treatment with 10^{-5} M BPA. BLM and RTS mRNA levels increased significantly and reached similar levels at 48 hr and 72 hr, but the levels of WRN, RecQ5 and RecQL1 mRNAs only moderately increased by BPA treatment. Figure 2C shows immunoblot analysis of the protein levels of RecQ helicase family in MCF-7 cells at 72 hr after BPA treatment. Consistent with the increased mRNA, BLM protein increased dose-dependently as we observed with the results shown in Fig. 2A. The RTS protein increased after treatment with a high dose of BPA, but only slightly, and the protein levels of the three other helicases were almost unaffected.

Kawabe *et al.*[23] showed differential expression of human RecQ helicases in cell transformation and cell proliferation. This study supported our previous conclusions[7] that estrogenic agents stimulate cell proliferation by interaction with ER and also stimulate the expression of BLM helicase participating in DNA repair during the cell cycle. However, whether this stimulation is the direct cosequence of E2 signalling affecting BLM transcription or due to an indirect effect by DNA damage induced by E2-based oxygen radicals remains to be studied. BLM helicase colocalizes with γH2AX in nuclear foci at the site of DNA damage induced by treatment with estrogenic agents, suggesting that part of BLM helicase is associated with DNA damage by estrogenic agents.[11] BLM and WRN helicases function coordinately[19–21] under certain conditions. However, our study showed no sign of colocalization of WRN

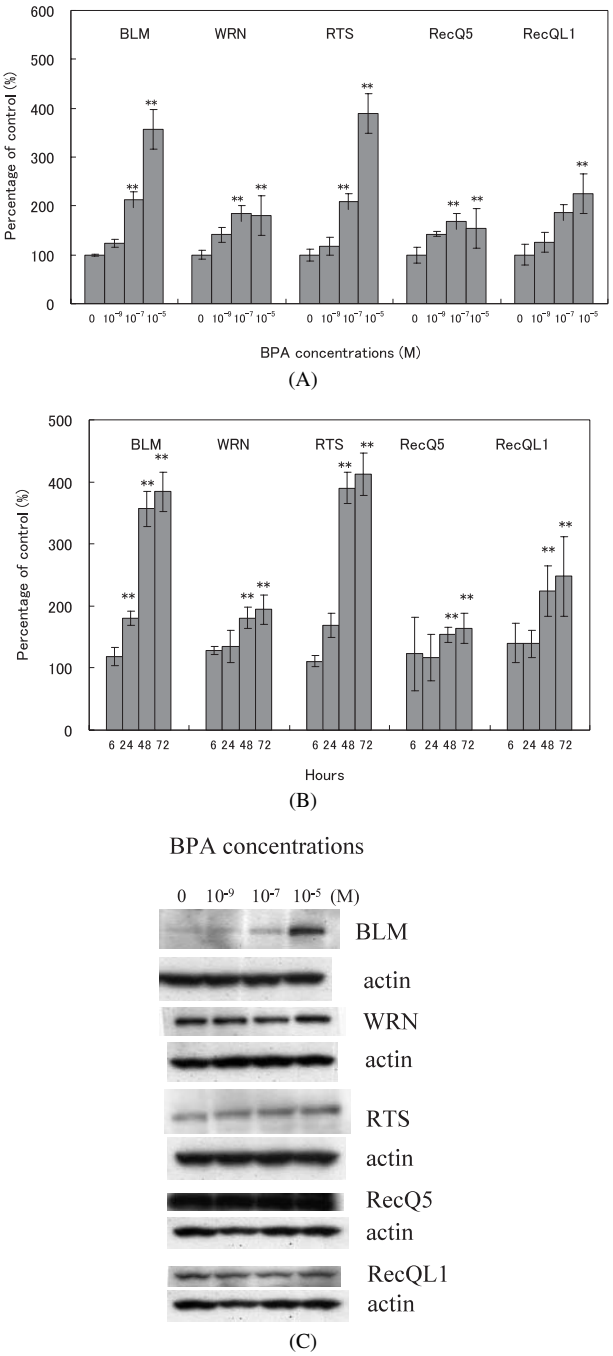

Fig. 2. Expression of mRNA and Its Product of RecQ Helicases in MCF-7 Cells after Treatment with BPA

(A) Concentration-dependent effect of BPA for 48 hr on MCF-7 cells. See also Fig. 1 legend. (B) Time course after treatment with 10^{-5} M BPA. See also Fig. 1 legend. (C) Expression of RecQ helicase proteins in MCF-7 cells as assessed by immunoblotting. MCF-7 cells were treated with BPA at the indicated concentrations for 72 hr. See also Fig. 1 legend.

and BLM helicases with estrogenic agents in MCF-7 cells (data not shown). RTS helicase has been assumed to fuction as a genome caretaker.[28] Woo *et al.*[29] reported that RTS changes subcellular localization in response to oxidative stress, suggest-

ing that RTS helicase may have a role in DNA repair after oxidative damage. Consistent with this report, RTS expression was stimulated after E2 or BPA treatment in our study, but further study is necessary to understand the RTS function. These results suggest that RecQ helicases are differentially regulated against the genotoxic effect by estrogenic agents and that BLM helicase may have an important role in ER-containing cells to cope with DNA damage induced by estrogenic agents. This study supports the importance of BLM helicase in the repair of DNA damage caused by estrogenic agents.

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REFERENCES

1) Liehr, J. G. (2000) Is Estradiol a genotoxic mutagenic carcinogen? *Endocr. Rev.*, **21**, 40–54.
2) Cavalieri, E., Frenkel, K., Liehr, J. G., Rogan, E. and Roy, D. (2000) Estrogens as endogeneous genotoxic agents — DNA adducts and mutations. *J. Natl. Cancer Inst. Monogr.*, **27**, 75–93.
3) Bhat, H. K., Calaf, G., Hei, T. K., Loya, T. and Vadgama, J. V. (2003) Critical role of oxidative stress in estrogen-induced carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 3913–3918.
4) Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S. L., Patil, K. D., Gross, M. L., Gooden, J. K., Ramanathan, R., Cerny, R. L. and Rogan, E. G. (1997) Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 10937–10942.
5) Frasor, J., Danes, J. M., Komm, B., Chang, K. C. N., Lyttle, C. R. and Katzenellenbogen, B. S. (2003) Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology*, **144**, 4562–4574.
6) Soulez, M. and Parker, M. G. (2001) Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. *J. Mol. Endocrinol.*, **27**, 259–274.
7) Iso, T., Futami, K., Iwamoto, T. and Furuichi, Y. (2007) Modulation of the expression of Bloom helicase by estrogenic agents. *Biol. Pharm. Bull.*, **30**, 266–271.
8) Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L. and Feldman, D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology*, **132**, 2279–2286.
9) Roy, D., Palangat, M., Chen, C.-W., Thomas, R. D., Colerangle, J., Atkinson, A. and Yan, Z.-J. (1997) Biochemical and molecular changes at the cellular level in response to exposure to environmental estrogen-like chemicals. *J. Toxicol. Environ. Health*, **50**, 1–29.
10) Hillard, C. A., Armstrong, M. J., Bradt, C. I., Hill, R. B., Greenwood, S. K. and Galloway, S. M. (1998) Chromosome aberrations *in vitro* related to cytotoxicity of nonmutagenic chemicals and metaboloic poisons. *Environ. Mol. Mutagen.*, **31**, 316–326.
11) Iso, T., Watanabe, T., Iwamoto, T., Shimamoto, A. and Furuichi, Y. (2006) Bisphenol A causes DNA damages by the estrogenic activity. *Biol. Pharm. Bull.*, **29**, 206–210.
12) Ellis, N. A., Groden, J., Ye, T.-Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M. and German, J. (1995) The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell*, **83**, 655–666.
13) Yu, C. E., Oshima, J., Fu, Y. H., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G. M., Muligan, J. and Schellenberg, G. D. (1996) Positional cloning of the Werner's syndrome gene. *Science*, **272**, 258–262.
14) Kitao, S., Shimamoto, A., Goto, M., Miller, R. W., Smithson, W. A., Lindor, N. M. and Furuichi, Y. (1999) Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat. Genet.*, **22**, 82–84.
15) Kitao, S., Ohsugi, I., Ichikawa, K., Goto, M., Furuichi, Y. and Shimamoto, A. (1998) Cloning of two new human helicase genes of the RecQ family: biological significance of multiple species in higher eukaryotes. *Genomics*, **54**, 443–452.
16) Puranam, K. L. and Blackshear, P. J. (1994) Cloning and characterization of RECQL, a potential human homologue of the *Escherichia coli* DNA helicase RecQ. *J. Biol. Chem.*, **269**, 29838–29845.
17) Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., Yamaoka, T., Hoshino, S., Ozawa, K., Eki, T., Nogami, M., Okumura, K., Taguchi, H., Hanaoka, F. and Enomoto, T. (1994) Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to *Escherichia coli* RecQ helicase and local-

ization of the gene at chromosome 12p12. *Nucleic Acids Res.*, **22**, 4566–4573.
18) Tada, S., Yanagisawa, J., Sonoyama, T., Miyajima, A., Seki, M., Ui, M. and Enomoto, T. (1996) Characterization of the properties of a human homologue of *Escherichia coli* RecQ from xeroderma pigmentosum group C and from HeLa cells. *Cell Struct. Funct.*, **21**, 123–132.
19) Bischof, O., Kim, S.-H., Irving, J., Beresten, S., Ellis, N. A. and Campisi, J. (2001) Regulation and localization of the Bloom syndrome protein in response to DNA damage. *J. Cell Biol.*, **153**, 367–380.
20) Machwe, A., Xiao, L., Groden, J. and Orren, D. K. (2006) The Werner and Bloom syndrome proteins catalyze regression of a model replication fork. *Biochemistry*, **45**, 13939–13946.
21) Karmakar, P., Seki, M., Kanamori, M., Hashiguchi, K., Ohtsuki, M., Murata, E., Inoue, E., Tada, S., Lan, L., Yasui, A. and Enomoto, T. (2006) BLM is an early responder to DNA double-strand breaks. *Biochem. Biophys. Res. Commun.*, **348**, 62–69.
22) Doherty, K. M., Sharma, S., Uzdilla, L. A., Wilson, T. M., Cui, S., Vindigni, A. and Brosh, R. M., Jr. (2005) RECQ1 helicase interacts with human mismatch repair factors that regulate genetic recombination. *J. Biol. Chem.*, **280**, 28085–28094.
23) Kawabe, T., Tsuyama, N., Kitao, S., Nishikawa, K., Shimamoto, A., Shiratoi, M., Matsumoto, T., Anno, K., Sato, T., Mitsui, Y., Seki, M., Enomoto, T., Goto, M., Ellis, N. A., Ide, T., Furuichi, Y. and Sugimoto, M. (2000) Differential regulation of human RecQ family helicases in cell transformation and cell cycle. *Oncogene*, **19**, 4764–4772.
24) Shiratori, M., Suzuki, T., Itoh, C., Goto, M., Furuichi, Y. and Matsumoto, T. (2002) WRN helicase accelerates the transcription of ribosomal RNA as a component of an RNA polymerase I-associated complex. *Oncogene*, **21**, 2447–2454.
25) Steinmetz, R., Brown, N. G., Allen, D. L., Bigsby, R. M. and Ben-Jonathan, N. (1997) The environmental estrogen bisphenol A stimulates prolactin release *in vitro* and *in vivo*. *Endocrinology*, **138**, 1780–1786.
26) Gould, J. C., Leonard, L. S., Maness, S. C., Wagner, B. L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D. P. and Gaido, K. W. (1998) Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. *Mol. Cell. Endocrinol.*, **142**, 203–214.
27) Leffers, H., Næsby, M., Vendelbo, B., Skakkebæk, N. E. and Jørgensen, M. (2001) Oestrogenic potencies of zeraniol, oestradiol. diethylstilboestrol, bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. *Hum. Reprod.*, **16**, 1037–1045.
28) Macris, M. A., Krejci, L., Bussen, W., Shimamoto, A. and Sung, P. (2006) Biochemical characterization of the RECQ4 protein, mutated in Rothmund-Thomson syndrome. *DNA Repair* (*Amst.*), **5**, 172–180.
29) Woo, L. L., Futami, K., Shimamoto, A., Furuichi, Y. and Frank, K. M. (2006) The Rothmund-Thomson gene product RecQL4 localizes to the nucleolus in response to oxidative stress. *Exp. Cell Res.*, **312**, 3443–3457.