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TERT mRNA Expression is Up-Regulated in MCF-7 Cells and a Mouse Mammary Organ Culture (MMOC) System by Endosulfan Treatment

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Endosulfan is one of the organochlorine pesticides, which are well-known endocrine disruptors (EDs), and it acts as an estrogen agonist. Estrogen is a group of hormones that play an important role in mammary gland function and are implicated in mammary carcinogenesis. In the present study, we studied the effects of endosulfan on nodule like alveolar lesion (NLAL) formation in mouse mammary gland development using a mouse mammary gland organ culture (MMOC) system. Although endosulfan-treated mammary glands did not form NLALs, more alveolar buds were formed in this group than in the negative control (vehicle-treated) group. In addition, telomerase reverse transcriptase (TERT) mRNA expression levels were increased in endosulfan-treated mammary glands in a dose-dependent manner. Telomerase can be activated by estrogen, therefore, we examined the effects of endosulfan on telomerase activity, and found that the telomerase activity in estrogen receptor-positive MCF-7 cells was up-regulated by endosulfan treatment. Moreover, this activation was accompanied by the up-regulation of the TERT mRNA expression. Also, transient expression assays using CAT reporter plasmids containing various fragments of the TERT promoter showed that this imperfect palindromic estrogen-responsive element is almost certainly responsible for the transcriptional activation by endosulfan. These results may help elucidate the endocrine disrupting mechanism of endosulfan.

Key words: Endosulfan, Telomerase, Mouse mammary organ culture (MMOC)

INTRODUCTION

Estrogens have mitogenic effects on breast tissue including the mammary gland and they play a pivotal role in the development of mammary carcinoma (Muller *et al.*, 2001; Marcantonio *et al.*, 2001). Many endocrine disruptors (EDs) show the estrogenic effect and have an environmental presence, and as such, may adversely affect both human health and wildlife (Schmitt *et al.*, 2001). Endosulfan is a cyclodiene organochlorine widely used as an insecticide throughout the world. It has been found to induce neurotoxicity, renal toxicity, hepatotoxicity, haematologic toxicity, respiratory toxicity and reproductive toxicity in mammals (Wade *et al.*, 1997). Endosulfan can act as an estrogen agonist and an androgen antagonist (Anderson, *et al.*, 2002). Proliferative and estrogen-like effects have been found in MCF-7 cells at a dose of 10 mM of endosulfan (Soto *et al.*, 1994). As well, it has been reported that endosulfan acts as an estrogen receptor agonists in Eker rat uterine myometrial cells (Hodges *et al.*, 2000). The current report intends to explain these observations by characterizing the estrogen mimicking properties of endosulfan.

The mammary gland is a highly complex hormone-dependent organ, which has an unlimited capability of repeatedly undergoing a sequence of reorganization (Richert *et al.*, 2000). Mammary glands are composed mainly of epithelial cells, myoepithelial cells and stromal cells. Mouse mammary glands in whole organ culture undergo organ development, functional differentiation and alveolar involution (regression), thus mimicking the pro-

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cesses that occur *in vivo* (Mehta *et al.*, 1997). Hormonal manipulation of this *in vitro* system brings about full lobuloalveolar development, lactogenesis and alveolar regression (Mehta *et al.*, 1997). Mammary glands from young virgin female mice respond dramatically to hormones in serum-free medium. When these glands are exposed to 7,12-dimethylbenz[a]anthracene (DMBA) for 24 h in culture, they develop hyperplastic alveolar nodule-like mammary lesions under appropriate hormonal conditions (Mehta *et al.*, 2000).

Telomerase is a cellular reverse transcriptase, which catalyzes the synthesis and extension of telomeric DNA (Greider and Blackburn, 1989). This enzyme is specifically activated in the majority of malignant tumors, but is usually inactive in normal somatic cells (Kim *et al.*, 1994). Moreover, a mechanism of maintaining telomere stability is required for cells to overcome replicative senescence, and telomerase activation may be a rate-limiting or at least a critical step in cellular immortality and oncogenesis (Harley, 1995). Recent studies have identified three major components of human telomerase: the template RNA (TR), telomerase-associated protein and the reverse transcriptase TERT subunit (Feng *et al.*, 1995; Harrington *et al.*, 1997; Nakamura *et al.*, 1997). TR and TERT are known to be sufficient to reconstitute telomerase activity *in vitro* (Weinrich *et al.*, 1997). Recent success in cloning the TERT promoter enabled innovative investigations into the mechanism controlling the transcriptional activity of TERT (Wu *et al.*, 1999; Takakura *et al.*, 1999). Specifically, TERT is both a direct and indirect target of estrogen, which implies the existence of hormone dependent mechanisms that control telomerase activity and a direct interaction between the activated estrogen receptor (ER) and an imperfect estrogen response element (ERE) in the TERT promoter (Kyo *et al.*, 1999).

Therefore, we examined the effects of endosulfan on mammary gland development and the TERT mRNA expression in the MMOC system. To clarify the estrogen mimicking action of endosulfan, we examined the effects of endosulfan on TERT transcription as well as telomerase activity by using the estrogen receptor-positive MCF-7 cell line. In addition, a CAT assay was performed to confirm the transcriptional activity of TERT by endosulfan.

MATERIALS AND METHODS

Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, USA) and were grown in DMEM with 5% FBS in the presence of 5% CO_2 at 37°C. In induction assays with 17β-estradiol (E2) and endosulfan, cells were grown in phenol red-free media containing 5% dextran-coated charcoal-treated FBS for 48 h prior to treatment.

Mouse mammary gland organ culture (MMOC)

Waymouth's medium 752/1, E2, progesterone, hydrocortisone, aldosterone, and insulin were purchased from Sigma (USA). Female 21 to 28 day old Balb/c mice were purchased from Daehan Biolink (Korea). The animals were pretreated daily with E2 (1 μg) and progesterone (1 mg) for 9 days. The procedure used for culturing whole mammary glands of the mice was followed as previously described by Metha *et al.* (1997). Briefly, mice were killed by cervical dislocation, and the inguinal pair of mammary glands were dissected out on silk rafts and incubated in Waymouth MB752/1 medium at 37°C in a 95% O_2 and 5% CO_2 environment. The medium was supplemented with 350 mg/mL of glutamine, antibiotics and growth-promoting hormones (5 μg of insulin, 5 μg of prolactin, 1 ng of E2, and 1 μg of progesterone per milliliter of medium). DMBA (10 μM) was added to the medium on day 3. To examine the effects of endosulfan, DMBA treatment was replaced with various concentrations of endosulfan (5 μM, 50 μM and 500 μM) or E2 (10 μM) treatment, and control dishes contained DMSO as a vehicle solvent (final 0.1% in culture media). On day 4, DMBA, endosulfan or E2 were removed from the media and the glands were then rinsed with fresh medium not containing a carcinogen and further incubated for 6 days. The glands were maintained for an additional 14 days in a medium containing only insulin (5 μg/mL). The glands were then fixed with formalin and stained with alum carmine and whole mounts were prepared. Ductal development was quantified as previously described by Barlow *et al.* (1997). Areas of development from the center of the fat pad to its periphery were photographed for each gland.

TRAP assay

The TRAP assay was performed using a TRAPEZE® telomerase detection kit (Oncor) (Brien *et al.*, 1997). MCF-7 cells were treated with endosulfan (1 and 10 μM) and E2 (100 nM) for 24 h and then cells were harvested for use in the TRAP assay. PCR products were electrophoresed on a 7% polyacrylamide gel and visualized with SYBR green I (FMC BioProducts).

TERT mRNA expression in MMOC and MCF-7 cells

The expression of TERT mRNA in the cultured mouse mammary glands and the MCF-7 cells was determined by RT-PCR. Mouse mammary glands were treated with endosulfan (5, 50, 500 μM) or E2 (10 μM), and MCF-7 cells were treated with endosulfan (1 and 10 μM) or E2 (10 nM) for 24 h. Then, TERT and β-actin transcripts were amplified by RT-PCR. Briefly, TERT and β-actin mRNAs were amplified using the following primer pairs: 5'-

CTCAGGAACACCAAGAAGTTCATC-3' (sense primer) and 5'-GGATGAAGCGGAGTCTGG A-3' (anti-sense primer) for TERT, and 5'-GACCCAGATCATGTTTGAGA-3' (sense primer) and 5'-GCTTGCTGATCCACATCTGC-3' (anti-sense primer) for β-actin. Total RNA was extracted using an Ultraspec™ solution (BIOTECX, USA) according to the manufacturer's instructions, and cDNA was synthesized from 2 μg of RNA using a Titan™ one tube RT-PCR Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions with some modification. Briefly, a mixture of 2 μg of RNA and 1 μL of an antisense primer (20 pmol) was heated at 95°C for 5 minutes then cooled rapidly on ice. After adjusting the mixture to a volume of 50 μL, containing 1X RT-PCR buffer, 0.2 mM of deoxynucleotide triphosphate (dNTP), 5 mM of DTT, 5 units of RNase inhibitor, 20 pmol sense primer and 1 μL of enzyme mix, the reaction mixture was subjected to RT-PCR using the following conditions: 50°C for 30 min and then 35 cycles at 94°C for 1 min, 60°C for 2 min, and 68°C for 2 min. PCR products were electrophoresed in 12% agarose gel and stained with SYBR green I (FMC BioProducts).

Plasmid construction

Genomic DNA from MCF-7 cells was extracted by using a Wizard™ Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions, and used as a template for the TERT promoter PCR. The PCR mixture contained 1×PCR buffer, 0.2 mM deoxynuclotide triphosphate (dNTP), 20 pmol of a sense (5'-AAGTGCCTCCGGGCAAGGGCA-3') and an antisense primer (5'-TGCGTCCCGGGCACGCACA-3'), 2.5 units of TaKaRa Ex Taq Polymerase, and 100 ng of template DNA, in a total volume of 100 μL. After denaturation at 94°C for 5 min, the reaction mixture was subjected to 35 cycles of reaction, which consisted of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and 68°C for 3 min, and this was followed by a final extension at 68°C for 7 min. Five microliters of the first amplification products were used as template DNA for the nested-PCR. The primer pairs used for the nested-PCR were as follows: 5'-TTGTTGCCCAGGCTGGAGTGCAGC-3' (sense primer) and 5'-TCCCTGGGCTGTCACGCTCGC-3' (antisense primer); and 5'-CCGCCCCTTTGCCCTAGTGGCAGA-3' (sense primer) and 5'-TGCGCAGCAGGGAGCGCACG-3' (antisense primer). The amplified PCR products were cloned into the pGEM T easy vector (Promega, USA). After confirming the sequence, each plasmid was digested with *Xba*I and *Sac*I and cloned into the pCAT3 reporter vector (Promega, USA). The structures of the TERT promoter-CAT constructs are shown in Fig. 4B. Various lengths of the DNA fragments upstream of the initiating ATG codon of the TERT gene were PCR-amplified and inserted into the CAT reporter vector (Promega, USA).

CAT assay

The transient transfection of Chloramphenicol acetyltransferase (CAT) reporter plasmids was performed using FuGene™ 6 Transfection Reagent (Boehringer Mannheim, Germany) according to the manufacturer's instructions. The control plasmid pCMV (1 μg) (Clontech, USA) was co-transfected to monitor the transfection efficiency. The CAT activity was normalized for transfection efficiency after determinating the β-galactosidase activity of the same sample. Various deletion mutants of TERT-promoter reporter plasmids were transfected into the MCF-7 cells and then treated with endosulfan (10 mM) or E2 (10 nM) for 48 h. The cells were harvested and the CAT assay was performed by the liquid scintillation counting (LSC) method upon the CAT reaction products (Gorman *et al.*, 1982). Each transfection was carried out in triplicate and the experiments were repeated at least four times.

Statistical analysis

All experimental data are expressed as means ± standard deviation of independent samples by using Student's *t*-test.

RESULTS AND DISCUSSION

Pesticides with xenoestrogenic activity are environmental contaminants that are suspected of promoting human diseases, such as cancers. Several natural and synthetic xenoestrogens have been evaluated at the cellular and molecular levels for their ability to mimic estrogen action (Coumoul *et al.*, 2001). The mouse mammary gland culture system has been widely used as an *in vitro* model to study the transformation of mammary cells by chemical carcinogens (Mehta *et al.*, 1997). By using the organ culture system of the whole mammary gland, treatment of the gland in culture with hydrocarbon carcinogens (DMBA) induces nodule-like alveolar lesions (NLALs) (Mehta *et al.*, 1997). This strategy appears to mimic the *in vivo* formation of female hormone related cancers, such as breast cancer. Therefore, using this method, we examined whether endosulfan has the ability to form precancerous lesions. DMBA-treated mammary glands developed alveolar buds and NLALs (Fig. 1B). NLALs are morphologically similar to presumptive preneoplastic lesions termed HANs (hyperplastic alveolar nodules), which arise in mice and chemical mammary tumorigenesis (Madina 2000). Compared with the DMSO control (Fig. 1A), the estradiol-treated (Fig. 1C) and the endosulfan-treated mammary glands (Fig. 1D, E, F) were not reversed to the original state. The control (DMSO-treated) glands, however, underwent regression from the alveolar structure to the original stage (Fig. 1A).

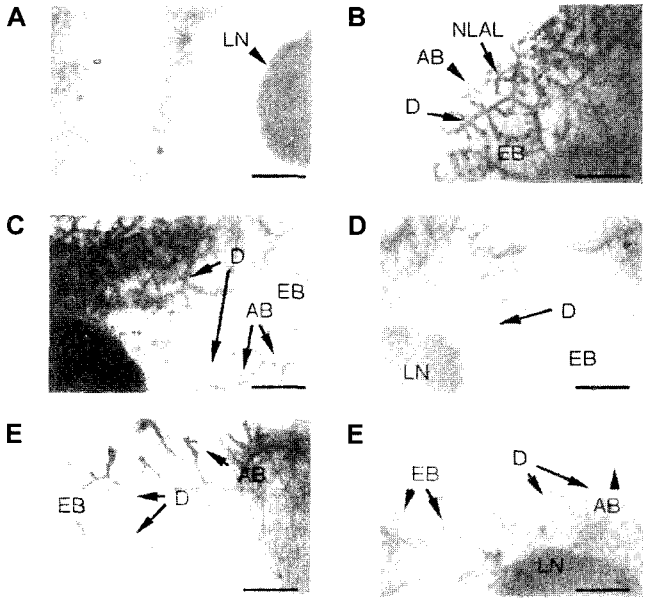

Fig. 1. Photographs of mouse mammary gland whole mounts stained with alum carmine. After treatment with E2 (1 μg) and progesterone (1 mg) for 9 days, the inguinal mammary glands were dissected. Five mammary glands per petri dish were cultured in Waymouth MB752/1 medium containing insulin, prolactin, aldosterone and hydrocortisone. On the 3rd day of mammary glands organ culture, endosulfan, E2 or DMBA were treated for 1 day. After an additional incubation for 6 days, mammary glands were allowed to regress in medium containing insulin alone for an additional 14 days. The glands developed ductal trees [(A) DMSO, (B) DMBA (10 μM), (C) E2 (10 μM), (D) endosulfan (5 μM), (E) endosulfan (50 μM) and (F) endosulfan (500 μM)]. Mammary structures are identified as follows: D, duct; EB, end bud; LN, lymph node; AB, alveolar bud; and NLAL, nodule like alveolar lesion. 20 X original magnification; the bar equals 200 μm.

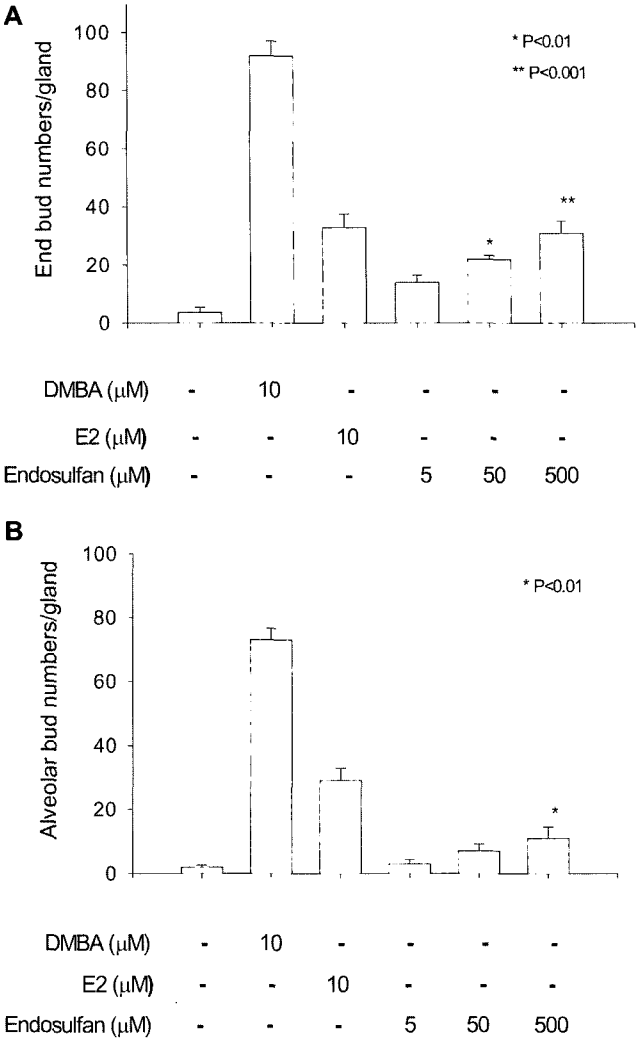

Fig. 2. Effects of endosulfan (5, 50 and 500 μM) on the development of an end bud (A) and alveolar (B) structures in a cultured mouse mammary gland organ. * indicates P<0.01 and ** indicates P<0.001 compared with the control group. Ten inguinal glands per each sample were used to get statistical results in this experiment. The results are expressed as means±SD of determinations.

The end bud (P<0.001) and the alveolar bud (P<0.01) formations were altered (regressions were disturbed) by endosulfan-treatment in a dose dependent manner (Fig. 2A, B). These results suggest that endosulfan can affect alveolar structure development by some unknown mechanisms.

Over the last few years, information has been accumulated on the role of telomerase in tumor development and progression (Holt *et al.*, 1996; Kim *et al.*, 1994). It has been shown that high telomerase activity is observed in practically all human breast cancers (Hiyama *et al.*, 1996); furthermore, the levels and number of cells expressing telomerase increase during mammary carcinogenesis (Kolquist *et al.*, 1998). An increase in telomerase activity has also been observed in rat mammary carcinomas (Varon *et al.*, 1997). It was also reported that telomerase activity in the human endometrium is regulated in a menstrual phase-dependent manner and that prolonged exposure to estrogen is one of the risk factors for the development of endometrial cancer (Kyo *et al.*, 1997; Sutton, 1990). Although the effects of estrogen on the proliferation of endometrial cells are complex, the activation of TERT and subsequent telomerase activation may contribute to estrogen-induced endometrial carcinogenesis. We examined TERT mRNA expression levels in endosulfan-treated mammary glands, and found that the TERT mRNA expression was increased in a dose-dependent manner (Fig. 3A).

To examine the effect of endosulfan on telomerase activity, ER-positive MCF-7 cells were cultured in the absence, or in the presence of endosulfan, at various concentrations to estimate telomerase activity. As shown in Fig. 3C., telomerase activity was found to be up-regulated by endosulfan treatment. The RT-PCR assay was performed to examine whether the activation of

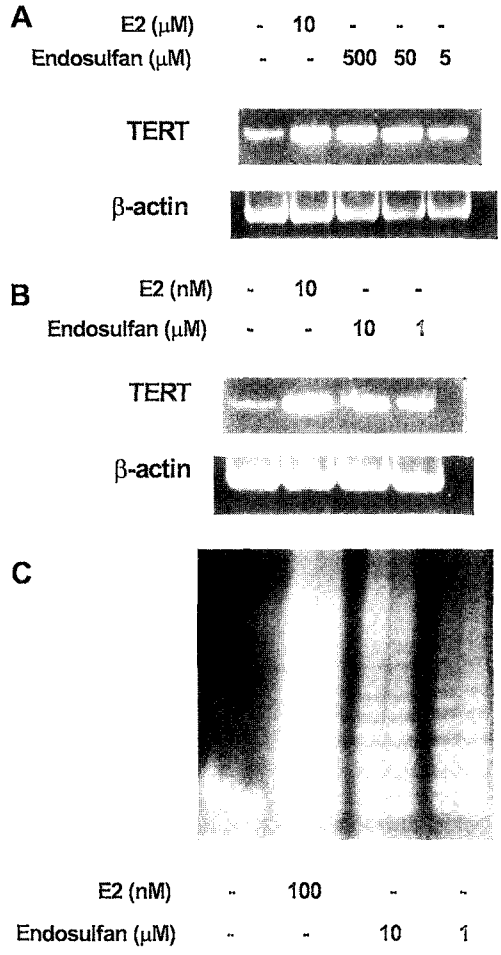

Fig. 3. (A) Identification of the TERT mRNA expression in a cultured mouse mammary gland by RT-PCR. Mouse mammary glands were treated with endosulfan (5, 50 and 500 μM), or E2 (10 μM) as the same schedule of the MMOC system. Total RNA was extracted from the mouse mammary gland samples and then TERT mRNA was amplified using RT-PCR. The PCR products were electrophoresed in 12% agarose gel and stained with SYBR green I (FMC BioProducts). (C) Endosulfan activates telomerase activity in MCF-7 cells. The MCF-7 cells were treated with endosulfan (1 and 10 μM) and E2 (100 nM) for 24 h and then cells were harvested. The TRAP assay was then performed. PCR products were electrophoresed on a 7% polyacrylamide gel and visualized with SYBR green I (FMC BioProducts). (B) Endosulfan activates the TERT mRNA expression in MCF-7 cells. RT-PCR assays to detect TERT mRNA are shown. The MCF-7 cells were treated with endosulfan (1 and 10 μM) and E2 (10 nM) for 24 h. TERT and â-actin mRNAs were amplified by RT-PCR. The PCR products were electrophoresed in 12% agarose gel and stained with SYBR green I (FMC BioProducts).

telomerase by endosulfan is due to the up-regulation of the TERT expression. Treatment of the MCF-7 cells with endosulfan led to the up-regulation of the TERT mRNA expression (Fig. 3B). Telomerase activation was accompanied by the mRNA up-regulation of the telomerase catalytic subunit. Recent studies have identified promoter sequences of TERT; therefore, we examined its transcriptional regulation with a reporter gene expression system. These studies demonstrated that the expression of TERT is regulated mainly at the transcriptional level, and the core promoter of the TERT gene is essential for transcriptional activation in cancer cells and immortalized cells (Wu *et al.*, 1999; Takakura *et al.*, 1999). As shown in Fig. 4A, the TERT core promoter encompasses the proximal 181 bp region upstream of the transcription start site. c-Myc and Sp1 specifically bind to the consensus motifs within this region and play essential roles in the transactivation of TERT (Wu *et al.*, 1999; Takakura *et al.*, 1999).

It has been demonstrated that estrogen activates telomerase via direct and indirect effects on the hTERT promoter (Kyo *et al.*, 1999). To examine the effect of endosulfan on the transcriptional activity of the TERT promoter, we cloned the TERT promoter region from MCF-7 genomic DNA, and constructed a serial-deletion TERT promoter-CAT reporter vector (Fig. 4B). Transient expression assays using CAT reporter plasmids containing various fragments of the TERT promoter showed that the CAT reporter plasmid, containing an imperfect palindromic ERE, is associated with telomerase activation. A 2.5-fold increase of transcription activation was observed with endosulfan treatment in pCAT3-2847, but the deletion mutants, pCAT3-1343 and pCAT3-706, which lacked the imperfect palindromic ERE, exhibited a decrease in transactivation (Fig. 4C).

Conclusively, in the MMOC system, TERT mRNA was increased by endosulfan treatment and the regression of mammary glands was hampered in a dose dependent manner. In addition, the telomerase activity in the estrogen receptor-positive MCF-7 cells was up-regulated by endosulfan treatment, and this activation was accompanied by the up-regulation of the TERT mRNA expression. The imperfect palindromic estrogen-responsive element was probably responsible for the transcriptional activation of TERT mRNA by endosulfan. Our results show that endosulfan affects the TERT mRNA expression, and this may help elucidate the mechanisms of endosulfan.

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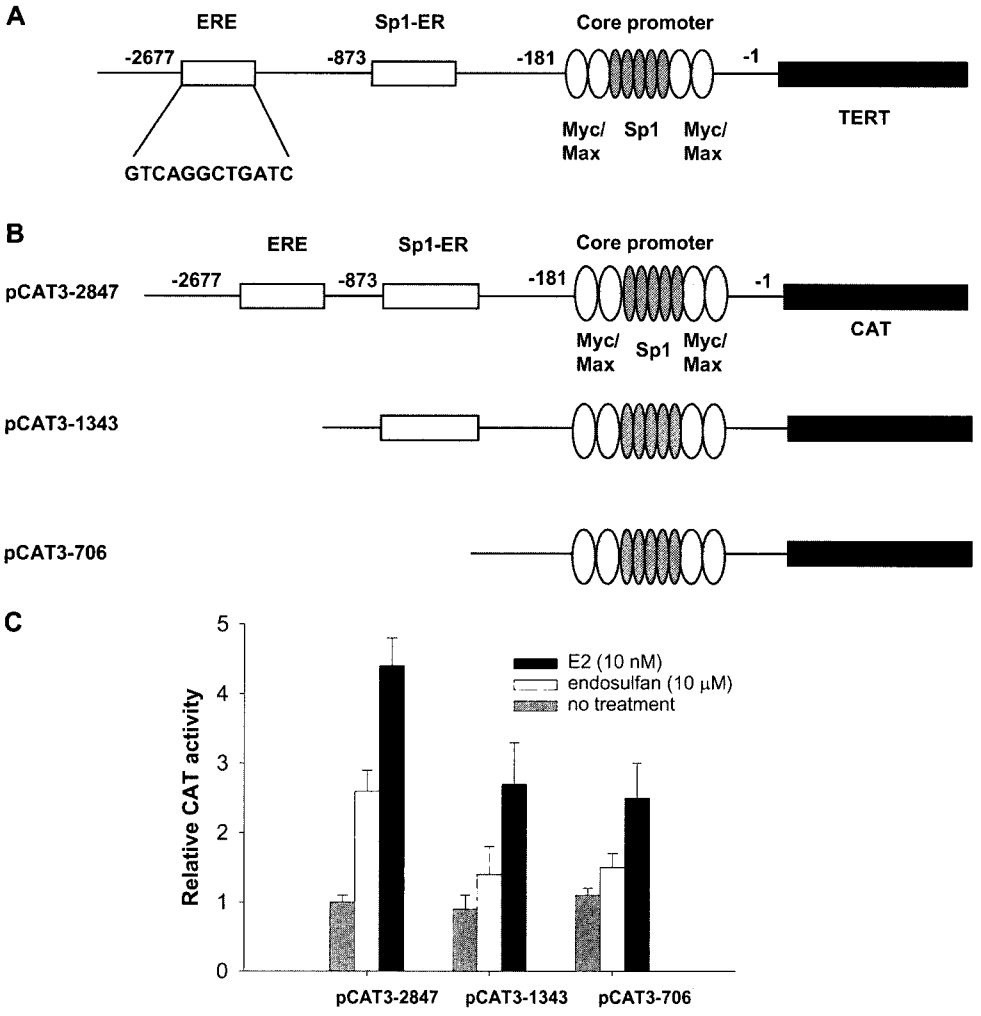

Fig. 4. (A) Schematic diagram of the TERT promoter. The sequence of an imperfect palindromic ERE at -2677 and the sequence of the Sp1/ER site at -873 are shown in the open boxes. (B) A schematic diagram of CAT reporter constructs. Various deletion mutants of the TERT-promoter plasmids were prepared as described in Materials and Methods. Plasmid constructs (pCAT3-2847, pCAT3-1343 and pCAT3-706) contained the CAT reporter gene under the control of the core promoter (-181 to -1 bp). (C) Various deletion mutants of theTERT-promoter reporter plasmids were transfected into the MCF-7 cells and then treated with endosulfan (10 μM) or E2 (10 nM) for 48 h. Cells were harvested and the CAT assay was performed by the liquid scintillation counting (LSC) method. The results are expressed as means ± SD of determinations.

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