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Characterization and Implications of Estrogenic Down-Regulation of Human Catechol-*O*-Methyltransferase Gene Transcription[1]

TAO XIE, SHU-LEONG HO, and DAVID RAMSDEN

Division of Neurology, University Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong (X.T., S.L.H.); and Department of Medicine, University of Birmingham, Queen Elizabeth Hospital, United Kingdom (D.R.)

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ABSTRACT

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) is a ubiquitous enzyme that is crucial to the metabolism of carcinogenic catechols and catecholamines. Regulation of human COMT gene expression may be important in the pathophysiology of various human disorders including estrogen-induced cancers, Parkinson's disease, depression, and hypertension. The gender difference in human COMT activity and variations in rat COMT activity during the estrous cycle led us to explore whether estrogen can regulate human COMT gene transcription. Our Northern analyses showed that physiological concentrations of 17-β-estradiol (10^{-9}–10^{-7} M) could decrease human 1.3-kilobase COMT mRNA levels in MCF-7 cells in a time- and dose-dependent manner through an estrogen receptor-dependent mechanism. Two DNA fragments immediately 5′ to the published human COMT gene proximal and distal promoters were cloned. Sequence analyses revealed several half-palindromic estrogen response elements and CCAAT/enhancer binding protein sites. By cotransfecting COMT promoter-chloramphenicol acetyltransferase reporter genes with human estrogen receptor cDNA and pSV-β-galactosidase plasmids into COS-7 cells, we showed that 17-β-estradiol could down-regulate chloramphenicol acetyltransferase activities, and COMT promoter activities dose-dependently. Functional deletion analyses of COMT promoters also showed that this estrogenic effect was mediated by a 280 base pair fragment with two putative half-palindromic estrogen response elements in the proximal promoter and a 323-base pair fragment with two putative CCAAT/enhancer binding protein sites in the distal promoter. Our findings provide the first evidence and molecular mechanism for estrogen to inhibit COMT gene transcription, which may shed new insight into the role of estrogen in the pathophysiology of different human disorders.

Catechol-*O*-methyltransferase (COMT) is a ubiquitous enzyme that catalyzes the transfer of the methyl group from the coenzyme *S*-adenosyl-L-methionine (SAM) to one of the hydroxyl groups of catechols in the presence of Mg^{2+} (Guldberg and Marsden, 1975). There are two isoforms of COMT of similar function: soluble and membrane-bound (MB). They are encoded by two transcripts [1.3 and 1.5 kilobase (kb) in human] regulated by the proximal and distal promoters, respectively (Tenhunen et al., 1994). The structural differences between these two human transcripts are a 5′ extension of 150 base pairs (bp), which codes for a signal-anchor domain to direct the MB-COMT polypeptide to membranes, and the presence of a 5′ noncoding region in the 1.5-kb transcript (Tenhunen et al., 1994).

COMT may play an important role in the pathophysiology of different human disorders including estrogen-induced cancers, Parkinson's disease, depression, and hypertension, because the substrates of COMT are catechol estrogens (e.g., carcinogenic 4-hydroxyestradiol), indolic intermediates in melanin metabolism, xenobiotic catechols (e.g., carcinogenic flavonoids), catechol neurotransmitters (e.g., dopamine and noradrenaline), and drugs (e.g., levodopa; Guldberg and

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[1] Database deposition: Human proximal (P1) and distal (P2) promoter regions have been deposited in Genbank/USA with accession numbers U97652 and AF001102, respectively.

ABBREVIATIONS: COMT, catechol-*O*-methyltransferase; ERE, estrogen response element; CEBP, cCAAT/enhancer binding protein; CAT, chloramphenicol acetyltransferase; SAM, *S*-adenosyl-L-methionine; MCF-7, human breast carcinoma cell line; HeLa, human cervix carcinoma cell line; COS-7, African green monkey kidney cell line; E2, 17-β-estradiol; bp, base pairs; kb, kilobase; P1, cloned proximal promoter; P2, cloned distal promoter; pCMV-hER, human estrogen receptor expression plasmid; hCOMTP1-CAT, chimeric human COMT P1-CAT reporter gene; hCOMTP2-CAT, chimeric human COMT P2-CAT reporter gene; Del, deletion fragment; hCOMTP-CAT, chimeric human COMT promoter-CAT; MB, membrane-bound.

Marsden, 1975; Kopin, 1985; Cavalieri et al., 1997). Although the regulation of human COMT gene by endogenous compounds would be of particular interest because of the functional importance of this enzyme, there has been a lack of studies on this issue. Nevertheless, altered COMT activities at different physiological status were observed both in animal and human studies. A significant decrease in COMT activity was found in rat liver and rabbit adrenal gland during pregnancy (Parvez et al., 1975; Parvez et al., 1976). COMT activity in pooled rat brain and adrenal gland was found lowest during the pro-estrus phase and highest during the estrus phase (Parvez et al., 1978). Women have a 20 to 30% lower COMT activity compared with men (Fahndrich et al., 1980; Floderous et al., 1981; Boudikova et al., 1990). The underlying mechanism for these observations has never been explored. Based on higher estrogen levels during pregnancy, and in women compared with men, and in rats during the pro-estrus phase in contrast with lower estrogen levels during the estrus phase (Shaikh, 1971), we hypothesized that estrogen might be the endogenous agent that inhibits COMT activity by down-regulating COMT gene expression.

The aim of this study was to explore our hypothesis by: 1) characterizing the effects of estrogen on human COMT mRNA expression in different cell models, 2) cloning and sequencing the structure of the COMT gene further upstream of the published promoter sequences (Tenhunen et al., 1994; Genbank accession numbers Z26491 and Z26490), and 3) determining the functional significance of the regulatory elements found in the new sequences. We have, for the first time, shown that estrogen can specifically down-regulate human COMT gene transcription in a time- and dose-dependent manner in estrogen receptor-positive human breast carcinoma cell line (MCF-7) cells, but not in estrogen receptor-negative human cervix carcinoma cell line (HeLa) cells. This estrogenic effect was mediated via a 280-bp fragment with two half-palindromic estrogen response elements (EREs) in the proximal promoter and a 323-bp fragment with two putative cCAAT/enhancer binding protein (CEBP) sites in the distal promoter. This study showed evidence and molecular mechanism for a novel link between estrogen exposure and altered COMT gene expression, which may provide a new insight into the role of estrogen in the pathophysiology of different human disorders.

Experimental Procedures

Materials. MCF-7 (ATCC HTB-22), HeLa (ATCC CCL2), and African green monkey kidney cell line (COS-7; ATCC CRL1651) were obtained from the American Type Culture Collection (Rockville, MD). The TA Cloning kit (Version 2.1) was purchased from Invitrogen Corporation (Carlsbad, CA). The PromoterFinder kit, human β-actin cDNA control probe, and *Sac*II were obtained from Clontech Laboratories (Palo Alto, CA). Thermoprime plus DNA polymerase was purchased from Advanced Biotechnologies (Epsom, Surrey, UK). *Xba*I, *Hin*dIII, *Eco*RV, *Sal*I, *Bst*EII, *Ava*I, T4 ligase, DNA polymerase I large (Klenow) fragment, T4 polymerase, ampicillin, penicillin, streptomycin, insulin, bovine serum albumin, lipofectin, and *Escherichia coli* DH5α competent cells were obtained from Life Technologies (Gaithersburg, MD). *AvrII* and T4 ligase were purchased from New England Biolabs (Beverly, MA). Primer-a-Gene Y labeling system, promoterless basic pCAT vector, pSV-β-galactosidase control vector, chloramphenicol acetyltransferase (CAT) enzyme assay system, and β-galactosidase enzyme assay system were purchased from Promega (Madison, WI). Protein assay kit (II) was obtained from Bio-Rad Laboratories (Hercules, CA). RNeasy mini kit, gel extraction kit, and plasmid midi kit were obtained from Qiagen (GmbH, Hilden, Germany). Dulbecco's modified Eagle's medium with or without phenol red indicator and OPTI-MEM I reduced serum medium were purchased from Life Technologies (Grand Island, NY). Charcoal-stripped bovine calf serum was obtained from Sigma (St. Louis, MO). Oligonucleotide primers were purchased from Alta Bioscience (University of Birmingham, Birmingham, UK). 1-Deoxy[dichloroacetyl-1-^{14}C]chloramphenicol and [^{32}P]dCTP were obtained from Amersham Life Science (Amersham International plc, Amersham, UK). Human estrogen receptor expression plasmid (pCMV-hER) was a gift from Dr. R. J. Miksicek (Cancer Center, Michigan State University, East Lansing, MI).

Cells and Cell Culture. MCF-7 cells were grown in 90% phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% calf serum depleted of exogenous estrogen, antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin), 2-mM L-glutamine, and 1 μg/ml insulin at 37°C in humidified air containing 5% CO_2. HeLa cells were grown in the same conditions as MCF-7 cells but without insulin. COS-7 cells were grown in the same conditions as MCF-7 cells but in humidified air containing 10% CO_2 without insulin. Serum-free medium (OPTI-MEM I) was used for the transient transfection assays in COS-7 cells as described below. MCF-7 or HeLa cells (2×10^6) were seeded onto 100-mm culture dishes and incubated in fresh medium for 48 h. The media were removed, and the cells were incubated in fresh media with different physiological concentrations of 17-β-estradiol (E2) at either 10^{-9} M, 10^{-8} M, or 10^{-7} M in 0.1% (v/v) ethanol, or without E2 (control) for different periods, ranging from 4 to 72 h, for dose-response and time course studies.

Northern Blot Analysis. Total RNA was extracted from the cells using the RNeasy mini kit according to the manufacturer's protocol. Probes [β-actin and human COMT gene probe produced by PCR using primers 5′-CTGCACAGGCAAGATCGTGGA-3′ and 5′-TCCAG GTCTGACAACGGGTCA-3′ as described previously (Xie et al., 1997)] were radiolabeled with α^{32}P dCTP using a random primer labeling kit. Extracted total RNA (10 μg/lane) was used for Northern analyses as described (Sambrook et al., 1989). The sequence of the human COMT gene probe is homologous to that of exon 4 (Fig. 1), and therefore it could hybridize with both 1.3- and 1.5-kb transcripts. The intensity of the blots was analyzed using a phosphoimager (Molecular Dynamics, Sunnyvale, CA) and expressed as a percentage of mRNA levels in E2-treated cells relative to nonE2-treated cells after correction for RNA loading using β-actin.

Genomic Cloning and Sequencing of Human COMT Cloned Proximal Promoter (P1) and Cloned Distal Promoter (P2). Primers (25 μM; U1) 5′-ACATCTGCTTTGCTGCCG-3′ in exon 2 and (D1) 5′-AGGATGCGCTGCTCCTTG-3′ in exon 3 (Fig. 1), and genomic DNA (0.5 μg) were used to amplify the P1 in "hot-start" PCR at 96°C for 5 min before adding *Taq* polymerase (1.5 U) followed by 95°C for 1 min, 52°C for 1 min, 72°C for 2 min, for 30 cycles, and a final extension time of 10 min at 72°C. The 1.5-kb PCR product was cloned into the Invitrogen TA vector. Sequencing was performed in an automated DNA sequencer. Putative regulatory elements in P1 were determined using TRANSFEC database (NCBI). P2 was produced by "gene walking" using Clontech Promoter-Finder kit and gene-specific downstream primers (D2) 5′-CCACATACCGGCCTCT-TGGTCTAGTCTT-3′ and (D3) 5′-TCTTGCTCAGGAGTCCGGGCT-GCTT-3′ at the distal promoter in the two stage amplification according to manufacturer's instructions (Fig. 1). The corresponding up-stream primers were provided in the kit. One library (Library 3) produced a single 1.5-kb product on the second amplification. This product was also cloned into the Invitrogen TA vector, sequenced, and analyzed for putative regulatory elements.

Construction of Chimeric Human COMT P1-CAT Reporter Gene (hCOMTP1-CAT) and Chimeric Human COMT P2-CAT Reporter Gene (hCOMTP2-CAT). Full length fragments from the

above P1 region were amplified by polymerase chain reaction with primers (U4) 5′-GCTCTAGAACATCTGCTTTGCTGCCG-3′ and (D4) 5′-GCTCTAGAGAGCAGGTTGTGGATGGG-3′ containing *Xba*I sites at the 5′ ends (Fig. 1). The products were digested with *Xba*I and then ligated into the linear *Xba*I site of Promega pCAT Basic Vector. Full length fragments from the above P2 region were amplified by polymerase chain reaction with primers (U5) 5′-ACGCGTCGACGCTCCTCTGGCGGAAAGGA-3′ and (D5) 5′-ACGCGTCGACCTCTCCCGCGACGGCCCG-3′ containing *Sal*I sites at the 5′ ends (Fig. 1). The products were digested with *Sal*I and then ligated into the linear *Sal*I site of pCAT Basic Vector. Ligated circular DNAs were transformed into competent *E. coli* DH5α cells. Colonies containing plasmids with inserts in the correct orientation (confirmed by sequencing) were cultured. Plasmid DNA was isolated using QIAFilter plasmid midi kits according to the manufacturer's protocol.

Construction of Serial 5′-End Deletion hCOMTP1-CAT and hCOMTP2-CAT. The bp number delineating the DNA fragments in our promoter sequences was based on the previous labeling system (Tenhunen et al., 1994). hCOMTP1-CAT plasmid containing insert (−1323 – +150) was doubly digested with *Hin*dIII and either *Avr*II, *Bsu*36I, *Bst*EII, or *Ava*I to give 5′ deletion fragments (Dels) −991 to +150 (1.1 kb), −799 to +150 (0.9 kb), −475 to +150 (0.6 kb), and −195 to +150 (0.3 kb), respectively. hCOMTP2-CAT plasmid containing insert (−1405 – +112) was digested with *Hin*dIII to give a fragment −1083 to +112 (1.1 kb), and doubly digested with *Hin*dIII and either *Apa*I, *Ban*II, or *Eco*RV to give 5′ Dels −716 to +112 (0.8 kb), −558 to +112 (0.7 kb), and −384 to +112 (0.4 kb), respectively. Blunt ends were created by reaction with either Klenow or T4 DNA polymerase. Dels containing the CAT vector were gel-purified, and then ligated with T4 DNA ligase for 4 h at 16°C. Ligated circular DNA was transformed into competent *E. coli* DH5α cells. Colonies containing plasmids with inserts in the correct orientation (confirmed by sequencing) were cultured. Plasmid DNA was then isolated using QIAFilter plasmid mid kits according to manufacturer's protocol.

Transient Transfection, CAT, β-Galactosidase, and Protein Assays. The promoter activities of various constructs were determined in transiently transfected COS-7 cells. In each transfection experiment, COS-7 cells were seeded into 60-mm culture dishes (6×10^6 cells/dish) and incubated for 24 h in fresh medium before transfection. Cells were then cotransfected with CAT reporter construct (3 μg each), pCMV-hER (0.8 μg), and Promega pSV-β-galactosidase (1 μg) using a lipofectin-mediated procedure in serum-free medium as described previously (Felgner et al., 1987). After incubation for 5 h at 37°C, the medium was removed, and the cells were incubated in fresh medium with the addition of E2 at 10^{-8} M in 0.1% (v/v) ethanol, or without E2 (control). After 48 h incubation, the cells were harvested. CAT and pSV-β-galactosidase activities were determined using the CAT and β-galactosidase enzyme systems, respectively, according to the supplier's protocol. Protein concentration was determined by the Bradford test kit. CAT activity was expressed after normalization for β-galactosidase activity and protein concentration.

Statistical Analysis. ANOVA (SPSS for Window 8.0) was used to determine the statistical significance in the difference in CAT activity between the different promoter fragments in the cotransfection assay. Comparisons in CAT activities were made among hCOMTP1 and Del 1 to 4, and among hCOMTP2 and Del 5 to 8, respectively.

Results

Estrogenic Inhibition of COMT Transcription Analyzed by Northern Blot. We performed a Northern blot analysis on estrogen receptor-positive MCF-7 (Brooks et al., 1973) and estrogen receptor-negative HeLa (Galien et al., 1996) cells to test if estrogen can down-regulate human COMT transcription, and if the estrogen receptor is needed for this regulation. When the MCF-7 cells were incubated with E2 for 48 h, the expression of 1.3-kb transcripts in MCF-7 cells was reduced by 20% at 10^{-9} M of E2 and by 50% at 10^{-8} M, relative to nonE2-treated cells (Fig. 2). E2 did not significantly affect COMT transcription in HeLa cells (control). These results indicate that estrogen can decrease levels of the human 1.3-kb COMT transcript in estrogen receptor-positive, but not receptor-negative cells. Time course and dose-dependent studies were then performed to determine the time point and estrogen concentration to achieve optimal inhibition of COMT transcription in MCF-7 cells. Inhibition by E2 on the expression of the 1.3-kb transcript was observed after 16 h, and lasted at least 72 h, being maximal at 48 h (Fig. 3). A dose-dependent inhibition was also observed on the expression of the 1.3-kb transcript, with a maximum effect at 10^{-7} M E2.

Genomic Cloning, Sequencing, and Structural Analysis of Human COMT P1s and P2s. We cloned and sequenced two more fragments (Fig. 4; Genbank Accession no.U97652 and AF001102) upstream to the existing promoter regions (Tenhunen et al., 1994; Genbank Accession no. Z26491 and Z26490) of the gene. Our sequences overlapped with these published sequences. A number of putative transcription factor binding elements that can affect both constitutive and tissue-specific expression of this gene, such as

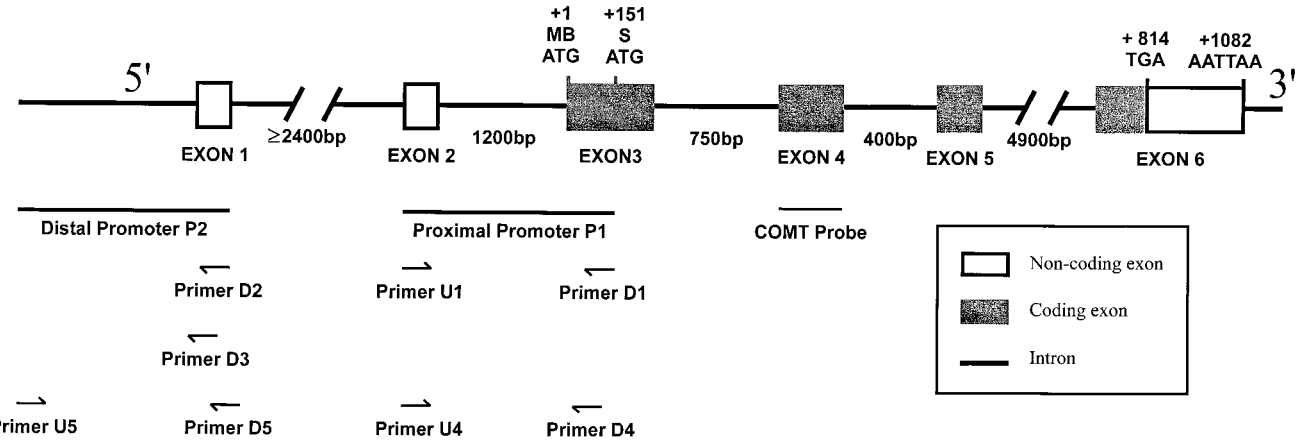

Fig. 1. Human COMT gene. The exon-intron organization is not to scale. The location of translation-start codons (MB-ATG and soluble-ATG), translation-stop codons (TGA), putative polyadenylation signal (AATTAA), COMT probe used in the Northern analysis, promoter regions, and primers are shown.

TATA and CAAT boxes, half-palindromic EREs, and CEBP sites, were found in both P1 and P2. The presence of CEBP sites in P1 and P2 is consistent with the fact that both isoforms of COMT are richly expressed in the liver. The presence of five and three half-palindromic EREs in P1 and P2 respectively, indicated that these two promoters could potentially respond to E2.

Estrogenic Inhibition of COMT Promoter Activities in Reporter Gene Expression. CAT-reporter gene assays were used to test the response of these promoters to E2. Chimeric human COMT promoter-CAT (hCOMTP-CAT) constructs containing P1 or P2 (each about 1.5 kb in size) cloned to the CAT-reporter plasmid were transiently cotransfected into COS-7 cells together with pCMV-hER and pSV-β-galactosidase (internal control for transfection efficiency). Physiological concentrations of E2 (10^{-9}–10^{-7} M) at 48 h decreased P1- and P2-CAT activities in a dose-dependent manner (Fig. 5). At E2 concentrations of 10^{-9}, $10^{-8,}$ and 10^{-7} M, P1-CAT activities were 64%, 32%, and 6.5%, respectively, relative to nonE2-treated control. At E2 concentrations of 10^{-9}, 10^{-8}, and 10^{-7} M, P2-CAT activities were 59%, 29%, and 4.5%, respectively, relative to nonE2-treated control. There was no detectable estrogenic effect on COMT gene expression without cotransfection of pCMV-hER (data not shown). These findings confirmed that down-regulation of the human COMT gene expression by estrogen were mediated by the two promoter regions (P1 and P2) in the presence of estrogen receptors.

Functional EREs and CEBP Sites Confer Estrogenic Inhibition. We then explored the role of the individual half-palindromic EREs found in P1 (ERE 1 to ERE 5) and P2 (ERE 6 to ERE 8) using hCOMTP-CAT constructs containing serial 5′-deleted fragments of either P1 or P2 cloned to the CAT-reporter plasmid which were transiently cotransfected into COS-7 cells together with pCMV-hER and pSV-β-galactosidase. In P1 and P2, the deletions did not affect ($p > .05$) promoter activity in nonE2-treated cells (data not shown). However, in E2-treated cells, the promoter activities in constructs hCOMTP1-CAT, Del 1, Del 2, and Del 3 were significantly ($p < .05$) reduced compared with Del 4 (Fig. 6A). ERE 1 and ERE 2 appeared to be crucial to the estrogenic inhibitory effect of P1. No significant differences ($p > .05$) were found in the comparisons of CAT activities among hCOMTP1-CAT, and Del 1 to 3. In P2, the promoter activities of constructs Del 5, Del 6, Del 7, and Del 8 were significantly ($p < .05$) increased compared with hCOMTP2-CAT, and also when Del 7 was compared with Del 8, in E2-treated cells (Fig. 6B). No differences ($p > .05$) were found in the comparisons of CAT activities among Del 5, 6, 7, and Del 5, 6, 8 in response to E2. The region (−1405 to −1083) upstream to the most distal ERE (ERE 8) appeared to be crucial for conferring estrogenic suppression of P2 activity. Further scrutiny of this distal fragment revealed the presence of two CEBP sites (Fig. 4B). ERE 6 appeared to enhance CAT activity because deletion of the fragment containing this element caused a significant decrease ($p < .05$) in promoter activity in response to E2.

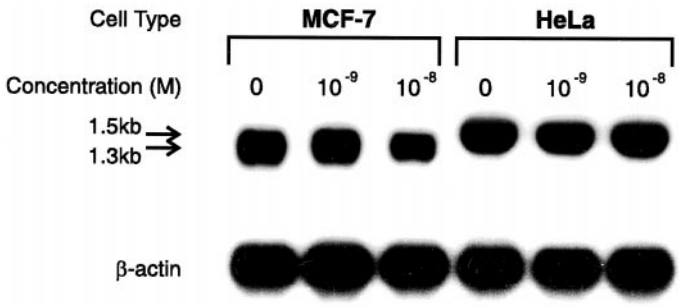

Fig. 2. Northern analyses of estrogenic effects on COMT gene expression in estrogen receptor-positive MCF-7 and estrogen receptor-negative HeLa cells after incubation with E2 at various concentrations for 48 h. Extraction of total RNA and Northern analysis were performed as described in *Experimental Procedures*. Top, blot hybridized with human COMT probe, (from left to right): MCF-7 cells cultured without E2, with E2 at 10^{-9} M, and with E2 at 10^{-8} M, respectively, HeLa cells cultured without E2, with E2 at 10^{-9} M, and with E2 at 10^{-8} M, respectively. Bottom, blot hybridized with human β-actin probe as an internal control for RNA loading.

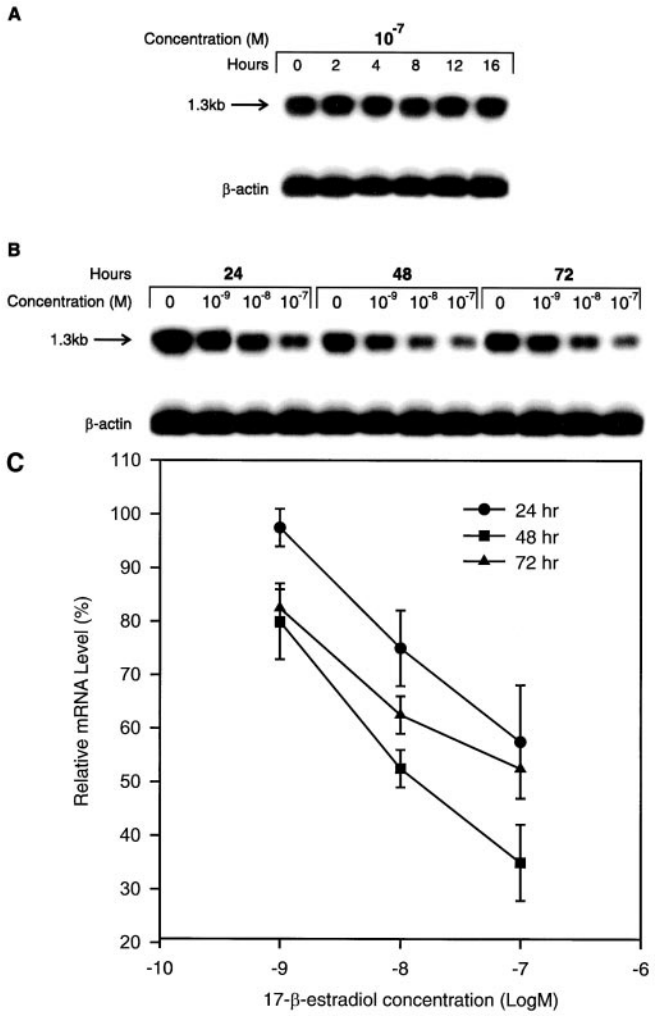

Fig. 3. Time course and dose-response inhibition of COMT gene expression by E2. A, MCF-7 cells were treated with 10^{-7} M of E2 for up to 16 h (0 h as control). B, MCF-7 cells were treated with E2 (10^{-9} to 10^{-7} M) for 24, 48, and 72 h (0 M as control). C, intensity of the blots in (B) was analyzed using a phosphoimager and expressed as a percentage of mRNA levels in E2-treated cells relative to nonE2-treated cells after correction for RNA loading using β-actin. The mean percentage values ± S.D. of at least two separate Northern blots are shown. Inhibition by E2 on the expression of the 1.3-kb transcript was observed after 16 h and lasted at least 72 h, being maximal at 48 h. A dose-dependent inhibition was also observed on the expression of the 1.3-kb transcript, with maximum effect at 10^{-7} M E2.

Discussion

Our Northern analyses showed that E2 can reduce COMT transcription in estrogen receptor-positive MCF-7 cells but not in estrogen receptor-negative HeLa cells. Our findings showed that this estrogenic effect required the presence of estrogen receptors, and was both dose- and time-dependent. This estrogenic inhibition was not likely due to inhibition of cell growth because estrogen stimulates the growth of MCF-7 (Lippman et al., 1976). Because MCF-7 cells can only express the 1.3-kb transcript in Northern analyses, we could not test this effect on the 1.5-kb transcript by this method. We are not aware of any estrogen receptor-positive cell line that expresses only 1.5-kb transcript. HeLa cells were used as a negative control even though it expresses mainly the 1.5-kb transcript because we are not aware of any estrogen receptor-negative cell line that expresses mainly the 1.3-kb transcript. However, a similar inhibition of the 1.5-kb transcript expression as in the 1.3-kb transcript (as shown in our CAT reporter gene assays) suggests a similar expression mechanism for both transcripts. The promoter-CAT reporter gene assays were performed in a nonE2-responsive cell line (i.e., COS-7 cells) that was transfected with an expression vector for estrogen receptors (i.e., pCMV-hER). Transfection of COMT promoter-reporter constructs into estrogen-responsive cells of different tissues would have allowed us to explore the possibility that promoter usage and effects of other regulatory elements might be different due to the presence of different coactivator proteins. However, COS-7 is recognized as a good host cell line for cotransfection CAT-reporter gene assay especially for COMT promoter function because of its high transfection efficiency (Tenhunen, 1996). The inhibition of COMT transcription at promoter level was estrogen receptor-dependent because CAT activity was not inhibited in estrogen-treated COS-7 cells without cotransfection of pCMV-hER. Estrogen antagonists (to block estrogen receptors) were not used to test the specificity of this estrogenic effect because pure antagonists (such as ICI 164384) are not commercially available, and the commonly used tamoxifen is a partial antagonist. However, the lack of estrogenic effect on COMT gene expression without estrogen receptors, as shown

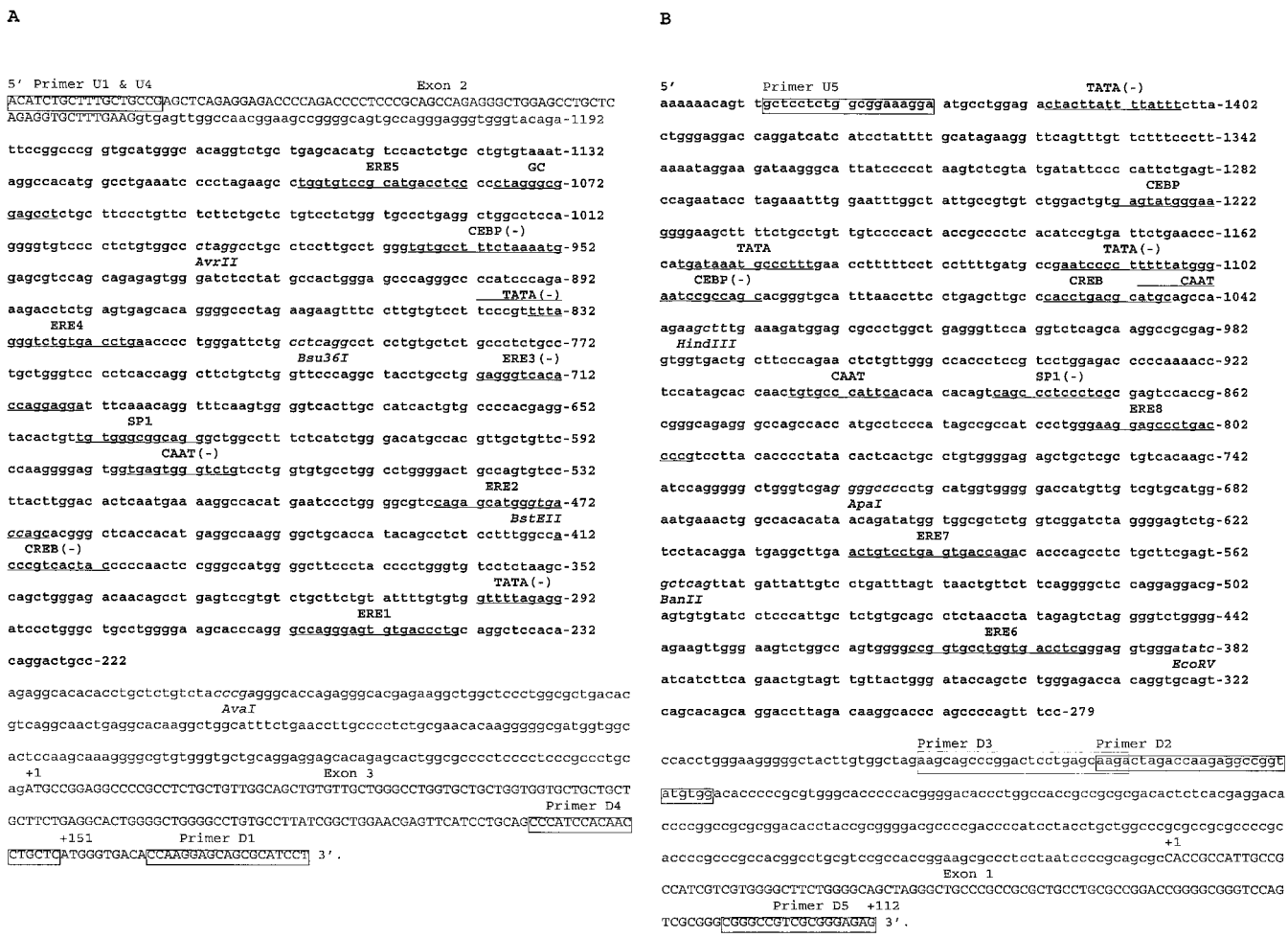

Fig. 4. Nucleotide sequences and putative regulatory elements of human COMT P1 (A) and P2 (B). The newly acquired sequences are indicated in bold [Genbank accession number U97652 (A) and AF001102 (B)] overlapped with the previous bordering sequence (Genbank accession numbers Z26491 and Z26490) shown without bold. Exon and intron regions are depicted in uppercase and lowercase, respectively. The putative regulatory elements are underlined or overlined. The (−) after the putative elements represent a reverse orientation of the elements. The restriction sites used in cloning are shown in italics. The locations of the primers are enclosed in boxes. The restriction site sequences that were added upstream to primers U4, D4 (*Xba*I) and U5, D5 (*Sal*I) for cloning into plasmids are not shown. EREs 1 to 5 in P1 (A) and EREs 6 to 8 in P2 (B) is consecutively arranged from 3′ to 5′. A, translation start position for 1.5-kb transcript is at +1, for 1.3-kb transcript is at +151 in exon 3. B, position of 5′-end of the longest 1.5-kb transcript is at +1 in exon 1.

in our Northern analyses and CAT reporter gene assays, achieved the same objectives as estrogen antagonists.

Although the EREs in the COMT promoters are half-sites of the perfect ERE palindrome (GGTCANNNTGACC), there is strong evidence that EREs can function in more loosely structured sequence motifs (Klein-Hitpass et al., 1986). Half-palindromic ERE (TGACC), which are more than 100 bp apart, can act synergistically to confer estrogen inducibility either to proximal ovalbumin gene promoter or to heterologous promoters (Kato et al., 1992). Even a single, noncanonical ERE is capable of estrogen inducibility in human pS2 gene (Berry et al., 1989). Multiple half-palindromic EREs in the COMT promoters provide the structural basis for estrogenic regulation of COMT transcription. To characterize the EREs responsible for the estrogenic effect on COMT transcription, we performed deletion analyses using cotransfection CAT reporter gene assays. Deletions in the promoters did not significantly affect COMT promoter activities in nonE2-treated cells, indicating that Del 4 (−195 to +150) in P1 and Del 8 (−384 to + 113) in P2 were sufficient for constitutive function in proximal and distal promoters, respectively. Although fragments upstream to Del 4 in P1 and Del 8 in P2 do not appear to be important in its constitutive function by the lack of significant differences in COMT promoter activities in nonE2-treated cells, they were important to the estrogenic regulation of COMT promoter function. Deletions of these fragments affected both promoter activities in E2-treated cells. In P1, there was a significant difference ($p < .05$) in CAT activities in response to E2 between Del 3 and Del 4, but no significant differences ($p > .05$) among hCOMTP1-CAT, and Del 1 to 3. This indicates that the 280-bp fragment (−475 to −195) with ERE 1 and 2 was crucial to the estrogenic inhibition of P1. In P2, there was a significant difference ($p < .01$) in CAT activities in response to E2 between hCOMTP2-CAT and Del 5. The 323-bp fragment distal to ERE 8 (−1405 to −1083) was crucial in estrogenic inhibition of P2 because CAT activity was not inhibited without this region even with ERE 6 to 8. With further scrutiny, this region contained two CEBP sites but without any known EREs (Fig. 4B). E2 can inhibit human interleukin-6 transcription via such sites (Stein and Yang, 1995; Galien et al., 1996). CEBP sites are essential for E2 inhibition of the IL-6 expression in HeLa cells (Galien et al., 1996) and can interact specifically with human estrogen receptor (Stein and Yang, 1995). Hence, COMT transcription may be similarly influenced by estrogen via CEBP sites in P2. ERE 6 in P2 appeared to enhance CAT activity because deletion of the fragment (−558 to −384) can significantly ($p < .05$) decrease promoter function in response to E2. The other putative EREs did not appear to be functional.

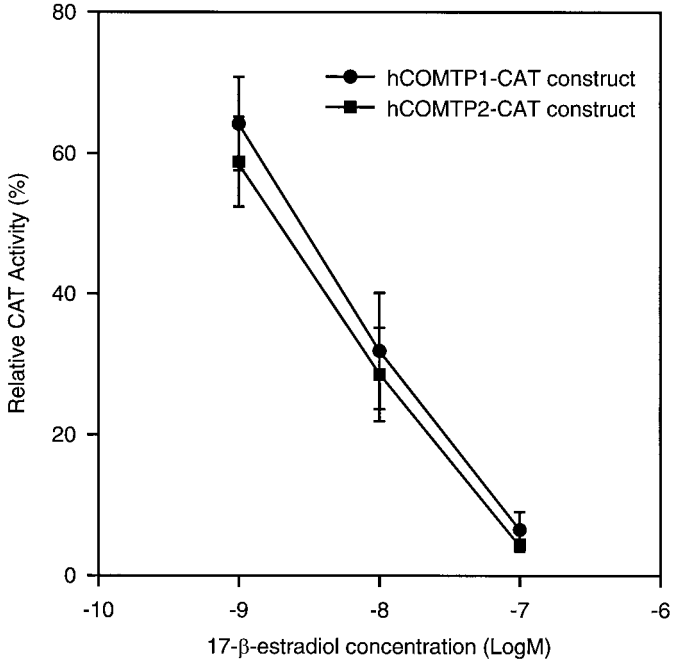

Fig. 5. Estrogenic inhibition of CAT activity in COS-7 cells cotransfected by hCOMTP-CAT constructs, pCMV-hER, and pSV-β-galactosidase. Constructs were cotransfected into COS-7 cells and incubated with E2 (10^{-9}–10^{-7} M) or without E2. After 48 h, the cells were harvested. CAT and β-galactosidase activities were determined as described in *Experimental Procedures*. The results were expressed as a percentage of CAT activity in E2-treated cells relative to nonE2-treated cells after correction for transfection efficiency using β-galactosidase. The mean values ± S.D. of at least three separate transfections are shown.

The structural and expressional features of rat and human COMT genes are similar (Tenhunen, 1996). Most tissue-specific protein binding sites in rat COMT proximal promoter are near the MB-ATG codon, similar to ERE 1 and 2 in human P1. Although no hormone response elements were found in rat COMT proximal promoter, functional CEBP binding sites were (Tenhunen, 1996). Thus, by analogy, the two most distal CEBP sites in human COMT P2 might be functional.

Our in vitro study is reflected in vivo. The degree of reduction in COMT expression by estrogen is comparable to the gender difference in its activity in humans (Fahndrich et al., 1980; Floderous et al., 1981; Boudikova et al., 1990). Estrogen, its receptors, and COMT expression are all found in brain tissues (Lloyd et al., 1975; Henry et al., 1991; Bixo et al., 1995). The ubiquitous role of COMT in catechol metabolism makes it a possible candidate in the pathophysiology and/or treatment of various human disorders.

Catechol estrogens are precursors of both catechol estrogen-2,3- and catechol estrogen-3,4-quinones, which have been hypothesized to be endogenous tumor initiators in breast cancer (Yager and Liehr, 1996; Cavalieri et al., 1997). O-methylation by COMT is a major inactivation pathway for catechol estrogens (Creveling, 1994; Yager and Liehr, 1996). COMT inhibition in a hamster kidney model enhanced estrogen-induced carcinogenesis, and was associated with high catechol estrogen levels (Zhu and Liehr, 1994). The low activity variant of COMT was associated with an increased risk for breast cancer (Lavigne et al., 1997). Chronic estrogen exposure resulting in liver and kidney cancers in rodents was associated with decreased erythrocytes and liver COMT activities (Li et al., 1989). Our results suggest that high estrogen levels can lower COMT expression and activity, partly contributing to higher concentrations of carcinogenic catechol estrogen metabolites.

However, estrogen also markedly increased the extent and intensity of COMT immunostaining in cytoplasm and cause a translocation of soluble COMT to the nucleus in normal hamster renal epithelial cells, unlike estrogen-induced renal cancer cells, which were nonCOMT-immunoreactive (Weisz et al., 1998). Our findings and others (Li et al., 1989) are consistent with the nonCOMT immunoreactivity found in renal cancer cells because estrogenic down-regulation of COMT

may have reduced the intensity of immunostaining of COMT to undetectable levels. Increased COMT levels in immunostained healthy renal cells were proposed to represent a normal response to a threat to the genome by catechol estrogens, which can be blocked by their O-methylation (Weisz et al., 1998). There may possibly be different pathways for estrogenic regulation of COMT, which needs to be further explored.

Parkinson's disease is characterized by degeneration of the nigrostriatal system, which leads to dopamine depletion. The most effective treatment remains replacing dopamine levels using levodopa. Women appear to require a lesser levodopa dose compared with men (Parkinson Study Group, 1996; Lyons et al., 1998). Transdermal estrogen in postmenopausal Parkinson's disease patients reduced the levodopa dosage required for an anti-parkinsonian response (Blanchet et al., 1998). Our results suggest that lower COMT activity related to higher estrogen level in women may explain these observations.

Depression is thought to be related to catecholamine deficiency (particularly norepinephrine) and low levels of SAM in cerebrospinal fluid (Bell et al., 1988; Cooper et al., 1991). Estrogen can reduce depressive symptoms (Henderson et al., 1991; Gregoire et al., 1996) but its mechanism is unknown. Our results suggest that estrogenic inhibition of COMT can help depressive symptoms by sparing endogenous norepinephrine and SAM.

Hypertension during pregnancy is related to elevated maternal plasma catecholamine levels (Davey and MacNab, 1981). Lower COMT activities were found in term placenta of hypertensive pregnant women compared with normotensive pregnant women (Barnea et al., 1988). Estrogenic inhibition of COMT may result in higher catecholamine levels during pregnancy, contributing to hypertension.

In summary, our study provides the first evidence and molecular mechanism that estrogen can directly down-regulate human COMT transcription, mediated via a 280-bp fragment with two putative half-palindromic EREs in proximal

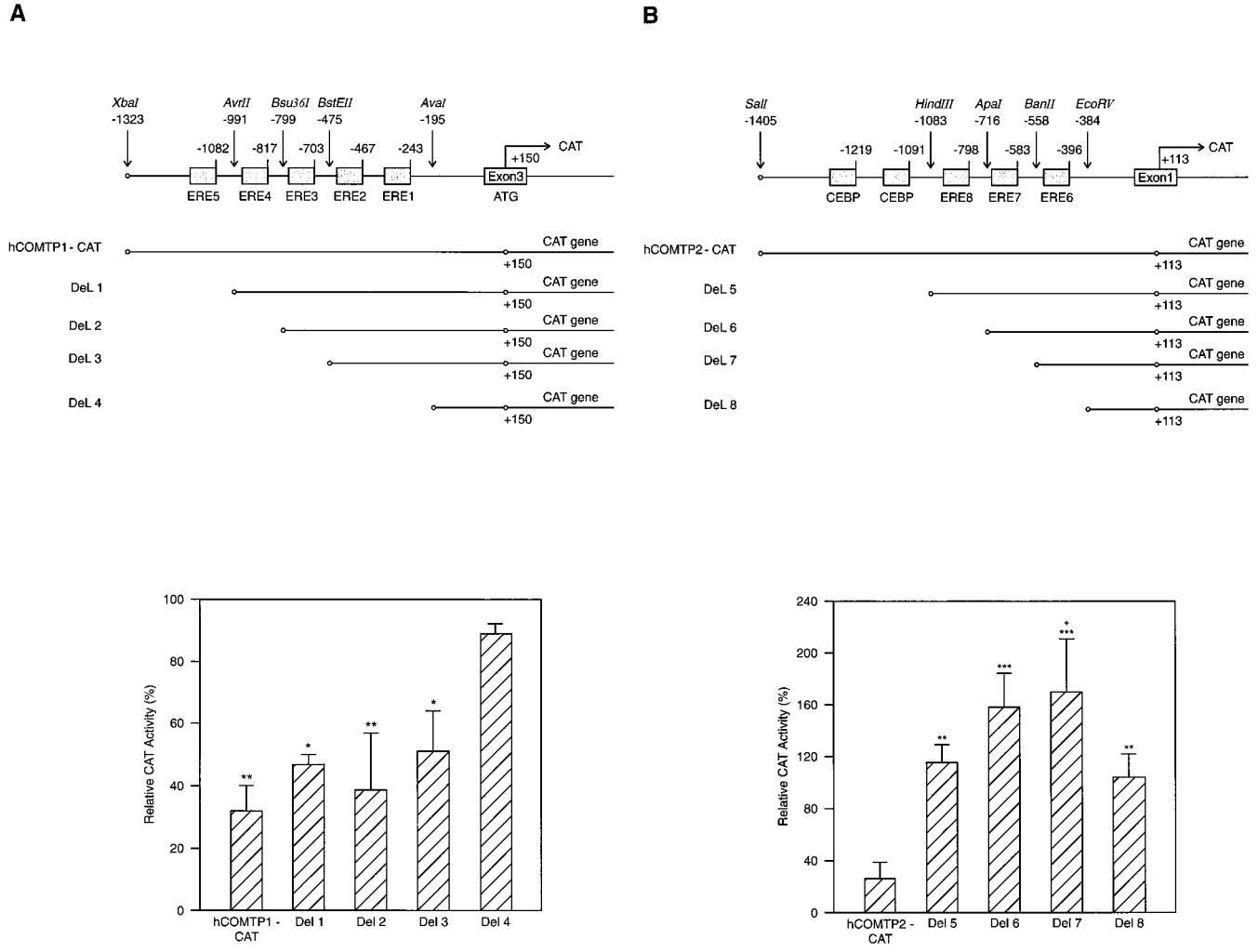

Fig. 6. Localization of regulatory elements responsible for the estrogenic inhibition on COMT gene transcription. Schematic diagrams of COMT gene promoters P1 (hCOMTP1-CAT) and P2 (hCOMTP2-CAT), their serial Dels (Del 1 to Del 8), and relative activities after E2 exposure for P1 (A) and for P2 (B), respectively. The bent arrows represent the transcription start sites of the human COMT gene. Fragments of the COMT 5′-flanking region of different lengths were fused to CAT reporter gene, cotransfected into COS-7 cells with pCMV-hER and pSV-β-galactosidase, and incubated with E2 (10^{-8} M) or without E2. After 48 h, the cells were harvested. CAT and β-galactosidase activities were determined as described in *Experimental Procedures*. The results were expressed as a percentage of CAT activity in E2-treated cells relative to nonE2-treated cells after correction for transfection efficiency using β-galactosidase. Mean values ± S.D. of at least three separate transfections are shown. Significant differences in relative CAT activity of each promoter fragment, determined by ANOVA, are expressed as $^{*}p < .05$ and $^{**}p < .01$, compared with Del 4 (A), $^{**}p < .01$ and $^{***}p < .001$, compared with hCOMTP2-CAT (B), and $+p < .05$ compared with Del 8 (B).

P1 and a 323-bp fragment with two putative CEBP sites in distal P2. Site-direct mutagenesis would be helpful to further confirm the estrogenic effect on these response elements.

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Send reprint requests to: Dr. S.L. Ho, Division of Neurology, University Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong. E-mail: slho@hkucc.hku.hk