Identification of an Estrogen Response Element Activated by Metabolites of 17β-Estradiol and Raloxifene

Na N. Yang,* Murali Venugopalan, Sushant Hardikar, Andrew Glasebrook

17β-Estradiol modulates gene transcription through the estrogen receptor and the estrogen response element in DNA. The human transforming growth factor–β3 gene was shown to be activated by the estrogen receptor in the presence of estrogen metabolites or estrogen antagonists. Activation was mediated by a polypurine sequence, termed the raloxifene response element, and did not require the DNA binding domain of the estrogen receptor. Interaction of the estrogen receptor with the raloxifene response element appears to require a cellular adapter protein. The observation that individual estrogens modulate multiple DNA response elements may explain the tissue-selective estrogen agonist or antagonist activity of compounds such as raloxifene.

The ovarian estrogen 17β-estradiol (E_2) regulates reproductive tissue function by modulating gene transcription through the estrogen receptor (ER) (*1*). ER-mediated gene transcription is initiated by binding of the DNA binding domain of the receptor to a consensus palindromic DNA sequence, the estrogen response element (ERE) (*2*). Subsequent transcriptional activation of the target gene is thought to be mediated through two distinct trans-activation domains of the receptor, termed AF-1 and AF-2 (*3*). Several classes of chemicals, represented by tamoxifen, raloxifene, and ICI 164,384, have been developed as antiestrogens, which antagonize estrogen actions in reproductive tissues. These compounds inhibit E_2-induced activation of ERE-containing genes to various extents (*4*, *5*).

Estrogen also has important effects on nonreproductive tissues. Loss of estrogen results in reduced bone mass and increased risk of cardiovascular disease in postmenopausal women. Estrogen replacement therapy prevents bone loss and provides cardiovascular protection in these women (*6*). In contrast to their antagonistic activities in the uterus and breast, raloxifene and tamoxifen function as estrogen agonists to preserve bone mineral density and reduce serum cholesterol concentrations in both animals and humans (*7*).

In investigating the tissue-selective activity of agents such as raloxifene, we discovered that certain key estrogen regulatory events in bone appear to be mediated through pathways independent of EREs in which antiestrogens function as agonists (*5*). Thus, the gene encoding transforming growth factor–β3 (TGF-β3), an important regulator of bone remodeling, is activated by estrogen and raloxifene in intact bone and cultured bone cells through a promoter-mediated and ER-dependent mechanism. However, activation of the TGF-β3 gene demonstrated a ligand preference distinct from that of an ERE-containing gene; that is, the antiestrogen raloxifene was more active than E_2 in the TGF-β3 system.

To elucidate the mechanism of ER-mediated TGF-β3 gene promoter activation, we cotransfected human osteosarcoma MG63 cells with the pTGF-β3CAT reporter plasmid, containing the human TGF-β3 promoter sequence [nucleotides (nt) −301 to +110] upstream of the chloramphenicol acetyltransferase (CAT) gene, and an expression plasmid (pCMVER) encoding the human ER. E_2 increased CAT activity in a dose-dependent manner, although high concentrations (>100 nM) of this ligand were required (Fig. 1A). In contrast, raloxifene increased CAT activity at nanomolar concentrations. Three antiestrogens—raloxifene, ICI 164,384, and tamoxifen—increased CAT activity to a greater extent than did E_2. The median effective doses of these ligands in inducing CAT expression

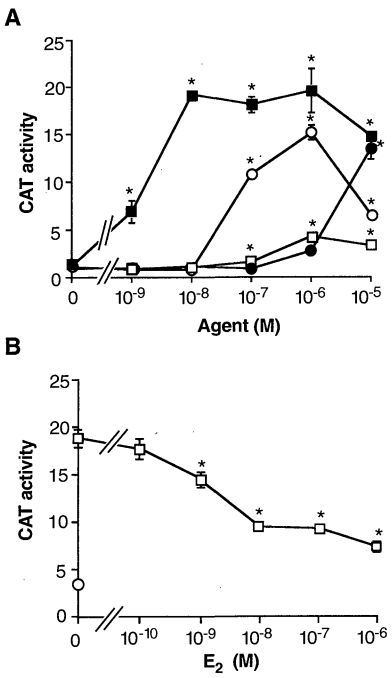

Fig. 1. Ligand preference of ER-mediated TGF-β3CAT activation. (**A**) Human MG63 cells were transfected with pTGF-β3CAT, pCMVER, and the pSV-β-galactosidase reference plasmid (*9*). Cells were exposed to E_2 (□), raloxifene (■), tamoxifen (●), or ICI 164,384 (○) at the concentrations indicated for 24 hours. *$P < 0.01$ versus vehicle control. (**B**) Transfected cells were exposed to 10 nM raloxifene and the indicated concentrations of E_2 for 24 hours. Control cells received ethanol (○). *$P < 0.01$ versus either 10 nM raloxifene alone or vehicle control.

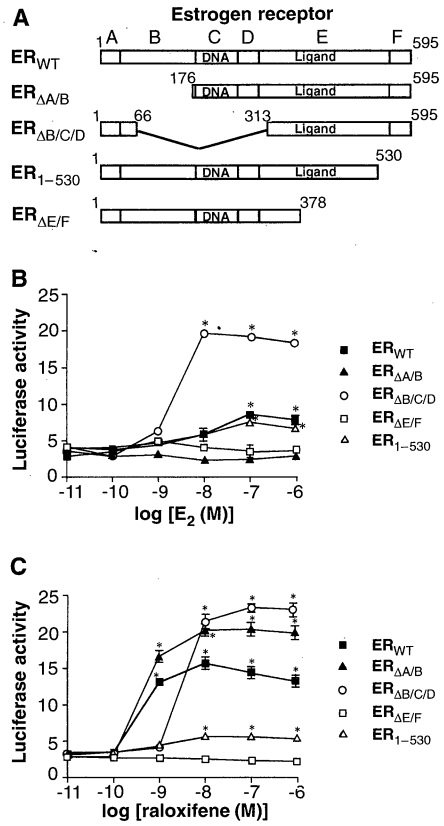

Fig. 2. Role of functional domains of the ER in activation of the TGF-β3 gene promoter. (**A**) Schematic illustration of the wild-type and deleted ERs. Numbers indicate amino acid positions. Respective DNA and ligand binding domains are shown. (**B** and **C**) MG63 cells were cotransfected with each of the ER expression plasmids individually and the pTGF-β3LUC reporter plasmid, and then exposed to the indicated concentrations of E_2 (B) or raloxifene (C). *$P < 0.01$ versus vehicle control.

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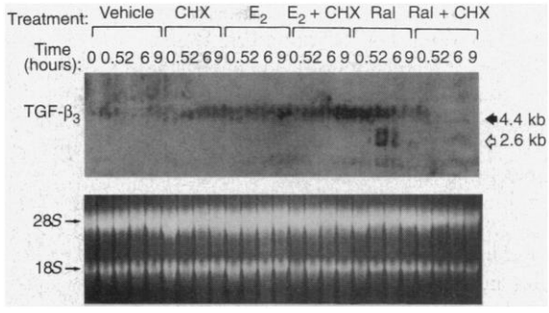
correlated with their binding affinities to the ER (8). E_2 partially inhibited the activity of 10 nM raloxifene in a dose-dependent manner (Fig. 1B).

We next examined the role of ER functional domains in mediating the unusual pattern of estrogen and antiestrogen activation in the TGF-β3 system. $ER_{\Delta A/B}$, $ER_{\Delta B/C/D}$, ER_{1-530}, and $ER_{\Delta E/F}$ represent ERs in which the AF-1, DNA binding, AF-2, or ligand binding domains, respectively, have been deleted from the wild-type receptor (ER_{WT}) (Fig. 2A) (9). Deletion of the ligand binding domain of the ER ($ER_{\Delta E/F}$) abolished both E_2- and raloxifene-induced TGF-β3 promoter activation (measured as luciferase activity) at all concentrations tested (Fig. 2, B and C). Deletion of the AF-1 domain ($ER_{\Delta A/B}$) abolished E_2-induced, but not raloxifene-induced, TGF-β3 promoter activation. In contrast, deletion of the AF-2 function ($ER1-_{530}$) had no effect on E_2-induced, but markedly inhibited raloxifene-induced, promoter activation. Deletion of the DNA binding domain ($ER_{\Delta B/C/D}$) of the ER enhanced the ability of the receptor to activate the TGF-β3 promoter in response to E_2; $ER_{\Delta B/C/D}$ mediated a fivefold increase in luciferase activity in response to either E_2 or raloxifene at the minimum effective concentration of 10 nM.

To identify the DNA response element for raloxifene, we systematically deleted the promoter sequence of the TGF-β3 gene in either CAT or luciferase reporter constructs and tested the resulting plasmids in cotransfection assays. Deletion of the promoter from nt −499 to −38, or from nt +75 to +110, had no effect on raloxifene induction of reporter gene expression, whereas deletion from nt +35 to +75 abolished raloxifene-induced activation (Fig. 3A). This loss of function defined the raloxifene response element (RRE) (Fig. 3B). The RRE shows no similarity to either the palindromic ERE or the AP-1 binding site, but comprises a polypurine sequence. Transfer of the RRE to a heterologous (SV40) promoter (RRE-pGL2) resulted in a twofold increase in luciferase activity induced by raloxifene or E_2 in MG63 cells (Fig. 3C). When a preceding GT repeat sequence from the TGF-β3 promoter was included in addition to RRE in the SV40 construct (GT-RRE-pGL2), raloxifene induced a threefold increase in expression of the luciferase reporter gene (Fig. 3C). Therefore, the RRE may be essential, but not sufficient by itself, to mediate full hormonal regulation of the TGF-β3 gene.

We then investigated whether raloxifene, acting through the RRE, regulates the expression of the endogenous TGF-β3 gene in MG63 cells. TGF-β3 mRNA was not detectable in vehicle-treated MG63 cells by Northern analysis (10) (Fig. 4). Raloxifene transiently induced the 4.4-kb TGF-β3 mRNA within 30 min and the 2.6-kb TGF-β3 mRNA (11) after 2 hours of treatment. E_2 increased the amount of the 4.4-kb TGF-β3 mRNA after 9 hours of treatment, but had no effect on the 2.6-kb transcript. A high concentration of cycloheximide (50 μM) blocked the raloxifene-induced, but not the E_2-induced, increase in the amount of TGF-β3 transcripts. In similar experiments, acting as a potent estrogen antagonist, raloxifene inhibited the E_2-induced increase in the expression of the ERE-containing progesterone receptor gene (8). Thus, two ER regulatory pathways, mediated by the ERE and RRE, appear to control endogenous gene expression in cultured cells by distinct mechanisms.



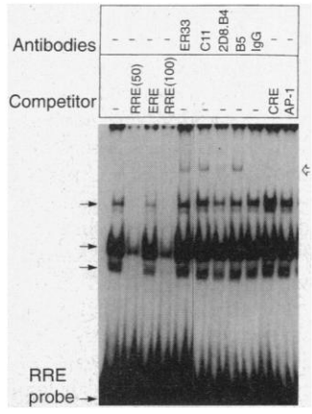
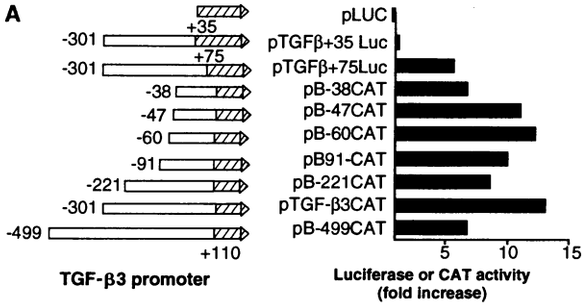
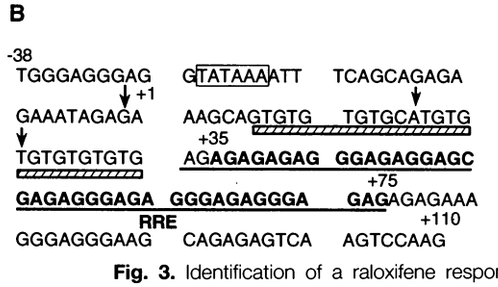


Fig. 4 (left). Effects of raloxifene and E_2 on TGF-β3 mRNA expression in MG63 cells. (Upper panel) Total RNA (20 μg) extracted from MG63 cells treated with ethanol (vehicle), 50 μM cycloheximide (CHX), 10 nM E_2, E_2 plus cycloheximide, 10 nM raloxifene (Ral), or raloxifene plus cycloheximide for the indicated times was subjected to Northern analysis (10). The 4.4- and 2.6-kb TGF-β3 transcripts are indicated by filled and open arrows, respectively. (Lower panel) The corresponding ethidium bromide–stained gel demonstrating equal loading of RNA samples. The positions of 28S and 18S ribosomal RNA are indicated. **Fig. 5 (right).** Interaction of the ER with the RRE DNA sequence via cellular adapter proteins. In a gel mobility shift assay (14), a ^{32}P-labeled RRE oligonucleotide probe was incubated with 6 μg of soluble protein extracted from MG63 cells. Unlabeled RRE probe (50 or 100 ng), or 100 ng of ERE, CRE, or AP-1 binding site oligonucleotides, was added as a competitor as indicated. Monoclonal antibodies to the ER DNA binding domain (ER33) or to the ER ligand binding domain (C11, 2D8.B4, and B5), or a nonspecific immunoglobulin G, were also included as indicated. Unlabeled arrows on the left indicate specific RRE-protein complexes. The open arrowhead indicates the band shifted by ER-specific antibodies. The position of free probe is also indicated.





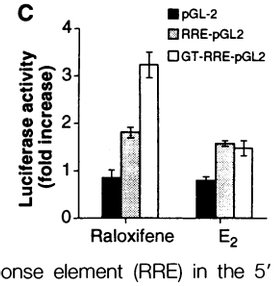


Fig. 3. Identification of a raloxifene response element (RRE) in the 5′ untranslated region of the TGF-β3 gene. (**A**) MG63 cells were cotransfected with one of the reporter plasmids containing the TGF-β3 promoter sequence (open boxes) with various deletions, or the cloning reporter vector pLUC (striped arrow), together with pCMVER (9). The fold increase in luciferase or CAT activity induced by 24 hours of raloxifene treatment is shown on the right. (**B**) The RRE sequence (boldfaced and underlined) in its promoter context. The TATA sequence is indicated by an open box. Arrows indicate major (+1) or minor transcription initiation sites. A GT repeat sequence is underlined by a hatched bar. Nucleotide positions are indicated above the sequence. (**C**) MG63 cells were cotransfected with pGL-2, RRE-pGL2, or GT-RRE-pGL2 reporter plasmids (9) and pCMVER. The fold increase in luciferase activity induced by 24 hours of 100 nM raloxifene or 100 nM E_2 treatment is indicated.

Activation of the TGF-β3 gene promoter by an ER lacking the DNA binding domain suggested a mechanism of ER interaction with the RRE different from that with the ERE. In gel mobility shift assays, no interaction was detected between a ^{32}P-labeled RRE probe and purified ER protein, which bound to an ERE probe with high affinity under the same conditions (*8*). However, with extracts from MG63 cells, which contain ~3000 ER molecules per cell (*8*), multiple RRE-protein complexes were detected (Fig. 5). The formation of three of these complexes was specifically inhibited by excess unlabeled RRE, but not by unlabeled ERE, cyclic AMP response element (CRE), or AP-1 binding site oligonucleotides. Inclusion of individual monoclonal antibodies to the ER—specific for the DNA binding domain (ER33) or the ligand binding domain (C11, 2D8.B4, or B5)—resulted in the appearance of a faint band of lower mobility; this band was not apparent with control immunoglobulin G. These data indicate that the ER may contribute to one of the RRE-protein complexes. Binding of the ER33 antibody to the complex suggests that the DNA binding domain of the ER was not in direct contact with the RRE. Although such "supershifted" complexes were reproducibly detected in this in vitro system with multiple antibodies to the ER, their low abundance suggests that the configuration of the ER in the complex may prevent high-affinity interaction between antibodies and the receptor.

To search for an endogenous ligand that mediates RRE activation, we examined the effects of human metabolites derived from E_2 in transient transfection assays. Some metabolites, such as 2-hydroxyestrone, were incapable of activating luciferase expression from the TGF-β3 promoter (Fig. 6). Metabolites such as 4-hydroxyestradiol activated luciferase expression only at high concentrations. Other metabolites such as 16-keto-17β-estradiol and 17-epiestriol were more potent in activating the TGF-β3 promoter. The factor of 10^4 difference in potency among these metabolites for TGF-β3 promoter activation did not reflect the relative binding affinities of these ligands for the ER, which differed by only a factor of 20 (*8*).

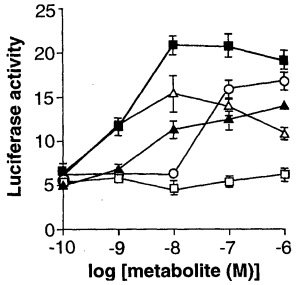


Fig. 6. Effects of estrogen metabolites on TGF-β3 promoter activity. MG63 cells were transfected with TGF-β3LUC and pCMVER and subsequently exposed to the estrogen metabolites 4-hydroxyestradiol (○), 17-epiestriol (△), 16-keto-17β-estradiol (▲), 2-hydroxyestrone (□), or raloxifene (■) for 24 hours at the concentrations indicated.

In summary, by comparing estrogen and antiestrogen regulation of the TGF-β3 gene, we revealed a new pathway of ER-mediated gene activation. The observation that the ER, in combination with different estrogen entities, regulates more than one DNA response element, the ERE and RRE, might explain the wide spectrum of estrogen effects in humans, especially in nonreproductive tissues. It has been observed that, although E_2 accounts for estrogenic effects on reproduction in vivo, other forms of estrogen exhibit distinct biological functions (*12*). A search of the GenBank database revealed several genes, including the osteonectin gene, the urokinase-type plasminogen activator gene, the neuron-specific growth-associated protein GAP-43 gene, and the proto-oncogene c-MYC (*13*), that contain RRE-like sequences. These genes, some of which are known to be regulated by estrogen, encode proteins that have important roles in bone, the cardiovascular system, and the central nervous system. Together, observations made in this study may suggest that the effects of estrogen in different tissues are regulated by different estrogen entities through specific pathways. Furthermore, the distinct effects of selective ER modulators (SERM), such as raloxifene at the ERE and RRE, may allow therapeutic tissue-selective hormone replacement.

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9. Cell culture conditions and the transfection procedure have been described previously (*5*). For the luciferase assay, cells were lysed in 0.1 M potassium phosphate buffer (pH 7.8) containing 1% Triton X-100, 2 mM EDTA, and 1 mM dithiothreitol (DTT), and lysate (40 μl) was mixed with 100 μl of reagent A [4 mM adenosine triphosphate, 15 mM $MgSO_4$, 30 mM Tricine (pH 7.8), 10 mM DTT], and 50 μl of reagent B [1 mM LH_2 luciferin (pH 6.0 to 6.4)]. Light output was measured for 10 s. All CAT and luciferase activities were normalized by β-galactosidase activity. Data are presented as means ± SD (n = 3). Statistical analysis was performed by one-way analysis of variance followed by a least significant difference multiple comparison test (Dunnett). Data are representative of at least three independent experiments. Plasmids pTGF-β3CAT (pB-301), pB-499, pB-221, pB-91, pB-60, pB-47, and pB-38 were provided by A. Roberts and M. Sporn [R. Lafyatis *et al.*, *J. Biol. Chem.* **265**, 19128 (1990)]. Plasmids pTGF-β3LUC, pTGFβ+75Luc, and pTGFβ+35Luc were generated by inserting nt −301 to +110 (Xba I–Hind III fragment of pB-301), nt −301 to +75, or nt −301 to +35 (obtained by polymerase chain reaction amplification of pB-301) of the TGF-β3 gene, respectively, into the pLUC luciferase reporter plasmid (Lilly Research Labs). RRE-pGL2 and GT-RRE-pGL2 were generated by inserting synthetic DNA sequences corresponding to nt +35 to +75 and +8 to +75 of the TGF-β3 gene, respectively, upstream of the SV40 promoter in the pGL-2 vector (Promega, Madison, WI). Kpn I and Mlu I restriction endonuclease sites were introduced flanking both the .RRE and GT-RRE sequences for cloning purposes. Plasmids pCMVER, $pCMVER_{\Delta A/B}$, and $pCMVER_{1-530}$ have been described [C. K. Wrenn and B. S. Katzenellenbogen, *J. Biol. Chem.* **268**, 24089 (1993); H. Cho and B. S. Katzenellenbogen, *Mol. Endocrinol.* **7**, 441 (1993)]. Plasmids $pCMVER_{\Delta B/C/D}$ and $pCMVER_{\Delta E/F}$ were prepared by J. C. Reese and B. Katzenellenbogen at the University of Illinois. For preparation of $pCMVER_{\Delta B/C/D}$, pCMVER was digested with Xma III, which cuts at codons 66 and 312, and then religated. The predicted deletion encompasses amino acids 67 to 312. The predicted molecular size of 39 kD of the encoded protein was confirmed by immunoblot analysis. For preparation of $pCMVER_{\Delta E/F}$, pCMVER was digested with Xba I, which cuts at codon 378, and then religated. The predicted deletion includes all amino acids after Leu^{378}, although eight unrelated amino acids are added at the COOH-terminus due to a cloning artifact. The predicted molecular size of the encoded protein (43 kD) was also confirmed by immunoblot analysis.
10. Total RNA from MG63 cells was prepared as described (*4*). A TGF-β3 cDNA probe (nt 200 to 5663) was generated by polymerase chain reaction amplification with the oligonucleotides 5′-TGTGCTGGGGTCTCTTCC-3′ and 5′-GTGAGGTTTGTTGCTTGT-3′ as primers. A human glyceraldehyde phosphate dehydrogenase gene probe (nt 471 to 740) (GenBank accession number M17851) was used as an internal control.
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14. For gel-mobility shift assays, a ^{32}P-labeled RRE probe (1 ng) was incubated in binding buffer [10 mM tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 2 μg of sonicated herring sperm DNA] for 30 min at room temperature. MG63 cells were lysed in 250 μl of lysis buffer [0.4 M KCl, 20% glycerol, 2 mM DTT, and 20 mM Hepes-NaOH (pH 7.8)], and the lysate was centrifuged at 80,000*g* for 1 hour at 2°C. DNA oligonucleotides were synthesized as the following complementary sequences: RRE probe, 5′-TCGAGGAGAGAGAGGGAGAGGAGCGAGAGGGAGAGGGAGAGGGAGAGA-3′ and 5′-AGCTTCTCTCCCTCTCCCTCTCCCTCTCGCTCCTCTCCCTCTCTCTCC-3′; ERE oligonucleotide, 5′-CTA-

GAAAGTCAGGTCACAGTGACCTG-3′ and 5′-TATGATCAGGTCACTGTGACCTGACT-3′; and CRE oligonucleotide, 5′-TCGAGCAAAATTGACGTCATGGTAATTAC-3′ and 5′-TCGAGTAATTACCATGACGTCAATTTTGC-3′. The AP-1 binding site oligonucleotide was obtained from Oncogene Science. Monoclonal antibodies (500 ng) to the ER DNA binding domain (clone ER33; Affinity BioReagents) or to the ER ligand binding domain (C11, 2D8.B4, and B5; Eli Lilly and Co.), or a nonspecific immunoglobulin G2a antibody (Eli Lilly and Co.), were included in antibody mobility-shift experiments.

15. We thank D. L. Phillips and L. Short for providing the ER antibodies and estrogen metabolite binding data; B. Katzenellenbogen for the ER constructs; G. L. Greene for the human progesterone receptor cDNA clone pHPR-65; and J. D. Termine, J. Hock, and A. H. Tashjian Jr. for discussions and comments on the manuscript.

19 March 1996; accepted 9 July 1996

Tourette Syndrome: Prediction of Phenotypic Variation in Monozygotic Twins by Caudate Nucleus D2 Receptor Binding

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Tourette syndrome, a chronic tic disorder with autosomal dominant inheritance, exhibits considerable phenotypic variability even within monozygotic twin pairs. The origins of this variability remain unclear. Recent findings have implicated the caudate nucleus as a locus of pathology, and pharmacological evidence supports dopaminergic involvement. Within monozygotic twins discordant for Tourette syndrome severity, differences in D2 dopamine receptor binding in the head of the caudate nucleus predicted differences in phenotypic severity (r = 0.99); this relation was not observed in putamen. These data may link Tourette syndrome with a spectrum of neuropsychiatric disorders that involve associative striatal circuitry.

Georges Gilles de la Tourette originally described Tourette syndrome (TS) in 1885 as a neuropsychiatric disorder characterized by chronic motor and vocal tics that begin in childhood (*1*). The tics are not strictly involuntary but commonly involve a compelling urge to perform rapid, sudden movements or vocalizations. TS is found in all cultures and racial groups; although an accurate prevalence rate for TS has not been established, it is increasingly recognized as being a relatively common disorder (*2*). Family studies suggest that TS is inherited as a single autosomal dominant gene with incomplete penetrance (*3*). Twin studies reveal higher concordance rates for TS within monozygotic twin pairs than within dizygotic pairs, supporting a primary genetic contribution. However, discordance for symptom severity between identical twins with TS indicates that nongenetic factors, including possible prenatal influences, modify the clinical expression of this disorder (*4*).

Several lines of evidence suggest that dopaminergic function is a factor in the phenotypic expression of TS, including the observed efficacy of dopamine D2 receptor antagonists such as haloperidol and pimozide and the exacerbation of symptoms by dopamimetic agents (for example, L-dopa and methylphenidate) (*2*). Although research has focused on the basal ganglia as a likely locus of pathology (*2*), direct evidence for an abnormality of the striatal dopaminergic system is limited (*5*). Thus far, investigations have not revealed an alteration of dopamine D2 receptor density in TS patients compared with persons without the disease (*6*), and linkage analyses have not identified a major gene effect of the dopamine receptor loci or of genes involved in dopamine metabolism and transport (*7*). Although the cause of TS does not appear to be an abnormality of dopamine receptors, the efficacy of antidopaminergic drugs suggests that differences in receptor function, perhaps in specific brain regions, could be a factor in phenotypic variance between individuals. One brain area rich in D2 receptors that has recently been shown to be functionally and morphometrically abnormal in TS is the striatum (*8*).

We studied identical twins to explore the role of dopamine receptor function as a modifying factor that might account for differences in clinical expression of TS. Identical twins discordant for severity of TS provide a powerful statistical design to discern extragenetic factors that correlate with phenotypic variation because genetic factors are controlled for. If, for example, differences in striatal dopamine function modulate the clinical expression of TS, they should be more apparent in the more symptomatic twin.

Five sets of monozygotic twins concordant for the diagnosis of TS but discordant for symptom severity (*9*) (Table 1) were studied with single–photon emission computed tomography (SPECT). Striatal binding of [I^{123}]iodobenzamide ([^{123}I]IBZM), a potent D2 receptor antagonist (*10*), was measured through use of anatomical regions of interest (ROIs) drawn on coregistered magnetic resonance imaging (MRI) scans (*11*) (Fig. 1). To analyze the time course ROI data, we used an integral method that yielded a measure proportional to D2 receptor availability (*12*) (Fig. 2).

Iodobenzamide binding to D2 receptors in the caudate nucleus was greater in all five of the more affected TS patients compared with their less affected siblings (sign test, P = 0.03), and mean caudate nucleus binding values differed by 17% between twins (mean ± SEM: 1.49 ± 0.16 compared with 1.25 ±

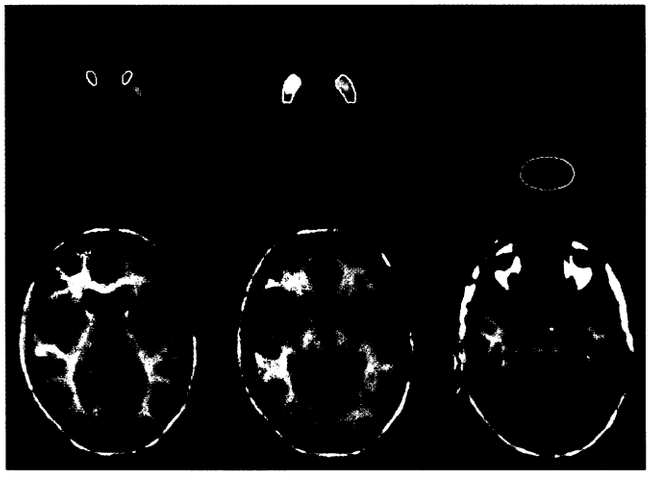

Fig. 1. Placement of ROIs for measurement of [^{123}I]IBZM binding. The 4-hour time-averaged SPECT image (top row) was used for coregistration of the MRI scan (bottom row). ROIs (in red), created on the MRI images for the head of the caudate nucleus (left), putamen (middle), and cerebellum (right), are shown transposed to the summed SPECT image (in white). Actual binding measurements were taken from individual time points (*11*).

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