Autoregulation of Cell-specific MAP Kinase Control of the Tryptophan Hydroxylase Promoter*

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The neurotransmitter serotonin controls a wide range of biological systems, including its own synthesis and release. As the rate-limiting enzyme in serotonin biosynthesis, tryptophan hydroxylase (TPH) is a potential target for this autoregulation. Using the serotonergic neuron-like CA77 cell line, we have demonstrated that treatment with a 5-hydroxytryptamine autoreceptor agonist, CGS 12066A, can lower TPH mRNA levels and promoter activity. We reasoned that this repression might involve inhibition of MAP kinases, since 5-HT1 receptors can increase mitogen-activated protein (MAP) kinase phosphatase levels. To test this hypothesis, we first showed that the TPH promoter can be activated 20-fold by mitogen-activated extracellular-signal-regulated kinase kinase kinase (MEKK), an activator of MAP kinases. This activation was then blocked by CGS 12066A. The maximal MAP kinase and CGS repression regulatory region was mapped to between −149 and −45 base pairs upstream of the transcription start site. The activation by MEKK appears to be cell-specific, because MEKK did not activate the TPH promoter in nonneuronal cell lines. At least part, but not all, of the MAP kinase responsiveness was mapped to an inverted CCAAT box that binds the transcription factor NF-Y. These data suggest a model for the autoregulation of serotonin biosynthesis by repression of MAP kinase stimulation of the TPH promoter.

Serotonin (5-hydroxytryptamine; 5-HT)1 is a monoamine neurotransmitter involved in diverse physiological functions including regulation of mood, aggression, anxiety, sleep, satiety, and sexual activity (1). Dysfunction in serotonergic systems has been implicated in the etiology of depression, aggressive behavior, and anxiety disorders (2, 3). The pathophysiological mechanisms behind these illnesses, however, are poorly understood, and very little is known about the control of serotonin levels in neurons.

Serotonin biosynthesis is restricted to serotonergic neurons in the brain raphe and gut, the pineal gland, enterochromaffin cells in the gastrointestinal tract, and rodent mast cells (4, 5). This is largely due to the cell-specific expression of tryptophan hydroxylase (TPH), the first and rate-limiting step in serotonin biosynthesis, which catalyzes the conversion of tryptophan to 5-hydroxytryptophan. As the rate-limiting enzyme, TPH is a potential target for control of serotonin levels. There is extensive evidence for post-translational regulation of TPH enzyme activity through phosphorylation by Ca2+/calmodulin-dependent protein kinase, cAMP-dependent protein kinase, and a member of the 14-3-3 protein family (6–8).

Another mechanism for the control of serotonin levels is through a negative feedback loop via activation of presynaptic 5-HT1 autoreceptors, which inhibit neuronal firing and serotonin release (5, 9). Stimulation of 5-HT1 autoreceptors also decreases the conversion of tryptophan to 5-hydroxytryptophan, suggesting that extracellular serotonin can regulate TPH activity (5). No studies to date directly demonstrate that 5-HT autoreceptor activation regulates TPH mRNA levels; however, in rats, administration of parachlorophenylalanine, an irreversible TPH inhibitor, depletes brain serotonin and causes significant increases in TPH mRNA (10). Furthermore, temporal changes in TPH mRNA are opposite and symmetric to changes in serotonin levels and TPH activity following parachlorophenylalanine treatment (11). Taken together, these data suggest that feedback control of serotonin biosynthesis may occur by transcriptional regulation of TPH gene expression.

A significant obstacle to the study of TPH transcription has been the absence of a good model system. Few neuronal cell lines express TPH. Previous studies of TPH promoter activity have been conducted in mouse mastocytoma cells (12–14), cultured pinealocytes (15), the RN46A raphe cell line (16), or non-TPH-expressing cell lines (12, 13, 15). Mastocytoma cells and pinealocytes are nonneuronal. RN46A cells are a good model but have limitations due to culture and transfection difficulties. We have used the rat CA77 thyroid C-cell line. Thyroid C cells share a similar ontogeny from the neural crest with TPH-expressing enteric neurons in the gut (17). CA77 cells express a number of serotonergic and neuronal markers, including TPH, the 5-HT1B autoreceptor, the 5-HT transporter, and regulated secretion of serotonin (17, 18). These features make CA77 cells a reasonable model for studying the regulation of serotonin biosynthesis in a neuronal setting.

In the present study, we demonstrate that the selective 5-HT1 agonist CGS 12066A (CGS) decreases TPH mRNA levels and represses TPH promoter activity. Previous studies have demonstrated that CGS acts through 5-HT1 receptors in CA77 cells (19, 20). Using transient transfection reporter gene assays, we also show that CGS blocks activation of a cell-specific MAP kinase-responsive element in the TPH promoter. In ad-
dition, we provide evidence for binding of the NF-Y transcription factor and at least one other, possibly cell-specific, factor to the MAP kinase-responsive element.

MATERIALS AND METHODS

Cell Culture—CA77 cells were maintained in Ham's F-12/Dulbecco's modified Eagle's medium (low glucose; 1:1) with 10% fetal bovine serum (FBS) at 37 °C and 7% CO2. CHO-IR/ER (CHO cell line stably transduced with the insulin and epidermal growth factor receptors) cells were kindly provided by Jeffrey Pessin (University of Iowa) (21). CHO-fected with the insulin and epidermal growth factor receptors) cells were kindly provided by John Harty (University of Iowa) and were maintained in Dulbecco's modified Eagle's medium (low glucose) with 10% FBS and 2 m M -glutamine at 37 °C and 5% CO2. N2A cells were maintained in Dulbecco's modified Eagle's medium (high glucose) with 10% FBS at 37 °C and 5% CO2. P815 cells were kindly provided by John Han (University of Iowa) and were maintained in RPMI 1640 with 10% FBS, 5 mM HEPES, 1.375 mM L-glutamine, and 50 μM 2-mercaptoethanol at 37 °C and 5% CO2. Adherent cell lines were subcultured by brief treatment with trypsin-EDTA (Life Technologies, Inc.). For 24 h prior to transfections and RNA isolation, all cells, except P815 cells, were subcultured in serum-free media supplemented with insulin, transferrin, and selenium (ITS; Collaborative Research Products) (18). N2A-5.15 cells maintained in serum-free conditions for 24 h prior to transfection yielded unusually low luciferase activities. Results obtained under these conditions, however, were similar to those from experiments without the serum-free period before transfection. All media contained 100 units/ml penicillin and 100 μg/ml streptomycin. CGS 12086A monomaleate (Research Biochemicals International) was prepared as a 10 mM solution in 0.1 N HCl and diluted to the desired concentration of 50–500 μM (Promega). Bovine β-galactosidase was provided by David Neilsen (National Institute of Mental Health) (26). 32P-labeled rabbit mitochondrial cytochrome oxidase II (COII) cDNA probe was generated from CA77 cells using the FastTrack 2.0 kit (Invitrogen). Samples were electrophoresed in a 1.3% agarose gel, blotted to nylon membrane, and hybridized as previously described (24). Bases 942–1338 of the rat TPH cDNA were cloned into XbaI-SmaI digested pGL3-Basic. TPH0.069-luc was cloned using the Elk1/Gal4 or ATF2/Gal4 reporter system, 2–3 kb of DNA 5' of the TPH promoter. For the forward primer was lost, yielding a construct with 69 bp of promoter sequence. TPHH2-SV40luc was constructed from complementary oligonucleotides from -70 to -41 bp with partial KpnI ends (5'-CTGGCG-CGCGCCGACAGTGGTG-3') and its complement 5'-cCGTCGAAGCCGCCTTGTCGCAAAG-3'. The oligonucleotides were phosphorylated on the 5' ends by T4 polynucleotide kinase, annealed, and ligated into KpnI-digested pGL3 promoter (Promega). TPH-7-luc of sequence from -151 to -65 bp with primers containing KpnI (forward) or BglII (reverse) sites (forward primer, 5'-aggattacTTCGCTTGCGATCGAGAG-3'; reverse primer, 5'-CTGGCGCGCCGACAGTGGTG-3'). The luciferase product was cloned into BglII/KpnI-digested pGL3-promoter (Promega). TPHmut0.15-luc was generated by PCR from TPH0.15-luc of two overlapping fragments containing the mutations. The first fragment extended from the RVprimer3 site in pGL3-Basic to -48 of the TPH promoter (forward primer, RVprimer3 (Promega); reverse primer (TPH), 5'-ACGCGTAAAGCCGCCGCGACGGTCT-3'). The second fragment extended from -61 of the TPH promoter to base pair 134 of pGL3-Basic (forward primer (TPH), 5'-CGCCCTTACTGCTTCTGAC- GGTAAGGATA-3'; reverse primer (pGL3), 5'-GGATAGAATGGCGCCGGCCGGCGCCGGCGACGGTCT-3'). Gel-purified PCR products were combined and amplified using the pGL3-Basic primers. The resulting product was cut with XbaI and EcoRI and cloned into XhoI-digested pGL3-Basic. TPH0.045-luc was generated by PCR amplification of TPH0.15-luc from TPH0.045-luc forward primer, 5'-TGACGATGCGCTTCTCCTAT-3'; reverse primer, 5'-GGATAGAATGGCGCCGGCCGGCGCCGGCGACGGTCT-3'). The luciferase product was cloned into pGL3-Basic (Promega). Luciferase gene from TPH0.15-luc (forward primer, 5'-CGCCCTTACTGCTTCTGACGGTAAGGATA-3'; reverse primer (pGL3), 5'-GGATAGAATGGCGCCGGCCGGCGCCGGCGACGGTCT-3'). The luciferase product was cloned into pGL3-Basic (Promega). Luciferase gene from TPH0.15-luc (forward primer, 5'-CGCCCTTACTGCTTCTGACGGTAAGGATA-3'; reverse primer (pGL3), 5'-GGATAGAATGGCGCCGGCCGGCGCCGGCGACGGTCT-3'). The luciferase product was cloned into pGL3-Basic (Promega). Luciferase gene from TPH0.15-luc (forward primer, 5'-CGCCCTTACTGCTTCTGACGGTAAGGATA-3'; reverse primer (pGL3), 5'-GGATAGAATGGCGCCGGCCGGCGCCGGCGACGGTCT-3'). Transfection and Reporter Gene Assays—CA77 cells (3–5 × 104) were transfected by electroporation in PBS at 220 mV, 960 microfarads as previously described (27) with 2.5 μg of reporter plasmid and 3.0 μg of MEKK using the Bio-Rad Gene Pulser apparatus. CHO cells were similarly transfected, but at 340 V with 0.5–10 μg of MEKK. In transfactions lacking MEKK, CMV5 vector control plasmid DNA was used to provide equal amounts of DNA in all transfections. In experiments using the Elk1/Gal4 or ATF2/Gal4 reporter system, 2–3 μg of pFR-Luc, 0.5–1.0 μg of pFA-Elk or pFA-ATF2, and 0.5–1.0 μg of pMEKK plasmid were used. To ensure equal transfection efficiency between CGS or MAP kinase inhibitor-treated cells and controls, electroporations were performed using pulses containing co-transfected control plasmid DNA. Cells were treated with 10 μg CGS or vehicle control (0.001 N HCl) for 24 h immediately following transfection except for the CGS time course, when cells were treated with 10 μg CGS for varying amounts of time immediately following transfection and the CGS-containing media were replaced with serum-free ITS media for the time remaining until harvest. For MAP kinase inhibitor experiments, cells were treated with 25 μg MEKK, 2.5 μg MAP kinase inhibitor-containing inhibitor plasmid, and 25 μg MEKK; no significant variations in transfection efficiency were observed. Luciferase activities were also normalized to pGL3-Basic or pGL3-promoter plasmid (pFR-Luc).

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Western Blot Analysis—CA77 cells were transfected with MEKK or CMV5 and harvested 24 h later. For sorbitol-treated controls, an equivalent amount of cells were incubated in 0.6 M sorbitol for 30 min before harvesting. Cells were washed in PBS, removed from dishes by scraping, and centrifuged to pellet. Whole cell extracts were prepared by lysis in 1× reporter lysis buffer (Promega) cell lysis buffer (MAP kinase assay kit; New England Biolabs) and removal of cellular debris by centrifugation. 10 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting as previously described (20). Polyclonal anti-active MAP kinase antibodies (Promega) were used at the following dilutions: ERK and JNK, 1:5000; p38, 1:2000. Membranes were stripped and reprobed with antibodies recognizing total (active and inactive) forms of ERK, JNK, or p38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:200.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotides used for EMSA were synthesized with partial BamHI ends (lowercase): TPH (wild type inverted CCAAT), 5′-gageCTGGCGCCGCGCCATTTGCGCCTCTTCTGAG Cg-3′, and its complement, 5′-gageCTGACAGAACCGCACAGGCCGGCCGGCAG-3′; TPHmut 7 (mutant inverted CCAAT), 5′-gageTGGCGCCGCGCCGGCCGGCAG-3′, and its complement, 5′-gageCTGACAGAACCGCACAGGCCGGCCGGCAG-3′ (mutations in boldface type). NF-κB, Sp1, and C/EBP consensus oligonucleotides were obtained from Santa Cruz Biotechnology. Oligonucleotides were annealed, labeled with [α-32P]dATP (20 μCi, 3000 Ci/mmole), and purified through an agarose (1%) and a G-25 column. Cold competitor oligonucleotides were similarly prepared in the absence of [α-32P]dATP. Nuclear extracts were prepared with a modified Dignam protocol (28). Binding reactions contained 0.02 pmol of labeled oligonucleotide (100,000–150,000 cpm), 3 μg of nuclear extract, 0.1 μg of poly(dI-dC) (Roche Molecular Biochemicals), and binding buffer (27). For some experiments, nuclear extracts were prepared from CA77 cells treated with CGS for 24 h. For competition assays, competitor oligonucleotides (1.0 pmol) and nuclear extract were preincubated for 15 min on ice prior to the addition of probe. For supershift assays, probe and nuclear extracts were preincubated on ice for 15 min prior to the addition of antibody (1–3 μl of Trans-Kit). The following rabbit polyclonal IgG antibodies were obtained from Santa Cruz Biotechnology, Inc.: p38 (1:1000), Sp1 (1:500), and C/EBP (1:200). Reactions were incubated on ice for 15 min and then resolved on a 6% polyacrylamide gel (29.1 acrylamide/bisacrylamide) in 0.25× TBE (Tris, borate, EDTA, pH 8.5) at 250 V for 2 h at 4 °C. Gels were dried and exposed to film with an intensifying screen for 2–20 h.

RESULTS

CGS Represses TPH mRNA Levels—To examine the effects of 5-HT1 agonists on TPH expression, CA77 cells were treated with the selective 5-HT1 receptor agonist CGS. Pharmacological studies have demonstrated that CGS preferentially activates 5-HT1B, 5-HT1D (29), and presumably 5-HT1F receptors. The selectivity of CGS for 5-HT1 receptors in CA77 cells has previously been demonstrated in CA77 cells using the 5-HT1 antagonist methiothepin (19). CA77 cells were treated for 24 h with CGS or vehicle control prior to isolation of poly(A) RNA. These conditions gave maximal effects of CGS on TPH promoter activity in transient transfection assays (data not shown). Northern blot analysis detected two TPH mRNA species of 4.0 and 1.8 kb, as previously identified in neuronal cells (18, 30) (Fig. 1A). To demonstrate specificity of CGS effects, blots were also hybridized with a cytochrome oxidase II (COII) probe, and TPH signals were normalized to COII. Total TPH mRNA levels were decreased by nearly one-half in CGS-treated cells, compared with levels in control-treated cells (p = 0.001) (Fig. 2A). A construct containing 69 bp of TPH 5′-flanking sequence (TPH0.069-luc) was repressed to 55% of control levels (p = 0.005). No significant differences in luciferase activity were observed with a construct containing the proximal 45 bp of the TPH promoter (TPH0.045-luc) or promoterless reporter gene vector (pGL3-Basic). These data indicate that the element mediating CGS responsiveness is located between −69 and −45 bp (Fig. 2B).

CGS Represses MEKK Stimulation of the TPH Promoter—Next, we investigated the mechanism of CGS repression of TPH promoter activity. Two pieces of data suggested that MAP kinase signal transduction pathways may be involved in CGS regulation of the TPH promoter. First, osmotic shock of RN46A cell lines stably transfected with a 3.1-kb TPH promoter-luciferase plasmid resulted in a 50-fold increase in luciferase activity (31). Osmotic shock is a strong activator of ERK, JNK, and p38 MAP kinase pathways (32). Second, treatment of CA77 cells with CGS results in increased levels of MAP kinase phosphatase-1 (MKP-1), a repressor of MAP kinase activity (20). Together, these data suggested that MAP kinases might stimulate TPH gene transcription.

To test this hypothesis, the effects of MAP kinases on TPH promoter activity were examined. MAP kinase signal transduction pathways in CA77 were activated by transfection of a plasmid encoding amino acids 380–672 of MEKK. This truncation of MEKK deletes the regulatory NH2-terminal domain to create a constitutively active protein that acts as an upstream activator of the three major MAP kinases: ERK, JNK, and p38 (32, 30). Western blot analysis with phosphospecific antibodies showed increased levels of the active forms of ERK, JNK, and p38 in cells transfected with MEKK plasmid compared with control plasmid (Fig. 3A). Phosphorylation of ERK by MEKK was considerably less pronounced than activation of JNK and p38 due to much higher basal levels of ERK phosphorylation. These data confirm that MEKK transfection can activate all three MAP kinase pathways in CA77 cells. CA77 cells were also cotransfected with MEKK and a Gal4/Elk1 or Gal4/ATF2 reporter system. These synthetic reporters contain a luciferase gene with Gal4 DNA-binding sites (pFR-Luc) and a plasmid encoding the Elk1 transactivation domain (pFA-Elk) or ATF2 transactivation domain (pFA-ATF2) linked to the yeast Gal4 DNA-binding domain. The transcription factor Elk
can be activated by all three major MAP kinases, while ATF2 is activated by JNK and p38 (34). Consistent with the Western blot data, the pFA-Elk transactivator stimulated pFR-Luc promoter activity 50-fold when cotransfected with MEKK in CA77 cells (p < 0.001), and the pFA-ATF2 construct stimulated the pFR-Luc promoter about 700-fold (p = 0.002) (data not shown). Previous studies in CA77 cells have described CGS effects on MAP kinase activity. When transfected with plasmids encoding constitutively active MEK1, CGS represses ERK phosphorylation (20). In cells cotransfected with MEKK plasmid, pFR-Luc, and pFA-Elk or pFA-Jun, a similar construct encoding the c-Jun transactivation domain, CGS, blocks transactivation of the reporter by both the Elk and Jun constructs (20). The effects of CGS on p38 have not been directly tested, but MKP-1, the effector of CGS, dephosphorylates all three major MAP kinases in other cell lines (34, 35).

Next, CA77 cells were transiently cotransfected with TPH luciferase reporter gene plasmids and MEKK plasmid or vector control. Following transfection, cells were treated with CGS or vehicle control for 24 h. When MEKK and TPH0.15-luc was cotransfected, a 20-fold increase in transcriptional activity was observed over vector control cotransfected cells (p < 0.001) (Fig. 3B). MEKK activation was also observed with reporter gene constructs containing the proximal 3100, 1400, and 700 bp of the TPH promoter, although to a lesser degree (4–12-fold) (data not shown). A reporter with only 69 bp of the TPH promoter (TPH0.069-luc) was activated 6–10-fold by MEKK (p = 0.02) (Fig. 3B). Hence, maximal activation was observed with the 149-bp TPH promoter fragment. In contrast, the construct containing only 45 bp of the TPH promoter was only slightly stimulated by MEKK. This latter activation was variable and not statistically significant. As controls, MEKK did not significantly activate the promoterless luciferase vector (Fig. 3B) or an SV40 promoter β-galactosidase reporter construct (data not shown). These data show that the TPH promoter is stimulated through MAP kinase signal transduction cascades that require elements between 149 and 45 bp for maximal activation.

As predicted, CGS treatment significantly repressed the stimulatory effects of MEKK on TPH0.15-luc and TPH0.069 to 20% (p < 0.001) and 38% (p = 0.04), respectively, of MEKK-stimulated levels (Fig. 3B). To ensure that we used the optimal length of CGS treatment time for maximal repression of MEKK activation of the TPH promoter, a time course was performed. CA77 cells were cotransfected with a 716-bp TPH promoter luciferase plasmid (TPH0.7-luc) and MEKK. Immediately following transfection, cells were divided equally between dishes and treated with CGS or vehicle control for 1.5–24 h. After the specified period, CGS was washed from the cells and replaced with serum-free media for the remaining time until harvest 24 h after transfection. CGS repression of MEKK activation increased with the length of time of CGS treatment (Fig. 3C). Importantly, considerable repression to about half that of MEKK-stimulated levels was seen after only 1.5 h of CGS treatment. These results demonstrate that CGS can repress MEKK activation of the TPH promoter and that repression can be detected following a relatively short treatment time.

Multiple MAP Kinase Pathways Activate the TPH Promoter—Because Mkp-1, the effector of 5-HT1 receptor activation (20), inactivates ERK, JNK, and p38, all three MAP kinase pathways are potential regulators of TPH transcription (34). We sought to elucidate the MAP kinase pathway mediating the TPH promoter response to CGS by two approaches. First, CA77 cells were cotransfected with TPH promoter reporter gene constructs and a plasmid encoding constitutively active MEK1. Unlike MEKK, MEK1 is a protein kinase highly specific for ERK (36). Following transfection, cells were treated with CGS or vehicle control for 24 h prior to harvest. MEK1 cotransfection resulted in a 2.7-fold increase in luciferase activity with the TPH0.15-luc reporter (p = 0.004) (Fig. 4). CGS treatment blocked MEK1 activation (p = 0.01). Similar to MEKK, no significant effects were observed with the 45-bp minimal TPH promoter construct. Therefore, MEK1 activates the TPH promoter, but only weakly compared with the 20-fold stimulation by MEKK, suggesting that other pathways are also important.

As an alternative approach to evaluate the contributions of the three major MAP kinases on TPH promoter activation, pharmacological inhibitors of MAP kinase activity were used. CA77 cells were cotransfected with TPH0.15-luc and MEKK. Eighteen hours following transfection, cells were treated with SB203580, U0126, or U0124 for 6 h before harvesting. SB203580 is a potent inhibitor of p38 kinase (37). U0126 is an inhibitor of MEK1 and MEK2, highly specific activators of ERK, and U0124 is an inactive analog of U0126 and a negative control (23). Because luciferase has a half-life of 3 h in mammalian cells, 6 h of inhibitor treatment was judged to be sufficient time for luciferase turnover, while minimizing effects of cellular compensatory mechanisms (38).

In cells cotransfected with MEKK and TPH0.15-luc, MEKK stimulation was partially repressed by both SB203580 and U0126 to 71% (p = 0.009) and 55% (p = 0.03) of MEKK-stimulated levels, respectively (Fig. 5A). This demonstrates
that both ERK and p38 pathways contribute to regulation of the TPH promoter and is consistent with the MEK1 cotransfection experiments. As a control, cells were cotransfected with MEKK and the Gal4/Elk reporter system, which is activated by all three major MAP kinases. Blots were stripped and reprobed with antibodies specific for total (active and inactive) ERK, JNK, or p38 to ensure equal loading. B, TPH promoter luciferase reporter plasmids were transiently cotransfected into CA77 cells with a plasmid encoding constitutively active MEKK or vector control and then treated with Cgs (10 μM) or vehicle (0.0001 N HCl) for 24 h prior to harvest. Promoter constructs from top to bottom are as follows: pGL3-Basic, TPH0.045-luc, TPH0.069-luc, and TPH0.15-luc. Luciferase activity is expressed as relative light units per 20 μg of protein ± S.E. Data represent at least three independent experiments. C, CA77 cells were transiently transfected with a TPH promoter luciferase vector containing 716 bp of the murine TPH promoter and MEKK plasmid. Immediately following transfection, cells were treated with Cgs (10 μM) or vehicle control (0.0001 N HCl) for varying amounts of time. Luciferase activity was normalized for protein and is expressed as a percentage of the mean MEKK-activated activity ± S.E. for all five time points.

MAP Kinase Responsiveness of the TPH Promoter Is Cell-specific—We also investigated whether the MAP kinase regulation of the TPH promoter may occur via multiple pathways. 

MAP Kinase Responsiveness of the TPH Promoter Is Cell-specific—We also investigated whether the MAP kinase regulation of the TPH promoter was cell-specific using the nonneuronal and nonserotonergic CHO, GH3, and NIH3T3 cell lines; the neuronal but nonserotonergic N2A cell line; and the serotonergic but nonneuronal P815 mastocytoma cell line. Cells were cotransfected with the 149-bp TPH promoter (TPH0.15-luc) or SV40 (SV40-luc) luciferase reporter gene construct and increasing amounts of MEKK. When reporter gene constructs were electroporated with up to 10 μg of MEKK plasmid in CHO cells, no significant difference in promoter activity was detected (Fig. 6A). This is in contrast to CA77 cells, where 3 μg of MEKK caused a 20-fold increase in reporter gene activity. GH3, 3T3, N2A, and P815 cells were transfected by lipid; thus, proportionally smaller amounts of cells and plasmid were used. No
difference in promoter activity between TPH and SV40 control was observed with MEKK cotransfection in GH3 (Fig. 6B), 3T3 (Fig. 6C), or P815 (Fig. 6E) cells. In N2A cells, however, statistically significant 3-fold activation of the TPH promoter construct was observed with 25 μg of MEKK plasmid (p<0.02) (Fig. 6D). As a positive control, cells were cotransfected with MEKK and the Gal4/Elk1 reporter system. A significant increase in luciferase activity was observed in each cell line, indicating that the lack of response with the TPH promoter in CHO, GH3, 3T3, and P815 cells was not due to a lack of MEKK signal transduction. Therefore, the TPH promoter response to MEKK appears to be specific to neuron-like cells.

MAP Kinase Regulation Requires Multiple Elements—Our data demonstrate that maximal MAP kinase responsiveness requires the proximal 149 bp of the TPH promoter. MEKK activation and CGS responsiveness, however, are retained with 69 bp of the promoter and lost when the promoter is truncated to 45 bp, suggesting the presence of crucial regulatory elements between −69 and −45. Additional MAP kinase-responsive or enhancer elements upstream of −69 could account for the maximal MAP kinase stimulation observed with TPH0.15-luc. These two regions of the TPH promoter were evaluated separately for the presence of MAP kinase-responsive elements. It is notable that there are no known MAP kinase-responsive elements, such as consensus sites for AP1 or Elk, within the promoter region required for maximal MAP kinase activation. The sequence spanning −151 to −65 was cloned into a luciferase reporter vector upstream of the SV40 promoter (TPH9-SV40luc). Two tandem repeats of the sequence from −70 to −41 were also cloned into the same vector (TPH7x2-SV40luc). Reporter constructs were cotransfected into CA77 cells with MEKK or control plasmid. As expected, MEKK cotransfection strongly stimulated activity with the TPH0.15-luc reporter. Surprisingly, the TPH7x2-SV40luc reporter was not activated to a greater degree than the parent vector (SV40-luc) (Fig. 7).

In addition, no significant activation was noted with TPH9-SV40luc. Basal activity of both TPH7x2-SV40luc (p = 0.03) and TPH9-SV40luc (p = 0.03) was increased nearly 4-fold over SV40-luc, indicating the presence of transcriptional elements. Therefore, these experiments imply that MAP kinase regulation of TPH transcription may require two or more interdependent promoter elements.

Mutation of an Inverted CCAAT Box Only Partially Reduces MAP Kinase Stimulation—Because MAP kinase responsiveness was lost upon transfer of the sequence between −71 and
plasmids were constructed by inserting the TPH sequence from −151 to −65 or two tandem repeats of the sequence from −70 to −41 bp into an SV40 promoter luciferase reporter plasmid (pGL3-promoter), yielding TPH9-SV40luc and TPH7x2-SV40luc. TPH promoter luciferase reporter plasmids or SV40-luc (pGL3-promoter) were transiently cotransfected into CA77 cells with a plasmid encoding constitutively active MEKK or vector control. Promoter constructs diagrammed from top to bottom are as follows: SV40-luc, TPH7x2-SV40luc, TPH9-SV40luc, and TPH0.15-luc. Luciferase activity is expressed as relative light units per 20 μg of protein ± S.E. Data represent at least three independent experiments.

An inverted CCAAT box between −57 and −49 bp had been previously identified (26), and it lies within the smaller MEKK-responsive region (Fig. 2B). Interestingly, the inverted CCAAT box has been shown to mediate cAMP responsiveness of the human promoter in pinealocyte cultures and PC12 cells (15). Increasing evidence supports cross-talk between MAP kinase and cAMP signal transduction pathways (39–41). In addition, a recent report demonstrated ERK activation of C/EBP, a CCAAT box-binding transcription factor (42). Therefore, we suspected that the inverted CCAAT box may also mediate MEKK responsiveness of the TPH promoter. To test this hypothesis, a 2-bp mutation previously shown to disrupt protein binding (15) was introduced in the inverted CCAAT box in the 149-bp luciferase reporter (TPHmut0.15-luc). We compared MEKK-stimulated promoter activity between TPH0.15-luc and TPHmut0.15-luc in transient transfection assays (Fig. 8). MEKK activation was partially reduced in the CCAAT mutant in comparison with the wild-type promoter sequence (p = 0.02). CGS repression of the MEKK stimulation was comparable for both the wild-type and mutant CCAAT element promoters (20 and 21%, respectively, of MEKK-stimulated levels). Furthermore, no significant differences in basal promoter activity or CGS repression of basal activity were detected between wild-type and the mutant CCAAT promoter constructs. The wild-type and mutant CCAAT promoters were repressed by CGS treatment to 56% (p = 0.002) and 52% (p = 0.007), respectively, of control levels. Thus, basal promoter activity and CGS repression do not require the inverted CCAAT box. These data suggest that the inverted CCAAT box contributes to the regulation by MAP kinases, but it is not the only MAP kinase-responsive regulatory element, which could account for the ability of CGS to still repress the mutant CCAAT promoter.

The Inverted CCAAT Box Mediates DNA-Protein Interactions—EMSA s were used to evaluate protein interactions with the MEKK- and CGS-responsive region of the TPH promoter. The minimally responsive region of −70 to −41 bp was used as a probe (TPH7) (Fig. 2B). This region encompasses the inverted CCAAT box. CA77 extracts yielded three distinct bands (CA1, CA2, CA3) (Fig. 9). All three complexes were competed in a dose-dependent manner by unlabeled TPH7 oligonucleotide, indicating specific DNA-protein interactions. Band CA2 varied greatly in intensity between experiments and was at least partially competed by all oligonucleotides. This suggests that it is a low affinity, nonspecific complex. In contrast to the three complexes observed with CA77 nuclear extracts, CHO nuclear extracts generated two distinct bands (CH1, CH2) when incubated with radiolabeled TPH7. Complex CH1 migrated at approximately the same rate as CA1; however, complex CH2 migrated slightly slower than CA3. Both complexes were competed in a dose-dependent manner with excess unlabeled TPH7.

Binding of these complexes to the inverted CCAAT box sequence was then demonstrated by competition assays using an unlabeled competitor oligonucleotide identical to the TPH7 probe but containing the 2-bp mutation in the inverted CCAAT box (mut7) (Fig. 9). CA1 and CH1 were not competed by the mutant oligonucleotide, and CA3 and CH2 were only weakly competed. When mut7 was radioactively labeled and used as a probe in similar experiments, the formation of CA1, CA3, CH1, and CH2 was almost completely absent (data not shown). Thus, the inverted CCAAT box is necessary for formation of these DNA-protein complexes.

NF-Y (CBF, CP1) Interacts with the Inverted CCAAT Box—CCAAT boxes have been demonstrated to bind a number of transcription factors including NF-Y (also called CBF or CP1), CP2, NF-1, and C/EBP (43, 44). In addition to the CCAAT box, the region between −70 and −41 bp was used as a probe contains two GC boxes that commonly bind Sp1, a relatively ubiquitous transcription factor. The murine and human TPH promoter inverted CCAAT box has been shown to bind the transcription factor NF-Y in mastocytoma and non-TPH-expressing cell line nuclear extracts (12, 13). Because the MAP kinase responsiveness is neuron-specific, alternative or additional cell-specific factors may bind this region of the TPH promoter. Competition with unlabeled Sp1, NF-Y, and C/EBP oligonucleotides was performed in an attempt to identify the complexes forming with the TPH7 probe in the neuron-like serotonergic CA77 cells (Fig. 9). When a 50-fold molar excess of NF-Y consensus oligonucleotide was used, CA1 and CH1 were strongly competed, implying that NF-Y is a component of these complexes. CA3 and CH2 were unaffected by NF-Y competitor. This suggests that the protein competed away from the probe by the NF-Y competitor is not necessary for formation of CA3 and CH2 despite the fact that they both appear to bind the inverted CCAAT box. The addition of a 50-fold molar excess of Sp1 or C/EBP consensus oligonucleotides had no significant effect on any of the complexes formed with CA77 or CHO nuclear extracts, with the exception of CA2, which, as previously discussed, we suspect is due to nonspecific interactions.

To further test the identities of proteins forming these complexes, Sp1 and NF-Y antibodies were added to the binding reactions following incubation of the probe and nuclear extract.
NF-Y antibody generated a clear supershift of complexes CA1 and CH1. Sp1 antibody did not generate a supershifted complex, suggesting that Sp1 is not a constituent of any of the observed complexes. Thus, the transcription factor NF-Y or an NF-Y-like protein binds to the MAP kinase-responsive region of the TPH promoter.

FIG. 8. Mutation of the inverted CCAAT box partially blocks MEKK activation. A TPH promoter luciferase reporter plasmid containing a 2-bp mutation in the inverted CCAAT box (TPH-mut0.15-luc) was transiently cotransfected into CA77 cells with plasmid encoding constitutively active MEKK or vector control and then treated with CGS (10 μM) or vehicle (0.0001 N HCl) for 24 h prior to harvest. Data for TPH0.15-luc from the same experiments are shown for comparison. Luciferase activity is expressed as relative light units per 20 μg of protein ± S.E. Data represent at least three independent experiments.

DISCUSSION

We have demonstrated that a selective 5-HT1 receptor agonist, CGS, represses TPH mRNA levels and promoter activity in a neuron-like cell line. This observation suggests a mechanism by which serotonin neurotransmitter levels may be controlled in the brain. Many enzymes are subject to end product inhibition. For example, tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is inactivated by the binding of catecholamines to its active site, providing a physiologically important negative feedback loop (45). However, TPH enzyme activity is not directly regulated by serotonin in a similar manner (5). By acting at autoreceptors coupled to signal transduction pathways that regulate the activity of transcription factors, we propose that serotonin could negatively control its own biosynthesis by modulating the transcription of TPH. We propose the following model by which serotonin regulates TPH gene transcription (Fig. 11). Extracellular stimuli acting through MAP kinases stimulate transcription of TPH to increase serotonin biosynthesis. As the neuron fires and long term serotonin levels increase, 5-HT1 autoreceptors are activated, and MKP-1 levels rise. MAP kinases are dephosphorylated and inactivated, reducing activation of the TPH promoter. TPH mRNA and enzyme levels decline, and serotonin biosynthesis falls, completing the negative feedback loop.

The mechanism of 5-HT1 autoreceptor feedback regulation is at least in part conveyed through repression of MAP kinase signal transduction pathways. Traditionally, 5-HT1 receptors have been classified as Gi-coupled receptors that inhibit adenylyl cyclase activity (9, 46); however, 5-HT1 receptors also increase intracellular calcium (47, 48). In CA77 cells, CGS and other 5-HT1 agonists do not affect cAMP levels, but they do cause a prolonged pertussis toxin-independent increase in intracellular calcium that leads to an increase in the levels of MKP-1 (19, 20). This rise in MKP-1 would account for the repression of MAP kinase activation of the TPH promoter that we have observed.

This is the first report that the TPH promoter is stimulated by MAP kinases. A variety of stressors are well known activators of MAP kinase signaling, and these pathways play physiologically important roles in mediating responses to stress in rat models (29, 34). For example, activity of the MAP kinase JNK is increased in selected brain structures by immobilization stress (49). Importantly, stress appears to modulate TPH activity. Sound stress in rats increases TPH activity in the brain (50). Immobilization stress has been shown by Sabban and co-workers (51) to significantly increase TPH mRNA levels in the rat raphe nuclei. Interestingly, this increase was not seen in the pineal gland, suggesting a neuron-specific mecha-
nism. Although extrapolation of in vitro data using osmotic shock and MEKK transfections to whole animal models of stress is risky, in vitro studies are valuable tools in guiding studies in more complex paradigms. Our data imply there may be a connection between the observed stress-induced increases in MAP kinase signaling and TPH mRNA and activity. The stress-activated p38 kinase was shown to contribute to MAP kinase stimulation of the TPH promoter. In addition, residual stimulation following treatment with both mitogen-activated protein kinase/extracellular signal-regulated kinase and p38 inhibitors may be due to stress-activated JNK, the MAP kinase elevated by immobilization stress. MAP kinases may coordinate a cell’s response to stress, including increased serotonin production via stimulation of TPH transcription. Because repression of MAP kinase activation was maintained for almost 24 h after a relatively short 1.5 h of CGS treatment, 5-HT1 receptor activation could be a biologically significant mechanism for long term regulation of TPH expression in response to stress-induced serotonin levels. In addition, the observed maximal repression after 24 h of CGS treatment is in agreement with a mechanism requiring increased synthesis of MKP-1.

Given the highly restricted nature of TPH expression and the diverse functions of the different cell types expressing TPH, cell-specific gene regulation could be crucial for a cell to respond appropriately to stress. The lack of a MEKK response in CHO, GH3, 3T3, and P815 cells argues that the MAP kinase regulation is cell-specific, perhaps operating through a cell-specific transcription factor. Because MEKK induced a modest increase in TPH promoter activity in N2A cells, the response may be in part neuron-specific. The lack of MEKK responsiveness in P815 cells, a serotonergic mouse mastocytoma cell line, suggests distinct mechanisms for TPH transcriptional regulation in neuronal and nonneuronal serotonergic cells. The maximal activation occurring in CA77 cells may result from additional serotonergic neuron-specific elements.

We narrowed the maximal MEKK-responsive element to between bases −149 and −45 of the TPH promoter with modest responsiveness maintained by the −69 to −45 bp region. When transferred to a heterologous promoter, this region did not facilitate MEKK activation, although it did confer a severalfold increase in basal promoter activity. Several reasons may account for the apparent discrepancy between the absence of MEKK responsiveness of TPH7x2-SV40-luc and the 6–60-fold MEKK activation of TPH0.069-luc. For example, spacing with the TATA box may be crucial or downstream elements may be necessary for stimulation by MAP kinases. The absence of MEKK responsiveness of TPH9-SV40-luc suggests that sequence downstream of −65, such as the inverted CCAAT box, is required. Mutations in the inverted CCAAT box located in this minimal region (−69 to −45 bp) caused only a partial reduction in activation by MEKK. The lack of complete inhibition may be due to the involvement of multiple MAP kinase-responsive elements between −149 and −45. Consistent with the reten-

FIG. 11. Model for the autoregulation of serotonin biosynthesis through transcriptional regulation of TPH. In response to extracellular stimuli, MAP kinase phosphorylation of transcription factors activates transcription of the TPH gene, increasing serotonin biosynthesis. As synaptic levels of serotonin increase, 5-HT1 autoreceptor stimulation increases MAP kinase phosphatase-1 in the cell. MAP kinases are dephosphorylated, the activation of the TPH promoter is reduced, and TPH gene transcription declines.

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