0026-895X/95/050915-08$3.00/0

MOLECULAR PHARMACOLOGY, 47:915–922 (1995).

Regulation of Rat 5-Hydroxytryptamine Type 2 Receptor Gene Activity: Identification of *Cis* Elements that Mediate Basal and 5-Hydroxytryptamine-Dependent Gene Activation

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Received December 6, 1994; Accepted February 10, 1995

SUMMARY

The 5-hydroxytryptamine type 2 receptor gene is transcriptionally induced by 5-HT-mediated activation of the 5-HT_2 receptor in rat myometrial smooth muscle cells. We recently cloned the promoter of the rat 5-HT_2 receptor gene and showed that a 1.4-kilobase promoter construct transfected into myometrial smooth muscle cells displays both constitutive and serotonin-dependent promoter activity. We have examined a series of deletional mutants of this promoter for their transcriptional activity. Deletions from base pair (bp) −1314 to bp −184 (with respect to the major transcriptional start site) resulted in no changes in constitutive or 5-HT-dependent transcriptional activity. A substantial loss of serotonin-dependent transcriptional activation was observed with a promoter construct from which the bp −184 to −108 sequence was deleted. A sequence [termed the serotonin-1 (S1) element], 5′-AGGTTnnnnnnnnAACCT-3′ (where n represents any deoxynucleotide), containing a novel dyad repeat is contained within this region. In addition to the S1 element, two simian virus 40 promoter factor 1 (SP-1) sites contiguous to this site, as well as an initiator element, appear to be important. Deletion of both the S1 and SP-1 sites resulted in an almost total loss of activity. Myometrial smooth muscle cells contain nuclear proteins that interact specifically with the S1 and SP-1 elements. Thus, multiple elements appear to be involved in serotonin-dependent induction of promoter activity. Analysis of the promoter elements that direct constitutive (i.e., serotonin-independent) activity revealed the involvement of a different region. Deletions from bp −1314 to bp −75 resulted in only minor increases in basal promoter activity. Deletion to bp −50 resulted in a 2.5-fold increase in basal promoter activity, whereas deletion to bp −25 resulted in a 5-fold increase in promoter activity. These results suggest that the basal promoter unit includes bp −25 to +1 and that upstream sequences act to repress basal promoter activity.

The 5-HT_2 receptor subtype plays a major role in mediating the effects of serotonin in a wide variety of tissues. The 5-HT_2 subtype is found in high concentrations in the frontal cortex of the brain, as well as in blood vessels and gastrointestinal and uterine smooth muscle cells, among others (1, 2). The 5-HT_2 receptor cDNA has been cloned, and the structure of the receptor as a typical seven-transmembrane region protein has been confirmed (3, 4). Alterations in the apparent number of 5-HT_2 receptors have been observed in several central nervous system disorders, such as schizophrenia, Parkinson's disease, and Alzheimer's disease (2). Complex regulation of the levels of 5-HT_2 receptors has been observed in the central nervous system, in which selective agonists and antagonists both down-regulate levels of 5-HT_2 receptors (5, 6). This is inconsistent with the traditional adaptive patterns of regulation of brain monoamine receptors, in which agonists typically down-regulate and antagonists typically up-regulate receptor numbers. In the developing mouse brain, on the other hand, coordinate changes in both 5-HT_2 receptor number and mRNA levels have been observed (7). In cultured rat myometrial smooth muscle cells, 5-HT_2 agonists were shown to activate the transcription of the 5-HT_2 receptor gene, whereas specific antagonists inhibited this induction (8). In those experiments, however, no changes in receptor numbers were observed. Taken together, these results suggest some inconsistencies among the various studies reported to date and suggest that the 5-HT_2 receptor may have unique regulatory mechanisms.

To study the mechanisms by which 5-HT_2 receptor gene regulation is accomplished in our cultured myometrial smooth muscle cell system, we have recently isolated the

This work was supported by United States Public Health Service Grants HD29961 and HD05291 and by the American Red Cross Blood Services, Greater Upstate New York Region.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; kb, kilobase(s); Inr, initiator; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; PCR, polymerase chain reaction; bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CAT, chloramphenicol acetyltransferase; SP-1, simian virus 40 promoter factor 1; AP, activator protein; S1, serotonin-1; YY1, yin-yang[1].

promoter of the rat 5-HT_2 receptor gene (9). Using a 1.4-kb fragment of the promoter to drive the transcription of the bacterial reporter gene CAT, we found that the promoter fragment displayed both constitutive and serotonin-inducible activities in uterine smooth muscle cells (9). The serotonin-dependent transcriptional activity was dependent upon the presence of functional 5-HT_2 receptors. The promoter is TATA-less but contains an Inr element contiguous to one of the multiple transcriptional start sites identified in the construct. Multiple potential regulatory sites were identified upstream of this start site, including an AP-1 site and several AP-2 and SP-1 sites. To gain further insight into the regulation of this promoter, we have evaluated a number of *cis* elements in the promoter, to determine their roles in both constitutive and 5-HT-dependent transcriptional activity. The results indicate a complex interaction of multiple *cis* elements in both regulatory pathways, involving SP-1 sites, a basal promoter unit, and a novel palindromic *cis* element that is apparently unique to the 5-HT_2 promoter.

Materials and Methods

Cell culture of rat uterine smooth muscle cells. Primary cultures of myometrial cells from 4-day postpartum rat uteri were prepared as described in detail previously (10). Cells were equilibrated to serotonin-free conditions by culturing for 3–4 days in medium containing serum that had been adsorbed with dextran-coated charcoal (11). Cells were treated with fresh serotonin-free medium at least 30 min before transient transfection.

5′ Deletion of the 5-HT_2 receptor gene promoter and CAT assays. Deletional analysis of the 5-HT_2 receptor promoter was performed by PCR amplification using different upstream primers, corresponding to the sequences in the appropriate 5′ region of the promoter. The downstream (3′) primer was the same for all constructs and was designed to extend 64 bases downstream of the major transcriptional start site (designated +64) (9). In both primers, a *Pst*I restriction site was incorporated at the 5′ end. PCR amplification products were ligated into the TA cloning vector (Invitrogen, San Diego, CA) and selected on kanamycin-containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside/agar plates by blue/white selection. Cloned fragments were cleaved with *Pst*I and cloned into the *Pst*I site of the promoterless pCAT-Basic vector (Promega, Madison, WI). Correct orientation of the promoter constructs was confirmed by restriction enzyme digestion. A commercial kit (Qiagen, Chatsworth, CA) was used to isolate large-scale plasmid preparations for transient transfection.

Transfection and CAT assays were performed as described in detail previously (9). Briefly, transfection was performed by the calcium phosphate method for 5 hr. Approximately 20 μg of pCAT plasmid (5.55 pmol) containing the appropriate construct were used for each transfection. After transfection, cells were washed with prewarmed serotonin-free medium and then were incubated with either medium containing 5 μM DOI (to assess 5-HT-dependent transcriptional induction) or serotonin-free medium (to assess constitutive transcription). Cell extracts were prepared 48 hr after transfection, by washing the cells twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and scraping the cell monolayers from the flasks with a rubber policeman. Cells were disrupted by three cycles of freeze-thawing and were incubated for 10 min at 65° to inactivate endogenous acetylases. Protein concentration was measured by a commercial micro-Bradford assay (Bio-Rad, Richmond, CA). The CAT activity of 20 μg of cell extract protein was measured by a diffusion method (12), utilizing [^{3}H]acetyl-coenzyme A (DuPont-NEN). Radioactivity in [^{3}H]acetylated chloramphenicol was determined in a liquid scintillation counter for at least three time points. In every case the reaction was linear for at least 6 hr. All experiments were performed in duplicate flasks and were repeated at least three times. Results are expressed as the mean ± standard error of three or four experiments. Specific activity in the absence of DOI for the longest construct (bp −1314/+64) was standardized as 1 unit of CAT activity. Transfection efficiency was assessed by co-transfection with pSV-β-Gal (Promega) for the same times as used for the experimental plasmids. The activity of β-galactosidase was measured by enzymatic assay using a commercial kit (Promega). In all cases, transfection efficiency was unaffected by treatment with serotonin or DOI and varied <10% between treatments.

PCR and recombinant PCR. Standard PCRs contained 20 mM Tris·HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 10 mM dithiothreitol, and 200 nM primers, in a final volume of 50 μl. Samples were denatured first at 95° for 5 min and then at 80° for 3 min. *Thermus aquaticus* DNA polymerase (1.2–1.5 units) was then added to the reaction mixture and PCR was performed under the following conditions: 30 cycles of 93.5° for 50 sec, 58° for 30 sec, and 72° for 1 min, with final extension at 70° for 10 min.

To specifically delete the Inr element and SP-1 sites, recombinant PCR was utilized as described previously (13). The deleted SP-1 sites encompass bp −107 to −89, and the putative Inr element (5′-TATTCTTCT-3′) is found at bp −9 to −1. After recombinant PCR, deletions were confirmed by dideoxy chain termination sequencing using a commercial kit (Sequenase; United States Biochemicals, Cleveland, OH).

Gel mobility shift analysis of DNA-protein interactions. Gel mobility shift assays were performed essentially according to the protocol of a commercial kit (Gel Shift System; Promega). Briefly, 15–25 μg of nuclear extract protein isolated from uterine smooth muscle cells were incubated with poly(dI·dC)·poly(dA·dT) (50 μg/ml) and specific or nonspecific competitor oligonucleotides for 5 min at room temperature. Promoter DNA fragments (approximately 0.5–1 ng) were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and were then added and incubated for 20 min. Protein-DNA complexes were electrophoretically resolved on 4% nondenaturing polyacrylamide gels in 0.5× Tris/borate/EDTA at 110–150 V, in a cold room (4°). Gels were dried at 85° and exposed to X-ray film at −80° for periods ranging from 2–3 hr to overnight.

Double-stranded SP-1 oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) and double-stranded Inr oligonucleotide (5′-CTGACTTATTCTTCTGGAAAT-3′), which spans the region from bp −15 to bp +6 around the predominant transcriptional start site, were labeled with [γ-^{32}P]ATP for use as probes in the SP-1 site or Inr element gel shift assays. The optimal buffer for the binding of YY1 to the Inr element (14) was used in the Inr gel shift assays. In the S1 element gel shift assays, [γ-^{32}P]ATP-end-labeled PCR products spanning bp −183 to −108 were used and the specific competitor was a double-stranded 17-bp oligonucleotide (5′-AGGTTCTCAATTAACCT-3′) corresponding to the S1 element. Specific and nonspecific competitor oligonucleotides were added at 25–50-fold molar excess over labeled nucleotides.

Nuclear extracts from cultured uterine smooth muscle cells were prepared according to methods described previously (15). The final nuclear extract was dialyzed against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, at 4°, divided into aliquots, and stored at −80°. Protein concentration was measured by the micro-Bradford assay (Bio-Rad). All experiments were repeated at least three times, using three different preparations of nuclear extracts.

Results

Effect of 5′ deletions on serotonin-dependent transcriptional activity of the 5-HT_2 receptor promoter. We showed previously that a 1.4-kb (bp −1314/+64) fragment of the 5-HT_2 receptor promoter can activate CAT gene expression in transiently transfected primary cultures of rat myo-

metrial smooth muscle cells (9). This serotonin-dependent activation of the promoter was approximately 2–3-fold above the constitutive level and required correct orientation of the construct; transfections in which the direction of the insert was reversed resulted in no promoter activity. To determine the *cis* elements required for serotonin-dependent promoter activity, we made a series of nested deletions of the original promoter construct. These deletions are illustrated in Fig. 1; also depicted in Fig. 1 are the known *cis*-acting elements in the constructs used. Serotonin-dependent (i.e., inductive) transcriptional activation of the CAT gene, as shown in Fig. 2, remained essentially unchanged with all deletions from bp −1314 to bp −184. Such deletions eliminated the single AP-1 site and several AP-2 sites present in the promoter construct. When the region from bp −184 to bp −108 was deleted, the inductive activity was reduced by >2-fold, to 45% of control levels (control activity is that of the bp −1314/+64 construct). Further deletion from bp −108 to bp −88 resulted in no further decrease in inductive transcriptional activity. Deletion from bp −88 to bp −75, however, resulted in a decrease in serotonin-induced transcriptional activity to approximately 25% of control. Deletion to bp −50/+64 or to bp +1/+64 completely abolished serotonin-inducible promoter activity. It should be noted, however (as described more fully below), that the level of constitutive transcriptional activity of some of these shorter constructs was considerably higher than that of the bp −1314/+64 control promoter construct.

These results indicate that the major *cis* elements responsible for serotonin-dependent transcriptional activity in the test construct lie in the region spanned by nucleotides −184 and +1. The bp −108 to −88 region contains two SP-1 sites, whereas the bp −184 to −108 region contains a novel 17-

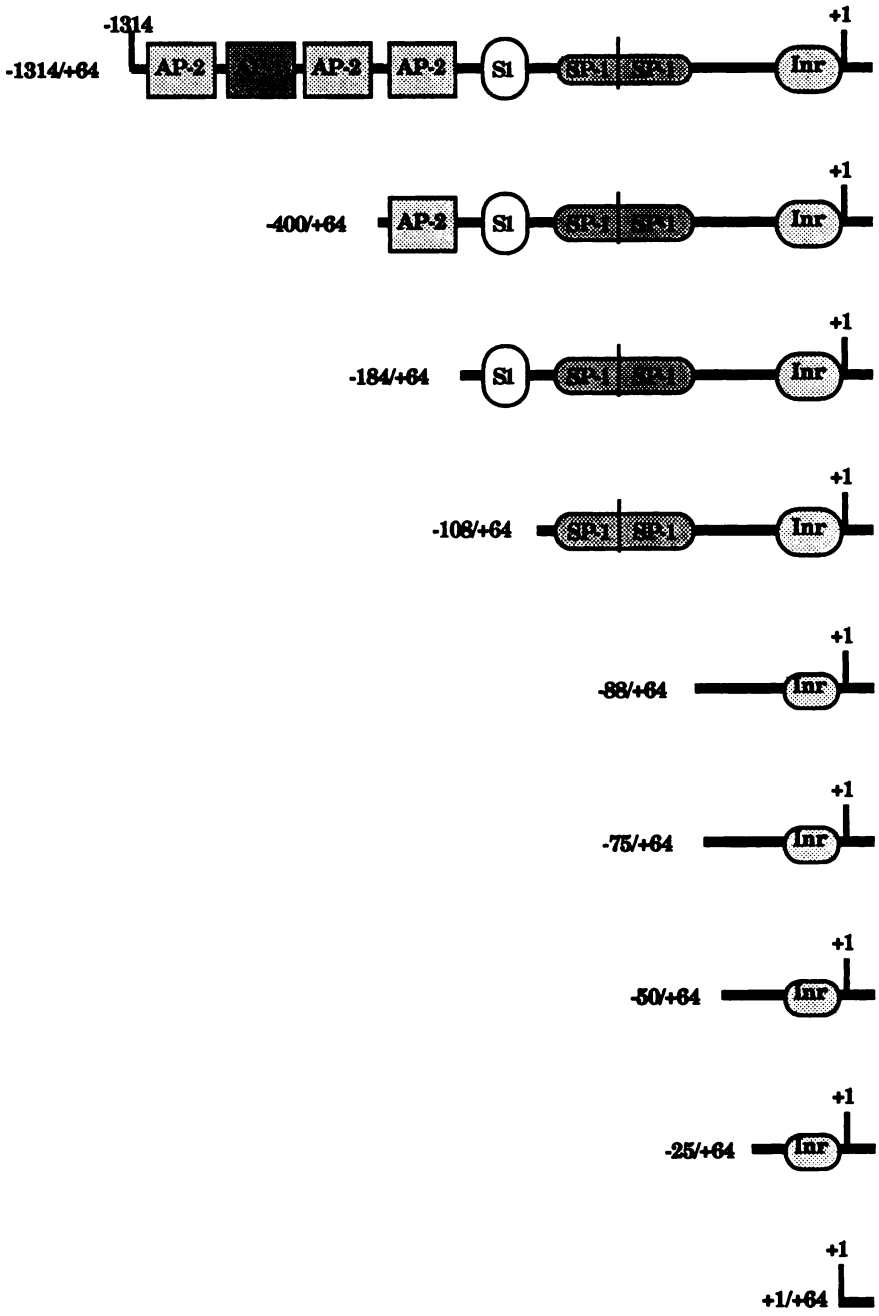

Fig. 1. 5′ Deletions of the 5-HT$_2$ receptor promoter. *Numbers to the left* indicate the length of each construct (in bp), relative to the major transcriptional start site (bp +1). The relative positions of *cis* elements are indicated. The longest construct is from bp −1314 to bp +64. Each construct has the same downstream boundary (bp +64).

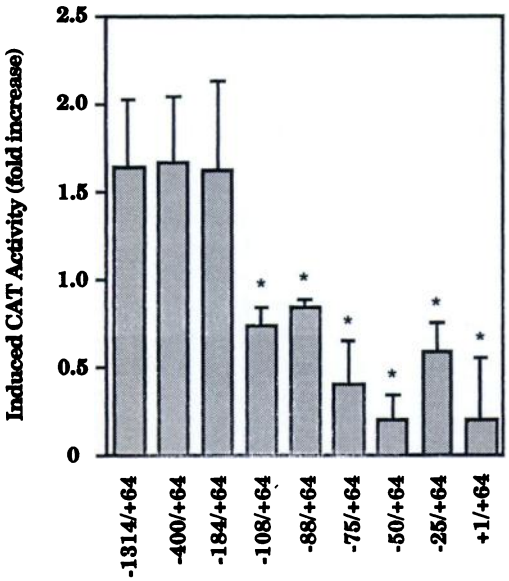

Fig. 2. Effects of 5′ deletions on the inducible transcription of the 5-HT_2 receptor gene promoter. An agonist of the 5-HT_2 receptor, DOI (5 μM), was used as the inducer. For each construct, the result is expressed as the fold increase in induction, as determined by using the equation (cpm of CAT in the presence of DOI/cpm of CAT in the absence of DOI) − 1. The bp −1314/+64 construct was used as a standard in each experiment and the activity generated by this construct was normalized as 1 unit of CAT activity. The CAT activities of other constructs were calculated by comparison with this internal control. Each experiment was performed using duplicate 75-cm^2 flasks. The data represent the mean ± standard error of three or four independent experiments. Statistical significance of the differences between each construct and the control (bp −1314/+64) was evaluated by Student's *t* test. *, Significantly different from control ($p < 0.05$).

nucleotide inverted repeat, 5′-AGGTTnnnnnnnAACCT-3′ (where n represents any deoxynucleotide), between bp −154 and −137, as shown in Fig. 3. A search of the GenBank database revealed only one such sequence, in a similar region of the promoter of the mouse 5-HT_2 receptor gene (16). The inverted repeat is identical in the two species, and four of the seven intervening nucleotides are also identical. Additionally, Garlow *et al.* (17) have recently reported the sequence of the rat 5-HT_2 receptor promoter and confirmed the existence of this sequence in the rat promoter. We have provisionally designated this sequence the "S1" sequence. The fact that this sequence has, to date, been identified only in the 5-HT_2 promoter regions of two species suggests a conserved role for the element. It will be of great interest, as promoter sequences for other serotonin receptor subtypes become available, to determine whether this element is indeed highly conserved between species.

To further examine the effects of specific *cis* elements in the promoter region on inductive transcriptional activity, specific elements were deleted. The effects of such deletions

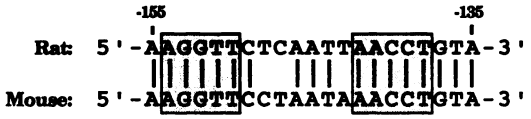

Fig. 3. Sequence of the S1 element in both the rat (*top*) and mouse (*bottom*) 5-HT_2 receptor promoters. The inverted repeat sequence is *shaded and boxed*, and the position relative to the transcriptional start site in the rat sequence is indicated (*numbers at the top*).

on serotonin-dependent CAT activation are illustrated in Fig. 4. As described above, deletion of the S1 sequence resulted in a nearly 2-fold reduction in transcriptional activity (Fig. 4). When a construct containing an intact S1 sequence but lacking the SP-1 sites between bp −108 and bp −88 was examined, a reduction in activity very similar in magnitude to the activity of the S1 deletion construct was observed (Fig. 4). Deletion of both S1 and SP-1 sites (bp −75/+64) resulted in a construct that exhibited a level of activity approximately equal to that of constructs in which either S1 or SP-1 was deleted (Fig. 4). Of further interest, deletion of the Inr element from the bp −184/+64 construct resulted in a loss in activity of approximately 40% (Fig. 4). In contrast, as we have reported previously (9), the removal of the Inr element from the shorter, bp −75/+64 construct failed to further reduce the transcriptional activity of this construct (Fig. 4).

DNA-protein interactions. To examine whether nuclear proteins from myometrial smooth muscle cells bind to the *cis* elements identified in the deletion studies, gel mobility shift experiments were performed. Either specific double-stranded oligonucleotides were synthesized or DNA containing the appropriate *cis* elements was amplified by PCR, labeled with [γ-^{32}P]ATP, and used as a probe. The results, displayed in Fig. 5, A and C, showed that proteins are present in the nucleus that specifically bind to the S1 (Fig. 5A) and SP-1 (Fig. 5C) sites. Addition of 25-fold excess unlabeled probe abolished the shift of the labeled DNA. Protein binding to the SP-1 oligonucleotide displayed no dependence on the presence of serotonin in the medium in which the cells were cultured. In some but not all cases, the level of protein binding to the S1 oligonucleotide appeared higher in nuclear extracts from cells cultured with serotonin. An example of such a difference is represented in Fig. 5B. In the experiment illustrated, the apparent serotonin-dependent difference in protein-oligonucleotide complex levels was 2.3-fold. Efforts are currently underway to purify the protein, to more precisely investigate its relationship to serotonin-mediated pro-

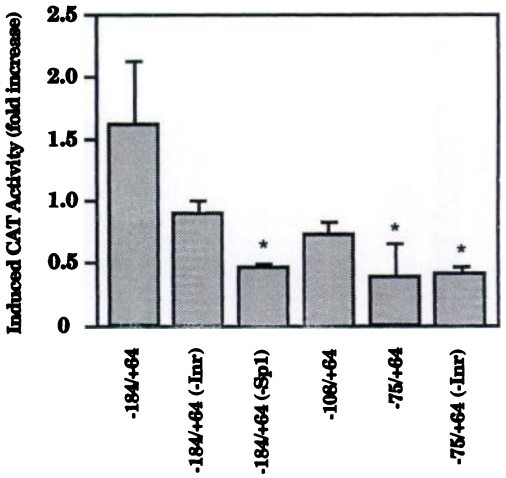

Fig. 4. Effects of specific deletion of the S1, SP-1, or Inr regions on the inducible transcription of the 5-HT_2 receptor gene promoter. Deletion of specific elements in constructs was achieved by recombinant PCR. DOI (5 μM), a selective agonist of the 5-HT_2 receptor, was used as the inducer. The results are expressed as described in Fig. 2. The results shown represent the mean ± standard error of three independent experiments performed in duplicate 75-cm^2 flasks. Statistical significance was assessed with Student's *t* test. *, Significantly different from the control construct (bp −184/+64) ($p < 0.05$).

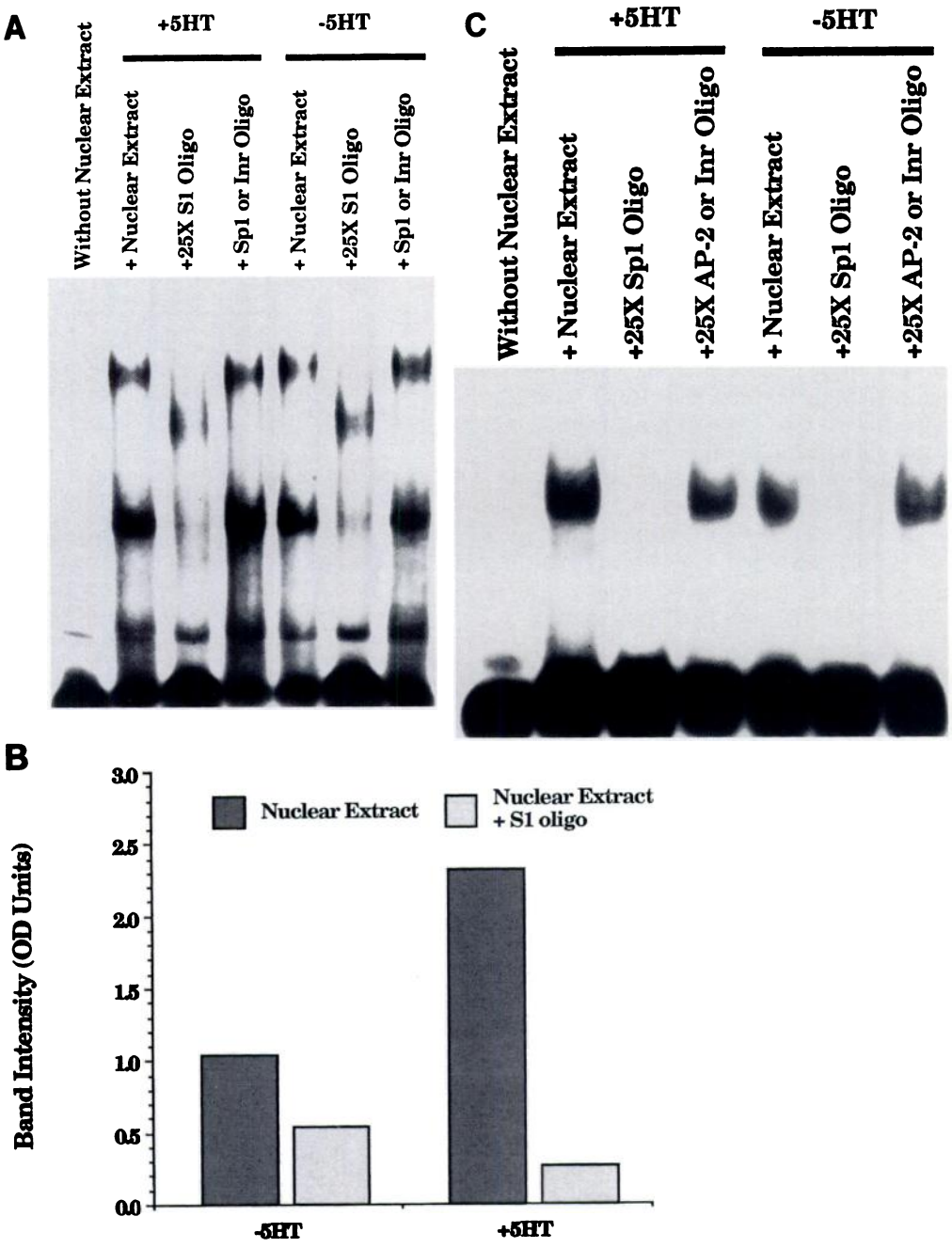

Fig. 5. Gel mobility shift experiments indicating the binding of nuclear proteins to an SP-1 oligonucleotide and the S1 region of the 5-HT$_2$ promoter. A, The bp −183/−108 fragment was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract (25 μg) of cultured uterine smooth muscle cells cultured in the presence (*+5HT*) or absence (*−5HT*) of 5 μM serotonin was used in each reaction. The double-stranded S1 element oligonucleotide (5′-AGGTTCTCAATTAACCT-3′) was used as the specific competitor in the assay. The concentrations of specific or nonspecific competitors were in 25-fold excess, compared with the labeled probe. The experiment was repeated three times with different preparations of nuclear extracts from cultured rat uterine smooth muscle cells. B, Image analysis of an experiment using the S1 probe is shown. Gel shift signals were scanned and quantified by digital image analysis, using the BioImage System (Millipore, Bedford, MA). Values are the integrated absorbance of the autoradiograph. ■, Lanes containing nuclear extract plus labeled probe; □, lanes also containing a 25-fold excess of unlabeled S1 oligonucleotide. C, The SP-1 oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) was end-labeled as described for the S1 probe. Nuclear extract (23 μg) isolated from cells cultured in the presence or absence of 5 μM serotonin was used in each reaction. Specific or nonspecific competitors were added at concentrations in 25-fold excess, compared with the labeled probe. Each experiment was repeated three times with three different preparations of nuclear extracts from rat uterine smooth muscle cells.

moter regulation. Interestingly, no binding of nuclear extract protein to the Inr oligonucleotide could be observed; furthermore, purified YY1 protein (14) also failed to bind to this oligonucleotide (data not shown).

Effect of 5′ deletions on constitutive transcriptional activity. A series of truncated constructs was prepared as described in Materials and Methods, and each was assessed for constitutive (serotonin-independent) activity in transient

transfection assays. The results are displayed in Fig. 6. Deletions from bp −1314 to bp −75 displayed small but perceptible increases above the constitutive activity of the full length promoter, which itself is approximately 8-fold above plasmid-only or reverse orientation control levels (9). Deletion to bp −50/+64 and to bp −25/+64, however, resulted in increases of 2.5- and 5-fold, respectively, above control promoter activity. Indeed, the activity of the bp −25/+64 construct was the highest ever observed in these studies, i.e., approximately 40-fold greater than background activity. Finally, removal of all promoter sequences upstream of bp +1 resulted in a return of promoter activity typical of that displayed by the bp −1314/+64 construct.

Further examination, by deletional analysis, of the role of specific promoter elements in the maintenance of constitutive activity (Fig. 7) showed that, to a first approximation, none of the elements implicated in serotonin-dependent transcriptional induction plays a major role in constitutive activity. Finally, deletion of the Inr element from the promoter construct failed to affect basal promoter activity, calling into question the role of this element in basal activity of the 5-HT_2 receptor promoter.

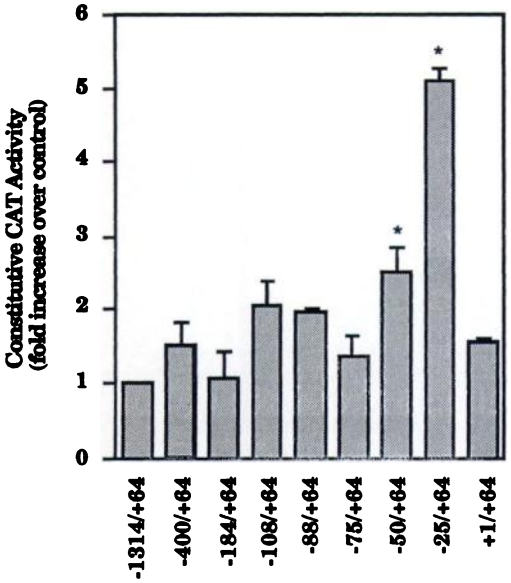

Fig. 6. Effects of 5′ deletions on the constitutive (serotonin-independent) transcriptional activity of the 5-HT_2 receptor promoter. The CAT activity of the promoterless pCAT vector in the absence of 5-HT was designated as 1 unit of CAT enzyme activity. The data represent the mean ± standard error of three or four independent experiments. Each experiment was performed in duplicate 75-cm^2 flasks. Statistical significance of the differences between each construct and the control (bp −1314/+64) was evaluated by Student's *t* test. *, Significantly different from control ($p < 0.05$).

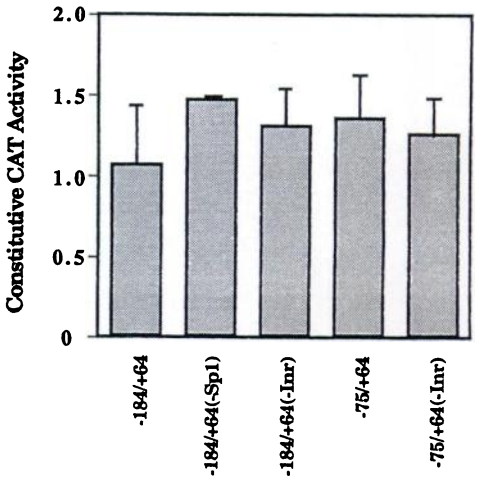

Fig. 7. Effects of specific mutations on the constitutive (serotonin-independent) transcription of the 5-HT_2 receptor promoter. Deletion of specific elements in the constructs was achieved by recombinant PCR. The CAT activity obtained from the cellular extract derived from transfection of the full length construct (bp −1314/+64) was assigned a value of 1 unit of CAT activity. Results are the mean ± standard error of three independent experiments. Each experiment was performed in duplicate 75-cm^2 flasks.

Discussion

In this study we have examined the role of potential *cis* regulatory elements in the 5-HT_2 receptor promoter in modulating the transcriptional activity of this gene in rat myometrial smooth muscle cells. Our previous and currently ongoing studies suggest a central role for serotonin in the biology of these cells (8–11). All of the serotonin-dependent gene activations so far identified in these cells are mediated by the 5-HT_2 receptor subtype. Furthermore, the regulation of steady state mRNA levels for the 5-HT_2 receptor is itself dependent upon the presence of the receptor protein; this finding prompted the studies described in this manuscript. To examine the regulation of the 5-HT_2 promoter in our cell system, we cloned and sequenced 1.4 kb of promoter region (9). It should be noted that Garlow *et al.* (17) have also isolated and sequenced the rat 5-HT_2 promoter. The sequences obtained by us and by Garlow *et al.* are essentially identical; agreement, for example, exists on the sequence and location of the element that we have termed S1. A major difference to be resolved between the two studies, however, is the issue of the location of the transcriptional start site(s). We have identified multiple transcriptional start sites in the promoter, both in brain and in myometrial smooth muscle cells. In this respect, our data correlate well with those of Ding *et al.* (16) on the mouse 5-HT_2 receptor promoter, with respect to both start sites and locations of regulatory elements. Garlow *et al.* (17), however, presented evidence for a single transcriptional start site in the rat 5-HT_2 gene, upstream from all of those that we observed. This site would put a number of the regulatory elements identified in our study downstream of the site of transcription initiation, presumably in the 5′ untranslated region of the mRNA. Further comparative studies are clearly needed to resolve this issue.

Transient transfection studies using our 1.4-kb 5-HT_2 receptor promoter construct showed that this construct possessed basal, or constitutive, activity as well as serotonin-dependent activity. Both pathways of transcriptional activation require that the promoter construct be in the correct orientation to activate the transcription of the reporter gene. Northern analysis clearly showed that transfection failed to increase the level of 5-HT_2 mRNA in myometrial smooth muscle cells (data not shown). Thus, the increase in basal transcriptional activity is not the result of the transfection itself. In the studies described here, we sought to identify the elements involved in both of these transcriptional activation pathways. Our findings indicate that the

regulation of either pathway is not a simple one at the level of *cis* element involvement.

In the case of the serotonin-dependent pathway, four *cis* elements, i.e., two SP-1 sites between bp −108 and bp −88, an Inr sequence at bp −9 to +1, and a new *cis* element we have termed the S1 element, an inverted repeat at bp −154 to −137, all appear to be involved. None, however, appears to be uniquely necessary for serotonin-dependent activation of promoter activity. Rather, the three elements appear to act cooperatively. Thus, for example, deletion of the S1 region reduces 5-HT-induced activity by approximately 45%, and further deletions of the SP-1 or Inr sites result in little or no additional loss of activity. Conversely, deletion of either the SP-1 or Inr motif in constructs containing an intact S1 region also results in a loss in activity of similar magnitude. Truncation of the promoter construct so that both the S1 and SP-1 regions are missing results in minimal serotonin-dependent promoter activation. Thus, deletion of each of these elements results in substantial reduction in inductive promoter activation, but further deletions have little or no effect. We interpret these findings as suggesting cooperative interaction between these promoter elements in the serotonin-dependent activation of the 5-HT$_2$ promoter.

In the shortest constructs used (bp −75/+64 and shorter), the reduced ability of serotonin to induce activity must be viewed in the context of the increased constitutive activity displayed by these short constructs (Fig. 6). The bp −25/+64 construct displays the highest transcriptional activation observed with any construct examined in these studies. The possibility thus exists that the constitutive activity displayed by such constructs represents a maximal level of basal promoter activity.

Common to both the rat and mouse 5-HT$_2$ receptor promoters is the sequence we have termed the S1 sequence, a 17-bp inverted repeat. In our studies, this sequence appears to play a substantive role in modulating the serotonin-mediated activation of the receptor promoter. A search of the GenBank database indicated that, to date, this sequence has not been reported to exist in the promoter of any gene except that of the mouse 5-HT$_2$ receptor (16). Taken together with the apparent existence of a nuclear protein from myometrial smooth muscle cells that binds specifically to the S1 element, the data suggest a role for this element in regulating the transcriptional activity of the promoter of this gene. Our preliminary results suggest the possibility that the levels of S1-binding protein are up-regulated by serotonin; this appears not to be the case for the SP-1-binding protein. Additionally, we could detect no binding of myometrial smooth muscle cell nuclear proteins to the Inr element. As an example of the complexity that may be required for proper regulation of this gene, a study by Lee *et al.* (18) has shown that a direct interaction occurs between SP-1-binding protein and YY1 (the protein that has been shown to bind to the Inr site). In the present case, such an interaction may be necessary for the binding of YY1 to the Inr element; if such is the case, then purified YY1 would not be expected to bind to the Inr oligonucleotide used in our gel shift experiments. Such interactions would also be consistent with our overall observation that the interaction of multiple elements is required for the regulation of the 5-HT$_2$ gene. It should be noted that none of the regions that appear to participate significantly in the regulation of 5-HT$_2$ promoter activation are involved in the regulation, by serotonin, of the rat interstitial collagenase promoter in these same cells. Our studies with this serotonin-dependent gene activation have shown that the AP-1 site is required for serotonin-mediated induction.[1] In contrast, the AP-1 site in our 1.4-kb 5-HT$_2$ promoter construct appears to be without effect on transcriptional activity in this setting. Thus, serotonin appears to regulate the two genes by two quite different pathways.

Taken together, all of our data on 5-HT$_2$ promoter activation suggest a complex set of regulatory mechanisms in which several *cis* elements participate, very likely in a cooperative way. To generalize, in a model such as that shown in Fig. 8, most if not all of the upstream elements examined here serve to repress, to a greater or lesser extent, the basal level of promoter activity represented by the bp −25/+64 construct. Serotonin-dependent induction, then, would represent a relief of this repression by upstream elements. Such a model would be consistent, in a short range sense, with the findings of Ding *et al.* (16) on the mouse 5-HT$_2$ promoter and of Garlow *et al.* (17) for the rat. The findings of both of these groups indicate that elements in the promoter well upstream from the 5′ end of the construct used here (bp −1314/+64)

[1] E.L. Weisberg, B.D. Wilcox, J.J. Jeffrey, Serotonin activation of the rat interstitial collagenase gene: a hormonal utilization of the AP-1 site. Submitted for publication.

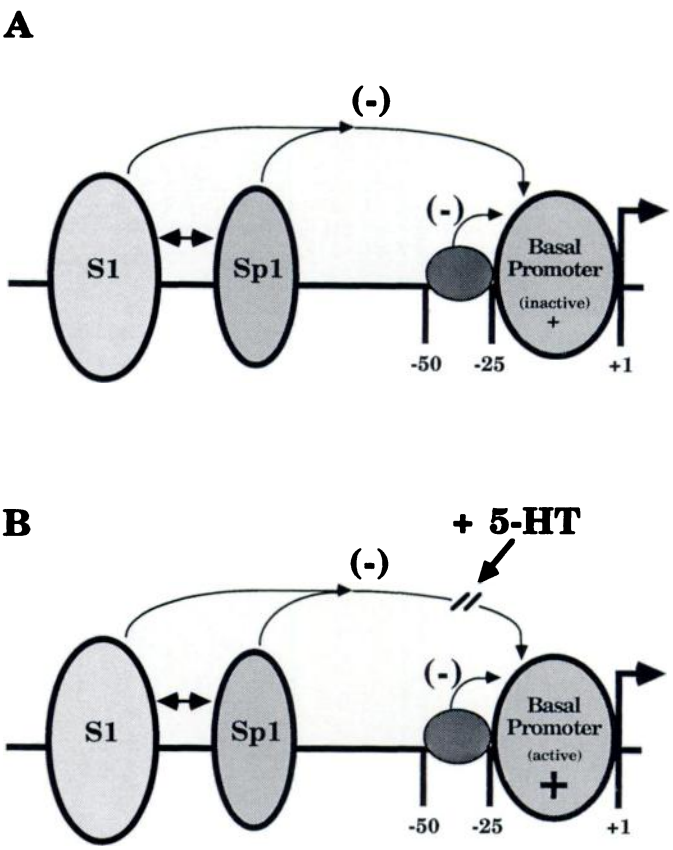

Fig. 8. Schematic model depicting the relationships and interactions of *cis* elements in the promoter of the 5-HT$_2$ receptor gene. Our results are consistent with the presence of a basal promoter (bp −25/+1) with high levels of constitutive activity. A, In the absence of serotonin the basal promoter is repressed by upstream 5′ regulatory elements. B, The addition of serotonin at least partially inhibits the ability of the upstream regions to repress basal promoter activity, resulting in a derepressed or "active" promoter.

appear to have repressive effects on promoter activity. In the studies of Garlow *et al.* (17) in rats, there also appear to be cell-specific differences in regions that repress and activate transcription. There may be, therefore, multiple elements spread over 2–3 kb of promoter DNA that, depending upon the cellular context, serve to activate or repress transcription of the 5-HT_2 receptor. Clearly, much more definition must be brought to the question of how these elements interact, and for what biological purpose. Our evidence suggests, for example, that progesterone, which completely prevents the 5-HT_2-mediated induction of interstitial collagenase by serotonin in our cultured smooth muscle cell system, is without effect on the activation of the 5-HT_2 gene (8). Preliminary evidence indicates that phorbol esters, which are powerful inducers of collagenase in these cells, have little or no effect on 5-HT_2 message levels.[2] It seems likely, in view of the limited results reported to date, that cell- and gene-specific mechanisms will emerge, with respect to both the use of particular transcriptional start sites in the 5-HT_2 receptor promoter and the use of what appears to be a menu of regulatory elements, positive or negative, in the promoter of the gene for this receptor.

Acknowledgments

The authors are grateful to Vera Gray for preparation of cultured cells.

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[2] B. D. Wilcox and J. J. Jeffrey, unpublished observations.

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