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Characterization of the Human 5-HT_{2A} Receptor Gene Promoter

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The regulation of 5-HT_{2A} receptor (5-HT_{2A}R) expression has been implicated in a variety of pathological processes and has been shown to be extremely complicated and controversial. In order to understand the mechanisms of regulation of this receptor, it is important to characterize its promoter. In this report, the 5′ end of the human 5-HT_{2A}R gene was cloned and characterized. Anchored PCR mapped multiple transcription initiation sites at nucleotides −1157, −1137, −1127, and −496. Transfection of chimeric growth hormone plasmids containing various DNA fragments into 5-HT_{2A}R-positive human cell lines (SHSY-5Y, neuroblastoma; HeLa, cervix carcinoma) showed that the 0.74 kb HaeIII/PvuII fragment, which encompasses the initiation sites between −1157 and −1127 and 5′ of the downstream initiation site (at −496), exhibited significant promoter activity. This promoter activity was not affected by the sequence upstream of the 0.74 kb fragment. The sequence downstream (the 0.45 kb PvuII/SmaI fragment) strongly repressed this promoter activity, suggesting the presence of a silencer. Sequence analysis combined with gel retardation and Dnase 1 footprinting assay identified multiple cis and trans elements for this fragment, including Sp1, PEA3, cyclic AMP response element (CRE)-like sequence, and E-boxes. Two novel transcription factors have been detected by gel retardation and DNase 1 footprinting assay; one of them may be specific for human. The transcription factors and promoter activities were low in the negative cell line NCI-H460 (human lung large cell carcinoma). Interestingly, the 0.39 kb fragment, isolated from the 3′ end of the 0.74 kb fragment, exhibited the highest promoter activity. The possibility that this 0.39 kb fragment may be an alternative promoter is discussed. These new data are essential for further study of the regulation of 5-HT_{2A}R gene expression.

[Key words: serotonin, human 5-HT_{2A} receptor, promoter, human growth hormone, anchored PCR, gel retardation, DNase 1 footprinting, transcription factor, Sp1, PEA3, CRE]

Serotonin (5-hydroxytryptamine, 5-HT) plays important roles in regulating diverse biological processes in nervous, cardiovascular, and gastrointestinal systems. These different functions are presumably mediated through specific receptors. Physiological, biochemical, and pharmacological studies have defined at least 12 receptor subtypes, which molecular cloning has confirmed. All 5-HT receptors are G-protein coupled, except 5-HT_3 receptor, which is a ligand-gated ion channel (for recent reviews, see Hoyer et al., 1994; Shih et al., 1994).

It has been shown that 5-HT_{2A} receptor (5-HT_{2A}R) is expressed in diverse tissues such as brain, vascular smooth muscles, and platelets. Like the 5-HT_{2C} receptor, it mediates stimulation of inositol polyphosphate formation by serotonin (de Courcelles et al., 1985). This receptor is implicated in various processes such as smooth muscle contraction (Cohen et al., 1981; Leysen et al., 1984), aldosterone production (Matsuoka et al., 1984), and platelet aggregation (DeClerck et al., 1984), and is related to disorders such as migraine headaches (Humphrey et al., 1990), anxiety (Taylor, 1990), and mental depression (Meltzer and Lowy, 1987). The antidepressant mianserin (Sanders-Bush, 1990) and hallucinogenic drugs (Pierce and Peroutka, 1989; Sadzot et al., 1989) have been shown to interact with this receptor.

The cDNAs for 5-HT_{2A}R in rat (Pritchett et al., 1988; Julius et al., 1990), hamster (Chambard, 1990), mouse (Yang et al., 1992), and human (Saltzman et al., 1991) have been cloned. The human 5-HT_{2A}R gene is located on chromosome 13q14–q21 (Sparkes et al., 1991), consists of three exons separated by two introns, and spans over 20 kb (Chen et al., 1992).

The regulation of 5-HT_{2A} receptor has been related to a number of pathological and neurological states. Expression of this receptor in transfected mouse fibroblast activates phospholipase C–signaling pathway and promotes cellular transformation (Julius et al., 1990). However, the mechanisms of the regulation are poorly understood and controversial. Most G-protein-coupled receptors are downregulated by agonists and upregulated by antagonists. In contrast, 5-HT_{2A} receptors are downregulated by both agonists (e.g., 5-HT, DOI) and antagonists (e.g., mianserin) (Blackshear et al., 1983; Sanders-Bush et al., 1987). While the downregulation by mianserin has been observed in animals, this phenomenon was not reproducible in studies using cell lines. Mianserin downregulation was observed in rat C6 glioma cells (Toth and Shenk, 1994), but not in rat P11 cells (Ferry et al., 1993), indicating that this paradoxical downregulation requires a certain signal transduction pathway which may not exist in all cells. Toth and Shenk (1994) reported a decrease in 5-HT_{2A} receptor mRNA levels in C6 glioma cells after mianserin treatment, suggesting that the regulation is at the level of transcription. A mianserin response element has been implicated in the

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upstream sequence of the basal promoter. On the contrary, no mRNA level change was observed in rats injected with mianserin (Roth and Ciaranello, 1991). Recently, Du et al. (1994) reported that 5-HT stimulated 5-HT_{2A} receptor promoter activity in rat myometrial smooth muscle cells via the 5-HT_{2A} receptor. These controversial results suggest the existence of a complex regulatory mechanism.

In order to understand the mechanism for human 5-HT_{2A}R gene expression, the 5′ flanking sequence of this gene has been characterized for the first time. We have defined transcription initiation sites between −1127 and −1157 by anchored PCR. Promoter activity was detected in a 0.76 kb HaeIII/PvuII fragment which encompasses these transcription initiation sites. Transcription factors Sp1, PEA3, E-box binding proteins, and two novel transcription factors have been shown to interact with this fragment. We also detected a downstream initiation site (at −496) and found promoter activity in a DNA fragment 5′ of this site, which raised the possibility for an alternative promoter.

Materials and Methods

DNA cloning and sequencing. The DNA fragments used in this study were isolated from an 8.5 kb EcoRI-phage clone lSE-5, which contains exons 1 and 2 of human 5-HT_{2A}R gene, intron 1, part of intron 2, and about 3.7 kb sequence 5′ of the translation start codon ATG (Chen et al., 1992). All the 3.7 kb 5′ sequence has been determined by Applied Biosystems model ABI370a automated DNA sequencer, and manually by the dideoxy chain termination method (Sanger et al., 1977).

Three point eight kilobases of mouse 5-HT_{2A}R genomic DNA 5′ of the translation initiation start codon ATG was mapped and sequenced from the 9 kb EcoRI fragment in the genomic clone MS2cos54-E9 (Yang et al., 1992).

The comparison between human and mouse 5′ sequences was facilitated by the sequence analysis software of Genetics Computer Group (University of Wisconsin Biotechnology Center, Madison).

Serotonin-stimulated hydrolysis of phosphoinositol (PI). In order to select 5-HT_{2A}R-positive and -negative cell lines, serotonin-stimulated PI hydrolysis was performed. The procedure used was a combination of the methods of Serra et al. (1986), Roth et al. (1986), and Kursar et al. (1992). Cells were seeded into 12-well dishes at a density of 4×10^5 cells/well and grown in inositol-free DMEM with 10% dialyzed fetal bovine serum for 24 hr. Myo-^{3}H-inositol was added to each well (1 mCi/ml) and the cells were grown for another 24 hr. Before the addition of serotonin, the cells were treated with serum-free DMEM containing 10 mM LiCl and 10 mM pargyline for 20 min. Twenty milliliters of the appropriate amount of serotonin was added and the samples were incubated at 37°C for 60 min. The reaction was terminated by aspiration of the medium followed by the addition of 1 ml ice-cold 75% methanol. The cells were scraped and the cell suspensions were transferred to ice-cold tubes containing 1 ml of chloroform and 0.5 ml of double-distilled water. The contents were mixed vigorously and then centrifuged at 2000 rpm at 4°C for 15 min. One milliliter of the upper aqueous phase was added to 2 ml of ice-cold double-distilled water and the mixture was applied onto a 10 ml AG1-X8 anion exchange column. The column was washed three times with 5 ml of double-distilled water, then washed twice with 5 mM sodium borate/50 mM sodium formate. The ^{3}H-inositol monophosphate was eluted with 4 ml of 0.2 M ammonium formate/0.1 M formic acid. The eluate was mixed with 14 ml of scintillation cocktail (Budget-Solve, Research Products International) and counted for radioactivity.

Determination of transcription initiation sites by anchored PCR. In this experiment, a kit (5′ AmpliFINDER RACE kit) containing a human whole brain cDNA library from Clontech (Palo Alto, CA) was used in which a 35-mer oligonucleotide (anchor) was linked to the 3′ end of each cDNA molecule (5′-Race-ready cDNA). PCR was performed with a primer for the anchor and primers for human 5-HT_{2A}R cDNA according to the procedure recommended by the manufacturer. Four nested primers for human 5-HT_{2A}R cDNA were used. The most 3′ primer used (S21: 5′ CTGTAGAGCCTGGTGTCATC 3′) is complementary to the nucleotide +86 to +67 (the A of the translation start codon ATG is defined as +1). The second primer (S387R: 5′ GCAATCAGAAA-CAGTGGGG 3′) was complementary to the nucleotide −441 to −459. The third primer (S133R: 5′ AAGAGCTGAGCCACGTCCGC 3′) was complementary to the nucleotide −932 to −951. The most 5′ primer (S116R: 5′ GGGAAAGTAGGAAGAGCTG 3′) was complementary to the nucleotide −1083 to −1101. The PCR products were resolved on an agarose gel and the DNA bands were cloned into pT7Blue(R) vector (Novagen, Madison, WI) for sequence determination, using T7 DNA polymerase. The sequence obtained was compared with genomic DNA to define the 5′ ends of 5-HT_{2A}R mRNA.

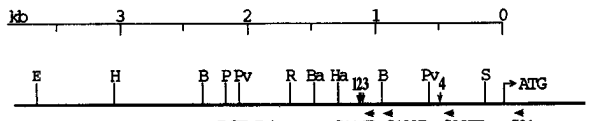
In order to map the 5′ end of 5-HT_{2A}R mRNA in SHSY-5Y (human neuroblastoma) and HeLa (human cervix carcinoma) cells, polyA$^+$ mRNA was isolated from these two cell lines with an mRNA isolation kit (version 1.2) from Invitrogen (San Diego, CA). These mRNA were reverse transcribed and ligated to the 35 bp anchor by single-strand ligation with T4 RNA ligase, as per the manufacturer's instructions (Clontech). PCR and product sequence determination were performed as with anchor-ready cDNA, with the same nested primers mentioned above.

Promoter activity measurement. Various DNA fragments (see Fig. 1) were isolated from the 3.7 kb 5′ sequence of human 5-HT_{2A}R gene and cloned into promoterless transient expression vector pOGH, which contains human growth hormone as the reporter gene (Selden et al., 1986). These constructs were then transfected into SHSY-5Y, HeLa, NCI-H460, (human lung large cell carcinoma) and MLg (mouse lung fibroblast) cells, using the low pH calcium phosphate coprecipitation method (Chen and Okayama, 1987). Promoter activity was assayed by measuring the human growth hormone secreted into medium by transfected cells with a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA) as described previously (Zhu et al., 1992).

Nuclear extracts. Nuclear extracts from HeLa and NCI-H460 cells were prepared essentially according to published procedures (Dignam et al., 1983), except that the cells were ruptured in buffer A in a polytron (Brinkman Homogenizer, model pcu 11, Luzern, Switzerland) at position 3, as described previously (Zhu et al., 1992).

Gel retardation assay. DNA fragments from the promoter regions detected by transient transfection assay were labeled with ^{32}P-dNTP and Klenow large fragment of DNA polymerase. Two and one-half micrograms of poly[dA-dT]·poly[dA-dT] and 0.25 μg of poly[dI-dC]·poly[dI-dC] were preincubated with 3 μg of the nuclear extracts for 10 min at room temperature in a mixture of 19 μl containing 20 mM Hepes-NaOH, pH 7.8, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol. When indicated, 250-fold excess of Sp1 consensus oligonucleotides (Promega, Madison, WI) was also included to replace Sp1 binding to the DNA fragments. One microliter of end-labeled DNA (about 2 fmol) was added and the incubation was continued for another 20 min at room temperature. After mixing with 2.5 μl of loading buffer (250 mM Tris-HCl, pH 7.8, 0.2% xylene cyanol, 0.2% bromophenyl blue, 40% glycerol), the mixture was loaded on a 5% nondenaturing acrylamide gel (acrylamide:bisacrylamide = 37.5:1) in 90 mM boric acid, 0.1 mM EDTA prerun at 10 mA for 1 hr. Gel electrophoresis was carried out at 10 mA in the same buffer for 1.5 hr. The temperature was never above 30°C, thus the protein–DNA complexes were not dissociated. The bands were visualized by autoradiography.

DNase1 footprinting analysis. In this experiment, one end of the DNA fragment was labeled with ^{32}P-dNTP. Since the 0.74 kb HaeIII/PvuII fragment is too long for such an assay, subfragments (the 0.35 kb HaeIII/BamHI fragment and the 0.39 kb BamHI/PvuII fragment; the latter was further divided into the 0.24 kb BamHI/HinpI fragment and the 0.15 kb HinpI/PvuII fragment; see Figs. 3, 5) were isolated for this purpose. For the labeling of the 0.35 kb HaeIII/BamHI fragment, the pOGH construct containing this fragment was digested with PstI (for the PstI site in the linker) and BamHI to isolate the 0.35 kb PstI/BamHI fragment. The sense strand was labeled at the BamHI site in a fill-in reaction with ^{32}P-dATP and Klenow large fragment of DNA polymerase 1 in the presence of cold dGTP to prevent digestion of the BamHI site by the exonuclease activity of Klenow large fragment. Cold dCTP was also included to prevent labeling at the PstI site by exchange reaction. The antisense strand was labeled at the PstI site by an exchange reaction with ^{32}P-dCTP and Klenow in the presence of cold dATP and dGTP to prevent further digestion at the PstI site and digestion at the BamHI site. In order to isolate the 0.24 and 0.15 kb fragments, the pOGH construct containing the 0.39 kb fragment was digested with BamHI to isolate a 0.39 kb BamHI/BamHI fragment (the 3′ BamHI site was from the polylinker). This fragment was then digested with HinpI to isolate the 0.24 kb BamHI/HinpI and 0.15 kb HinpI/BamHI fragments (see Fig. 5). These two fragments were labeled at the HinpI site with ^{32}P-



	Kb	SHSY-5Y	HeLa	NCI-H460	MLg
		Promoter activity (% of pXGH5 ±SD)			
E — S	3.5	N.D.	0.1 ± 0.1	–	–
H — S	3.0	N.D.	0.1 ± 0.1	–	–
P — S	2.0	N.D.	0.1 ± 0.1	–	–
Pv — S	0.45	N.D.	0.2 ± 0.1	–	–
B — B	1.40	2.6 ± 0.1	1.9 ± 0.4	0.3 ± 0.4	0.1 ± 0.1
R — B	0.70	4.2 ± 1.0	3.0 ± 0.1	–	–
Ba — B	0.53	4.5 ± 1.5	3.3 ± 0.3	–	–
Ha — B	0.35	4.3 ± 0.6	7.2 ± 1.4	1.3 ± 0.1	0.3 ± 0.1
Ha — Pv	0.74	4.5 ± 0.3	3.3 ± 1.7	–	–
Pv — Pv	1.60	4.3 ± 1.8	2.7 ± 0.2	–	–
Ha — S	1.20	N.D.	0.2 ± 0.1	–	–
B — Pv	0.39	16.0 ± 3.9	13.1 ± 0.1	1.7 ± 0.1	1.0 ± 0.1
pXHG5		100.0	100.0	100.0	100.0
cpm		2,055	75,367	32,656	17,315

Figure 1. Restriction enzyme map of the human 5-HT_2R promoter region and promoter activity measurement. A series of DNA fragments were inserted into the promoterless expression vector pOGH and transfected into four cell lines (SHSY-5Y, HeLa, NCI-H460, and MLg). The length of these fragments (in kilobases) is shown at the right side of each fragment. The promoter activity was measured by the amount of growth hormone produced. Plasmid pXGH5, which contains mouse metallothionein promoter, was used to estimate transfection efficiency and used as 100%. The background (the constructs with similar cpm as the cells without transfection) was about 150–200 cpm. *N.D.*, not detectable. Above these DNA fragments is the restriction enzyme map of 5-HT_{2A}R promoter. Only the restriction enzyme sites used for isolating DNA fragments for promoter activity measurements are shown. A length scale (in kilobases) above the map indicates the distance to the translation start codon ATG. The locations of the four primers used in anchor PCR (S116R, S133R, S387R, and S21) are indicated below the map by *arrows*. Restriction enzymes sites: *B*, BamHI; *Ba*, BalI; *E*, EcoRI; *H*, HindIII; *Ha*, HaeIII; *P*, PstI; *Pv*, PvuII; *R*, RsaI; *S*, SmaI.

dCTP with a mutant Klenow fragment which lacks exonuclease activity (Stratagene, La Jolla, CA).

For footprinting analysis, 43 μg of HeLa nuclear protein was first incubated with 2.5 μg of poly[dI-dC]·poly[dI-dC] (Sigma) for 10 min at room temperature in 20 mM Hepes-NaOH, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 10% glycerol in a total volume of 19 μl. One microliter of labeled DNA (3–5 fmol) was added and the mixture was incubated for another 20 min followed by DNase 1 digestion. The partial enzymatic cleavage was carried out at room temperature for 60 sec. The amount of DNase 1 (Promega) used was determined empirically. The digestion was terminated by the addition of 5 μl of 3 M ammonium acetate and 0.1 M EDTA. One hundred and twenty-five microliters of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) was added and the mixture was extracted with 75 ml of phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v). The phenol phase was extracted with 50 μl of TE. The two aqueous phases were combined and the DNA was ethanol precipitated, washed twice with 70% ethanol (−20°C), and air dried. The pellet was dissolved in 12 μl of 5 M urea, 5 mM EDTA, 50% formamide, 0.3% xylene cyanol, and 0.3% of bromophenyl blue. The samples were heated at 90°C for 20 min, then loaded immediately on a 7.5% acrylamide (acrylamide:bisacrylamide = 29:1)/8 M urea gel. Both a short and long run were performed to examine the proximal (relative to the labeled end) and the distal part of the fragments. MspI-digested pBR322 fragments were end labeled using ^{32}P-dCTP and Klenow polymerase, and were used as molecular weight standards. DNA bands were visualized by autoradiography.

Results

Mapping of transcription initiation sites by anchored PCR

Due to the low level of human 5-HT_{2A}R in human brain and cell lines, traditional methods for initiation site determination, such as primer extension and nuclease 1 protection assay, were not successful. In order to increase the sensitivity of detection, we used polymerase chain reaction (PCR) with "anchored" cDNA (Edwards et al., 1991), in which a 35-mer oligonucleotide (anchor) was ligated to the 3′ end of 5-HT_{2A}R cDNA molecules; PCR was then performed with a primer for the anchor and nested primers specific to human 5-HT_{2A}R cDNA to increase specificity.

In order to map 5-HT_{2A}R initiation sites, an anchored cDNA library from human brain (Clontech) was used. In addition, two human cell lines, SHSY-5Y (neuroblastoma) and HeLa (cervix carcinoma), were used. The presence of 5-HT_{2A}R in these cell lines was demonstrated by the complete block of 5-HT-stimulated phosphoinositol hydrolysis by nanomolar concentrations of ketanserin, a specific 5-HT_{2A}R antagonist (data not shown). PolyA$^+$ mRNA were isolated from these two cell lines and linked to the same 35 bp anchor.

Four nested primers for human 5-HT_{2A}R promoter were used (S21, S387R, S133R, and S116R; marked by arrows in Fig. 1, underlined in Fig. 2, top lines H). The first round of PCR was performed with primer S21, located in the coding region. From the PCR product, more upstream primers were used for the second round of PCR. The PCR products were cloned into pT7Blue vector and sequenced. By comparing the sequences of PCR products with 5′ flanking sequence of human 5-HT_{2A}R gene, the location of the transcription initiation site was determined.

PCR with anchored cDNA molecules has found initiation sites at similar regions in different cells. Site 1 (at nucleotide −1157; the A of the translation start codon ATG was defined +1) was found in brain and HeLa cells; site 2 (at −1137) was found in SHSY-5Y cells; site 3 (at −1127) was found in brain (see Fig. 1, sites 1, 2, 3, marked with vertical arrows; also Fig. 2, marked with thick arrows with the number above the initiation site).

An additional initiation site (site 4) was mapped to −496 in brain (Figs. 1, 2), 629 bp downstream from the site 3.

Identifying DNA fragments which exhibited promoter activity

For promoter activity measurement, various DNA fragments from the 5′ flanking region of the 5-HT_{2A}R gene were cloned

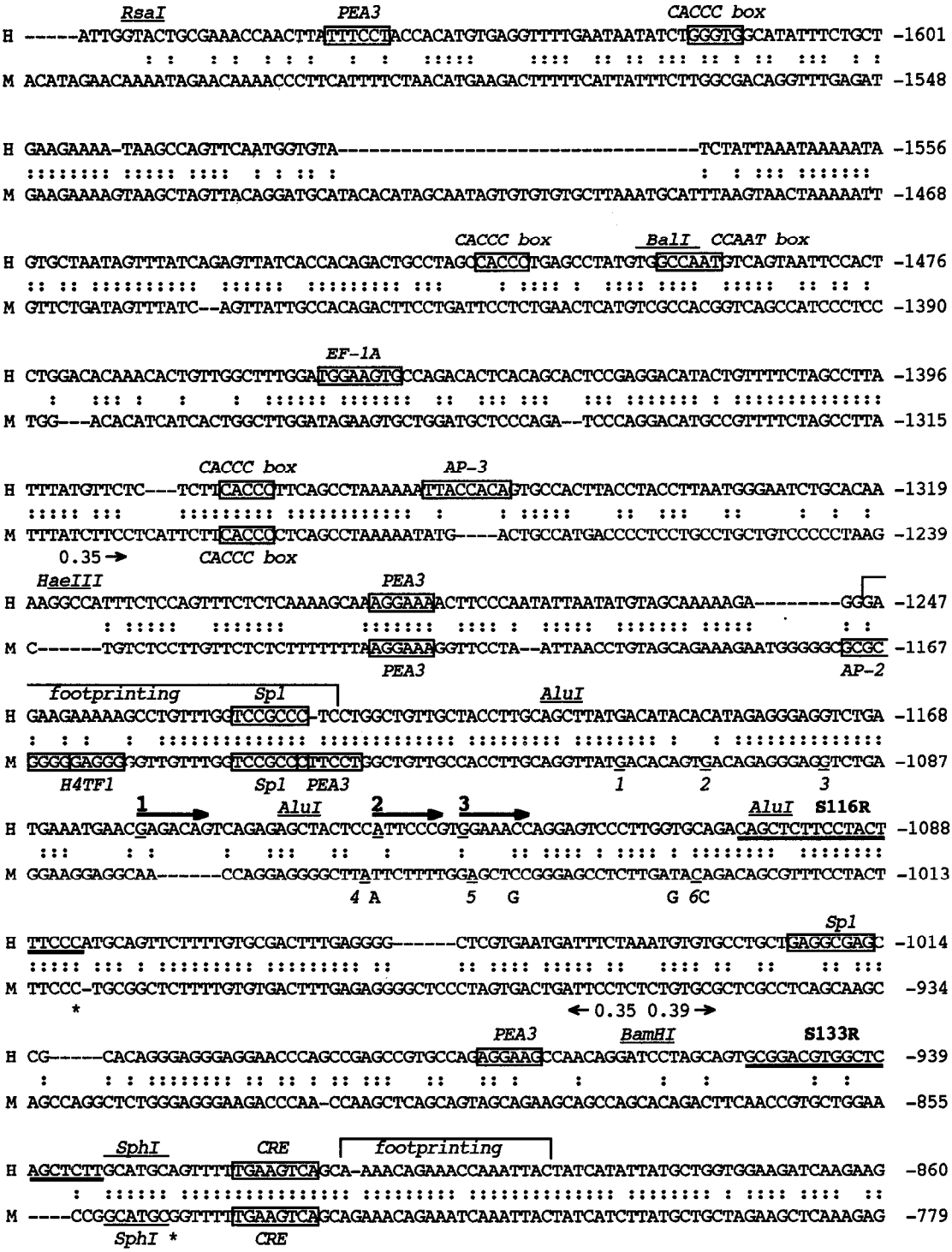


Figure 2. Promoter sequences of human and mouse 5-HT$_{2A}$ receptor genes. One thousand, six hundred and seventy-five base pair of human (*H*) sequence 5′ of the translation start codon ATG (the A of this codon is defined as +1 shown at the end of the sequence) are lined up with mouse (*M*) sequence. Gaps are introduced to obtain maximal alignment. Identical nucleotides are marked by *double dots* (:). The differences between the human sequences shown in this report and that published by Saltzman et al. (1991) are shown above the human sequence from −144 downstream. The differences between the mouse sequence reported in this article and that by Ding et al. (1993) are also shown: the nucleotides present in the sequence of Ding et al. but absent in our sequence are shown below the mouse sequence, between its flanking nucleotides. The nucleotides present in our sequence but missing in the sequence of Ding et al. are marked by *asterisks* (*) below. Potential transcription factor binding sites are boxed and marked. Three primers (S116R, S133R, S387R) used in anchored PCR for transcription initiation site mapping are *underlined* and *marked.* The fourth one (S21) is in the coding region and is not shown in this figure. The transcription initiation sites mapped are indicated by *thick arrows* and numbered *1–4* from 5′ to 3′; the position of the numbers indicates the exact location of the initiation sites. For comparison, the mouse initiation sites mapped by Ding et al. are marked by numbers *1–6* (in italic) below the sequence. Only the six most 5′ initiation sites are shown. The DNA sequences protected by nuclear proteins in DNase1 footprinting analysis are also marked. The boundaries of the 0.35 kb HaeIII/BamHI, the 0.39 kb BamHI/PvuII, and the 0.45 kb PvuII/SmaI fragments are indicated by a *short arrow* above the corresponding restriction enzyme sites.

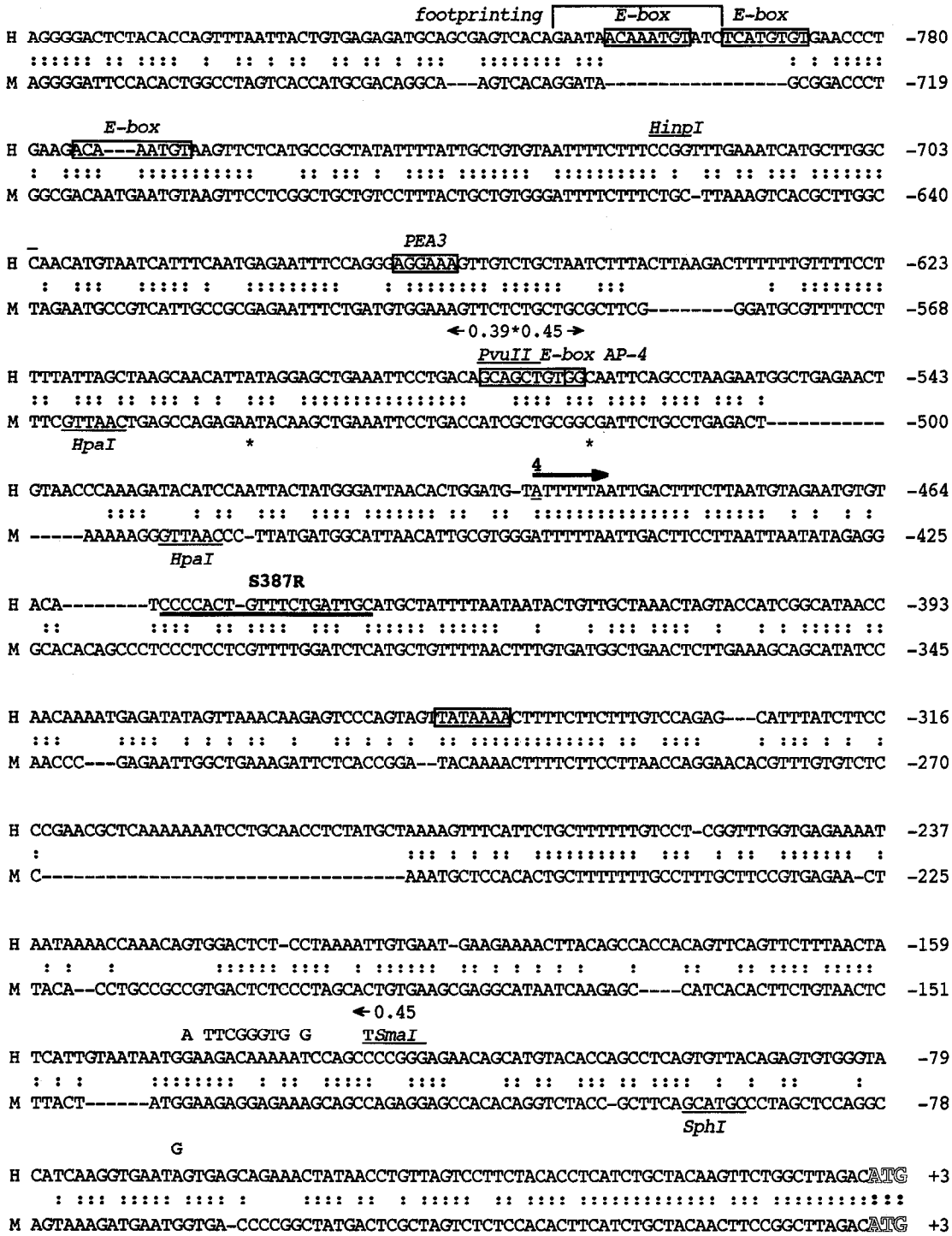


Figure 2. Continued.

into promoterless expression vector pOGH in which human growth hormone was the reporter gene. These constructs were transfected into SHSY-5Y and HeLa cells (presence of 5-HT_{2A}R), and NCI-H460 (human lung large cell carcinoma) cells (absence of 5-HT_{2A}R). A mouse cell line MLg (lung fibroblast), which expressed a high level of 5-HT_{2A}R as demonstrated by a sevenfold increase in PI turnover when stimulated by serotonin, was used for comparison. Promoter activity was determined by the production of the growth hormone and presented as the percentage of the activity of the control plasmid pXGH5, which contains a powerful mouse metallothionein promoter, and was taken as 100%.

The 0.74 HaeIII/PvuII fragment (Fig. 1, fragment 0.74), which contains initiation sites 1–3 and upstream the initiation site 4, exhibited promoter activity in 5-HT_{2A}R-positive SHSY-5Y (4.5%, 92 cpm) and HeLa (3.3%, 2512 cpm) cells. The low cpm for the 0.74 kb fragment in SHSY-5Y cells was due to low transfection efficiency in these cells, as indicated by the low cpm of the control plasmid pXGH5 (2055 cpm) in SHSY-5Y cells compared with pXGH5 in HeLa cells (75,367 cpm). Inclusion

of the upstream sequence (the 1.60 kb PvuII/PvuII fragment) had little effect (from 4.5% to 4.3% in SHSY-5Y cells and from 3.3% to 2.7% in HeLa cells).

The 0.35 kb HaeIII/BamHI fragment, a subfragment isolated from the 0.74 kb fragment (Fig. 1, the fragment 0.35, also marked in Fig. 2 with small arrows at both ends) and containing initiation sites 1–3, also exhibited promoter activity (4.3% in SHSY-5Y cells and 7.2% in HeLa cells). Inclusion of upstream sequences also had little effect (see fragments 0.53, 0.70, and 1.40). These results indicate that no enhancer was detected 5′ of the 0.74 or the 0.35 kb fragment.

When the sequence of the 0.74 kb fragment was extended to the SmaI site (the 1.20 kb HaeIII/SmaI fragment), a drastic drop in promoter activity was seen (to 0.2% in HeLa cells and not detectable in SHSY-5Y cells). This result suggests that the 0.45 kb PvuII/SmaI fragment may contain a silencer for 5-HT_{2A}R expression. The 0.45 kb fragment had little activity by itself (0.2% in HeLa cells and not detectable in SHSY-5Y cells), and all fragments which contain the 0.45 kb sequence (the 2.0 kb PstI/SmaI, 3.0 kb HindIII/SmaI, and 3.5 kb EcoRI/SmaI fragments) exhibited low promoter activity (0.1% in HeLa cells and not detectable in SHSY-5Y cells). The activities of these fragments also suggest that there was no enhancer in the 3.5 kb EcoRI/SmaI fragment.

The 0.39 kb BamHI/PvuII subfragment, isolated from the 3′ end of the 0.74 fragment (Fig. 1, fragment 0.39; Fig. 2, marked at both ends), exhibited the highest promoter activity in all four cell lines tested (16.0% in SHSY-5Y cells, 13.1% on HeLa cells, 1.7% in NCI-H460 cells, and 1.0% in MLg cells). Since initiation site 4 was downstream of this fragment, this result suggests that there may be an alternative promoter in this fragment. This fragment contains cyclic AMP response element 5′ TGAAGTCA 3′ (CRE, consensus sequence 5′ TGACGTCA 3′), a PEA3 site, and several E-boxes (Fig. 2, boxed and marked).

The validity of the observed promoter activity was supported by the experiments in which insignificant promoter activity was observed when the 0.35 kb (1.3%) and 0.39 kb (1.7%) fragments were transfected into the human 5-HT_{2A}R-negative NCI-H460 cells. Also interesting, the promoter activities of these two fragments were very low in mouse cell line MLg (lung fibroblast) (Fig. 1), which expresses high level of 5-HT_{2A}R. These low promoter activities were not due to low transfection efficiency, because the activity of pXGH5 was 32,656 cpm in NCI-H460 cells and 17,315 cpm in MLg cells (Fig. 1; compare cpm of pXGH5 in the four cell lines). Even though mouse 5-HT_{2A}R transcription initiation sites are in the same region as human initiation sites 1–3 (Fig. 2), this result implies differences in cis and trans elements between human and mouse 5-HT_{2A}R promoters (see Discussion).

Novel and known transcription factors bound to the 0.35 kb and the 0.39 kb fragment—gel retardation and DNase 1 protection assay

The promoter activity measurements have shown that the 0.74 kb HaeIII/PvuII fragment and its two subfragments, the 0.35 kb HaeIII/BamHI fragment and the 0.39 kb BamHI/PvuII fragment, exhibited promoter activity. In order to identify the transcription factors interacting with these fragments, gel retardation and DNase 1 protection analyses were performed. In gel retardation assay, nuclear proteins extracted from 5-HT_{2A}R-positive (HeLa) cells were incubated with the 0.35 and 0.39 kb fragments end labeled with ^{32}P-dNTP. The protein–DNA complexes formed and free DNA (with no protein bound) were resolved on a nondenaturing polyacrylamide gel.

Lane 1 in Figure 3 shows strong protein–DNA complex bands in the 0.35 fragment, suggesting binding of multiple nuclear proteins. The intensity of all these bands was greatly reduced in the presence of unlabeled 0.35 kb fragment (data not shown), indicating that these bands were specific. Competition with Sp1 consensus oligonucleotides abolished most of the binding in the high-molecular-weight region (Fig. 3, lane 2). Since Sp1 has been known to form multiple bands at this region (Zhu et al., 1994), effective displacement by Sp1 oligonucleotides indicates that Sp1 may be one of the transcription factors. This result confirms the sequence analysis, which revealed two Sp1 binding sequences in the 0.35 kb fragment (Fig. 2, boxed and marked; Fig. 3*A*, triangles). There were two protein–DNA bands not displaceable by a 250-fold excess of Sp1 oligos (Fig. 3, lane 2, bands a, b), suggesting that there may be two non-Sp1 transcription factors interacting with the 0.35 kb fragment.

To further define the region responsible for the non-Sp1 binding, the 0.35 kb fragment was digested with AluI to produce two subfragments (0.12 kb, 0.13 kb) which were used for gel retardation analysis (Fig. 3*A*). The 0.12 kb HaeIII/AluI fragment, the 5′ end of 0.35, contains one Sp1 binding sequence whereas the 0.35 kb fragment contains two. Thus, the Sp1 binding at a high-molecular-weight region was less in the 0.12 kb fragment than in the 0.35 kb fragment when the same amount of nuclear proteins was used (Fig. 3*B*, lanes 1, 3). In the presence of excess Sp1 oligos, there were two protein–DNA bands not displaceable, suggesting that there might be two non-Sp1 transcription factors binding to the 0.12 kb fragment (Fig. 3*B*, lane 4, bands a, b). These two non-Sp1 bands had similar mobilities (relative to the free DNA) on the gel as the two non-Sp1 bands in the 0.35 kb fragment (Fig. 3, lane 1), so possibly the non-Sp1 bands in lanes 2 and 4 represent the same transcription factors.

Another fragment, the 0.13 kb AluI/BamHI fragment in the 3′ region of 0.35, also contains an Sp1 binding sequence (Fig. 3*A*). The presence of Sp1 binding was shown by the effective displacement of the higher-molecular-weight protein–DNA complex band by Sp1 oligonucleotides (Fig. 3*B*, compare lanes 5 and 6). Interestingly, only one non-Sp1 band (band a) was present (Fig. 3*B*, lane 6). Since both the 0.12 and the 0.13 kb fragments contain a PEA3 binding site (Fig. 2, boxed and marked; Fig. 3*A*, circles), and the position of band a in the gel was the same in both fragments 0.12 kb and 0.13 kb, these results suggest that band a may represent PEA3 binding. Thus, band b may be a novel transcription factor interacting with the 0.12 kb fragment.

To accurately define the DNA sequences interacting with transcription factors, DNase1 footprinting experiments were performed in which the DNA sequence bound by protein was protected from DNase 1 digestion, thus leaving a "bleached" area in the ladder produced by DNase 1 partial digestion. DNase 1 footprinting assay with the 0.35 kb fragment and HeLa nuclear extract showed a 30 bp protected sequence in the 0.12 kb part of the 0.35 kb fragment (from −1248 to −1219; Fig. 3*A*, marked under both the 0.35 and the 0.12 fragments; Fig. 2, marked by "footprinting"):

SP1

5′ GAGAAGAAAAAGCCTGTTTGGTCCGCCCTC 3′

This protected DNA sequence was longer than the 17–18 bp required for a single Sp1 site (Jones and Tjian, 1985) and the

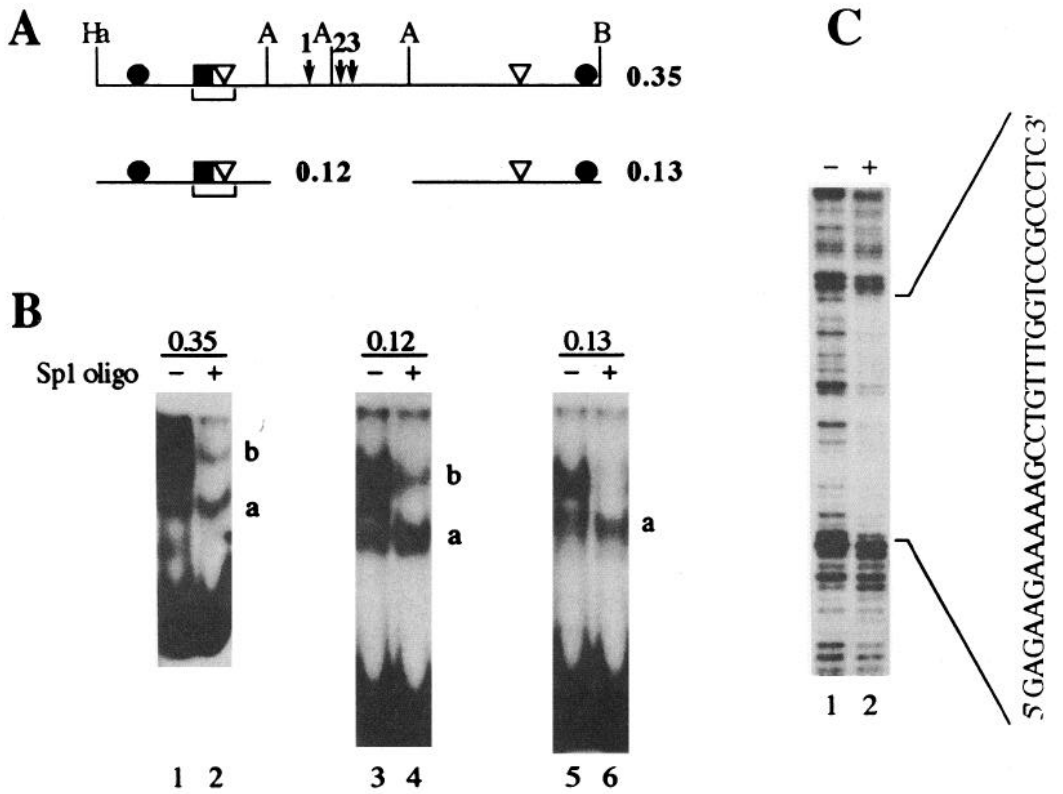


Figure 3. The restriction map (*A*), gel retardation (*B*) and DNaseI footprinting analysis (*C*) of the 0.35 kb fragment. *A,* The restriction map of the 0.35 kb fragment and the subfragments used. The lengths of the fragments are shown at right side of each fragment (in kilobases). The cis elements are also shown: Sp1, ▽; PEA3, ●; and the novel transcription factor binding site, ■. The DNA sequence protected by nuclear proteins is indicated by a *bracket* below the map. Restriction enzyme sites: *A*, AluI; *B*, BamHI; *Ha,* HaeIII. *B,* Gel retardation assay. The 0.35 kb HaeIII/BamHI (*0.35*), 0.12 kb HaeIII/AluI (*0.12*), and 0.13 kb Alu/BamHI fragments (*0.13*) were ^{32}P-end labeled and incubated with HeLa nuclear extract as described in Materials and Methods. *Lane 1* shows the binding of nuclear proteins to the 0.35 fragment. *Lane 2,* The same amount of nuclear extract was used together with Sp1 consensus oligonucleotides to remove binding by the transcription factor Sp1. Retarded protein bands not displaceable by Sp1 oligonucleotides are depicted as *a* and *b*. The same experiments were carried out for the 0.12 (*lanes 3, 4*) and 0.13 fragments (*lanes 5, 6*). *C,* DNaseI footprinting analysis. The 0.35 HaeIII/BamHI fragment was isolated from its pOGH construct as a PstI/BamHI fragment, labeled at one end, and then partially digested by DNaseI in the presence and absence of HeLa nuclear proteins. Competitive DNA (poly[dI-dC]) was used to remove nonspecific binding as described in Materials and Methods. Shown in this figure is the protection of the antisense strand labeled at the PstI site. *Lane 1* shows DNaseI partial digestion pattern in the absence (−) of nuclear protein. *Lane 2* shows the DNA sequence protected by HeLa nuclear proteins (+), with the protected sequence shown.

Sp1 site (in the reversed form CCGCCC) is located at the 3′ end (Fig. 3*A*, triangle in both 0.35 and 0.12 fragments). Therefore, it is likely that there is another protein binding site at the 5′ end. The protected sequence may be binding to a novel transcription factor, as the sequence has not been reported. The presence of this novel factor was consistent with gel retardation experiments in which band b was detected in the 0.35 and the 0.12 kb fragments (Fig. 3*B*), but absent in the 0.13 kb fragment. In addition, this factor may be specific for human, because this sequence is not conserved in mouse (Fig. 2), rat (Du et al., 1994), or Chinese hamster (Chambard et al., 1990).

No retarded bands were observed when the same amount of NCI-H460 nuclear protein was used for gel retardation experiments (Fig. 4, lane 3). This finding is consistent with the observation that there was no detectable 5-HT_{2A}R activity and low promoter activity in this cell line. This result suggests that these factors may be essential for 5-HT_{2A}R expression.

Gel retardation assay with the 0.39 kb fragment was not successful. The labeled DNA formed several bands in the absence of nuclear proteins and considerable DNA did not even enter the gel, possibly due to secondary structures in this fragment.

DNase 1 footprinting experiments with the subfragments isolated from the 0.39 kb fragment (Fig. 5*A*, the 0.24 kb BamHI/HinpI fragment and the 0.15 kb HinpI/PvuII fragment) detected two protein binding sequences. Lane 2 in Figure 5*B* shows that the protected sequence in the sense strand of the 0.24 kb fragment labeled at the HinpI site was

5′ AAAACAGAAACCAAATTAC 3′

This sequence was from nucleotide −891 to −1109 (Fig. 5*A*, shaded square; Fig. 2, marked by "footprinting"), located immediately 3′ of the CRE-like sequence (Fig. 5*A*, boxed diamond; Fig. 2, boxed and marked). This sequence is novel, suggesting the presence of another novel transcription factor.

Figure 5*B*, lane 4 shows an additional protected sequence in the 0.24 kb fragment:

E-box
5′ GAATAACAAATGTATC 3′

It was from −810 to −795 and contains one of the four E-boxes (Fig. 5*A*, vertical bars; Fig. 2, boxed and marked; the protected E-box is marked by "footprinting"), suggesting that this E-box may be used for transcription of 5-HT_{2A}R gene.

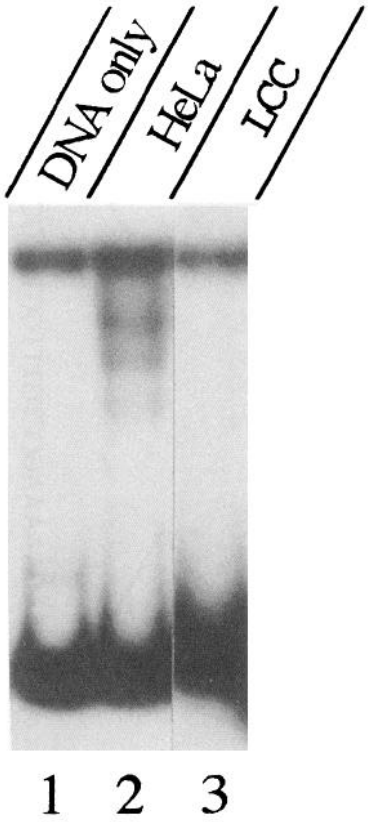


Figure 4. Comparison of concentration of transcription factors in HeLa and NCI-H460 cells. The ^{32}P-end-labeled 0.35 kb fragment was incubated with nuclear proteins extracted from HeLa (*lane 2*) and NCI-H460 (*lane 3*) cells as described in Materials and Methods. *Lane 1* shows free DNA of the labeled 0.35 kb fragment, without addition of nuclear proteins.

Discussion

Our results show that multiple transcription initiation sites (sites 1–3) are present in human 5-HT$_{2A}$R gene. They are located from 1127 to 1157 bp upstream of the translation start codon ATG. These locations are similar to the initiation sites found in mouse (the most 5′ one at −1111; Ding et al., 1993) and rat (the most 5′ one at −1120; Du et al., 1994). Since no TATA box or initiator (Inr; Smale and Baltimore, 1989) sequences were found in this region, multiple initiation sites are expected. On the other hand, the sequences following the initiation sites in these species are similar, suggesting that these sequences may be important. For example, the sequence following the most 5′ human initiation site (G, underlined) is similar to that of mouse initiation sites 1 and 2 (Ding et al., 1993):

human initiation site 1	5′ GAGACAGTCAGAGAG 3′
mouse initiation site 1	5′ GACACAGTGACAGAG 3′
mouse initiation site 2	5′ GACAGAGGGAGGTCT 3′

The sequence around human initiation sites 2 and 3 is similar to that of mouse initiation sites 4 and 5 (Ding et al., 1993), rat initiation site 1 (Du et al., 1994), and the HIP1 initiation sequence in 3-phosphoglycerate kinase and osteonectin gene promoter (Means and Farnham, 1990):

human initiation site 2 and 3	5′ ATTCCCGTGGA 3′
mouse initiation site 4 and 5	5′ ATTCTTTTGGA 3′
rat initiation site 1	5′ ATTCTTCTGGA 3′
HIP1	5′ ATTC−−−TGCA 3′

Another interesting phenomenon for the transcription initiation sites in both human and mouse is that the distances between these sites are very close to integral times of 10.5 bp (20 bp and 10 bp between human initiation sites 1 and 2, 2 and 3, respectively; 8 bp between mouse sites 1 and 2; 11 bp between sites 2 and 3; 31 bp between sites 3 and 4; 10 bp between sites 4 and 5; 22 bp between sites 5 and 6) required for one turn of B-type DNA (Wang, 1979). This suggests that these initiation sites are on the same side of the DNA. The significance of this phenomenon remains to be studied.

The 0.74 kb fragment encompasses human initiation sites 1–3, upstream initiation site 4, and exhibited significant promoter activity (Fig. 1). Since the upstream sequence had little effect on its promoter activity, it is suggested that the 0.74 kb fragment may contain sufficient cis elements for 5-HT$_{2A}$R expression. Sequence analysis, gel retardation, and footprinting experiments showed that multiple transcription factors may interact with this fragment. They are Sp1, PEA3, E-box, and CRE binding proteins, and two novel transcription factors (Figs. 2, 3, 5).

A 30 bp protein binding area has been detected in the 0.74 kb fragment by DNase 1 footprinting experiments, 61 bp upstream of the initiation sites 1–3 (Figs. 2, 3), suggesting that this region may contain the key cis elements for 5-HT$_{2A}$R expression. This region consists of as Sp1 binding sequence and a novel transcription factor binding sequence. The juxtaposition of these two binding sequences suggests that the bound Sp1 and the novel transcription factor may interact with each other, forming a more effective promoter structure. Comparison of this sequence with the corresponding sequence in mouse and rat 5-HT$_{2A}$R promoter showed that the Sp1 site is conserved in all three species, but the sequence immediately 5′ of this Sp1 site is different. In mouse, there is an H4TF1 binding sequence (5′ GGGGAGGG 3′) overlapped with an AP-2 binding sequence (Fig. 2, boxed and marked). In rat, there is an additional Sp1 sequence GGGCGGT (Du et al., 1994). The different transcription factors found in this region may explain the low promoter activity of the 0.35 kb human DNA fragment in mouse cells.

Gel retardation experiments (Fig. 4) have shown that NCI-H460 cells contained little, if any, of the transcription factors in HeLa cells, suggesting that the cellular concentration of the transcription factors may be important for 5-HT$_{2A}$R promoter activity. No other retarded band was seen on the gel, thus the low promoter activity observed in NCI-H460 cells was not due to the presence of repressors.

The presence of the downstream initiation site (site 4) in human brain is interesting. It is 629 bp downstream of the initiation site 3. This site was not reported in mouse (Ding et al., 1993) and rat (Du et al., 1994), probably because all the probes used for nuclease protection assays and the PCR primer used for 5′ RACE analysis were upstream of the human initiation site 4, and thus this site could not be detected. Northern analysis with human brain RNA showed a major 5-HT$_{2A}$R mRNA band (approximately 4.5 kb) and a minor transcript about 600 bp smaller

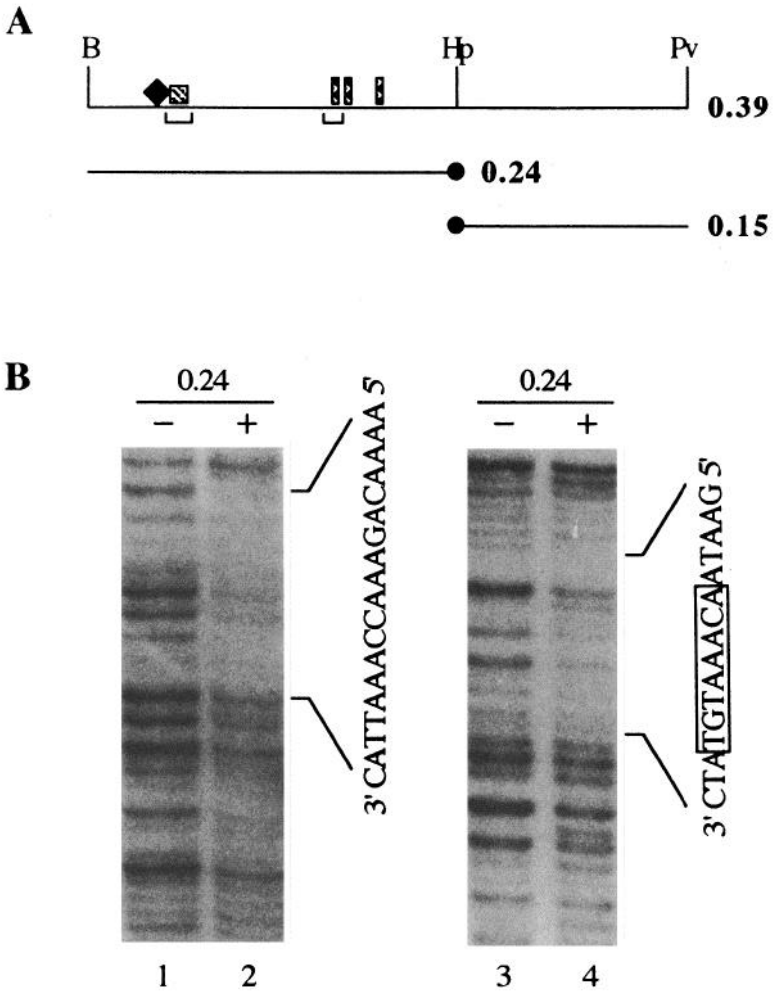


Figure 5. DNase1 footprinting analysis of the 0.39 kb fragment. *A*, Restriction enzyme map of the 0.39 kb fragment and the DNA fragments used in footprinting experiments. The length of these fragments is shown at the *right* (in kilobases). The *black dot* at the end of each DNA fragment indicates the end labeled with ^{32}P. The CRE-like sequence is represented by ♦; the three E-boxes are marked by the *smaller dark rectangles to the right.* ▨ shows the novel protein binding site detected by DNase1 footprinting assay. The protected regions are marked below the map. Restriction enzyme sites in the map: *B*, BamHI; *Hp*, HinpI; *Pv*, PvuII. *B, Lanes 1* and *3* show different parts of the partial digestion pattern of the sense strand 0.24 kb fragment labeled at the HinpI site (see *A* for the map and labeling), in the absence of nuclear proteins (−). *Lanes 2* and *4* show the protected sequences by HeLa nuclear proteins (+), with the protected sequences shown. The E-box in the protected sequence shown in *lane 4* is boxed. The 0.15 fragment revealed no protected region (not shown).

(data not shown). A similar minor band about 400–500 bp shorter than the major 5-HT_{2A}R mRNA band has also been seen in mouse brain RNA (Chen et al., 1992), and rat frontal cortex and smooth muscle mRNA (Julius et al., 1990; Rydelek-Fitzgerald et al., 1993; Du et al., 1994). Further, the highest promoter activity was detected upstream of this initiation site, but downstream of initiation sites 1–3 (Fig. 1). Similar high promoter activity has been detected in a corresponding mouse fragment (the 0.32 kb SphI/HpaI fragment, data not shown). Taken together, these results suggest that the 0.39 kb fragment might contain an alternative promoter.

The 0.39 fragment contains a cyclic AMP response element (CRE)-like sequence, three E-boxes, and a PEA3 site. In addition, a new transcription factor binding sequence has been detected next to the CRE element by DNase1 footprinting analysis (Fig. 5; also marked in Fig. 2). The factor bound to this site may interact with the CRE binding factor. The other protein binding sequence detected by footprinting experiments contained an E-box (Figs. 2, 5). E-boxes have been identified as enhancer elements of immunoglobulin genes (Church et al., 1985; Lenardo et al., 1987; Hagman et al., 1990) and many nonimmunoglobin genes (Buskin et al., 1989; Meister et al., 1989; Piette et al., 1990; Lin et al., 1991; Therrien and Drouin, 1993; etc.). The E-boxes are the binding site for a family of transcription factors containing a basic helix-loop-helix (bHLH) domain. However, none of the E-boxes found in human 5-HT_{2A}R promoter are conserved in rodents (Fig. 2), indicating that these E-boxes may be a unique characteristic of the human 5-HT_{2A}R gene. The structure of the 0.39 kb fragment is completely different from that of the 0.35 kb fragment. If it indeed contains an alternative promoter, the regulation of 5-HT_{2A}R gene expression would be mediated by different mechanisms. Although the 0.39 kb fragment exhibited the highest promoter activity when tested alone (Fig. 1), its activity is strongly repressed by the 0.35 kb fragment immediately upstream (Fig. 1; compare the 0.39 kb and the 0.74 kb fragment). This repressed activity of the 0.39 kb fragment may explain in part why the shorter transcript was only a minor one.

At this time, the possibility that site 4 may be an artifact due to premature termination cannot be excluded. Fold and stem search in the 5′ untranslated region showed that site 4 (underlined) is located in the middle of a 13 bp stem:

```
   GAT     CGC          4         AATTGA 3′
  G    TAA     TGGATGTATTTTT
G      : : :    : : : : : : : : : : :
  T    ATT     ACCTACATAGAAA
   ATC      A                      CCCAAT 5′
```

This stem may hinder the reverse transcriptase, resulting in termination. Thus, whether the 0.39 kb fragment contains an alternative promoter remains to be clarified.

The 5-HT_{2A} receptor level is low in most cells. We have

shown that the 0.45 kb PvuII/SmaI fragment, located in the 5′ untranslated region of human 5-HT_{2A}R gene, significantly repressed reporter gene expression. This fragment contains 11 ATG sequences which may act as cryptic translation start sites, thus rendering the translation less efficient. In addition, this fragment contains a typical sequence for a TATA box, TATAAAA (Fig. 2, boxed), which might compete for the binding of TFIID for the assembly of transcription complex, thus lowering transcription efficiency. Moreover, the stems (such as the one shown above) and other secondary structures revealed in our folding studies (not shown) may influence mRNA stability or translation efficiency.

In summary, our results have shown that the 0.74 kb fragment contains the promoter for human 5-HT_{2A}R gene. This fragment encompasses initiation sites 1–3 and upstream of initiation site 4, and may interact with multiple transcription factors, including Sp1, PEA3, E-box, and CRE binding proteins, and two novel transcription factors. One of them may be specific for human. The 0.45 kb fragment in the 5′ untranslated region may contain the silencer for the gene expression. The 0.39 kb fragment exhibited the highest promoter activity and was 5′ of a initiation site (−496), suggesting that there might be an alternative promoter. Further studies are needed to clarify this point.

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