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Isolation and Expression of a Mouse CB1 Cannabinoid Receptor Gene

COMPARISON OF BINDING PROPERTIES WITH THOSE OF NATIVE CB1 RECEPTORS IN MOUSE BRAIN AND N18TG2 NEUROBLASTOMA CELLS

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ABSTRACT. The predominant animal model in which the pharmacology of cannabinoids is studied is the mouse. Nonetheless, the structure and functional expression of the mouse cannabinoid receptor (CB1) gene have not been reported. We have cloned and expressed the gene for the mouse CB1 receptor and compared its properties with those of native mouse CB1 receptors in brain and N18TG2 neuroblastoma cells. The mouse CB1 gene was isolated from a mouse 129 strain genomic library. Sequence analysis of a 6-kb *Bam*HI fragment of the mouse CB1 genomic clone indicates 95% nucleic acid identity between mouse and rat (99.5% amino acid identity) and 90% nucleic acid identity (97% amino acid identity) between mouse and human. Examination of the 5′ untranslated sequence of the mouse CB1 genomic clone revealed a splice junction site approximately 60 bp upstream from the translation start site, indicating the possibility of splice variants of the CB1 receptors. The coding region of the mouse CB1 receptor was stably expressed in 293 cells, and binding by [^{3}H]SR 141716A and [^{3}H]CP-55,940 was determined. The B_{max} and K_d values obtained with [^{3}H]SR 141716A (921 ± 58 fmol/mg and 0.73 ± 0.13 nM, respectively) were similar to those of native mouse CB1 receptors in brain (B_{max} of 1.81 ± 0.44 pmol/mg, K_d of 0.16 ± 0.01 nM) and N18TG2 cells (B_{max} of 197 ± 29 fmol/mg, K_d of 0.182 ± 0.08 nM). The mouse CB1 receptor genomic clone will be a useful tool for studying the function and regulation of the CB1 receptor in mice. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:207–214, 1997.

KEY WORDS. marijuana; tetrahydrocannabinol; DNA, recombinant; receptors; cell line; mice

Marijuana, the common name for the plant *Cannabis sativa*, is the most widely used street drug. Marijuana has prominent effects on the central nervous system as well as numerous peripheral effects. The primary psychoactive constituent in the marijuana plant is Δ^9-THC.† There are a number of well documented and potential therapeutic effects of Δ^9-THC and related cannabinoids, including antiemesis, analgesia, anticonvulsant action, and lowered intraocular pressure [1]. However, the central effects and abuse potential of Δ^9-THC have discouraged its therapeutic use. Thus, a number of synthetic analogs of Δ^9-THC have been designed in an attempt to find an agent that will dissociate euphoria from analgesia, for instance. This reasoning assumes that there are specific receptors that mediate the different actions of cannabinoids, and the pharmacology of some of these compounds suggests that there may indeed be subtypes of cannabinoid receptors (reviewed in Ref. 2).

Two cannabinoid receptors have been identified to date; one is localized predominantly in the central nervous system (CB1), whereas the other is located primarily in the immune system (CB2). The CB1 receptor cDNA was isolated from a rat brain library by a homology screen for G-protein-coupled receptors, and its identity was confirmed by transfecting the clone into CHO cells and demonstrating cannabinoid-mediated inhibition of adenylyl cyclase [3]. Shortly thereafter, the cloning of a human CB1 receptorcDNA was reported [4]. There is an excellent correlation between binding affinities at the cloned CB1 receptor and binding in brain homogenates using [^{3}H]CP-55,940 as the radioligand [5]. Previously, Devane *et al.* [6] reported that a selected series of analogs exhibited a high degree of correlation between antinociceptive potency and affinity for the site in brain homogenates. Compton *et al.* [7] extended this correlation to include 60 cannabinoids and several mouse behavioral measures including hypoactivity, antinociception, hypothermia, and catalepsy. A high degree of correlation was found between the K_i values and

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† *Abbreviations:* Δ^9-THC, (−)-Δ^9-tetrahydrocannabinol; CB1, cannabinoid receptor; CP-55,940, (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxyl propyl] cyclohexan-1-ol; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride; G418, geneticin; and PCR, polymerase chain reaction.

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in vivo potency in the mouse. Thus, this receptor appears to be sufficient to mediate many of the known pharmacological effects of cannabinoids. In addition, an antagonist (SR 141716A) with high affinity for the CB1 receptor has been discovered which blocks the behavioral effects of agonists like Δ^9-THC and CP-55,940 [8, 9].

Rodents, particularly mice, are the predominant animal model in which the pharmacology of cannabinoids is studied [10]. Nonetheless, the structure and functional expression of the mouse cannabinoid receptor gene have not been reported. Recently, we and others have sequenced the mouse CB1 gene and cDNA (GenBank accession numbers U22948, U40709, and U17985). The sequence of the mouse CB1 cDNA (U17985) has been published [11]. Sequence analysis of the mouse CB1 genomic and cDNA clones indicates 95% nucleic acid identity between mouse and rat (99.5% amino acid identity) and 90% nucleic acid identity (97% amino acid identity) between mouse and human [3, 4]. Rat CB1 probes can be used to detect mouse cannabinoid receptor mRNA [12], again indicating conservation among species. However, the human and rat sequences diverge about 60 bp upstream of the translation initiation codon. Furthermore, we have isolated a second rat CB1 cDNA clone [12] (and unpublished results) that is identical to the first sequence [3] in the coding region, but also diverges about 60 bp upstream of the translation codon. These data suggest the possibility of splice variants of the CB1 receptor as well as divergence of other regulatory sequences between these genes. The existence of CB1 receptor splice variants has been demonstrated recently for the human and rat receptors [13]. Elucidation of the 5′ untranslated sequence of the mouse cannabinoid receptor gene is necessary to evaluate this possibility for the mouse CB1 receptor. Furthermore, mouse-specific information will be essential for the development of transgenic animals. We report here the molecular cloning and expression of the mouse cannabinoid receptor (CB1) gene from a mouse strain 129 library. This receptor exhibits binding properties similar to those of the native mouse CB1 receptor, found in brain and in the mouse neuroblastoma cell line N18TG2.

MATERIALS AND METHODS

PCR

PCR was performed as follows: a mixture containing 1 μg of template DNA, 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 1 mM each of dATP, dCTP, dGTP, and TTP, 15 μM of designated primers, and 2.5 U of *Vent* polymerase (New England Biolabs, Beverly, MA) was denatured for 1 min at 95°, cooled to 60°, and then heated for 4 min at 60° (to allow optimal extension by the polymerase). This sequence was then repeated by heating again for 1 min at 95°, and so on for 25 cycles with a 6-min 72° extension at the final cycle.

Cloning of the Mouse CB1 Gene

Oligonucleotide primers homologous to bp 1–21 and bp 843–824 on the opposite strand of rat CB1 were chosen for use in the PCR to generate an 843 bp fragment in mouse genomic DNA. This fragment was cloned into the TA cloning vector (Invitrogen, San Diego, CA) and sequenced via the dideoxy method (US Biochemical, Cleveland, OH). This sequence information was used to design PCR primers homologous to bp 1–22 and 150–129 on the opposite strand of mouse CB1. The PCR primers were then supplied to Genome Systems, Inc. (St. Louis, MO) to screen their mouse strain 129 P1 plasmid library. Four clones were obtained from this screen. Southern blot analysis with the coding region of the rat CB1 receptor [12] revealed positively hybridizing fragments in all four clones. The 6-kb *Bam*HI fragment of clone 2841 was subcloned into the pBS-SK$^+$ vector (Strategene, La Jolla, CA) for sequencing and into the pcDNA3 vector (Invitrogen) for expression. However, very low levels of expression were obtained using this genomic fragment; therefore, we subcloned the coding region using a PCR-directed strategy. A 5′ primer containing a *Bam*HI site and the predicted translation initiation start site (5′ CGG GAT CCA TGA AGT CGA TCT TAG ACG GCC TTG) and a 3′ primer containing an *Eco*RI site and the predicted translation stop site (5′ CGG AAT TCA GCC ACA AAA GCA GCA GCT CA) were used in the PCR to amplify the ~1.4 kb coding region from the 2841-BS plasmid, which was subsequently subcloned into a (*Bam*HI/*Eco*RI digested) pcDNA3 vector.

Cell Culture and Transfection

Human embryonic kidney 293 cells obtained from the American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal clone II (HyClone, Logan, UT) and 5% CO_2 at 37° in a humidified incubator. Cell lines expressing the mouse CB1 cannabinoid receptor were established by cloning the coding region (described above) into the pcDNA3 mammalian expression vector and transfecting this plasmid into 293 cells by the Lipofectamine reagent (LTI, Gaithersburg, MD). Stable transformants were selected using growth medium containing G418 (0.8 mg/mL; LTI). Colonies of about 500 cells were picked (about 2 weeks post-transfection) and allowed to expand, then tested for expression of receptor mRNA by northern blot analysis. Cell lines containing moderate to high levels of receptor mRNA were tested for receptor binding properties. Transfected cell lines were maintained in DMEM with 10% fetal clone II plus 0.3–0.5 mg/mL G418 and 5% CO_2 at 37° in a humidified incubator.

Radioligand Binding

The current assay was modified from a brain membrane preparation [7]. Cells were harvested in phosphate-buffered saline containing 1 mM EDTA and centrifuged at 500 *g*. The cell pellet was homogenized in 10 mL of buffer A (320 mM sucrose, 50 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4). The homogenate was centrifuged at 1600

g (10 min), the supernatant saved, and the pellet washed once in buffer A with subsequent centrifugation. The combined supernatants were centrifuged at 100,000 g for 60 min. The pellet was resuspended in 3 mL of buffer B (50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, pH 7.4) to yield a protein concentration of approximately 1 mg/mL [14]. The tissue preparation was divided into equal aliquots, frozen on dry ice, and stored at −70°. Membranes from whole mouse brain were prepared similarly. Binding was initiated by the addition of 50–100 μg membrane protein to silanized tubes containing [^{3}H]CP-55,940 (102 Ci/mmol; NEN, Boston, MA) or [^{3}H]SR 141716A (43 Ci/mmol; Amersham, Arlington Heights, IL) and a sufficient volume of buffer C (50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, and 1–5 mg/mL fatty acid free BSA, pH 7.4) to bring the total volume to 1 mL. The addition of 1 μM unlabelled CP-55,940 or SR 141716A was used to assess non-specific binding. Following incubation (30° for 1 hr), binding was terminated by the addition of 2 mL of ice-cold buffer D (50 mM Tris–HCl, pH 7.4, plus 1 mg/mL BSA) and vacuum filtration through Whatman GF/C filters [pretreated with polyethyleneimine (0.1%) for at least 2 hr]. Tubes were rinsed with 2 mL of ice-cold buffer D, which was also filtered, and the filters were subsequently rinsed twice with 4 mL of ice-cold buffer D. Before radioactivity was quantitated by liquid scintillation spectrometry, filters were shaken for 1 hr in 5 mL of scintillation fluid. Saturation experiments were conducted with 5–7 concentrations of radioligand ranging from 20 pM to 5 nM. The B_{max} and K_d values obtained from Scatchard analysis of saturation binding curves [15, 16] were determined by the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ).

RESULTS

The mouse CB1 gene was isolated from a mouse strain 129 genomic library. Oligonucleotide primers homologous to bp 1–21 and bp 843–824 on the opposite strand of rat CB1 were chosen for use in the PCR to generate an 843 bp fragment in mouse genomic DNA. In addition, PCR using primers based on the entire translated region of the cannabinoid receptor resulted in a 1.4 kb fragment, suggesting the absence of introns in this region of the receptor gene. The 843 bp fragment was sequenced and this sequence information used to design mouse-specific PCR primers for use in screening a mouse strain 129 P1 plasmid library (see Materials and Methods). P1 clones contain larger inserts (50–100 kb) than λ phage and cosmid clones (25 and 40 kb, respectively), while still constituting representative libraries. The large insert size increases the odds that a full-length gene is contained in the library. Furthermore, there are significant advantages in utilizing plasmid clones; the DNA is easy to grow and isolation of pure insert DNA is simpler as well.

Four P1 clones were isolated, and the Southern analysis is shown in Fig. 1. These clones contained inserts ranging from ~38 to ~62 kb. With the coding region of a rat CB1 cDNA [12] used as a probe, each clone showed an identical hybridization pattern. Therefore, the 6 kb *Bam*HI fragment of clone 2841 was subcloned into the pBS-SK$^+$ vector for sequencing. The sequence of the mouse CB1 gene encompassing the coding region is shown in Fig. 2. Examination of the 5′ untranslated sequence of this mouse CB1 genomic clone indicated a splice junction site 60 bp upstream from the probable translation start site, as has been reported for the human and rat CB1 receptors [13]. This splice site

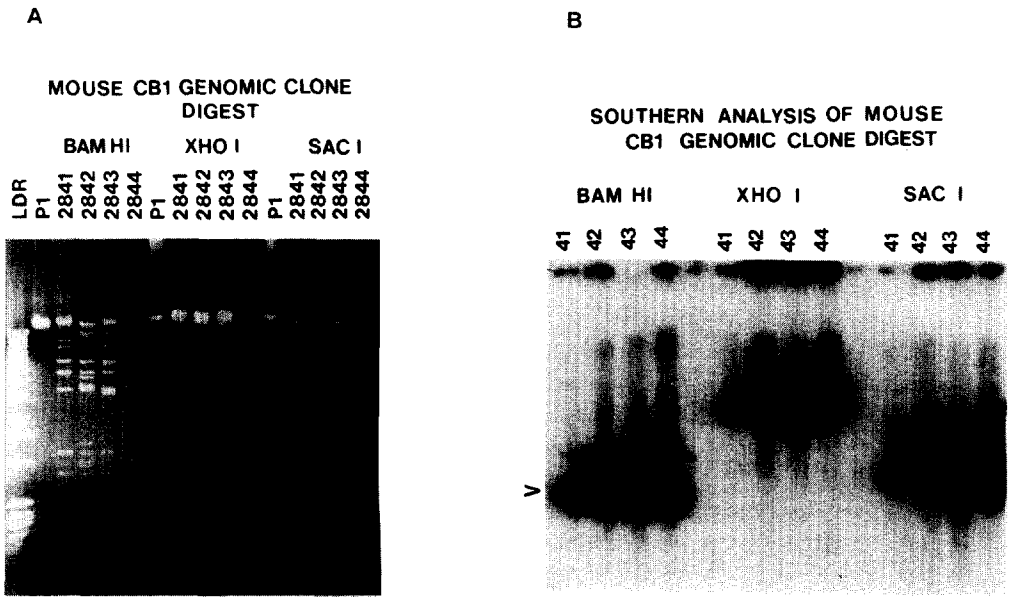

FIG. 1. Restriction digest and Southern analysis of mouse CB1 genomic clones. The parent plasmid (P1) and the four P1 clones (2841–2844) were digested with the indicated restriction enzymes. (A) Restriction analysis indicated that clones 2842 and 2844 are identical. (B) A probe containing the coding region of rat CB1 was used as a hybridization probe. The arrowhead marks the ~6 kb *Bam*HI fragment of clone 2841 isolated for sequencing of the coding region.

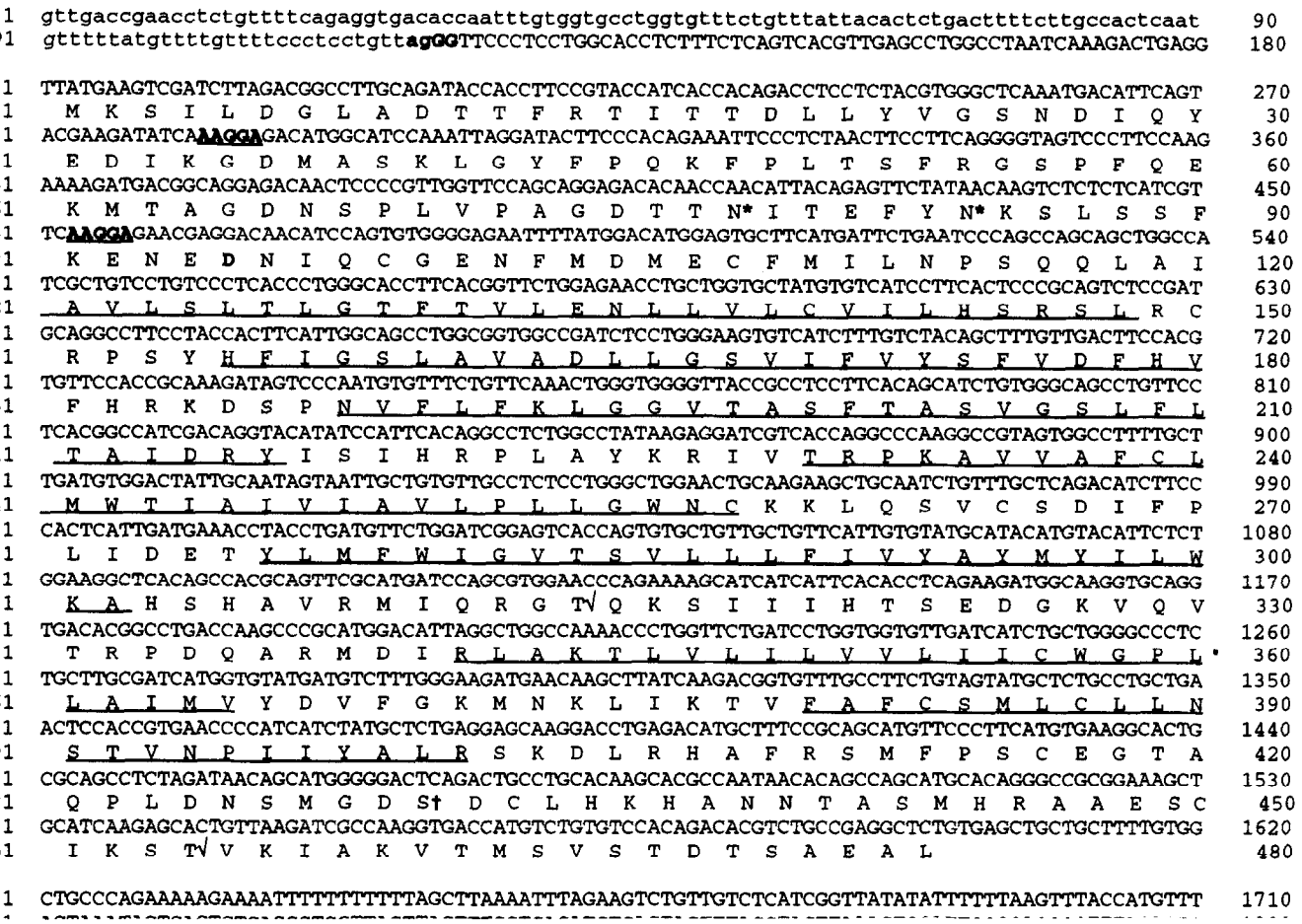

FIG. 2. Sequence analysis of the mouse CB1 gene. Sequences in the 5′ intron are denoted by lower case letters, and the splice junction site is indicated in bold. The sequences encompassing additional second splice junction sites are indicated in bold and underlined. The single amino acid difference between rat and mouse (E95D) is indicated in bold. The transmembrane domains are underlined according to Bramblett *et al.* [17]. The putative glycosylation, protein kinase C and G-protein-coupled receptor kinase phosphorylation sites are indicated by *, √, and †, respectively.

defines the 3′ end of an intron that is bordered by the exon shown (Fig. 2) and an untranslated 5′ upstream exon that will require further characterization (data not shown). There is a second splice junction site in the coding region for CB1 (which produces CB1A [13]), and this is also present in the mouse sequence shown (Fig. 2).

The mouse CB1 sequence is highly identical to the rat [3] (95% nucleic acid identity, 99.5% amino acid identity) and human [4] (90% nucleic acid identity, 97% amino acid identity) CB1 sequences. The rat and mouse sequences differ by only one amino acid and that is a conservative change (E95D, indicated in bold in Fig. 2). The transmembrane domains are designated (Fig. 2) according to Bramblett *et al.* [17]. The putative glycosylation, protein kinase C, and G-protein-coupled receptor kinase phosphorylation sites are indicated (Fig. 2).

Subsequent to our sequencing of the mouse gene encoding CB1, three other mouse CB1 sequences have appeared in the GenBank database (accession numbers U22948, U40709, and U17985). Our sequence is identical to U22948 (also a c129 strain P1 genomic clone, 100% over its 1654 bp) and to U40709 (a cDNA sequence from NG108–15 mouse neuroblastoma × rat glioma hybrid cells encompassing the coding region of CB1). These sequences are also highly identical to U17985 (from C57 strain mouse brain cDNA, 99% over 1459 bp).

The region of the mouse CB1 receptor gene that corresponds to the coding region was amplified using PCR and subcloned into the pcDNA3 expression vector. Stable cell lines in 293 cells were established which expressed the mouse CB1 receptor. No specific [^{3}H]SR 141716A or [^{3}H]CP-55,940 binding to 293 cells was found prior to transfection (data not shown). Specific binding in the mouse CB1 transfected cells was found to be linear at protein concentrations between 50 and 100 μg/mL, but dropped off at protein concentrations over 100 μg/mL (data not shown). Thus, 80 μg/mL of membrane protein was used in subsequent assays, where specific binding averaged 68%

at a radioligand concentration of 500 pM. Representative Scatchard analyses from a mouse CB1-293 cell line are shown (Figs. 3 and 4A).

Saturable, high-affinity binding was obtained with membranes prepared from the transfected cells, compatible with a single site (Hill coefficients of 0.99 and 1.05, Figs. 3 and 4A, respectively). Using [^{3}H]CP-55,940 as a radioligand, K_d values of 0.90 ± 0.13 nM and B_{max} values of 908 ± 45 fmol/mg were found (a representative experiment is shown in Fig. 3). When [^{3}H]SR 141716A was used as a radioligand, K_d values of 0.73 ± 0.13 nM and B_{max} values of 921 ± 58 fmol/mg were obtained (an example of this is shown in Fig. 4A). The level of receptor binding and the affinity for SR 141716A in these stably transfected cells were comparable to those found in the mouse brain (Fig. 4B with average K_d values of 0.16 ± 0.012 nM and B_{max} values of 1.81 ± 0.44 pmol/mg).

The mouse neuroblastoma cell line N18TG2 has been used extensively as a model system for examining the effects of cannabinoids on cellular signal transduction (reviewed in Ref. 18). However, it has been difficult to demonstrate the presence of cannabinoid receptors in these cells using radioligand binding, probably because they express low levels of CB1 receptors. While screening transfected cell lines for CB1 receptor expression, we found that we could detect lower levels of expressed receptors by using [^{3}H]SR 141716A as a radioligand. Therefore, we examined [^{3}H]SR 141716A binding to membranes prepared from N18TG2 cells. The results are shown in Fig. 4C. N18TG2 cells express low levels of CB1 binding sites (B_{max} = 197 ± 29 fmol/mg protein) as compared with mouse brain membranes (B_{max} = 1.81 ± 0.44 pmol/mg). Thus, while specific CB1 binding sites on N18TG2 cells could not be detected using [^{3}H]CP-55,940 as a radioligand (data not shown), they could be demonstrated using [^{3}H]SR 141716A. Furthermore, the affinity of CB1 receptors in N18TG2 cells was nearly identical to that in mouse brain (K_d values of 0.182 ± 0.08 and 0.16 ± 0.01 nM, respectively).

DISCUSSION

The isolation and expression of the mouse CB1 receptor gene allow further opportunity to probe the structure and regulation of this neuronal receptor. High levels of CB1 receptors can be stably expressed in 293 cells, which will facilitate the screening of novel drugs. We found the binding characteristics of the transfected cells expressing the mouse CB1 receptor gene to be very similar to those in mouse brain and in N18TG2 neuroblastoma cells expressing native receptor. Felder *et al.* [5] had shown previously that the binding properties of the human CB1 receptor genomic clone transfected into CHO cells were similar to native cannabinoid receptors in rat brain. Since the mouse receptor shares 97% amino acid identity with the human clone (and 99.5% identity with the rat clone) [3, 4], similar binding properties would be expected.

Sequence analysis of the mouse genomic clone indicates a splice junction site in the 5′ untranslated region 60 bp upstream from the probable translation start site. Thus, the intron/exon structure of the CB1 gene is conserved among species, as this site has also been reported for the human

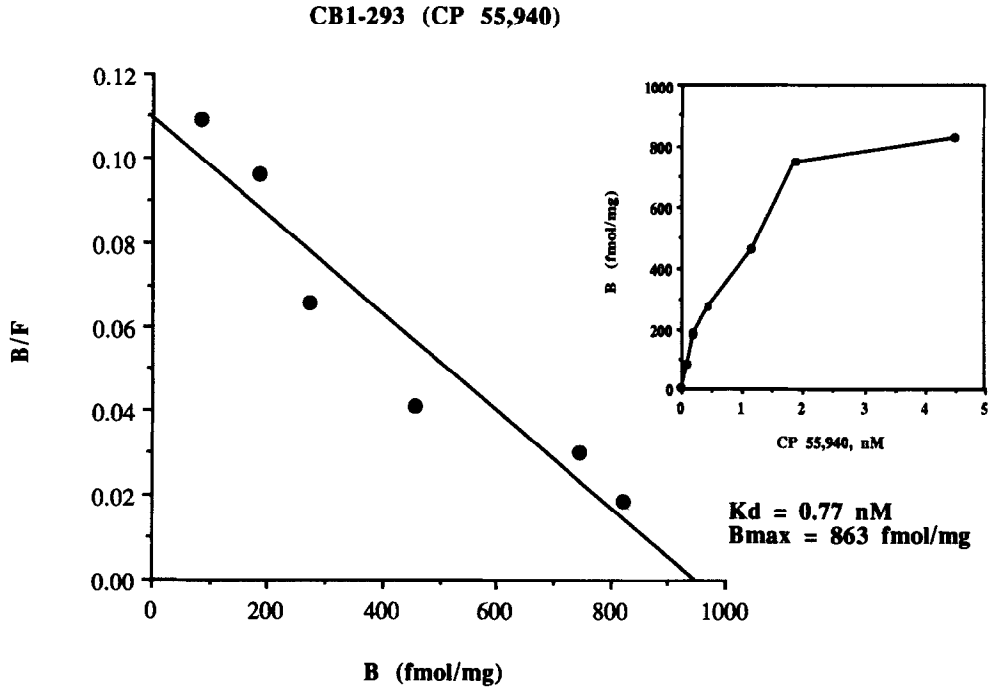

FIG. 3. Representative Scatchard and saturation (inset) analysis of [^{3}H]CP-55,940 binding to membranes prepared from transfected 293 cells expressing mouse CB1. In this experiment, K_d and B_{max} values were 0.77 nM and 863 fmol/mg protein for CB1. These data are representative of three experiments performed in triplicate.

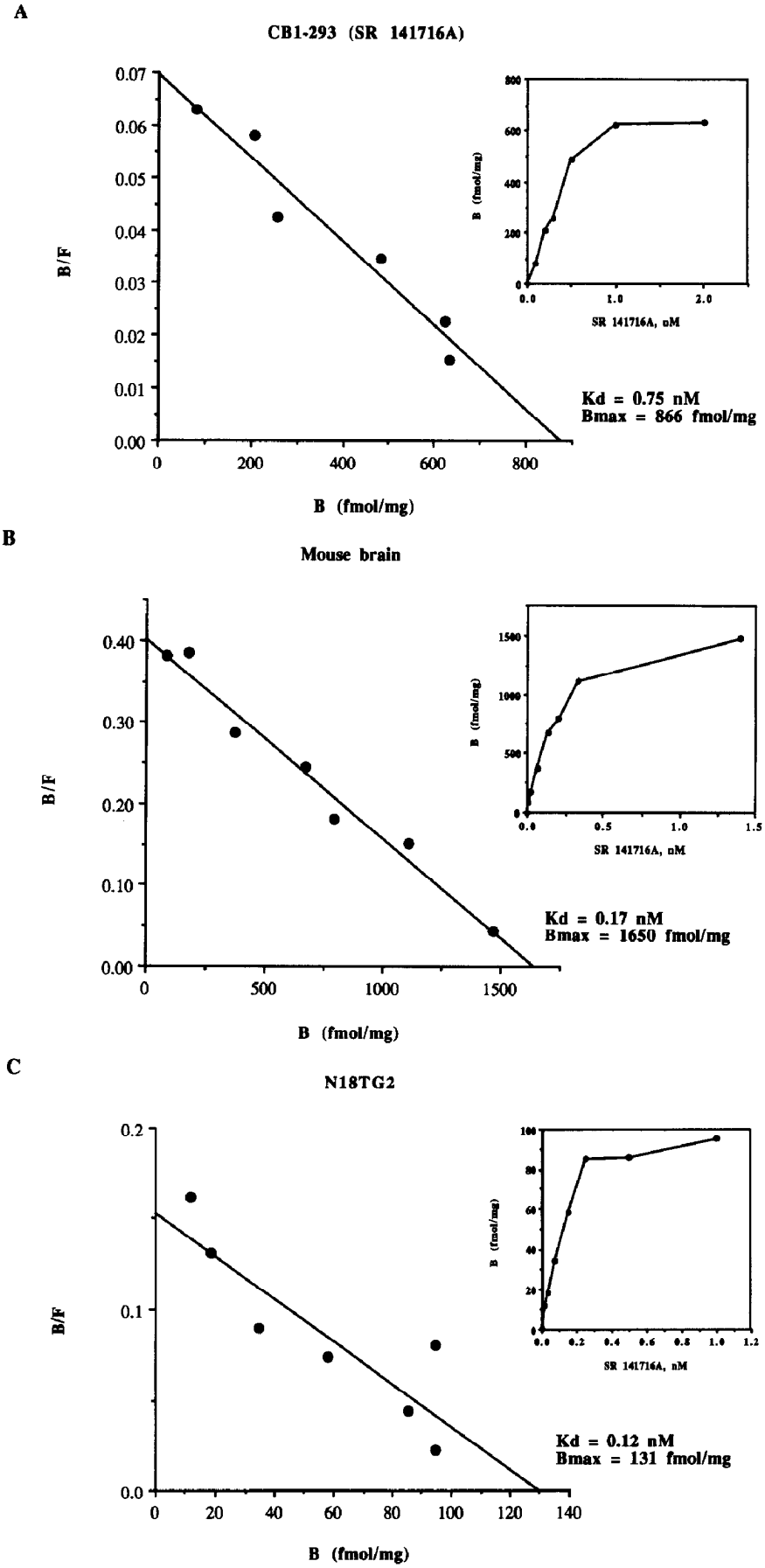

FIG. 4. Comparison of Scatchard and saturation (inset) analyses of [^{3}H] SR141716A binding in membranes prepared from: (A) transfected 293 cells expressing mouse CB1; (B) mouse brain, and (C) N18TG2 neuroblastoma cells. These data are representative of at least three experiments performed in triplicate.

and rat CB1 receptors [13]. There may be heterogeneity in the 5′ upstream untranslated exon, as the human and rat cDNA sequences diverge about 60 bp upstream of the translation initiation codon [3, 4]. In addition, we have isolated a rat CB1 cDNA clone that is identical to the published sequence in the coding region, but diverges about 60 bp upstream of the translation codon ([12] and unpublished results). These data suggest the possibility of splice variants of the CB1 receptor as well as divergence of other regulatory sequences between these genes.

There is a second set of splice junction consensus sites in the coding region of the mouse CB1 genomic sequence which could generate CB1A [13]. CB1A was isolated as a PCR amplification product from a human lung cDNA library and found to lack 167 base pairs of the coding region of the CG1 receptor between these putative splice junction sites [13]. This alternative splice form (CB1A) is unusual in that it is generated from the mRNA encoding CB1, and not from a separate exon [13]. If expressed, mouse CB1A would translate to a receptor truncated by 80 amino acids with 9 amino acids altered at the NH_2-terminal, which could lead to a receptor with altered ligand binding properties. However, our binding data suggest that the CB1 receptor expressed in 293 cells is consistent with a single binding site with an affinity similar to that of native CB1 receptors. Thus, if CB1A is produced in this cell line, it is expressed at very low levels relative to CB1.

We have also demonstrated the presence of specific CB1 binding sites on N18TG2 mouse neuroblastoma cells by using [^{3}H]SR 141716A as a radioligand. Although N18TG2 cells have been shown to possess cannabinoid receptors as evidenced by their high potency in mediating signal transduction with cannabinoids [18–23], it had not been possible to demonstrate specific binding sites on these cells. There is significant non-specific binding with [^{3}H]CP-55,940 [24], which is particularly evident when low amounts of receptor are expressed. Our initial binding studies indicated that non-specific binding was lower with [^{3}H]SR 141716A as compared with [^{3}H]CP-55,940, allowing us to detect lower levels of expressed receptors in transfected cells and in the N18TG2 cell line. Thus, it may be useful to examine [^{3}H]SR 141716A binding in other cell lines and tissues that are thought to possess CB1 receptors for which binding sites have not been described previously.

The elucidation of the structure and functional expression of the mouse CB1 receptor will facilitate the search for new and improved therapeutic agents that act through the cannabinoid system.

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