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Regulation of Mouse Neuropeptide Y Y_1 Receptor Gene Transcription: A Potential Role for Nuclear Factor-κB/Rel Proteins

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SUMMARY

We previously isolated a 1.3-kb genomic fragment in the 5′-flanking region of the murine neuropeptide Y (NPY) Y_1 receptor gene, which is able to drive the expression of *LacZ* reporter gene in neuronal cells. We determined the ability of deletion mutants of this region to modulate transcription of the heterologous luciferase gene in the Y_1 receptor-expressing neuroblastoma/glioma NG108-15 cells and the Y_1 receptor-deficient 293 cells. Results suggest the presence of a cell type-specific core promoter (−399 to −218 from the initiator ATG) and, upstream, of two positive and two negative regulatory elements. Sequence analysis of the Y_1 receptor promoter identified two decameric sequences corresponding to consensus binding sites for nuclear factor-κB/Rel proteins. Gel shift analysis indicated that a 29-bp oligonucleotide comprising the two putative κB sites, which we refer to as Y_1-κB sequence, specifically binds κB-related complexes in nuclear extracts from rat brain areas, NG108-15 cells, and the murine T cell clone A.E7. In nuclear extracts from A.E7 and NG108-15 cells, the Y_1-κB sequence specifically binds an additional complex whose molecular nature remains to be elucidated. Through transient transfection studies, we also demonstrated that the Y_1-κB sequence acts as an enhancer element, inferring its potential role in regulation of the Y_1 receptor gene expression.

NPY is the most abundant and widely distributed neuropeptide within the central nervous system, where it participates in the control of a large number of physiological functions, including effects on food intake, psychomotor activity, and central endocrine secretion and potent vasoactive effects on the cardiovascular system (1, 2). Two major subtypes of NPY receptors, Y_1 and Y_2, have been defined on the basis of pharmacological criteria; the Y_1 receptor is considered to be a postjunctional receptor, and the Y_2 receptor is considered to be a prejunctional receptor (3). We and others previously reported the molecular cloning of the Y_1 receptor cDNA from rat (4, 5), mouse (6), and human (7, 8) tissues; its primary structure shows that it belongs to the superfamily of G protein-coupled receptors. In peripheral tissues, the Y_1 receptor is found predominantly at the sympathetic postjunctional sites in blood vessels, where it mediates the contractile response to NPY of vascular smooth muscle, both directly and indirectly by potentiating the action of other pressure agents, such as norepinephrine (3). In the central nervous system, the Y_1 receptor has been linked with different physiological processes, including stimulation of feeding behavior (9), stimulation of luteinizing hormone-releasing hormone release (9), a sedative anxiolytic effect (10, 11), and modulation of inflammation and nociception (12, 13).

Recent studies have shown that a marked plasticity in the expression of the Y_1 receptor and its mRNA can be induced under different circumstances. For example, peripheral tissue inflammation evokes up-regulation of Y_1 receptor mRNA in dorsal root ganglia; in the same tissue, peripheral axotomy changes expression of the Y_1 receptor mRNA level (12, 13). The molecular mechanisms responsible for regulation of Y_1 receptor expression are unknown; however, like the mechanisms for β_2 receptors and other G protein-coupled receptor genes (14–16), they may result from alteration of the transcriptional regulatory pathway.

We recently cloned the murine gene of the Y_1 receptor, and we isolated a 1.3-kb genomic fragment of the 5′ flanking region that is able to drive the expression of the *lacZ* reporter gene in the mouse neuroblastoma/rat glioma NG108-15 cell line and in rat corticostriatal neuron primary cultures but not in the Y_1 receptor-deficient rat glial and human embryonic kidney 293 cells (6). Sequence analysis of this region

ABBREVIATIONS: NPY, neuropeptide Y; Y_1R-LUC, Y_1 receptor/luciferase fusion gene; κB-Y_1-LUC, Y_1-κB/Y_1 receptor/luciferase fusion gene; Y_1-κB, wild-type κB site(s) from the Y_1 receptor gene; mY_1-κB, mutated κB site(s) from the Y_1 receptor gene; DTT, dithiothreitol, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL, interleukin; Ig-κB, κB sequence from the immunoglobulin κ light chain enhancer region; AP-1, activator protein-1.

revealed the presence of several potential recognition sequences for known transcription factors, including two decameric sequences corresponding to consensus sites for members of the NF-κB/Rel family of transcription factors, three AP-1 sites, three half-palindromic estrogen-responsive elements, and one cAMP-responsive element (Fig. 1A).

In the current study, we further characterized the upstream promoter region of the murine Y_1 receptor gene through the use of transient transfection assays with NG108-15 and 293 cells. Analysis of Y_1-R/LUC constructs containing deletions of the Y_1 receptor regulatory region suggested the presence of a 181-bp cell type-specific core promoter spanning nucleotides −399 through −218 from the initiator ATG and, upstream of this region, of two positive and two negative regulatory elements. Furthermore, we present evidence that members of the NF-κB/Rel family of transcription factors may participate in regulation of the Y_1 receptor gene expression. The functional role of this family of inducible, ubiquitous transcription factors has been widely characterized in the periphery, where these proteins respond to a variety of signals and control expression of several genes mainly implicated in inflammatory and immune reactions (for reviews, see Refs. 17–19). More recently, several groups have shown that NF-κB is also abundant in brain, where it was found as both an inducible and a constitutively activated form (20–27). However, currently, very little is known about the role of NF-κB-related factors in regulation of the expression of genes whose products play a functional role in the central nervous system.

Materials and Methods

Cell culture. The mouse neuroblastoma/rat glioma NG108-15 cells were plated onto Falcon Petri dishes coated with 10 μg/ml poly-L-lysine (M_r 70–150 × 10^3) and were cultured in the minimum essential medium containing 10% fetal bovine serum and 1× HAT supplement (all from GIBCO, Grand Island, NY). The human embryonic kidney 293 cells were grown in minimum essential medium and 10% fetal bovine serum. Mouse fibroblast NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum (GIBCO). All culture media contained 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmid construction. A *Sal*I/*Bgl*II fragment from the original murine Y_1 receptor genomic clone (from nucleotides −1523 to −218 relative to the initiator ATG) was first subcloned into the polylinker of pBluescript SK^- (Stratagene, La Jolla, CA) (6) (Fig. 1B). The *Sal*I/*Bgl*II fragment was isolated by gel electrophoresis; then, the *Bgl*II-digested end (all restriction enzymes were from Boehringer-Mannheim Biochemicals, Indianapolis, IN) was filled in with the Klenow fragment of DNA polymerase I (Boehringer-Mannheim) and excess of dNTPs (0.4 mM). The resulting *Sal*I-blunt fragment was ligated into the *Sal*I/*Cla*I sites of pBluescript SK^- after the *Cla*I site of the plasmid was filled to obtain a blunt end. This construct (pBS-Y_1PR) contains 1305 bp of the 5′ flanking region of the murine Y_1 receptor gene and includes the first three sites of initiation of transcription (6). The Y_1R-LUC expression plasmids were con-

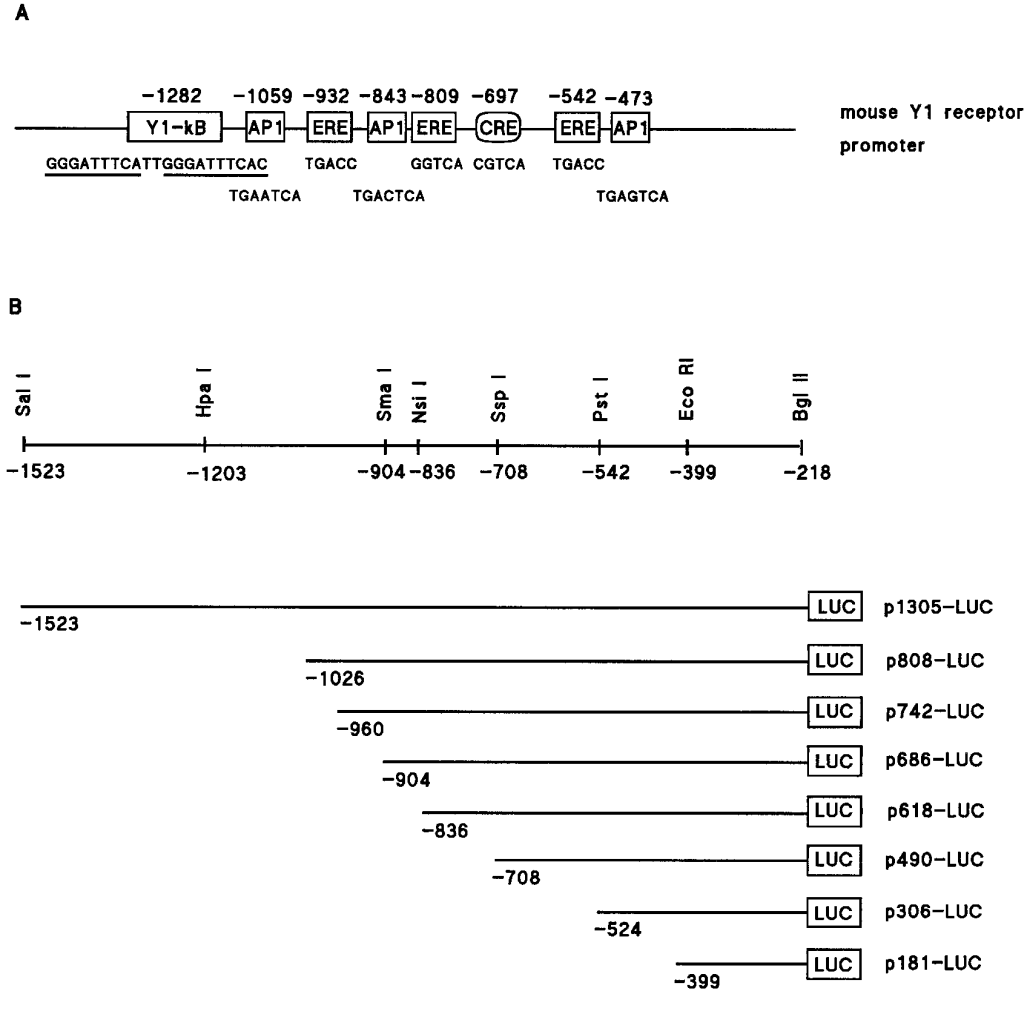

Fig. 1. Murine Y_1R-LUC expression plasmids. A, Putative *cis*-acting elements residing in the upstream region of the murine Y_1 receptor gene. *Above each box*, relative position of the proximal nucleotide in each motif in relation to the initiator ATG. *Below each box*, nucleotide sequences. *ERE*, estrogen-responsive element; *CRE*, cAMP-responsive element. *Underlined*, sequences in the Y_1-κB motif corresponding to the κB site. B, Luciferase fusion constructs containing deletion fragments of the murine Y_1 receptor 5′ flanking region. *Top*, restriction fragments that were used for deletion mutants.

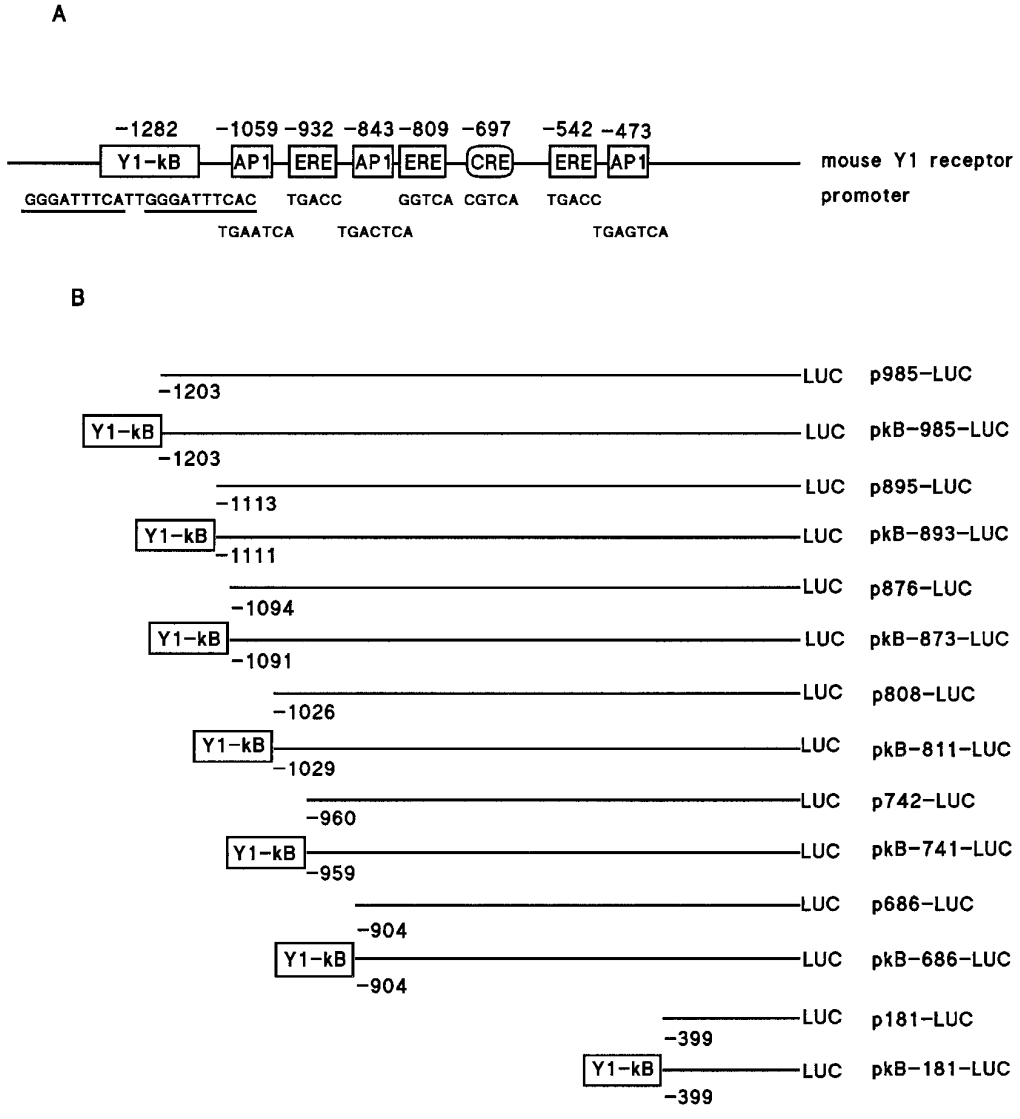

Fig. 2. κB-Y_1R-LUC expression plasmids. A, Putative regulatory elements residing in the 5′ flanking region of the Y_1 receptor gene. B, κB-Y_1R-LUC fusion genes containing the Y_1-κB motif ligated immediately upstream of different deletion fragments of the murine Y_1 receptor promoter. *Above each κB-Y_1R-LUC plasmid*, corresponding luciferase fusion constructs containing the deletion fragments of the murine Y_1 receptor 5′ flanking region. Mutant constructs were obtained as described in Materials and Methods and were confirmed by sequence analysis and restriction mapping. Restriction fragments that were used for deletion mutants are shown in Fig. 1.

structed through subcloning into the polylinker of pGL2-basic (Promega, Madison, WI) with the following restriction fragments from pBS-Y_1PR: a 1305-bp *Sal*I/*Hin*dIII fragment (p1305-LUC), a 985-bp *Hpa*I/*Hin*dIII fragment (p985-LUC), a 686-bp *Sma*I/*Hin*dIII fragment (p686-LUC), a 618-bp *Nsi*I/*Hin*dIII fragment (p618-LUC), a 490-bp *Ssp*I/*Hin*dIII fragment (p490-LUC), a 306-bp *Pst*I/*Hin*dIII fragment (p306-LUC), and a 181-bp *Eco*RI/*Hin*dIII (p181-LUC) fragment (see Figs. 1B and 2B). Constructs containing an upstream sequence between nucleotides −1113 and −960 (p895-LUC, p876-LUC, p808-LUC, and p742-LUC; see Figs. 1B and 2B) were obtained through digestion of pBS-Y_1PR with *Hpa*I and subsequent treatment with Bal31 enzyme (Boehringer-Mannheim). The plasmid DNA was then rendered blunt with the Klenow enzyme, digested a second time with *Hin*dIII, and subcloned into the *Sma*I/*Hin*dIII sites of pGL2-basic. The junctions between the insert DNAs and luciferase gene of the fusion constructs were confirmed through sequence analysis (28).

Oligonucleotides containing the Y_1-κB and mY_1-κB (see below) were synthesized with *Bgl*II overhangs, annealed, and inserted into the *Bam*HI site of pBluescript SK⁻, resulting in κB-pBS SK⁻ and mκB-pBS SK⁻.

To construct pκB-985-LUC, pκB-686-LUC, pmκB-686-LUC, and pκB-181-LUC plasmids, the deletion fragments of the Y_1 receptor 5′ flanking sequence were obtained from pBS-Y_1PR using the unique *Hin*dIII site from the pBluescript SK⁻ polylinker and the appropriate restriction sites in the upstream region of Y_1 receptor gene and were ligated immediately downstream of the wild-type and mutated κB sites of κB-pBS SK⁻and mκB-pBS SK⁻. The resulting plasmids were digested by *Xba*I, rendered blunt by Klenow enzyme, digested a second time with *Hin*dIII, and subcloned into the *Sma*I/*Hin*dIII sites of the pGL2-basic vector (see Fig. 2B). To obtain the pκB-893-LUC, pκB-873-LUC, pκB-811-LUC, and pκB-741-LUC plasmids, the pκB-686-LUC plasmid was digested by *Sma*I/*Hin*dIII to remove the 696-bp insert, resulting in κB-pGL2. The plasmid pBS-Y_1PR was then digested with *Hpa*I and subsequently treated with Bal31 enzyme. The plasmid DNA was rendered blunt by Klenow enzyme, digested a second time with *Hin*dIII, and ligated into the *Sma*I/*Hin*dIII sites of κB-pGL2 (see Fig. 2B). The sequences of the resulting κB/Y_1R-LUC expression plasmids were confirmed through restriction analysis and nucleotide sequence determination.

Sequencing of the Y_1R-LUC expression plasmids revealed seven nucleotide errors compared with the previously published 5′ flanking region of the Y_1 receptor gene.[1]

Transient transfection experiments. Transfection of reporter plasmids into NG108-15, 293 and NIH 3T3 cells was performed according to the calcium phosphate coprecipitation method (29). In all experiments, pSV-β-galactosidase control vector (Promega), containing the β-galactosidase gene linked to the simian virus 40 early

[1] The corrected DNA sequence is in the EMBL/GenBank database (accession No. Z18281).

promoter/enhancer, was included as an internal control for the different transfection efficiencies between experiments. When cells reached ~50% confluence, each 35-mm Petri dish received equimolar amounts (2.5 μg) of test plasmid and pSV-β-galactosidase. Cells were harvested 48 hr after transfection, and the activities of luciferase and β-galactosidase were assayed as previously described (30). As controls, the plasmid pGL2-basic, containing the promoterless luciferase gene, and the plasmid pGL2-promoter vector (Promega), containing the luciferase gene driven by the simian virus 40 promoter, were transfected into parallel cultures of each cell line. In all of the cell lines tested, the pGL2-basic was inactive, whereas the pGL2-promoter vector showed high levels of luciferase activity.

Pharmacological treatments of transiently transfected NG108-15 cells were performed 8 and 20 hr before processing of cells.

Nuclear extracts and electrophoretic mobility shift assays. Nuclear extracts from rat brain areas were prepared essentially as described by Kang *et al*. (31). Nuclear extracts from cell lines and from A.E7 cells were prepared according to a small-scale protocol (25) with minor modifications. Briefly, 5–10 $\times$ 10^6 cells were scraped into cold phosphate-buffered saline, washed once in phosphate-buffered saline, and pelleted for 10 sec in an Eppendorff centrifuge. Cells were then resuspended in 400 μl of cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride), allowed to swell on ice for 10 min, and vortexed for 10 sec. Samples were centrifuged for 10 sec, and the pellets were resuspended in 50 μl of cold buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min for high salt extraction. Cellular debris were removed by centrifugation for 2 min at 4°, and the supernatant was stored at −70°. Protein concentration was assessed by BioRad (Hercules, CA) Bradford assay according to the manufacturer's instructions. DNA binding reaction were initiated by the combination of 2 μg of nuclear extracts with 20,000 cpm (0.1 ng) of γ-^{32}P-labeled oligonucleotide probes in 1× lipage buffer (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol) containing 0.5 μg of poly(dI/dC) in a total volume of 10 μl. In competition experiments, indicated amounts of unlabeled competitor oligonucleotides were added with ^{32}P-labeled probes. Reactions were carried out for 20 min at room temperature, and protein/DNA complexes were resolved on nondenaturing 4% polyacrylamide gels in 1× Tris/glycine/EDTA buffer (1× = 50 mM Tris, 380 mM glycine, and 2.7 mM EDTA). Gels were then dried and subjected to autoradiography at room temperature.

Synthetic DNA oligonucleotides. Oligonucleotide sequences and their respective complementary strands were synthesized with a DNA synthesizer (Applied Biosystems, Norwalk, CT) and purified through denaturing gel electrophoresis. Oligonucleotides were annealed to complementary strands by heating to 68° and cooling slowly to room temperature. For gel shift analysis, double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP (> 7000 Ci/mmol; ICN Pharmaceuticals, Costa Mesa) and T4 polynucleotide kinase (Boehringer-Mannheim) to obtain a specific activity of $>10^8$ cpm/μg. The sequences of the oligonucleotides were as follows: Y_1-κB, 5′-GATCATGGGATTTCATTGGGATTTCACTT-3′ (sense); mY_1-κB, 5′-GATCCATctcATTTCATTctcATTTCACTT-3′ (sense); Ig-κB, 5′-CAGAGGGGACTTTCCGAGAGGC-3′; and octamer (octamer binding site from the IL-2 gene enhancer region), 5′-TATGTGTAATATGTAAAACATTTTGACACC-3′. Sequences corresponding to the κB site are underlined.

Statistical analysis. Statistical analysis was performed by using the Mann-Whitney *U* test.

Results

Analysis of the effect of progressive deletion within the Y_1 receptor promoter on heterologous gene expression. The ability of several deletion mutants of the 5′ flanking region of the murine Y_1 receptor gene to drive the expression of the luciferase reporter gene was analyzed in transient transfection experiments. As shown in Fig. 3, the 1.3-kb genomic fragment, spanning nucleotides −1523 through −218 relative to the initiator ATG (p1305-LUC), drives the luciferase activity in NG108-15 cells, whereas a very low enzyme activity was determined in 293 cells. Sequential deletion from nucleotides −1523 through −1026 (p985-LUC, p895-LUC, p876-LUC, and p808-LUC) had no significant effect on luciferase activity (Figs. 3 and 4). Further deletion to nucleotide −960 (p742-LUC) resulted in an ~2-fold increase in luciferase activity in NG108-15 cells but not in 293 cells, suggesting the presence of a negative regulatory element between nucleotides −1026 and −960 that is operative in NG108-15 cells. The promoter activity remained unchanged by removal of the region between −960 and −904 (p686-LUC), whereas further reduction of the upstream sequence to nucleotide −836 (p618-LUC) increased the luciferase activity by ~2-fold in NG108-15 cells but not in 293

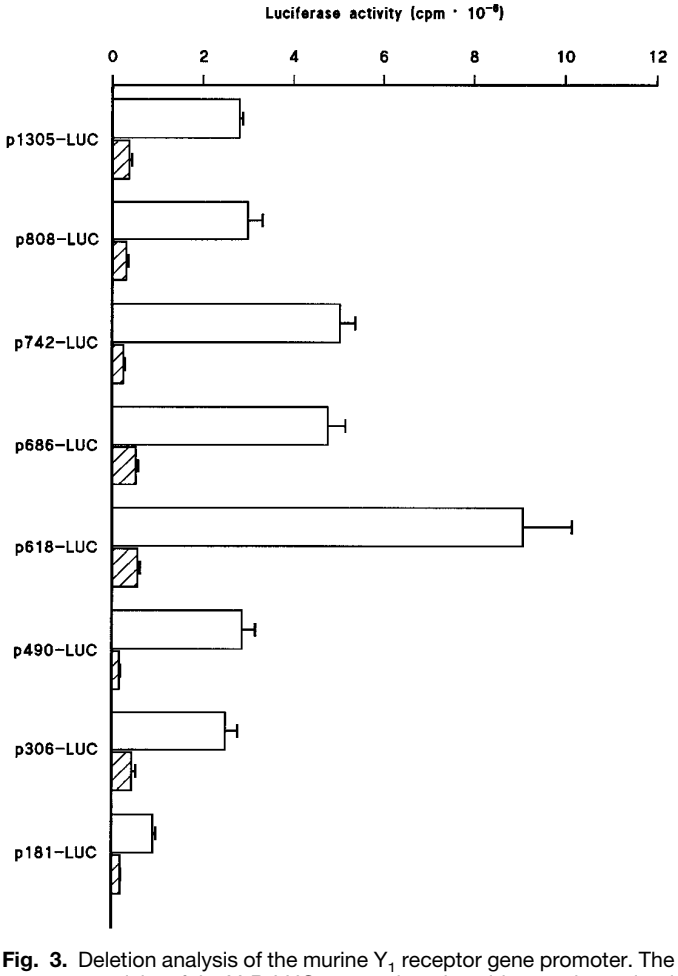

Fig. 3. Deletion analysis of the murine Y_1 receptor gene promoter. The promoter activity of the Y_1R-LUC expression plasmids was determined in (*open bars*) NG108-15 and (*hatched bars*) 293 cells. *Left*, tested reporter plasmids. The luciferase activity was normalized to β-galactosidase activity obtained by cotransfecting cells with the control plasmid pSV-β-galactosidase (in cpm). Values are mean ± standard error from eight or more transfection experiments, each performed in triplicate, with plasmid DNAs from at least two different preparations. Cultures transfected with the promoterless plasmid pGL2-basic had a mean luciferase activity of 0.16 ± 0.018 and 0.04 ± 0.003 cpm $\times$ 10^{-6} in NG108-15 and 293 cells, respectively.

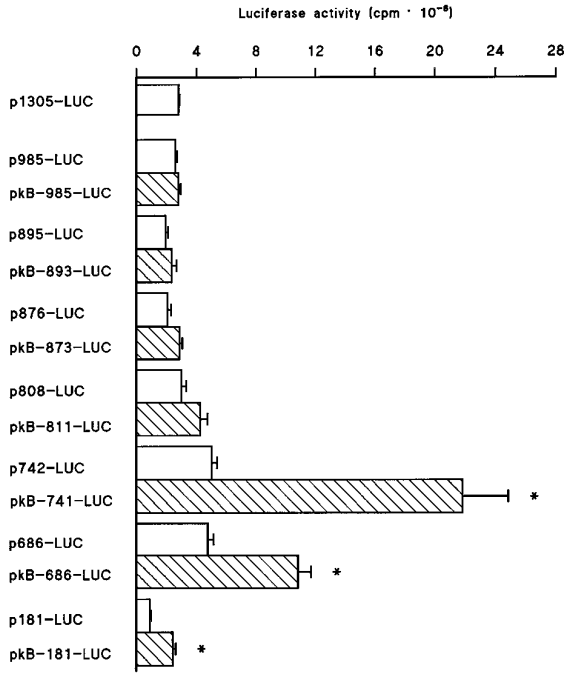

Fig. 4. Enhancer activity of the Y_1-κB sequence in NG108-15 cells. Histograms show the mean ± standard error of at least eight transfection experiments, each performed in triplicate. Each reporter plasmid tested (*left*) was independently prepared at least two times. The luciferase activity was normalized to β-galactosidase activity from a co-transfected internal control plasmid pSV-β-galactosidase (in cpm). The luciferase activity of pκB-741-LUC, pκB-686-LUC, and pκB-181-LUC was significantly more than that of p742-LUC, p686-LUC, and p181-LUC, respectively (*, $p < 0.05$).

cells, suggesting that the sequence between nucleotides −904 and −836 contains a negative regulatory element that contributes to lower Y_1 receptor gene expression in this cell type (Fig. 3). Further deletion to nucleotide −708 (p490-LUC) decreased the luciferase activity to the level driven by the undeleted Y_1 receptor promoter (p1305-LUC) in NG108-15 cells, suggesting the presence of a positive *cis*-acting element between nucleotides −836 and −708. Extension of 5′ deletion to nucleotide −524 (p306-LUC) did not affect the luciferase activity significantly, whereas further deletion to nucleotide −399 (p181-LUC) reduced the reporter gene expression by 2.8-fold in NG108-15 cells, suggesting the presence of a positive *cis*-acting element between nucleotides −524 and −399. The remaining 181 bp-genomic fragment, spanning nucleotides −399 and −218 of the Y_1 receptor gene, drives the luciferase activity in NG108-15 cells at a level significantly above the pGL2-basic (see legend to Fig. 3), indicating that it represents the minimal promoter region still capable of directing expression of the Y_1 receptor gene in the neuroblastoma/glioma cell line. It should also be pointed out that the same sequence is unable to drive luciferase activity in 293 cells. Furthermore, all of the Y_1 receptor deletion mutants/fusion constructs drove negligible luciferase activity when transiently transfected into the mouse fibroblast NIH 3T3 cell line (data not shown).

Binding of Y_1-κB sequences to members of the family of NF-κB transcription factors. Detailed sequence analysis of the Y_1 receptor promoter region reveals the presence of putative binding sites for known transactivating factors that may play a role in the regulation of the tissue-specific expression of this gene (Fig. 1A). Particularly, we focused our interest on the sequence located in position −1302 through −1282 in the Y_1 receptor gene regulatory region because it contains two decameric sequences corresponding to consensus sites for members of the NF-κB/Rel family of transcription factors (6). We performed experiments to verify whether the aforementioned sequences are indeed binding sites for transcriptional control proteins belonging to this family. A well-studied model of gene regulation mediated by κB/Rel proteins is represented by A.E7 cells, a $CD4^+$ murine T cell clone in which κB-mediated gene expression has been extensively analyzed. Kang *et al.* (31) demonstrated that these untransformed cells constitutively express at least two nuclear complexes belonging to the NF-κB/Rel family: the p50-p65 (relA) heterodimer and the p50 homodimer. In this well-characterized model, we initially studied binding properties of the putative κB sequences from the Y_1 receptor gene.

An oligonucleotide comprising the two κB sequences from the Y_1 receptor gene (Y_1-κB oligonucleotide) was synthesized, radioactively labeled at the 5′ end with T4 kinase, and incubated with nuclear extracts prepared from A.E7 cells. Sub-

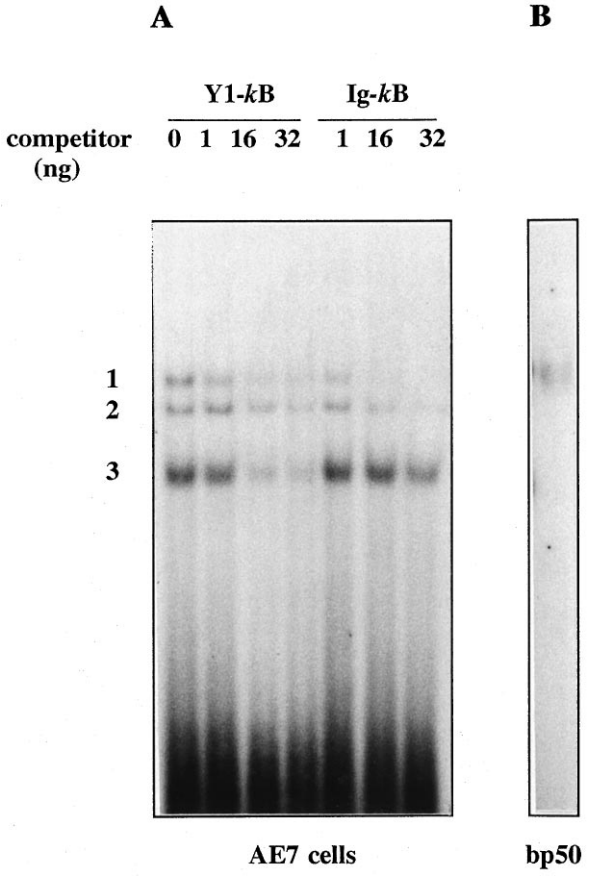

Fig. 5. Analysis of the binding properties of the Y_1-κB sequence on nuclear extracts from a T cell clone endowed with constitutive NF-κB/Rel activities and on the p50 subunit. A, Oligonucleotide sequence comprising the two κB sequences from the Y_1 receptor gene (Y_1-κB) binds three specific nuclear complexes in extracts from a murine T cell clone, A.E7. Electrophoretic mobility shift assay obtained through incubation of 2 μg of A.E7 nuclear extracts with ^{32}P-labeled Y_1-κB probe in the absence (0 ng) or in the presence of 1, 16, or 32 ng of unlabeled Y_1-κB or Ig-κB as competitors. B, Affinity purified, bacterially produced p50 subunit of the NF-κB/Rel family can bind the Y_1-κB oligonucleotide probe. *bp50*, bacterially purified p50.

sequently, binding reactions were analyzed in a gel shift assay; results are shown in Fig. 5A. The Y_1-κB probe could detect three nuclear complexes with different migration properties. All complexes proved to result from specific interaction with the DNA sequence because they were displaced, in a dose-dependent manner, by the unlabeled oligonucleotide Y_1-κB. To further analyze the relationship between these nuclear complexes and NF-κB/Rel proteins, an oligonucleotide sequence containing a classic κB site (Ig-κB) was tested for the ability to compete for binding to the Y_1-κB oligonucleotide. As shown in Fig. 5A, only the top two migrating complexes were competed, whereas the binding of the third complex was unaffected. These results suggested that the two higher-molecular-weight complexes able to bind the Y_1-κB sequence were indeed κB-related proteins, whereas the third complex, which was present in nuclear extracts from A.E7 cells, was also able to specifically bind the examined sequence, but as shown by the results of the competition analysis with the Ig-κB oligonucleotide, we could exclude that it was a κB-related protein or even a degradation product of the higher-molecular-weight κB complexes. To further prove the ability of the Y_1-κB sequence to bind κB-related complexes, we confirmed that bacterially expressed, affinity purified p50 protein, one of the members of the NF-κB/Rel family, was indeed able to bind the Y_1-κB probe in a gel shift assay (Fig. 5B).

Nuclear extracts were also prepared from NG108-15 cells and tested in a gel shift assay for binding to the ^{32}P-labeled Y_1-κB sequence. In analogy with what we observed in A.E7 extracts, three retarded complexes were detected (Fig. 6A, *lane 1*). When the same extracts were incubated with the Ig-κB oligonucleotide probe, two complexes were detected, comigrating with the top two complexes bound to the Y_1-κB probe (Fig. 6A, *lane 2*). A detailed competition analysis of the Y_1-κB bound complexes was performed (Fig. 6B). Increasing amounts (1–8 ng) of unlabeled Y_1-κB oligonucleotide (*lanes 2–4*) and Ig-κB (*lanes 5–7*) were used. The two higher-molecular-weight complexes were displaced by both competitors, whereas binding of the third complex seemed to be affected by the Y_1-κB but not by the Ig-κB oligonucleotide. None of the complexes were competed by an unrelated oligonucleotide containing the octamer protein binding site (*lanes 9–12*), even when a higher concentration (16 ng) of competitor was used (*lane 12*).

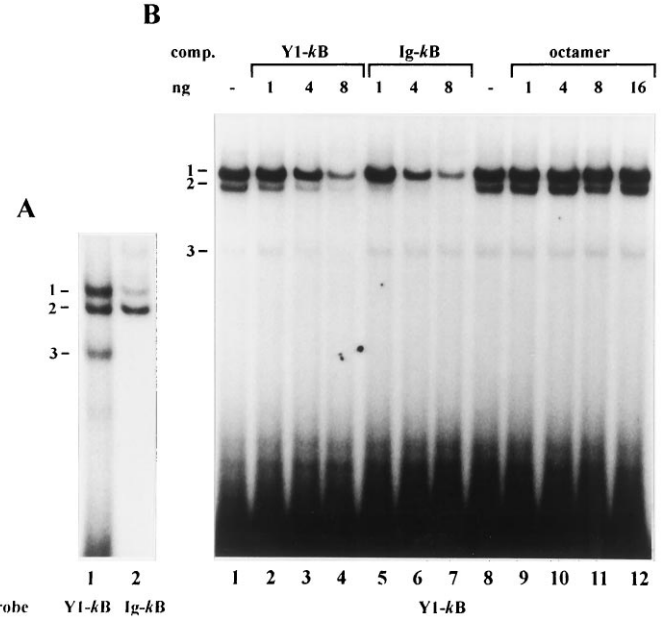

Fig. 6. Gel shift analysis of nuclear complexes specifically interacting with the Y_1-κB probe in NG108-15 cell extracts. A, Comparison of the migration properties of complexes intercepted by (*lane 1*) Y_1-κB and (*lane 2*) Ig-κB oligonucleotide probes in NG108-15 cell extracts. B, Competition analysis of the complexes bound by the Y_1-κB oligonucleotide probe. Competition was performed by adding the indicated amounts (in ng) of the unlabeled oligonucleotide sequences Y_1-κB (*lanes 2–4*) or Ig-κB (*lanes 5–7*) or the unrelated oligonucleotide sequence for octamer binding proteins (*lanes 9–12*). −, No competitor (*lanes 1 and 8*). Apparent discrepancies in the migration properties of complexes in A and B are due to differences in gel running length in the two different sets of experiments.

Interaction between the Y_1-κB sequences and DNA-binding factors was further investigated in nuclear extracts from several rat brain regions, including cortex, hippocampus, striatum, cerebellum, and olfactory bulb. Results of a representative gel shift assay are shown in Fig. 7. Surprisingly, in this situation, a single DNA binding activity was detected, which seemed to be specific because it could be competed by unlabeled Ig-κB sequence [Fig. 7, *lane 6*, shows competition on nuclear extracts from rat cortex, but the same results were obtained with extracts from the other rat brain regions (not shown)].

The Y_1-κB oligonucleotide sequence from the Y_1 receptor gene acts as enhancer element in NG108-15 cells. Although the deletion from nucleotides −1523 to −1203 (p985-LUC) in the Y_1 receptor sequence did not affect luciferase activity in our *in vitro* model (Fig. 4), based on the essential role of the NF-κB/Rel family of transcription factors for the expression of several genes (for reviews, see Refs. 17–19), we surmised that the Y_1-κB sequence may participate in the regulation of the transcriptional activity of this gene. To test this possibility, we constructed a series of expression plasmids (pκB-Y_1R-LUC) in which an oligonucleotide corresponding to the Y_1-κB sequence was placed immediately upstream of deletion fragments of the Y_1 receptor 5′ flanking region that did not contain the endogenous sites (Fig. 2B). Results indicated that the Y_1-κB sequence, when placed upstream of nucleotides −959, −904, and −399 from the initiator ATG (pκB-741-LUC, pκB-686-LUC, and pκB-181-LUC, respectively), enhances by >2-fold the luciferase activity in NG108-15 cells (Fig. 4). Specificity of the effect was demonstrated by the fact that mutation of selected nucleotides within the motifs, which abolished binding activity in gel shift assay (not shown), completely abolished enhancer activity (Fig. 8). Interestingly, the Y_1-κB sequence seemed to be functional in NG108-15 cells but not in 293 cells (Fig. 8). Furthermore, the Y_1-κB motif failed to increase luciferase activity when placed upstream of nucleotides −1203 (pκB-985-LUC), −1111 (pκB-893-LUC), −1091 (pκB-873-LUC), and −1029 (pκB-811-LUC) suggesting that the 70-bp sequence spanning nucleotides −1029 and −959 of the Y_1 receptor promoter contains a negative regulatory element that inhibits Y_1-κB enhancer activity in NG108-15 cells (Fig. 4). The κB-related factors were found as both inducible and constitutively activated complexes in the central nervous system (20–27). The κB nuclear activity interacting with the Y_1 receptor gene is constitutive. We verified whether specific extracellular signals might further activate the enhancer

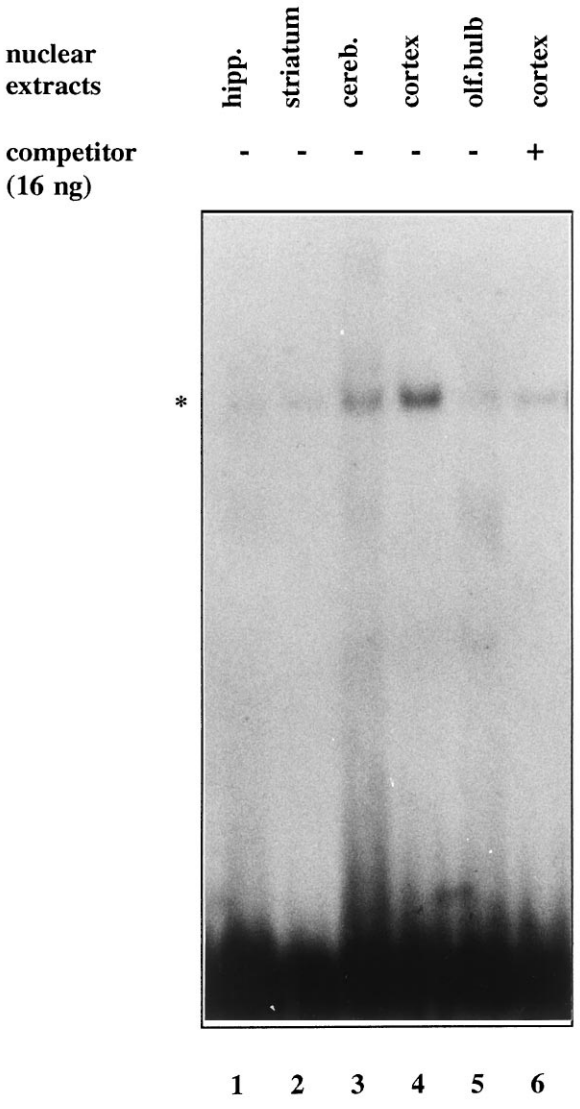

Fig. 7. The Y_1-κB oligonucleotide sequence binds a single κB-related complex in nuclear extracts from several rat brain regions. Protein extracts were from hippocampus (*lane 1*, *hipp.*), striatum (*lane 2*), cerebellum (*lane 3*, *cereb.*), cortex (*lanes 4 and 6*), and olfactory bulb (*lane 5*, *olf. bulb*). *Lane 6*, binding competition was performed with 16 ng of the unlabeled oligonucleotide Ig-κB and nuclear extracts from cortex.

activity of the Y_1-κB sequence. To investigate this possibility, we treated NG108-15 cells transfected with pκB-686-LUC or pκB-181-LUC plasmids with various agents that are known to modulate activity of these transcriptional regulators in either peripheral or central nervous system-derived cells (17, 22, 25–27). In particular, we tested cytokines, such as IL-1 (30 units/ml), IL-2 (2 nM), and tumor necrosis factor-α (100 ng/ml); lipopolysaccharide (25 μg/ml); concanavalin A (25 μg/ml); 12-*O*-tetradecanoylphorbol-13-acetate (0.1 μM); hydrogen peroxide (50 and 100 μM), KCl (30 mM); and glutamate (100 μM). However, none of these agents were able to further augment the transcriptional activity of pκB-686-LUC or pκB-181-LUC expression plasmids (data not shown).

Discussion

The Y_1 receptor subtype plays important roles in mediating NPY-induced control of several functions, including cardiovascular system activity, neuroendocrine secretion, food intake, and nociception (1, 2). *In situ* hybridization studies have shown that Y_1 receptor mRNA is indeed highly expressed in several regions of the rat forebrain, in the hypothalamus, and in dorsal root ganglia (4, 13, 32, 33). We previously isolated the murine gene encoding the Y_1 receptor and demonstrated that the 5′ flanking region of this gene contains a functional promoter that is active in neuronal cells (primary cultured neurons and NG108-15 cells) but not in glial or 293 cells (6).

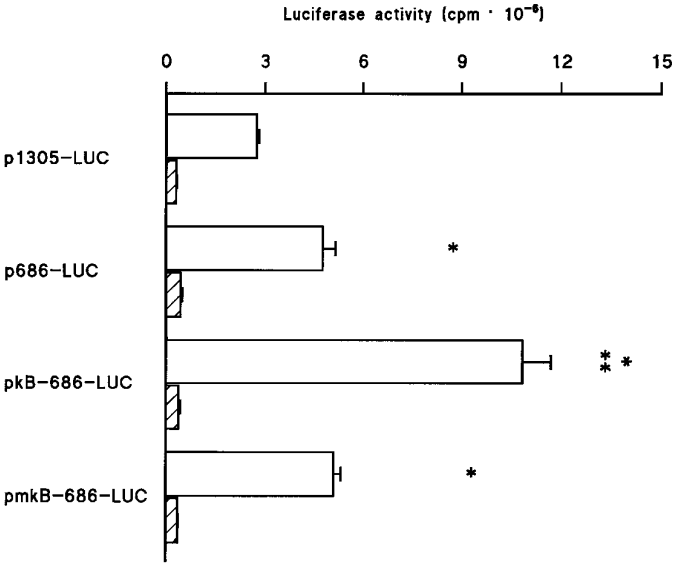

Fig. 8. Mutational analysis of the Y_1-κB sequence. A three-nucleotide mutation was made in each κB motif from the Y_1-κB oligonucleotide sequence as described in Materials and Methods. The pmκB-686-LUC fusion plasmid was prepared by ligating the mutated Y_1-κB motif (mY_1-κB) immediately upstream of the 686-bp (*Sma*I/*Hin*dIII) deletion fragment of the Y_1 receptor promoter. Promoter activity of the p1305-LUC, p686-LUC, pκB-686-LUC, and pmκB-686-LUC was determined in (*open bars*) NG108-15 and (*hatched bars*) 293 cells. The enhancer activity of the mutated Y_1-κB motif was depicted in NG108-15 cells. *, $p < 0.05$ versus p1305-LUC. **, $p < 0.05$ versus p686-LUC.

In the current study, we investigated which regions of the Y_1 receptor promoter contain potential negative or positive *cis*-acting elements participating in the regulation of gene expression. For this purpose, luciferase constructs comprising the 1.3-kb 5′ flanking regions of the Y_1 receptor or various 5′ deletions of it were transfected into a cell line that expresses the Y_1 receptor endogenously, NG108-15 cells, and in the Y_1 receptor-deficient 293 cell line. Deletional analysis has shown the presence of several potential areas of transcriptional regulation. Our data suggest the presence of at least two positive acting regulatory elements lying between nucleotides −836 and −708 and between nucleotides −524 and −399. Although the sequence of the fragment contained between −836 and −708 has no clear homology to any known regulatory element, it is noteworthy that an AP-1 binding site resides between nucleotides −473 and −479. Further mutational and deletional analysis will be required to define whether this site functions as a *cis*-acting element. In preliminary experiments, we were able to show that the Y_1 receptor promoter/luciferase reporter gene can be positively modulated by treatment with phorbol esters but that deletion of the sequence containing the AP-1 site (−473 to −479) fails to suppress this type of responsiveness (data not shown).

A second type of regulation that may exist for the Y_1 receptor gene expression involves silencer domains. Our work suggests the presence of at least two negative regulatory elements contained between nucleotides −1026 and −960 and between nucleotides −904 and −836. To assess the cell type specificity of these sequences, 293 and NIH 3T3 cells were transfected with the corresponding luciferase constructs. All deletion mutants were ineffective in modulating transcription of the heterologous luciferase gene in these cell lines, indicating that the negative regulatory elements of the Y_1 receptor gene are not responsible for repression in these cell lines.

Reporter gene assay also suggested that the core promoter (−399 to −218) of the Y_1 receptor gene exhibited substantial cell type specificity. Ball *et al*. (34) recently reported that the human Y_1 receptor gene is under the control of three promoters that are activated in a tissue-specific manner. It is noteworthy that the core promoter of the murine Y_1 receptor gene displays a high sequence homology with the corresponding region of the human promoter directing the expression of the most abundant Y_1 receptor transcript.

Detailed analysis of the sequence of the murine Y_1 receptor 5′ flanking region reveals the presence of many putative binding sites for known transcription factors (6) (Fig. 1A). We focused our attention on the murine Y_1 receptor promoter region that contains two decameric sequences located in tandem in position −1302 to −1282 bp, relative to the ATG. These sequences correspond to consensus sites for members of the κB-Rel family of transcription factors (17). In the current study, we showed that this sequence can indeed bind κB-related nuclear complexes in a specific manner and acts as an enhancer element in transiently transfected NG108-15 cells.

NF-κB/Rel proteins are constitutive and inducible transcription factors that are present in most cell types. Each κB complex corresponds to homodimers and heterodimers whose subunits belong to a superfamily that comprises at least five DNA binding proteins: p50, p52 (p50B), p65 (RelA), c-rel, and RelB (17–19). The inducible form of NF-κB contains an additional inhibitory subunit called IκB and can be activated in response to stimuli that mostly represent pathogenic conditions, including viruses, bacterial lipopolysaccharide, inflammatory cytokines, and oxidants. It was previously suggested that only a limited number of lymphoid cells contain constitutively active NF-κB-related factors (35, 36). More recent evidence, however, indicates that in the central nervous system, members of the κB family of transcription factors are constitutively active and are present in the nucleus of cultured neurons as well as in neurons *in vivo* (21, 24, 25).

Our data demonstrate that the Y_1-κB sequence binds with high affinity members of the κB/Rel family of transcription factors in nuclear extracts from rat brain areas, from the NG108-15 neuronal cell line, and from the murine T cell clone A.E7. Interestingly, different binding properties were observed in nuclear extracts from different sources. In nuclear extracts from rat brain regions, a single κB-related complex was detected. In nuclear extracts prepared from the cell line NG108-15, as well as in extracts from the murine T cell clone A.E7, three complexes with different migration properties interacted specifically with the Y_1-κB sequence, but only two of them seemed to be κB-related nuclear activities in competition experiments. The molecular nature of the third complex, specifically interacting with the Y_1-κB sequence, remains to be elucidated. Furthermore, detailed mutation studies are necessary to better define the binding requirements of each complex to the Y_1-κB sequence containing the two κB sites. Also, it will be interesting to clarify the significance of the differences in binding activities in rat brain extracts compared with extracts from NG108-15 cells and from the murine T cell clone A.E7. It is noteworthy that the single complex identified by the Y_1-κB oligonucleotide probe in rat brain extract is reminiscent of the binding specificity of a κB-binding site recently identified and characterized in the regulatory region of the amyloid precursor protein, which in rat brain extracts recognizes specific complexes that are either identical or very similar to p50 homodimers (25).

In transient transfection assays, we also demonstrated that the Y_1-κB sequence behaves as an enhancer element when placed upstream of deletion fragments of the Y_1 receptor regulatory region. Surprisingly, the Y_1-κB site does not enhances the transcriptional activity of the fusion gene constructs when placed ≥70 bases upstream of nucleotide −959 relative to the initiator ATG. These results suggest that the 70-bp region lying between −1029 and −959 bases of the 5′ flanking region of the Y_1 receptor gene might contain a negative regulatory element that is able to suppress the enhancer activity of Y_1-κB sequence in NG108-15 cells.

The NF-κB activity interacting with the Y_1 receptor gene promoter seems to be a constitutive activity, but it is well known that these transcriptional activators can be present as both activated and inducible forms in neurons (20, 22, 25–27). A wide variety of stimuli can modulate NF-κB/Rel activities, depending on the cell type (17–19). However, the treatment of NG108-15 cells with several agents, including inflammatory cytokines, oxidants, bacterial lipopolysaccharide, and neurotransmitters, failed to stimulate the transcriptional activity of the Y_1-κB sequence. It is possible that in NG108-15 cells, the intracellular signals that activate NF-κB proteins are coupled to specific membrane receptors that we were unable to identify, or that in this tumoral cell line, the Y_1-κB binding activity is maximally up-regulated. To answer these questions, we are analyzing the modulation of the Y_1-κB binding activity in primary cultures of neuronal cells.

The results that we report are, to our knowledge, the first demonstration that a κB-related motif contained in the regulatory region of a neuropeptide receptor gene binds κB-related nuclear proteins and might be activated by this family of transcription factors. The functional significance of these data remains to be elucidated. Kaltschmidt *et al*. (22) suggested that in neurons, the κB-related factors might participate in the normal physiology and development of the nervous system. Sequence analysis revealed the presence of putative κB-related sequences in the regulatory region of other neuropeptide receptor genes, such as the vasoactive intestinal peptide receptor (37) and the δ-opioid receptor (38). It is possible that this family of transcription factors participates in the control of neurotransmission by transcriptionally regulating the expression of neuropeptide and neurotransmitter receptor genes.

In most cell types, the κB-related proteins mediate an immediate-early response to stimuli that represent stress conditions. Vascular responsiveness to NPY was shown to be

increased in conditions that were occurring physiologically during prolonged stress or in disease states, such as hypertension (39). In addition, NPY seems to play a critical role in the transmission of stress-related information to the hypothalamic/hypophysial system and in the activation of neuroendocrine responses essential for the survival of the organism (40). An interesting possibility is that the Y_1 receptor for NPY may represent one of the κB site-containing genes that is modulated in the mammalian nervous system by κB-related factors in response to stimuli that require an immediate defensive response.

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