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Identification of a Promoter Region in the Rat Prion Protein Gene[1]

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We have demonstrated the presence of a rat prion protein (RaPrP) gene promoter upstream of multiple initiation sites. A 0.1-kb fragment upstream of the 5′-untranslated region contains specific DNA motifs characteristic of promoter elements including an AP-1 binding site, an inverted CCAAT motif and three inverted Sp-1 binding sites. This fragment directs transcription of a luciferase reporter gene in pheochromocytoma cells (PC12) and rat glioma cells (C6), suggesting that it contains the promoter for the RaPrP gene. To more precisely localize the transcription regulatory elements in this region, a series of 5′-deletion mutants were generated. Deletion analysis showed that an inverted CCAAT and adjoining Sp-1 binding sequences may play an important role in transcription of the RaPrP gene.

The prion protein (PrP) is a mammalian plasma membrane protein that is most closely associated with the development of neurodegeneration in prion-induced diseases in humans and animals (1, 2). The scrapie isoform of the PrP, PrP^{Sc}, is a necessary component of infectious scrapie particle, prion, which is a posttranslational modified form of a host-encoded cellular prion protein (PrP^{C}) (1, 3).

The rat PrP (RaPrP) gene spans 16 kb, and contains 3 exons encoding PrP by the third exon (4), which is similar to the genomic structure of the mouse PrP gene (5). On the other hand hamster (3) and human (6) PrP genes encompass two exons with the second exon encoding PrP. All previously reported PrP genes in animals including human, lack a TATA box in the 5′-flanking region upstream of transcriptional start sites (3–7). The rodent PrP genes appear to have multiple initiation sites, in contrast to those of the human and sheep in which a single and two major transcriptional start sites, respectively, have been observed (3–7). Although the genomic organization of the rodent PrP genes has been determined, the mechanisms controlling PrP gene expression are still poorly understood. The characterization of the PrP gene regulation is important for evaluating how the expression of this gene is related to the development and incidence of inherited or acquired prion diseases.

Recently, we determined the genomic structure of the RaPrP gene which contains 1 kb of the 5′-flanking region (4). In this study, to examine the transcriptional regulation of PrP gene, we determined the nucleotide sequence of 2.8-kb upstream of exon 1. We have demonstrated, for the first time, a specific region of the RaPrP gene that is involved in regulating transcription. Transient transfection in PC12 and C6 was used to investigate the RaPrP gene promoter activity.

MATERIALS AND METHODS

Cell Culture

The rat pheochromocytoma cell line, PC12, was maintained in DMEM supplemented with 10% FBS and 10% horse serum. The rat glioma cell line, C6, was cultured in DMEM with 10% FBS.

[1] The nucleotide sequence data reported in this in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number: D50092.

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Abbreviations: PrP, prion protein; RaPrP, rat prion protein; bp, base pair(s); kb, kilobase(s); DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; Luc, luciferase; RACE, rapid amplification of cDNA end.

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Construction of Plasmids for Promoter Analysis

The rat genomic-library, prepared from-liver DNA in the λDASH II phage vector (Stratagene), was screened as described previously (4). An 8.4-kb *Hin*d III fragment from the RaPrP genomic DNA clone (λRP2112) (4) covering the 5′-upstream region of the gene was subcloned into the pBluescript II KS+ plasmid (Stratagene) and sequenced on both strands using the Sanger dideoxy method in ALF DNA sequencer (Pharmacia). The plasmid containing a *Hin*d III fragment of the gene was used as a DNA template for PCR to generate deletion mutants of the RaPrP promoter. Two oligonucleotides were used as primers (PP166, 5′-ttaagtAAGCTTTTAAAGCCACTTCCTG-3′ and PN164, 5′-tgccaagcttCTGCCACCGACGCGAC-GCTCAG-3′) for PCR, were synthesized to amplify base pairs −2831 to +47 of the gene. The PCR product had the restriction enzyme site *Hin*d III on both ends and was inserted directionally into pGL2-Basic (Promega) in the sense orientation [praPrP(−2831)-luc plasmid] or in the reverse orientation [praPrP(−2831)r-luc plasmid]. Similarly, the oligonucleotides used as 5′ primers were 5′-taagtaagcTTTATATACAT-GGCATGGGAATA-3′, 5′-taagtaagcTTGGTGGTGTTGAC-CAGCCA-3′, 5′-taagtaagcTTG-GACAGCCTTGATTT-AAGTA-3′, and 5′-taagtaagcTTACGG-TGGTTTATGTATGATATC-3′ for praPrP(−2114)-luc, praPrP(−1481)-luc, praPrP(−1026)-luc and praPrP(−514)-luc, respectively. The 3′ oligonucleotide, PN164, was the same as that used to generate praPrP(−2831)-luc in all cases. Other series of 5′-deletion mutants, praPrP(−350)-luc to praPrP(+16)-luc (see Figure 2), were generated using DNA modifying enzymes (exonuclease III, mung bean nuclease, klenow fragment, and T4 DNA ligase) (8). All inserts were sequenced to verify proper orientation and fidelity of PCR and to confirm the 5′-end of each mutant.

Transient Transfections and Luciferase Assay

Transfection of plasmid DNA was carried out by electropolation using 20 μg of test plasmid and 10 μg of pSV/β-gal (Promega). Log-phase cells (2.0 × 106) were placed in 400 μl of DMEM with 20% FBS and electroporated by a ECM600 (BTX, CA, USA) at 700 μF and 220 V in a 0.2-cm cuvette. Luciferase (Luc) activity in cell lysates prepared 48 h post-transfection was measured as relative light units using a TD-4000 luminometer (Futaba Medical, Tokyo, Japan) and Luciferase Assay System according to the manufacturer's protocol (Promega). Beta-galactosidase activities present in each lysate, measured by a colorimetric enzyme assay using o-nitrophenyl-β-D-galactopyranoside as a substrate, were used to normalize the transfection efficiency between different experiments. The promoterless Luc vector (pGL2-basic) and SV40 promoter containing Luc vector (pGL2-control, Promega) were similarly transfected as negative and positive controls of transfection, respectively. All transfection experiments were repeated, at least, five times with two different DNA preparations.

RESULTS AND DISCUSSION

The cDNA sequence and genomic structure of the RaPrP gene has been reported previously (4, 9). However, the structure of the upstream regulatory region of the RaPrP gene was not completely characterized. The nucleotide sequence of the 5′-flanking region was determined between bp −2831 and +168 relative to the transcriptional start site indicated as 0 (Figure 1). Sequence analysis of this region revealed the presence of a CCAAT element (12) and several consensus binding sites for the transcription factors, AP-2 (10), AP-1 (11) and Sp-1 (13), which are required to promote constitutive or induced transcription from many viral or cellular eukaryotic promoters. However, the potential CCAAT element and three repeated Sp-1 binding sites were located in the reverse orientation (Figure 1). In this region, there were no consensus binding sequences for CRE, NF-κB and OTF-I in both orientations.

To study the mechanisms underlying transcriptional regulation of the PrP gene, chimeric Luc plasmids containing serial deletions, from −2,831 to +16 bp, of the 5′-flanking region of the RaPrP gene were prepared and transfected into PC12 and C6 cells. The resulting Luc activity was normalized with respect to the β-galactosidase activity expressed from a co-transfected plasmid (pSV-β-Galactosidase, Promega). The transient transfection of the fusion construct, praPrP(−2831)-luc, containing 2,871 bp of upstream sequence including an entire exon 1 efficiently directed the expression of ∼660-fold greater Luc activity than the promoterless pGL2-basic plasmid, whereas the same fragment in the reverse orientation, praPrP(−2831)r-luc, did not (Figure 2A, B and C). Thus, the 2,871-bp genomic fragment functioned as a promoter in an *in vitro* assay. To more precisely localize the most functionally regulatory domains of the RaPrP promoter involved in the basal transcriptional activity of this gene, a series of 5′-deletion mutants of the 2,871-bp region were generated in the pGL2-basic vector. Deletions of fragments at positions from −2,113

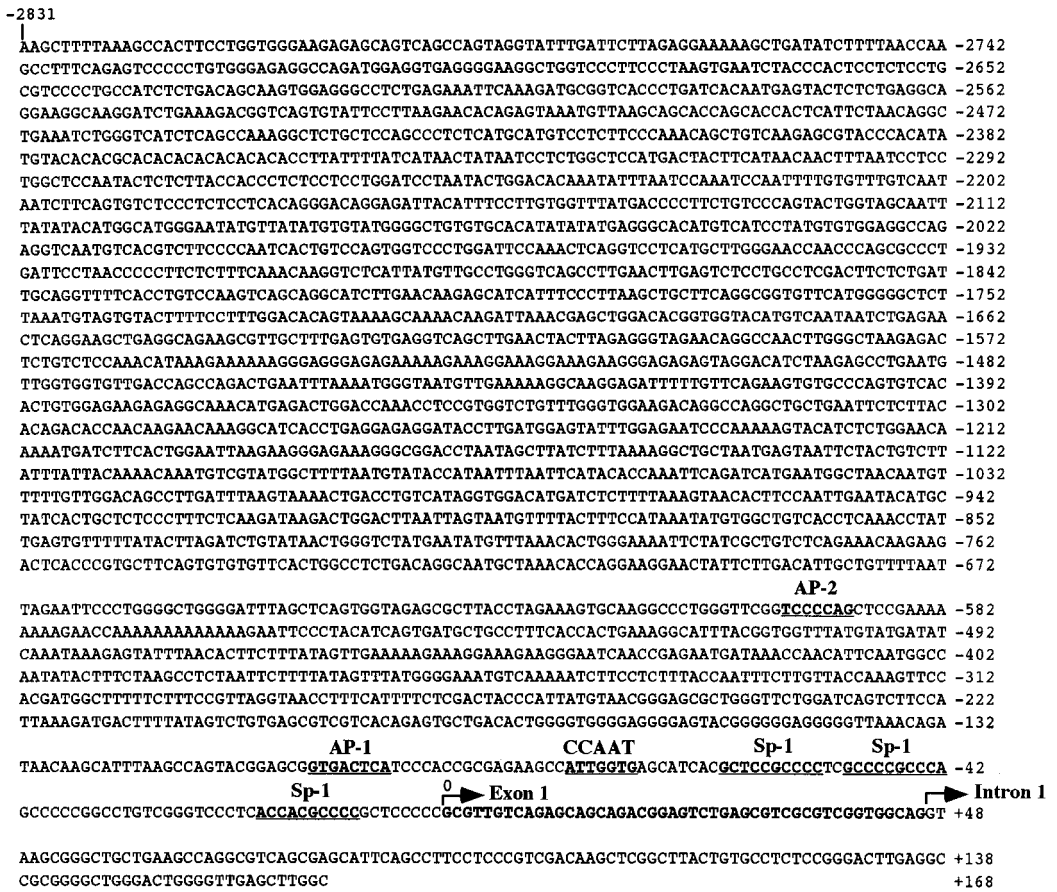

FIG. 1. Nucleotide sequence of the 5′-flanking region of the RaPrP gene. The transcriptional start site is designated as 0 based on the longest 5′-untranslated region among those identified by 5′-RACE as previously reported in ref. 4. The nucleotide sequence from position −2831 to +168 is numbered relative to the transcriptional start site. Several transcriptional factor binding sites are located as follows: AP-2 site (−597 to −591 bp), AP-1 site (−103 to −96 bp), CCAAT (−78 to −72 bp) and Sp-1 sites (−63 to −54 bp, −51 to−42 bp, and −18 to −9 bp).

to −90 bp of 5′-flanking region did not markedly reduce the Luc activity of the corresponding constructs, praPrP(−2113)-luc to praPrP(−90)-luc (Figure 2B and 2C). Thus, the proximal 90-bp upstream of the transcriptional start site contains significant region required for promoter function.

When all constructs were transfected into C6 cells, Luc activity was approximately 1.3-fold higher than those in PC12. However, deletion of nucleotides between bp −90 and −66 resulted in an approximately 60% decrease in Luc activity compared to that of praPrP(−2831)-luc in both PC12 and C6 cells. A region between bp −90 and −66 was identified as a conserved motif in the rodent PrP genes, containing an inverted CCAAT element (5′-ATTGGTG-3′) at a position between bp −78 and −72 (Figure 3). Many eukaryotic promoters possess a transcriptional regulatory element that contains the pentanucleotide sequence CCAAT between bp 60 and 80 upstream of the transcriptional start site (12). The RaPrP gene promoter containing a CCAAT element found in the same position also belongs to the category of genes that possess the CCAAT element. Moreover, longer deletions of fragments in the 25-bp region between bp −66 and −41 containing two Sp-1 binding sites resulted in a 98% decrease in Luc activity (Figure 2B and 2C). Remarkably, deletions of fragments from −51 to −41 bp strongly reduced Luc activity, which contained an entire Sp-1 binding site. The Luc activities of the constructs, [praPrP(−41)-luc to praPrP(+16)-luc], deleted

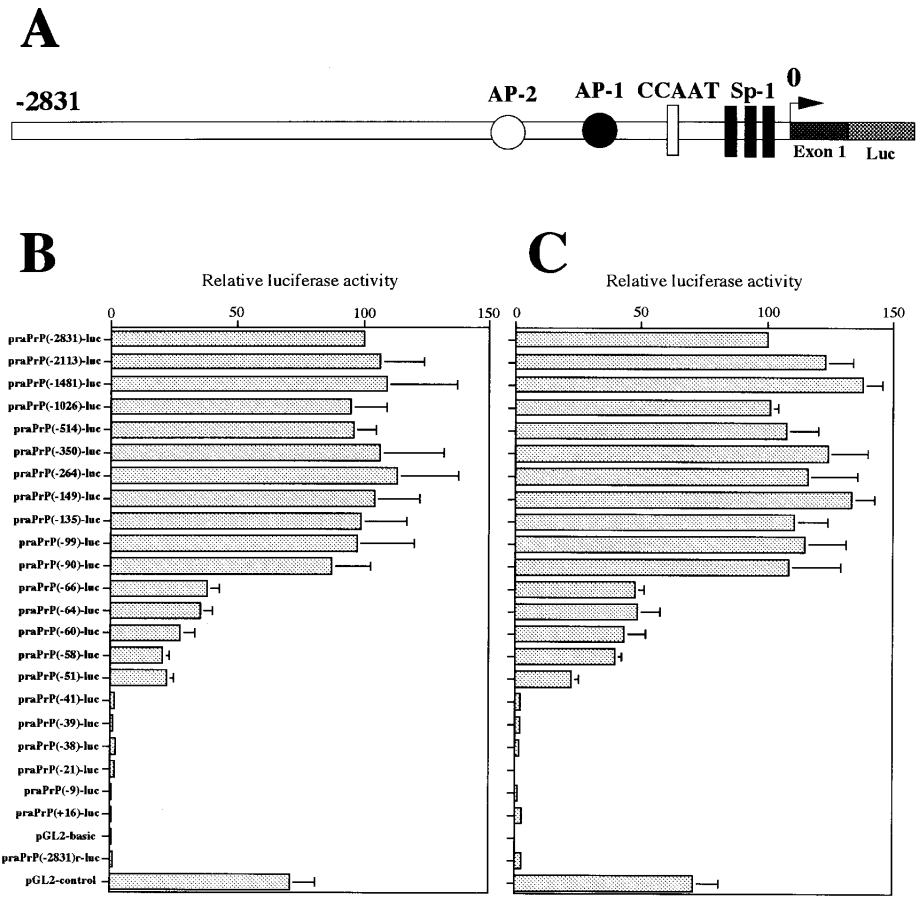

FIG. 2. Functional activity of the RaPrP promoter. (A) Schematic representation of the main features of reporter construct, praPrP(−2831)-luc, is shown. The arrow indicates the transcriptional start site. Constructs containing RaPrP upstream sequence were transiently transfected into PC12 (B) and C6 cells (C) by electroporation. Luc activity was measured by luminometry in cell lysates prepared 48 h posttransfection. Relative Luc activity (mean ± S.D.) for five or more replicate experiments of each mutant is shown in comparison to the praPrP(−2381)-luc plasmid (100%).

fragments from −41 to +16 bp were 97–99% lower than that of praPrP(−2831)-luc but 2 to 18-fold higher than the background level obtained with the promoterless Luc vector (pGL2-basic) or the construct praPrP(−2831)r-luc. These findings indicate the presence of a proximal promoter between bp −90 and −41. Furthermore, the location of the most functionally regulatory domains of the RaPrP gene promoter were found at positions between bp −90 and −66 containing an inverted CCAAT element and between bp −51 and −41 containing a potential Sp-1 binding site.

The 5′-flanking regions of the mouse and hamster PrP genes have been reported previously (3, 5). Comparison of 5′-flanking regions of PrP genes revealed a conserved region between bp −304 and −164 for RaPrP gene (5) (Figure 3). Within these conserved nucleotides, four motifs (Figure 3) are identified and are also observed in human and sheep PrP genes (5). To examine which of these identified conserved motifs are involved in the regulation of RaPrP gene transcription, several different constructs [praPrP(−350)-luc, praPrP(−264)-luc and praPrP(−149)-luc] were transfected into PC12 and C6 cells. The Luc activity expressed by these constructs showed that deletions of fragments in the region between bp −350 and −149 had no significant effect on RaPrP gene promoter activity (Figure 2B and 2C). The remarkably conserved motifs in the rodent PrP genes were identified between transcriptional start sites and four motifs (Figure 3). These included an

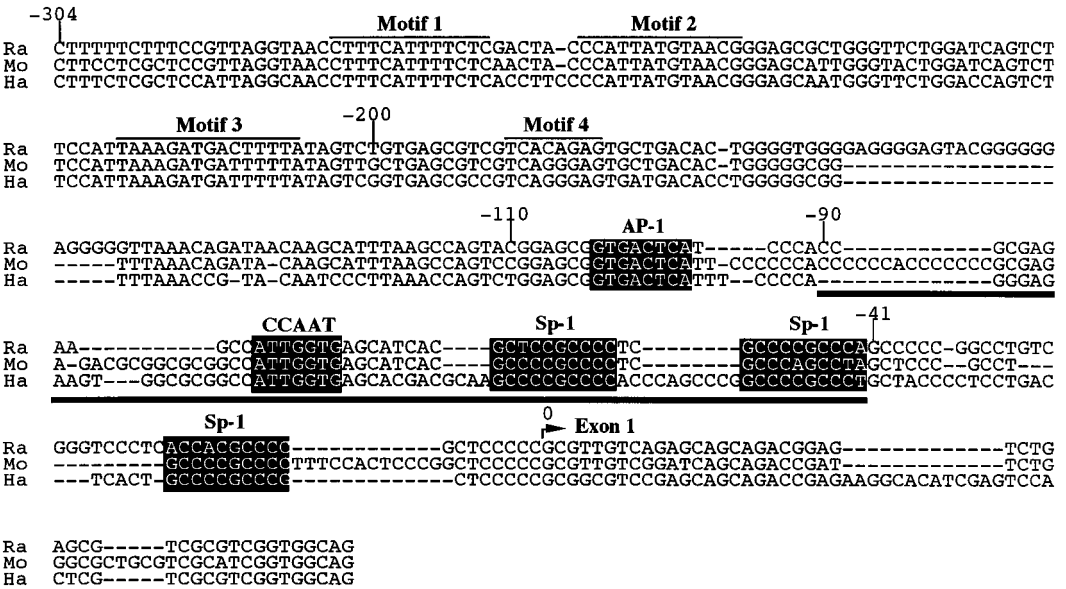

FIG. 3. Nucleotide sequence comparison of the rat, mouse, and hamster 5′-flanking region of Prp genes. Numbering for the RaPrP gene is as in Figure 1. Motifs 1 to 4 indicate conserved nucleotides in PrP genes as previously described in ref. 5. Several potential transcription factor binding sites are indicated by closed boxes: AP-1 site (−103 to −96 bp), CCAAT (−78 to −72 bp) and Sp-1 sites (−63 to −54 bp, −51 to −42 bp, and −18 to −9 bp) for RaPrP gene. Functionally active promoter regions (Fig. 2) are boldly underlined. Dashes indicate gaps introduced to maximize similarity. Ra, rat; Mo, mouse; Ha, hamster.

inverted CCAAT element and consensus binding sites for the transcription factors, AP-1 and Sp-1. The potential CCAAT element and three repeated Sp-1 binding sites are located in the reverse orientation in all rodent PrP genes. In RaPrP gene, three putative Sp-1 binding sequences, which are identical with the consensus sequence, 5′-(G/A)(C/T)(C/T)(C/A)CGCC(C/T)(C/A)-3′, were found at positions between bp −63 and −54, −51 and −42, −18 and −9 (Figure 3). Sequences (5′-GCCCAGCCTA-3′) in mouse PrP gene and (5′-GCCCCGCCCT-3′) and (5′-GCCCCGCCCG) in that of hamster, differ from the consensus sequence by one mismatch indicated in bold type. Analysis of the basal transcription by progressive deletions showed a 49-bp region from −90 to −41, which appears to be critical for optimal promoter activity in PC12 and C6 cells. Three potential regulatory elements were found between bp −90 and −41: a CCAAT element and two Sp-1 binding sites. The regions including an inverted CCAAT element and adjoining Sp-1 binding sites may play an important role in regulation of transcription of the rodent PrP gene. Sequences containing CCAAT element and Sp-1 binding site are involved in the regulation of transcription of numerous viral and eukaryotic genes (12, 13).

The nerve growth factor increases PrP mRNA level in developing hamster brain (14). Identification and characterization of the regulatory sequences conferring either up-regulation or tissue-specific expression will be important for understanding the pathophysiology of prion diseases. An experimental model established in this study will help us to investigate up-regulation as well as tissue-specific transcription in PrP genes.

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