Volume 297, number 3, 257–262 FEBS 10672 February 1992

Structural characterization and expression of a brain specific gene encoding chick kainate binding protein

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Received 17 October 1991; revised version received 2 December 1991

The gene encoding chick kainate-binding protein (c-KBP), a member of the non-NMDA ionotropic glutamate receptor family has been isolated and characterized. The c-KBP gene is at least 13 kilobases long and contains 11 exons interrupted by 10 introns. Primer extension and RNase protection studies identified a major transcription initiation site located 117 bases upstream from the initiation methionine codon ATG. Consensus TATA and CCAAT sequences were detected in the putative promoter region. The structure of the c-KBP gene is strikingly different from that of other members of neurotransmitter-gated ion-channels (cloned at present) although the topology of c-KBP consists of four membrane-spanning domains, a structural characteristic of ionotropic receptor subunits. The c-KBP gene was found to be expressed at high levels in chick cerebellar Bergmann glia and at extremely low levels in the forebrain. The limited expression of the c-KBP gene raises important questions concerning the mechanisms governing the regulation of c-KBP gene transcription.

Glutamate receptor; Kainate; Gene structure; Promoter; Transcription; In situ hybridization

1. INTRODUCTION

Kainate, a rigid analog of glutamate, has been used as a ligand to study the pharmacology of receptors mediating excitatory neurotransmission in the nervous system of several species [1]. Previous studies demonstrated that kainate-binding sites are particularly abundant in brains of lower vertebrates, such as frogs, birds and fish [2]. Purification of these sites from detergent extracts of chick cerebellar and frog brain membranes showed that the kainate binding proteins (KBPs) consist of 49 kDa and 48 kDa glycoproteins, respectively [3,4]. Recent molecular cloning of cDNAs for chick and frog KBPs and mammalian glutamate receptor subunits (GluRs) confirmed that KBPs are members of the ionotropic glutamate receptor family [5–7]. Although the molecular size of KBPs is about half that of GluRs, KBPs display a transmembrane organization similar to that of GluRs. Membrane fractions of mammalian cells transfected with KBP cDNA were shown to bind kainate [6]. However, electrophysiological studies on oocytes injected with KBP RNA synthesized in vitro could not demonstrate ion channel activity [6]. In contrast to the neuronal distribution of frog KBP in the frog brain [8], the c-KBP exhibited a glial distribution in the chick brain. The c-KBP was found predominantly in cerebellum, where it was exclusively localized in Bergmann-glia cell bodies and processes nesting to the parallel fiber/Purkinje cell contacts [9]. Bergmann glia are thought to play an important modulatory role on the efficacy of synapses between parallel fibers and Purkinje cell spines, leading to changes in plasticity of the excitatory synapse. The abundance of KBP in these cells suggests that c-KBP may be involved in the mechanism of synaptic plasticity [10]. Due to the predominant distribution of c-KBP in Bergmann glia, elements which take part in the regulation of c-KBP transcription are of particular interest. In an effort to define potential regulatory regions of the c-KBP gene, we isolated the genomic DNA for c-KBP and characterized its structure.

Abbreviations: c-KBP, chick kainate-binding protein; KBP, kainate-binding protein; GluR, glutamate receptor; PCR, polymerase chain reaction; nAChRs, nicotinic acetylcholine receptor.

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2. MATERIALS AND METHODS

2.1. *Screening of chick genomic library*

A chick genomic library (Clontech, Palo Alto, CA) constructed in the cloning vector λEMBL 3 was screened with a ^{32}P labeled c-KBP cDNA fragment (1.6 kb). The fragment was prepared by polymerase chain reaction (PCR) method [11] from a pool of chick cerebellum cDNAs using specific primers designed according to published sequences at positions 1–20 and 1631–1651 [5]. Hybridization was performed in a solution containing 5 × SSPE (1 × SSPE is 180 mM NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) at 60°C [12]. Filters were washed at high stringency conditions: 0.2 × SSPE at 60°C [12]. Positive clones were purified by three additional screenings. Phage DNAs were extracted by a standard method [12], and genomic

DNA inserts were isolated by *Sal*I partial digestion and subcloned into plasmid vector pGEM-3Z (Promega, Madison, WI).

2.2. *Characterization of chick KBP gene*

Intron/exon organization was determined by DNA sequencing using the dideoxy-mediated chain reaction termination method [13] and oligonucleotides which prime with c-KBP cDNA sequences spaced approximately 150 nucleotides apart. Additional gene-specific synthetic primers were used to sequence the 5′ flanking region of the c-KBP gene. Sizes of introns were estimated by PCR method utilizing primers designed from sequences present in close proximity to exon/intron boundaries. The restriction enzyme map of the chick KBP gene was determined by Southern blot analysis [14] using the full-length chick cDNA probe and 30 base oligonucleotide probes that correspond to the 5′ and 3′ ends (nucleotides 46–76 and 1628–1658, respectively) of the c-KBP cDNA published sequence [5].

2.3. *RNA isolation*

Total cellular RNA was extracted from 7-day-old chick cerebellum and forebrain by the guanidinium thiocyanate method [12] and purified by ultracentrifugation through a cesium trifluoro-acetate gradient (Pharmacia, Uppsala, Sweden).

2.4. *Determination of transcription initiation site by primer extension and RNase protection assays*

(A) Primer extension analysis: Five 20-base oligonucleotide primers complementary to the previously cloned c-KBP cDNA sequences [5] were synthesized:
5′-AGCAATTCCTTCAGCCTCTC-3′ (designated as P1, positions 57–76),
5′-ATTGGGCTTAATCATGGCAT-3′ (designated as P2, positions 203–222),
5′-CATGGCATCATCATTCCTCA-3′ (designated as P3, positions 191–210),
5′-AGAATCTCTCCAATCATCCC-3′ (designated as P4, positions 430–449) and
5′-CAAATCAATGCAGTATCCCT-3′ (designated as P5, positions 314–333).

These oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase [15] (Dupont/NEN, Wilmington, DE) to a specific activity of 10^8 cpm/μg. Extension reactions were performed according to an established method [14] using 50 μg of total RNA from chick cerebellum and forebrain.

(B) RNase protection assays: A 0.3 kb ^{32}P cRNA probe was generated from the c-KBP genomic clone by PCR using two oligonucleotide primers:
5′-TAATACGACTCACTATAGGGAGCAATTCCTTCAGCCTC-TC-3′ (the first 20 bases correspond to T7 promoter sequence, the last 20 bases are complementary to sequence 56–76 of the c-KBP cDNA) and a sense primer derived from the sequence of the c-KBP genomic 5′ flanking region 5′-ATCTGTATTTTCCGAGTCCC-3′ (positions −210 through −191 in Fig. 3). The PCR product was purified on a 0.8% agarose gel, and transcribed in vitro with an RNA transcription kit (Stratagene, San Diego, CA) using T7 RNA polymerase. RNase protection reactions were carried out according to a modification of a published method [15] using 30 μg chick cerebellum and forebrain RNA.

The resulting primer-extended fragments and the RNase-protected products from both assays were analyzed on 6% polyacrylamide-urea sequencing gel. Yeast tRNA (BRL, Gaithersburg, MD) was used as control RNA in the two methods. The chick genomic clone (c-KBP13) was sequenced with primer P1 to produce a sequence ladder electrophoresed in parallel and used to determine the sizes of reaction products. pBR322 DNA digested with *Hin*fI and *Eco*RI [15] was used as additional molecular weight markers to corroborate the length of detected bands.

2.5. *In situ hybridization*

^{35}S-labeled oligonucleotide probe (50-mer) complementary to the 3′ coding sequence of the c-KBP cDNA nucleotides (positions 1484–1534) was prepared and hybridized with cryostat sections (16 μm thick) of 7-day-old chick cerebellum as previously described [16]. Sections were exposed for 11 days to NTB-2 Kodak emulsion. Specificity

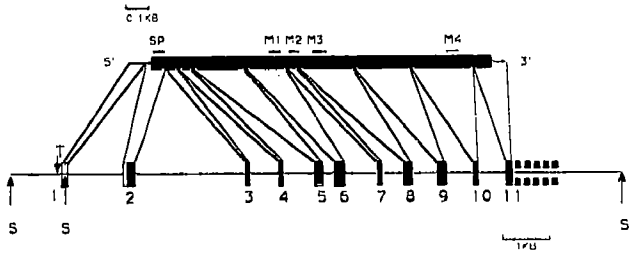

Fig. 1. Organization of the c-KBP gene. The positions of restriction endonuclease cleavage sites for *Sal*I (S) are indicated by arrows. The location of 11 exons in the c-KBP gene are designated by numbered boxes: 1–11. The black boxes represent the protein-coding sequences while the white boxes represent the non-coding sequences. Note that the 3′ end of exon 11 has not been determined. Transcription start site is designated as T. Shown above the genomic structure is a schematic representation of the c-KBP cDNA: the signal peptide (SP) and the four transmembrane domains (M1–M4) that are encoded by the cDNA are indicated.

Table I

Intron/exon organization of the chick kainate binding protein gene

Exon	Intron	Exon
1--(82bp)--GGAATTGCT/gtgagatgct-----	(1.1kb)	-------acctcaacag/GAAGTTTCC-2
2--(108bp)-GCCAGACAG/gtgagaatat-----	(2.4kb)	-------ttttttcag/GAGCAATGA-3
3--(32bp)--ATGATTAAG/gtatgtttaa-----	(0.6kb)	-------ttatttgtag/CCCAATGAC-4
4--(60bp)--ACAATCTTG/gtaaaagcag-----	(0.6kb)	-------gtttatgcag/GAAGATCCC-5
5--(180bp)-CTGAGACAG/gtaattttca ----	(0.2kb)	-------ctctttacag/GAAGCAGAC-6
6--(227bp)-CGTTGCCAG/gtataaatgt-----	(0.9kb)	-------ttcctcatag/GCTGAGCCC-7
7--(98bp)--CGTTGCAAG/gtgagctggg-----	(0.45kb)	-------ggtgtcgcag/GTGTCACCC-8
8--(218bp)-TTCTTCAAG/gtgcagaaaa-----	(0.4kb)	-------ggggttgcag/AACTCCAAG-9
9--(226bp)-CTGCCCAGG/gttagtcacc-----	(0.75kb)	-------ctgctgccag/CATCGCCAT-10
10-(248bp)-CATATCAAG/gtgggtgact-----	(0.6kb)	-------agagttccag/AAATCCTGT-11

Exons are numbered from 1 through 11 and their respective sizes are indicated in the parentheses appearing on the left hand side (the size of exon 11 was not determined). Capital letters represent exon sequences and lower-case letters are intron sequences across the intron/exon junctions. Estimated intron sizes are indicated in parentheses in the intron column.

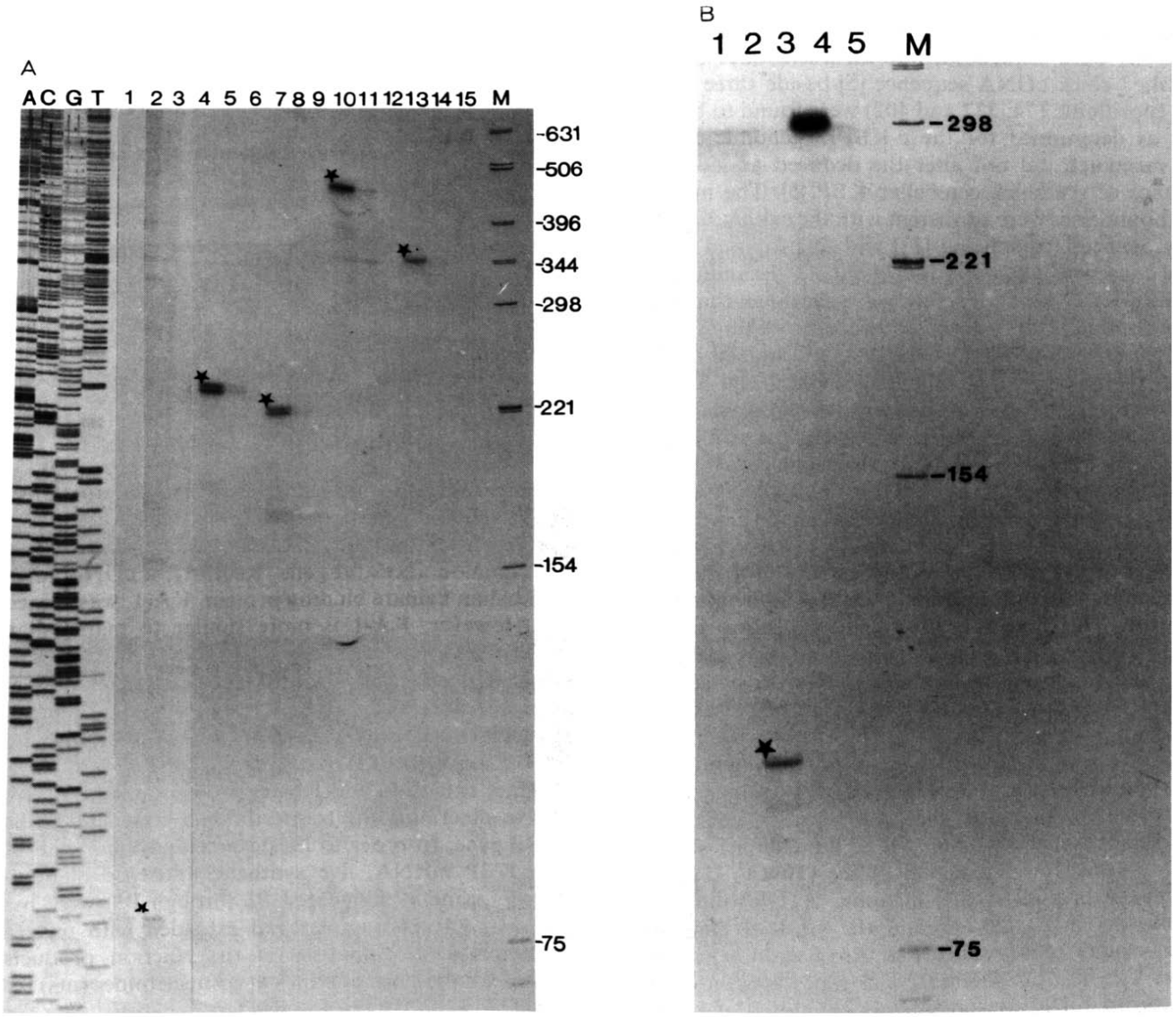

Fig. 2. Mapping of the transcriptional start site of c-KBP gene by primer extension analysis (A) and RNase protection assay. (B). (A) The primers used for extension correspond to lanes: 1,2,3=P1; 4,5,6=P2; 7,8,9=P3; 10,11,12=P4; and 13,14,15=P5. RNA samples are lanes: 1,5,8,11,14=chick forebrain RNA; 2,4,7,10,13=chick cerebellum RNA; 3,6,9,12,15=yeast tRNA. Lanes ACGT: P1 was used to prime dideoxy sequencing reactions of cKBP13 as template to produce the sequencing ladder. (B) Sources of cellular RNA: lane 1=chick forebrain; lane 2=yeast tRNA; lane 3=chick cerebellum. Lanes 4 and 5 represent non-digested and digested end-products of reactions (run in parallel) using the cRNA probe in the absence of RNA samples, respectively. Lane M in A and B: DNA molecular weight markers. Asterisks show major end products used for the identification of the transcription initiation site.

was determined by incubating the tissue sections with radiolabeled probe mixed with excess of unlabeled oligonucleotide.

3. RESULTS AND DISCUSSION

3.1. *Isolation and structural characterization of c-KBP gene*

Genomic clones encoding the c-KBP gene were identified by screening about 2×10^6 recombinants of a chick genomic library with a full-length chick cDNA as a probe. Positive clones were plaque-purified to homogeneity. Phage DNA from a positive clone was analyzed by digestion with *Sal*I and by successive hybridization with oligonucleotide probes from the 5′ and 3′ regions of the chick cDNA as well as with the full-length chick cDNA probe. The clone was found to contain a 13-kb *Sal*I-*Sal*I fragment that spans the entire protein-coding sequence. The insert of the clone, named c-KBP13, was subcloned into the *Sal*I site of pGEM-3Z to determine the c-KBP genomic structure.

Analyses of the c-KBP13 insert by detailed restriction enzyme mapping, Southern blot hybridization and DNA sequencing indicated that the c-KBP gene is encoded by at least 11 exons interrupted by 10 introns

ranging in size from 200 bp to 2.4 kb (Fig. 1, Table I). All exons were sequenced, and all nucleotides of the published chick cDNA sequence [5] beside three positions (positions: 174, 372 and 402) were found to be the same as determined for the c-KBP13 genomic clone. This mismatch did not alter the deduced amino acid sequence of the chick cerebellar KBP [5]. The intron/exon boundaries were consistent with the canonical GT-AG sequences for splicing [17] and fit proposed junctional consensus sequences [18] (Table I).[a] Examination of the c-KBP gene structure demonstrated that the signal peptide of the c-KBP is encoded by exon 2, and the proposed N-terminal extracellular domain is encoded by exons 3–5 and by part of exon 2 and 6. The putative intracellular loop present between transmembrane domains M3 and M4 is encoded by parts of exons 8, 9 and 10, and the proposed extracellular domain at the carboxy-terminus is encoded by parts of exons 10 and 11. The membrane-spanning domains M1, M3 and M4, are encoded by single exons (parts of exons 6, 8 and 10, respectively) while the coding sequence for the second membrane-spanning domain (M2) is interrupted by an intron. The 5′ untranslated sequence shown in the published chick cDNA [5] was present in the c-KBP 13 clone and was found to be encoded by exon 1 and by part of exon 2. Since putative kainate binding sites have not been yet identified in the KBP, it is at present difficult to say which exons correspond to functionally important structural domains of KBP. Comparing c-KBP gene structure with the genomic organization of other members of the ligand-gated ion-channel superfamily cloned thus far, including the $GABA_A$ [19] receptors, brain and muscle nicotinic acetylcholine receptors (nAChRs) [20,21], and the GluR-B receptor (partly cloned) [22] revealed that intron/exon organization is significantly different. The genes encoding the various brain nAChR subunits are composed of different numbers of exons from the gene encoding c-KBP. Although the number of exons in some of the muscle nAChR subunits is identical to that of the c-KBP gene, positions of the protein-coding sequence interrupted by each intron do not correspond to those of the c-KBP gene. In the GluR-B receptor, a small segment of the putative cytoplasmic loop between transmembrane domains M3 and M4 was found to be encoded by an alternative usage of the flip and flop exons, which were shown to be important determinants of the electrophysiological properties of GluR. Such small alternative exons were not found in the c-KBP gene. However, recent data indicated that the GluR-5 and GluR-6 genes (partially cloned) [23] and the c-KBP gene show the same split M1-M2 exon. Due to lack of further information regarding the organization of the glutamate receptor subunit genes, one cannot assume that they evolved via duplication and subsequent diversification of a common ancestral gene. Recently, a cDNA for a mammalian kainate binding protein, KA-1, was cloned [24]. However, KA-1 is more similar to mammalian GluRs than chick and frog KBPs. Thus, c-KBP gene may have diverged from GluR genes early in evolution.

[a]The presence of introns no. 5 and 9 in the c-KBP gene was brought to our attention in a presentation describing a similar c-KBP gene mapping given by Dr. P. Gregor et al. at the Annual Meeting of the Society for Neuroscience, and was confirmed by us through sequence analysis of our gene.

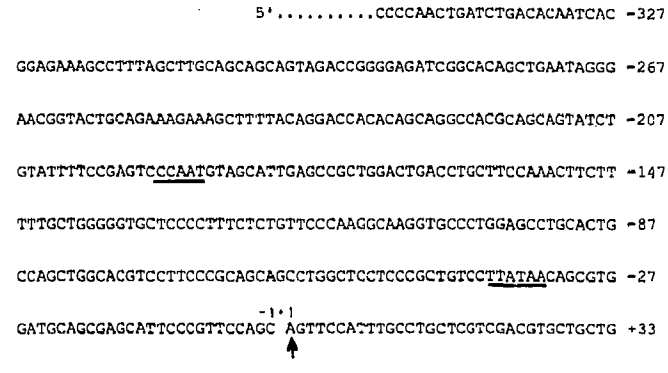

Fig. 3. Nucleotide sequence of the 5′-flanking region of the chick KBP gene. Nucleotides are numbered from the major transcriptional start site designated by arrow. The putative *cis*-acting regulatory elements, TATAA and CCAAT, are underlined.

3.2. *Determination of the c-KBP gene transcription initiation site and surrounding sequences*

Primer extension and RNase protection assays were used to determine the transcriptional start site of the c-KBP gene. In order to locate precisely the 5′ end of the c-KBP mRNA, five synthetic antisense oligonucleotide primers (designated P1 through P5) were hybridized to RNA samples and extended with reverse transcriptase. Gel analysis of the reaction products showed the presence of bands at 83 nucleotides (nts) for primer 1, 228–229 nts for primer 2, 216–217 nts for primer 3, ~460 nts for primer 4 and ~340 nts for primer 5 (Fig. 2A). These results indicated that a major starting point is located 116–117 bases upstream from the initiation methionine codon, ATG mapped to a dG and a dA residue, respectively. The intensity of the resulting extended products showed that KBP mRNA is highly and predominantly expressed in chick cerebellum (Fig. 2A). No extended products were observed when reactions were performed with samples of yeast tRNA.

RNA protection analysis was performed to confirm the location of the putative transcription initiation sites. An antisense cRNA probe of 300 bases was hybridized to RNA samples. Analysis of RNA:RNA duplexes protected from the digestion with RNase A and RNase T1 (Fig. 2B) showed a major protected band by cerebellar RNA with the size of 97 bases. In accordance with the primer extension experiments, a much lower amount of protected fragment of chick forebrain RNA:RNA duplex could be observed. No protected fragments were observed with yeast tRNA. The major transcription site

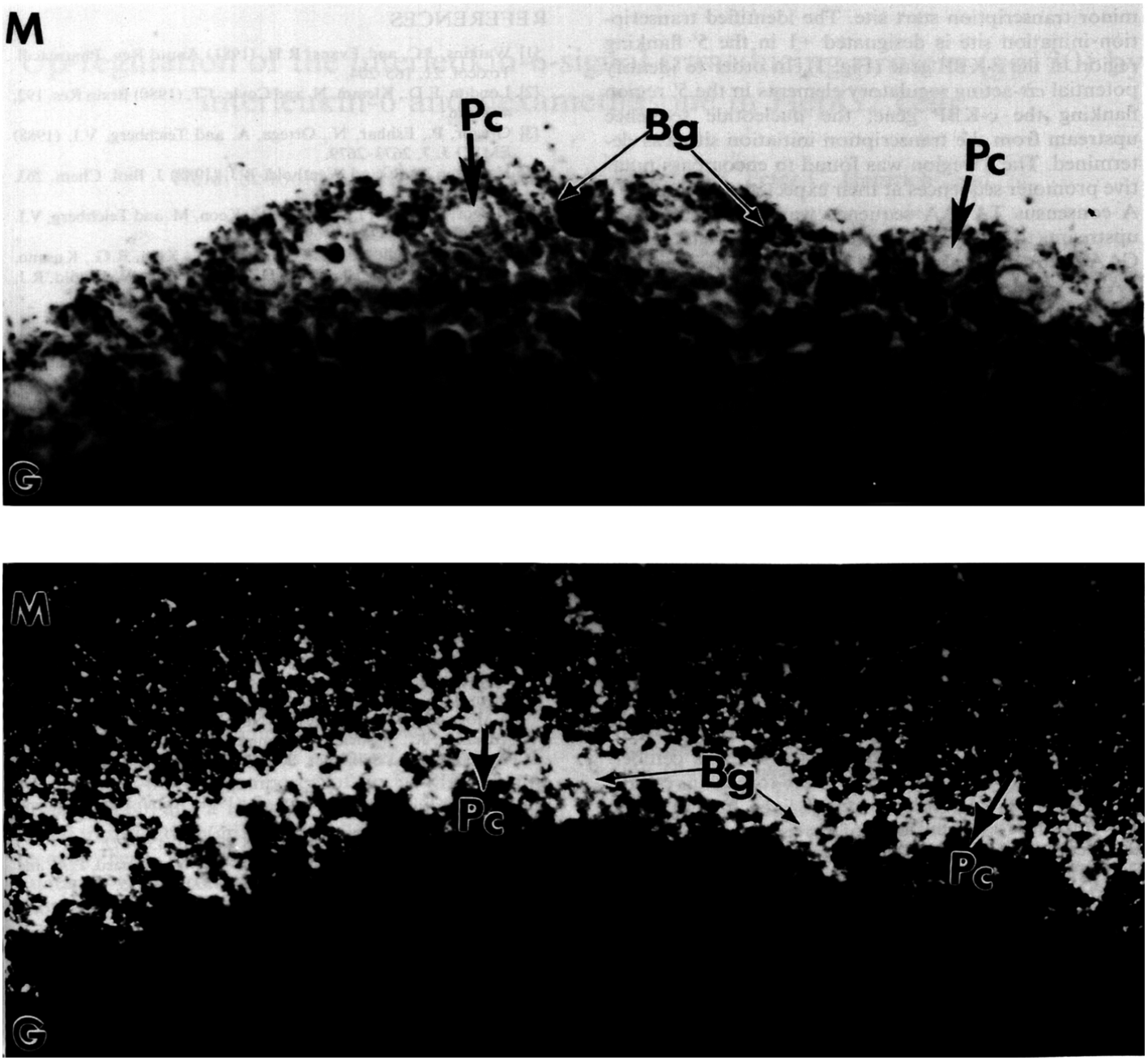

Fig. 4. Histochemical localization of KBP mRNA by in situ hybridization in a sagittal section of 7-day-old chick cerebellum. (Upper panel) Bright-field micrograph. The grains appear in black in the molecular layer (M). A dense labeling of KBP mRNA appears within Bergman glia cells (Bg) surrounding Purkinje cell bodies (Pc). The nebulous aspect of the granular layer (G) is due to the underlying thionin staining. (Lower panel) Dark-field micrograph of the same area. The grains appear in white in the Purkinje cell layer. Note the sparing of the Purkinje cell bodies (Pc) and the very small grain density in the granular cell layer (G).

detected by RNase protection assay is located 132 bases 5′ from the initiation methionine codon (ATG) and is thus slightly different from that determined by primer extension analysis. We attribute the discrepancy between the two data (15 bases) to the difference in migration mobility properties between RNA and DNA through polyacrylamide-urea gels (DNA is about 20% more mobile than RNA). Since adenosine residue is a preferred nucleotide for the capping of mRNAs in eukaryotes [17] and RNase protection analysis confirmed the presence of one protected band, we conclude that the major transcription site for the c-KBP gene is located 117 bases 5′ from the initiation codon ATG and that the dG residue next to the dA residue may be a

minor transcription start site. The identified transcription initiation site is designated +1 in the 5′ flanking region of the c-KBP gene (Fig. 3). In order to identify potential *cis*-acting regulatory elements in the 5′ region flanking the c-KBP gene, the nucleotide sequence upstream from the transcription initiation site was determined. The 5′ region was found to encompass putative promoter sequences at their expected positions [17]. A consensus TATAA sequence was located 38 bases upstream to the transcription initiation site and a CAAT sequence was mapped 154 bases upstream to the TATAA sequence (Fig. 3). These features suggest that the 5′ flanking region contains at least part of the promoter which has all of the hallmark characteristics of an RNA polymerase II transcription initiation element [17].

3.3. *Expression of the chick KBP gene in chick cerebellum*

We determined the distribution of the c-KBP transcripts through chick cerebellar sections by in situ hybridization. A high density of grains was observed exclusively over Bergmann glia surrounding the Purkinje cell bodies, sparing the Purkinje cell soma (Fig. 4). The very low grain density in the granular layer and the prominent expression of the c-KBP message in Bergmann glia is in agreement with the localization of KBP as determined by immunohistochemical studies [9,25]. As shown in previous studies [5] and current Northern blot analysis (data not shown) c-KBP transcript was predominantly located in chick cerebellum with a much lower level in chick forebrain. c-KBP transcript was not detected in chick liver, human brain, and in primary cell cultures of rat astrocytes and glia. Since cell specificity of expression of c-KBP differs from that of other members of the glutamate receptor family [6,16], different regulatory DNA elements may have been added to the genes in this family after gene divergence or duplication. Further investigations of promoter activity and transcriptional regulatory elements of the c-KBP gene are now feasible. Such studies will contribute to defining factors involved in the control of c-KBP gene expression.

Acknowledgements: We gratefully acknowledge the technical assistance of Thuc D. Vu in the preparation of sections used for in situ hybridization, and thank Dr. R.S. Petralia for his devoted help in editing the paper and thank members of the Laboratory of Neurochemistry, NIDCD for their comments on this manuscript. Especially, we thank Dr. J. Battey for his excellent advice and helpful suggestions regarding this work, and for his thoughtful review of this paper.

REFERENCES

[1] Watkins, J.C. and Evans, R.H. (1981) Annu. Rev. Pharmacol. Toxicol. 21, 165–204.
[2] London, E.D., Klemm, N. and Coyle, J.T. (1980) Brain Res. 192, 463–476.
[3] Gregor, P., Eshhar, N., Ortega, A. and Teichberg, V.I. (1988) EMBO J. 7, 2673–2679.
[4] Hampson, D.R. and Wenthold, R.J. (1988) J. Biol. Chem. 263, 2500–2505.
[5] Gregor, P., Mano, I., Maoz, I., McKeon, M. and Teichberg, V.I. (1989) Nature 342, 689–692.
[6] Wada, K., Dechesne, C.J., Shimasaki, S., King, R.G., Kusano, K., Buonanno, A., Hampson, D.R., Banner, C., Wenthold, R.J. and Nakatani, Y. (1989) Nature 342, 684–689.
[7] Dingledine, R. (1991) Trends Pharmacol. Sci. 12, 360–362.
[8] Dechesne, C.J., Oberdorfer, M.D., Hampson, D.R., Wheaton, K.D., Nazarali, A.J., Goping, G. and Wenthold, R.J. (1990) J. Neurosci. 10, 479–490.
[9] Somogyi, P., Eshhar, N., Teichberg, V.I. and Roberts, J.D.B. (1990) Neuroscience 35, 9–30.
[10] Ortega, A., Eshhar, N. and Teichberg, V.I. (1991) Neuroscience 41, 335–349.
[11] Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R. and Horn, G.T. (1988) Science 239, 487–491.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) Current Protocols in Molecular Biology, vol. 1, Greene Publishing Assoc., New York.
[15] Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic Methods in Molecular Biology, Elsevier, New York/Amsterdam/London.
[16] Keinänen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakmann, B. and Seeburg, P.H. (1990) Science 249, 556–560.
[17] Breathnach, R. and Chambon, P. (1981) Annu. Rev. Biochem. 50, 349–383.
[18] Mount, S.M. (1982) Nucleic Acids Res. 10, 459–472.
[19] Lasham, A., Vreugdenhil, E., Bateson, A.N., Barnard, E.A. and Darlison, M.G. (1991) J. Neurochem. 57, 352–355.
[20] Nef, P., Oneyser, C., Alliod, C., Couturier, S. and Ballivet, M. (1988) EMBO J. 7, 595–601.
[21] Deneris, E.S., Connolly, J., Rogers, S.W. and Duvoisin, R. (1991) Trends Pharmacol. Sci. 12, 34–40.
[22] Sommer, B., Keinänen, K., Verdoorn, T.A., Wisden, W., Burnashev, N., Herb, A., Köhler, M., Takagi, T., Sakmann, B. and Seeburg, P.H. (1990) Science 249, 1580–1585.
[23] Sommer, B., Köhler, M., Sprengel, R. and Seeburg, P.H. (1991) Cell 67, 11–19.
[24] Werner, P., Voigt, M., Keinänen, K., Wisden, W. and Seeburg, P.H. (1991) Nature 351, 742–744.
[25] Eshhar, N., Lederkremer, G., Beaujean, M., Goldberg, O., Gregor, P., Ortega, A., Triller, A. and Teichberg, V.I. (1989) Brain Res. 476, 57–70.