Analysis of the promoter region of the murine gephyrin gene

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Abstract The peripheral membrane protein gephyrin is essential for the postsynaptic localization of the inhibitory glycine receptor and is thought to anchor the receptor complex to the subsynaptic cytoskeleton. Since gephyrin transcripts are also found in non-neuronal tissues, we analyzed the 5′-flanking region of the gephyrin gene to identify its promoter region. This region contains several SP1 consensus sites, which may account for the widespread expression of gephyrin mRNA. Indeed, reporter gene constructs encompassing at least the two SP1 consensus sites upstream from the transcription start site were efficiently transcribed in both a fibroblast and a neuronal cell line. Our data are consistent with the widespread expression of the gephyrin gene in most mammalian tissues.

Key words: Gephyrin; Glycine receptor; Promoter; Receptor clustering

1. Introduction

Gephyrin was originally identified as a peripheral membrane protein of relative molecular mass 93 kDa that co-purifies with the mammalian inhibitory glycine receptor upon affinity chromatography [1–3]. Light and electron microscopic immunocytochemistry revealed that gephyrin decorates the cytoplasmic face of glycinergic and a subset of GABAergic postsynaptic membrane specializations in spinal cord and brain [4–8]. In cultured spinal neurons, the accumulation of gephyrin at developing postsynaptic sites precedes the synaptic clustering of the glycine receptor [9,10], and inhibition of gephyrin expression by antisense oligonucleotides prevents the formation of glycine receptor clusters in these cultures [9]. Gephyrin expression is therefore considered essential for the synaptic localization of the glycine receptor [9]. Interestingly, transcription of the gephyrin gene is not restricted to the central nervous system, since gephyrin transcripts were also found in liver, kidney, heart, and possibly lung [11]. We therefore characterized the putative promoter region of the murine gephyrin gene and found a 5′-flanking region harbouring multiple transcription factor binding sites suitable for driving strong and ubiquitous expression of the gephyrin gene. Our data demonstrate a high transcriptional activity of the proximal portion of the promoter region in both a neuronal and a non-neuronal cell line.

2. Materials and methods

2.1. Library screening

A mouse brain cDNA library constructed in the phage vector λ-ZAPII (Stratagene, Heidelberg, Germany) was used to isolate a full length mouse gephyrin cDNA according to standard procedures [12]. P1 clones harbouring the 5′ untranslated region (UTR) of the gephyrin gene were identified by PCR screening of a P1 library (Genome Systems Inc., St. Louis, USA) using oligonucleotide primers derived from genomic sequences upstream from the translation start site.

2.2. Chemicals and enzymes

Radiochemicals used for radiolabelling of DNA probes were purchased from Amersham (Braunschweig, Germany), and DNA-modifying enzymes and restriction endonucleases from Boehringer (Mannheim, Germany). Fine chemicals were obtained from Sigma (Deisenhofen, Germany).

2.3. RNA preparation and primer extension

Total RNA was prepared from mouse brain extracts by guanidine/caesium chloride extraction [13]. 50 μg of total RNA were used for primer extension with an oligonucleotide of 29 bases, antisense to positions +167 to +139 of the 5′ UTR of the p1 cDNA [11], and 'SuperScript' reverse transcriptase (Gibco, Eggenstein, Germany) according to standard protocols [14]. To prevent mismatch priming, annealing and extension were performed at 53°C.

2.4. Reporter gene constructs

Fragments of the 5′-flanking region were first subcloned into pBluescript SK+ and subsequently transferred into the *Hin*dIII/*Bgl*II sites of the luciferase vector pGL_2 (Promega, Heidelberg, Germany), taking advantage of the *Sma*I site at position +141 downstream from the transcription start point. Construct LUP2 was created by subcloning a 1.7 kb *Eco*RI/*Sma*I fragment of the 5′-flanking region, and the construct LUP1 was derived from a shorter *Pst*I/*Sma*I fragment of the same region corresponding to positions −534 to +140. Constructs LUP1c and LUP1d were generated taking advantage of a *Bss*HII site at position −234 and an *Nae*I site at position −159 for subcloning. The smaller fragments for constructs LUP1f and LUP1g were created by subcloning PCR-amplified fragments covering positions −74 to +140 and positions −23 to +140. Negative numbers correspond to bp upstream of the transcription start site.

2.5. DNA transfections and luciferase assays

Human 293 embryonic kidney (HEK 293; ATCC no. CRL1537) cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 in Minimal Essential Medium (Gibco, Eggenstein, Germany), and N18 neuroblastoma (N18TG2; ATCC no. DSM ACC103) cells were maintained in an atmosphere of 90% air/10% CO_2 in Dulbecco's Minimal Essential Medium (Gibco, Eggenstein, Germany). Both culture media were supplemented with 50 U penicillin/ml, 50 μg/ml streptomycin (Flow Laboratories), 2 mM L-glutamine (Boehringer) and 10% (v/v) fetal calf serum (Gibco, Eggenstein, Germany). Cells were grown to 80% confluency and subsequently transfected using the Ca^{2+} phosphate precipitation method [15] followed by a glycerol shock after 5 h [16]. To assess the efficiency of the transfections, cells were co-transfected with equal amounts of a pSV40 β-galactosidase vector (Promega, Heidelberg, Germany). 2 days after transfection, the cells were washed with phosphate-buffered saline and lysed in 150 μl of 1×Reporter Lysis buffer (Promega, Heidelberg, Germany). Aliquots (20 μl) of the lysate were assayed for luciferase activity in a Lumat luminometer (Berthold, Bad Wildbad, Germany). The efficiency of each transfection was determined by a colorimetric β-galactosidase assay according to the manufacturer's instructions (Promega, Heidelberg, Germany). The luciferase activity was determined from three individual culture dishes. To test the effect of metal ions on promoter activity, we added 50 μM $ZnCl_2$, 250 μM $CuSO_4$, or 2.5 μM $CdCl_2$ to the culture medium immediately after the glycerol shock and thereafter determined luciferase activities as described above.

2.6. DNA sequencing

Nucleotide sequences were determined according to the dideoxy

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chain termination method using either a T_7-sequencing kit (Pharmacia, Freiburg, Germany) or a DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems Inc., Langen, Germany) according to the manufacturer's instructions. Sequence-specific primers were synthesized using a Gene Assembler Plus (Pharmacia, Freiburg, Germany). All sequences were confirmed by analyzing both DNA strands.

3. Results

3.1. Identification of the transcription start site

In order to determine the transcription start site of the murine gephyrin mRNA, we performed primer extension experiments with antisense primers derived from different regions of the 5′ UTR. These experiments unequivocally located the transcription start point 401 bp upstream from the start codon (Fig. 1).

3.2. Characterization of the 5′-flanking region of the gephyrin gene

The 5′-flanking region was analyzed further by sequencing about 1 kb of genomic DNA upstream from the start codon. Comparison with the mouse cDNA isolated from a brain cDNA library (see Section 2) indicated no additional introns within this region of the 5′ UTR. GC-rich regions with 5 consensus sites for SP1 [17,18] and a CCAAT box like element [19] were identified at positions 255–260, 186–190, 172–177, 32–37, and 13–19, respectively, and between positions 178 and 182 bp upstream from the transcription start point (Fig. 2). In addition, we found consensus binding sites for the transcription factors AP-2 [20] (positions −91 to −80 and −260 to −251), PEA3 [21] (positions −443 to −438) and the muscle-specific transcription factor MyoD [22] (positions −331 to −336). Consensus sites for metal response elements MBF-1 CS and MRE CS [23,24] were found at positions −208 to −202 and −59 to −56 bp, respectively (Fig. 2). However, no consensus sites for neuron-specific transcription factors could be identified in this region of the gephyrin gene.

3.3. Transcriptional activity of the 5′-flanking region

To demonstrate that the 5′-flanking region contains a functional gephyrin promoter, we chose a DNA fragment (LUP1) encompassing positions −534 to +140 to drive luciferase expression in fibroblasts (HEK 293) and neuroblastoma (N18) cells. This construct produced 4–5-fold higher expression of the reporter gene than the SV40 promoter in both cell lines (Fig. 3). A second construct (LUP2), which extended approx. 1.7 kb further upstream, did not exhibit significantly higher activity. We therefore conclude that relevant regulatory elements of gephyrin gene expression are located within a region of the 5′-flanking sequence extending 534 bp upstream from the transcription start point. In order to further delineate sites important for promoter activity, we generated several deletion constructs. Construct LUP1c lacking the most 5′ SP1 consensus site and the consensus sites for PEA3 and MyoD gave decreased luciferase expression as compared to LUP1 or LUP2 (Fig. 3). Deletion of two additional downstream SP1 sites, two AP-2 sites, the CCAAT box, and one metal response element in construct LUP1d decreased the luciferase activity by another approx. 30% in HEK 293, but not in N18 neuroblastoma cells. The shortest construct (LUP1g) starting 23 bp upstream from the transcription start point and harbouring a single SP1 site showed no significant expression, but a construct only 51 bp longer, and therefore carrying a second

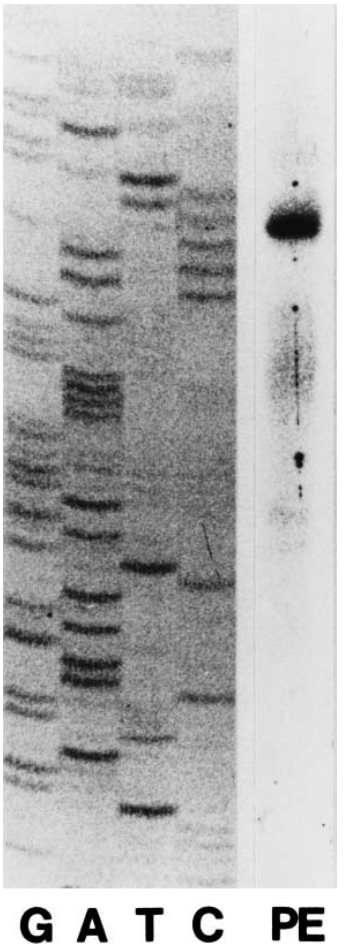

Fig. 1. Determination of the transcription start point. (Left) Autoradiograph of the ^{35}S-labelled gephyrin reference sequence primed with the same oligonucleotide as used for primer extension. Nucleotides are indicated at the bottom. (Right) Electrophoretic separation of the primer extension reaction (PE). Only a single band is detected, which indicates a transcription start point at position −401.

SP1 site and a metal response element (LUP1f), allowed the expression of the reporter gene at levels higher than those obtained with the SV40 promoter containing control plasmid (Fig. 3). Since two consensus sites for metal response elements were found in the 5′-flanking region, we also investigated the effect of di- and tri-valent metal ions on promoter activity. Treatment of transfected cells with Zn^{2+}, Cu^{3+} or Cd^{2+} ions did not lead to significant changes in β-galactosidase activity (not shown). Notably, the expression levels observed with the different constructs were similar in both cell lines, suggesting a lack of cell type specificity of the gephyrin promoter. We conclude from these data that the SP1 sites are important determinants of gephyrin promoter activity.

4. Discussion

We have analyzed the 5′-flanking region of the gephyrin gene and located the transcription start point 401 bp upstream of the start codon of the gephyrin cDNA. Comparison of the transcriptional activities of sequences extending 1.7 kb and 534 bp upstream from the transcription start point clearly showed that the shorter construct contained important pro-

Fig. 2. Sequence of the mouse gephyrin promoter region. About 1 kb of DNA upstream of the translation start site were sequenced (available under GenBank/EMBL database accession numbers Y10882 and Y10883). The sequence of the 5′-flanking region used to generate promoter constructs is shown. The 5′ ends of the segments used in the reporter plasmids are indicated by horizontal arrows followed by the name of the respective construct. The transcription start site (+1) is marked with an asterisk. Underlined sequences correspond to consensus binding motifs for transcription factors; the position of a CCAAT box is also indicated.

moter elements of the gephyrin gene. Computational analysis of this region revealed several consensus sites for transcription factors including five consensus sites for SP1 binding, two AP-2 consensus sites, two consensus sites for metal response elements, one CCAAT box like element, and one PEA3 and one MyoD consensus site, each. Our reporter construct assays show that the 5′ UTR of the gephyrin gene contains a highly efficient promoter region and that the proximal SP1 consensus

Fig. 3. Structure and activity of gephyrin promoter driven reporter gene constructs. (A) Schematic representation of the fragments from the 5′ upstream region of the gephyrin gene used for reporter plasmid construction. Consensus sites for transcription factor binding, promoter elements and the transcription start point (arrow) are indicated above the LUP2 fragment. Lines represent the 5′ segments used to construct additional plasmids for determining transcriptional activity by the luciferase assay. (B) The bars represent the luciferase activities obtained in HEK293 (▩) and N18 (□) cells transfected with a promoterless control plasmid (pGL2), a control plasmid containing the SV40 promoter (SV40), and the reporter constructs indicated on the left. Activity values represent the mean ± S.E.M. of three independent experiments as detailed under Section 2.

elements are particularly important for its activity. The transcription factor SP1 is found in many different cell types and organs [18]; this may account for the widespread expression of gephyrin transcripts in all rat tissues investigated [11].

The contribution of the other transcription factor binding sites was not analyzed in detail due to their close overlap with SP1 sites or the absence of the relevant transcription factors (MyoD) in the cell lines used. Surprisingly, deletion of the CCAAT box did not abolish transcription, as seen with promoters from other genes [25–27]; a modulatory influence of the CCAAT box on transcription rates, however, may have escaped detection by our assay system. The presence of metal response elements in the 5′ UTR suggests a modulatory effect of metal ions on the transcriptional activity of the gephyrin promoter. However, the addition of di- or tri-valent metal ions, such as Zn^{2+}, Cu^{3+}, and Cd^{2+}, did not lead to significant changes in reporter gene activity in the cell lines used.

Gephyrin transcripts have been demonstrated in a variety of organs including in brain, liver, heart and lung [11]. These findings are substantiated here by structural and functional features of the promoter region of the gephyrin gene. However, the physiological significance of the widespread expression of gephyrin outside the nervous system remains unclear. One might speculate that the known homology of the N- and C-terminal regions of the polypeptide to proteins involved in the biosynthesis of the molybdenum cofactor [11,28,29] implies a dual function. The targeted disruption of the gephyrin gene should clarify whether gephyrin contributes to molybdenum cofactor biosynthesis in addition to its role in anchoring inhibitory neurotransmitter receptors.

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References

[1] Pfeiffer, F., Graham, D. and Betz, H. (1982) J. Biol. Chem. 257, 9389–9393.
[2] Graham, D., Pfeiffer, F., Simler, R. and Betz, H. (1985) Biochemistry 24, 990–994.
[3] Schmitt, B., Knaus, P., Becker, C.-M. and Betz, H. (1987) Biochemistry 26, 805–811.
[4] Triller, A., Cluzeaud, F., Pfeiffer, F., Betz, H. and Korn, H. (1985) J. Cell Biol. 101, 683–688.
[5] Triller, A., Cluzeaud, F. and Korn, H. (1987) J. Cell Biol. 104, 947–956.
[6] Altschuler, R.A., Betz, H., Parakkal, M., Reeks, K. and Wenthold, R. (1986) Brain Res. 369, 316–320.
[7] Sassoè-Pognetto, M., Kirsch, J., Grünert, U., Greferath, U., Fritschy, J.M., Möhler, H., Betz, H. and Wässle, H. (1995) J. Comp. Neurol. 357, 1–14.
[8] Todd, A.J., Spike, R.C., Chong, D. and Neilson, M. (1995) Eur. J. Neurosci. 7, 1–11.
[9] Kirsch, J., Wolters, I., Triller, A. and Betz, H. (1993) Nature 366, 745–748.
[10] Béchade, C., Colin, I., Kirsch, J., Betz, H. and Triller, A. (1996) Eur. J. Neurosci. 8, 429–435.
[11] Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., Maulet, Y., Werner, P., Langosch, D., Kirsch, J. and Betz, H. (1992) Neuron 8, 1161–1170.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Chirgwin, J.M., Przybila, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5297.
[14] Kotewicz, M.L., Sampson, C.M., D'Alessio, J.M. and Gerard, G.F. (1988) Nucl. Acid Res. 16, 249–251.
[15] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2751.
[16] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G, Smith, J.A. and Struhl, K. (1992) Short Protocols in Molecular Biology, Wiley, New York.
[17] Jones, K.A. and Tjian, R. (1985) Nature 317, 179–182.
[18] Briggs, M.R., Kadonaga, J.T., Bell, S.T. and Tjian, R. (1986) Science 234, 47–52.
[19] Benoist, C., O'Hare, K., Breathnach, R. and Chambon, P. (1980) Nucl. Acids Res. 8, 127–142.
[20] Imagawa, M., Chiu, R. and Karin, M. (1987) Cell 51, 251–260.
[21] Martin, M.E., Piette, J., Yaniv, M., Tang, W.-J. and Folk, W.R. (1988) Proc. Natl. Acad. Sci. USA 85, 5839–5843.
[22] Anthony-Cahill, S.J., Benfield, P., Fairman, R., Wasserman, Z.R., Brenner, S.L., Stafford, W.F., Altenbach, C., Hubell, W.L. and DeGrado, W.F. (1992) Science 255, 979–983.
[23] Imbert, J., Muhammad, Z., Culotta, V.C., Gedamu, L. and Hamer, D. (1989) Mol. Cell. Biol. 9, 5315–5323.
[24] Culotta, V.C. and Hamer, D. (1989) Mol. Cell. Biol. 9, 1372–1380.
[25] McKnight, S., Gavis, E., Kingsbury, R. and Axel, R. (1981) Cell 25, 385–398.
[26] Wright, S., Rosenthal, A., Flavell, R. and Grosveld, F. (1984) Cell 38, 265–273.
[27] Wu, B.J., Kingston, R.E. and Morimoto, R.I. (1986) Proc. Natl. Acad. Sci. USA 83, 629–633.
[28] Kamdar, K.P., Shelton, M.E. and Finnerty, V. (1994) Genetics 137, 791–801.
[29] Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, H. and Mendel, R.R. (1995) Plant J. 8, 751–762.