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Identification of two promoter regions in the rat B-50/GAP-43 gene

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Abstract

To determine cis-acting elements controlling the rat B-50/GAP-43 gene expression, the genomic DNA encoding exon 1 and the 5′ flanking sequence was isolated. Sequence analysis of 1 kb 5′ untranslated region (UTR) revealed the presence of a (GA)-repeat and a (GT)-repeat. The size of the (GA)-repeat varied due to both an instability of phage lambda λ DNA in *E. coli* and genomic variation between rats. Transcription initiation sites were mapped in 8-day-old rat brain poly(A)$^+$ mRNA. Primer extension indicated multiple transcription start sites at −159 and −339/−342 nt upstream of the translation start site; reverse transcriptase coupled PCR showed that the most 5′ transcription start site is located between −465 and −440. Northern blotting demonstrated that ~90% of the B-50 mRNAs initiates at approximately −50. Promoter analysis by transient transfection assays in undifferentiated and retinoic acid-differentiated P19-EC cells revealed that the rat B-50 gene contains two promoters. P1 (located between −750 and −407) contains commonly observed promoter elements such as a TATA box and CCAAT boxes. P2 (located between −233 and −1) neither contains TATA boxes, CCAAT boxes nor consensus sequences of house-keeping gene promoters like GC-boxes. The activity of P1 is inhibited at neuroectodermal differentiation of P19-EC cells whereas the activity of P2 is stimulated. In 8 day old rat brain the majority of the B-50 mRNA transcripts are derived from P2. It is concluded that at this developmental stage P2 is the most important promoter.

Key words: B-50/GAP-43; Transcription initiation site; Luciferase; Transient transfection; Promoter activity; PC12 cell; P19-EC cell

1. Introduction

The phosphoprotein B-50/GAP-43 has been implicated in neuronal growth and signal transduction [5]. Expression of the protein and its messenger is largely restricted to the nervous system and is greatly enhanced during development and regenerative neurite outgrowth [25,57]. Under certain circumstances, B-50 is present in other cell types of the nervous system as well [14,15,19,30,60,64]. Recently, it has also been shown that B-50 is transiently expressed in non-neuronal cells of the developing chicken limb [58].

In mature neurons expression is mostly low, whereas neurons that are believed to be involved in ongoing synaptic remodelling, e.g. human associative brain areas [4,43–45], rat hippocampal pyramidal cells and olfactory areas [3,17,36,39,47,48,63] continue to express high levels of B-50.

On Northern blots, a single 1.4–1.5 kb rat B-50 mRNA species has been found [2,46,52]. One of the cDNA sequences reported extends to 216 bp upstream of the translation start codon [52], all others have a 5′ untranslated region (UTR) of 70 bp or less [2,9,35,46]. Recently, two cDNAs extending upstream of −216 were isolated by specific screening of a cDNA library with an oligonucleotide probe complementary to the 5′ end of the longest cDNA known. These clones extend to −355 and to −411, with respect to the translation start codon [42].

To study the regulation of B-50 gene expression, we cloned both the rat and human B-50 gene including upstream sequences and analyzed the transcription start sites. This report will only deal with the descrip-

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tion of the results obtained with exon 1 and upstream sequences of the rat gene. While this work was in progress the groups of Fishman [27] and Skene [42] reported on the presence of three exons in the rat B-50 gene. In both studies, multiple transcription starts were found. RNase protection mapping showed three major transcription start sites at −51/52, −55/56 and −80 [27]. Primer extension analysis resulted in multiple bands at −51/52, −55/56 and −80 [27] and multiple bands at −52, −81 and at −132 [42] as well. With a reverse transcriptase-coupled polymerase chain reaction (RT-PCR), the longest transcripts were detectable with a primer from −482 to −456 [42].

In primary cortical cell cultures, a core promoter fragment of 386 bp (−753 to −367) has been described. Inclusion of either the upstream GT-repeat or the downstream GA-repeat depresses the activity of the 386 bp promoter. In constructs where both elements are present, each counteracts the repressive influence of the other [42].

In this report, we describe the isolation and characterization of the rat B-50 exon 1 and upstream flanking sequences. The transcription start sites were mapped with both enzymatic and non-enzymatic methods. Promoter activity of the gene was determined in rat PC12 cells and murine P19 embryo carcinoma (EC) cells differentiated to a neuronal phenotype with NGF and retinoic acid (RA) respectively, by transient transfection using luciferase as reporter gene. In these cell lines, it was previously demonstrated that B-50 mRNA and protein were induced during neuronal differentiation [32,35,61]. Two elements containing promoter activity have been identified: P1 located between bp −750 and −407 and P2 located within the first 233 bp upstream of the translation start.

2. Materials and methods

2.1. Oligonucleotide synthesis, labelling of probes, blotting and hybridization

Oligonucleotides were synthesized on a Pharmacia/LKB Gene Assembler Plus and purified by gel filtration. Oligonucleotides were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Pharmacia). To obtain oligonucleotides of higher specific activity a short complementary oligonucleotide (A-180, for the positions of the oligonucleotides used, see Table 1) was annealed to a longer template oligonucleotide (S−214), and extended with Klenow poly-

Table 1
Position of the oligonucleotides

Code [1]	Sequence 5′ to 3′ [2]	Position [3]
C_{16}	cggatgtcgactcggaagcttCCCCCCCCCCCCCCCC	
S − 1036 [4]	AAGGAACCACAATGGTCAAACAAGC	−1036 to −1012
S − 792	CTCCACAATTATCAACTGCACACAA	−792 to −765
S − 554	TGGGTAATTGGGTCCAGATTGGAGG	−554 to −530
S − 509	TGGCAGGGGACTGGGAGGGGG	−509 to −489
S − 488	CGACGGTCTAGAAATGGGGGTAG	−488 to −466
S − 465	GGGCTATGGGAAGTGATTAGTCACT	−465 to −441
S − 440	GGAAGCTAGTGAACAATTCTGAGAA	−440 to −416
S − 410	tgctatCCCGGGCAGAAAGAAGAAAAAAGATT	−410 to −380
S − 242	AGAAATGCATATGCGGTGAGCAATA	−242 to −218
S − 214	GATCGCTGTAGACCTTACAGTTGCTGCTAACTGCCCT	−214 to −178
S − 175	TGTGTGTGAGGGAGAGAGAGGGA	−175 to −153
S − 34	GAGAAGGCAGGAAGAAGGCAGGGGAAGATACCACCAT	−34 to +2
S + 585	GAGAAAGAAGCTTAAGATGAAGCC	+585 to +609
A − 445	CTAATCACTTCCCATAGCCCCTACC	−445 to −469
A − 218	TATTGCTCACCGCATATGCATTTCT	−218 to −242
A − 180F	GGCAGTTAGCAGCAACTGTAAGGTCTACAGCGATC	−180 to −214
A − 180	GGCAGTTAG	−180 to −189
A − 161	ACACACCAGGGCAGTTAGCAGCAA	−161 to −194
A − 88	CTTGCTCACTCGCTCTCTCGCGCT	−88 to −111
A − 62	agcggatCCCCCTCTCCACCTCTTTTC	−62 to −81
A + 30	tggaattCTGTTTGGTTCTTCTCATACAGC	+30 to +8
A + 89	TTATCCTCCGGTTTGACACCATCTT	+89 to +65
A + 130	CCACGGAAGGTAGCCTGA	+130 to +112
A + 636	TGTCGGGCACTTTCCTTAGGTTTGG	+636 to +611
A + 813	CTCACAGACGTGAGCAGGACAG	+813 to +791

[1] The codes refer to the DNA strand (S = sense, identical to mRNA; A = antisense), and the position of the 5′ base of the oligonucleotide in the corresponding genomic sequence.
[2] Lower case represents non-homologous nt added to the 5′ end to introduce endonuclease recognition sites (underlined).
[3] The first base of the startcodon is numbered +1.
[4] Based on the sequence published by Nedivi et al. [42].

Table 2
Construction of genomic subclones and luciferase/LacZ expression hybrids

Name	Plasmids used	Insert size	Restriction enzymes used
$pG3(1.0E1.0)_{12}$ *	pGEM-3Z	2 kb $(GA)_{12}$ genomic insert (−957 to 1 kb intron 1)	*Hin*dIII
$pG3(1.0E1.0)_{41}$	$pG3(1.0E1.0)_{12}$/TA(−789 to +30)	2 kb $(GA)_{41}$ genomic insert (−1015 to 1 kb intron 1)	*Bst* X I/*Nhe*I
pGB_E	pGEM-3Z	cDNA of B-50 (−40 to +1085)	*Eco*RI
pGB_{ES}	pGB_E	cDNA of B-50 (−40 to +1085)	*Eco*RI and *Sph*I
pGB_{SX}	pGB_{ES}/TA(−750 to −53)	(−481 to +1085)	*Sal*I/*Xba*I and *Xho*I/*Xba*I
TA(−750 to −53)	TA-vector	PCR product of $pG3(1.0E1.0)_{41}$	
TA(−750 to −218)	TA-vector	PCR product of −1016Luc	
TA(−789 to +30)	TA-vector	PCR product of λGEM-11.4	
$pBlue_{XK}$	pBluescript II KS	PCR product of λGEM-11.2 (−410 to −1)	*Xma*I/*Kpn*I
$pBlue(8.4E3.4)_S$	pBluescript II KS	11.8 kb $(GA)_{41}$ genomic insert of λGEM-11.4	*Sac*I
−1016Δ(−321 to −263) to −112Luc	p19Luc		*Hin*dIII/*Nhe*I and *Sma*I
−1016 to −112Luc	$pG3(1.0E1.0)_{12}$		*Bst* X 1/Nhe
−1015 to −233Luc	−1015Luc		*Nsi*I/*Sac*I
−1015Δ(−321 to −263)Luc	$pBlue_{XK}$/−1015Δ(−321 to −263) to −112Luc		*Nsi*I/*Kpn*I
−1015Luc	$pBlue_{XK}$/−1015 to −112Luc		*Nsi*I/*Kpn*I
$-1015Luc_S$	−1015Luc/p19Luc		*Hin*dIII and *Sma*I
−1015Δ(−613 to −407) to −1Luc	$-1015Luc_S$		*Bst* X I/*Sma*I
$-1015\Delta(-321 \text{ to } -263)Luc_S$	$-1015Luc_S$/−1015Δ(−321 to −263)Luc		*Bst* X I/*Nhe*I
−750 to −407Luc	PCR on $-1015Luc_S$		*Hin*dIII/*Psp*AI
−613Luc	$-1015Luc_S$		*Hin*dIII/*Bst* X I
−613 to −407Luc	−613Luc		*Sma*I/*Sac*I
−407Luc	$-1015Luc_S$		*Hin*dIII/*Sma*I
−407Δ(−321 to −263)Luc	−1015Δ(−321 to −263)Luc		*Hin*dIII/*Sma*I
−233Luc	$-1015Luc_S$		*Hin*dIII/*Nsi*I
−233 to −112Luc	−233Luc		*Nhe*I/*Sac*I
−112Luc	$-1015Luc_S$		*Hin*dIII/*Nhe*I
−8.4kbLuc	$pBlue(8.4E3.4)_S$/$-1015Luc_S$		*Sal*I/*Nhe*I

* 1.0E1.0 = 1 kb upstream of the ORF, the coding part of exon 1 and the first 1 kb of intron 1 (total size 2 kb).

merase (Pharmacia) in the presence of [α-^{32}P]dCTP, followed by purification of the labelled strand (A−180F) on a denaturing polyacrylamide gel [40,59].

DNA probes were prepared by random-primed labelling according to the protocol supplied by the manufacturer (Boehringer Mannheim [24]) and by PCR using 20 cycles, 100 ng template, 3 μM dCTP and 50 μCi [α-^{32}P]dCTP followed by gel filtration on Nick columns (Pharmacia). Southern and Northern blots were prepared on nylon membranes (Hybond-N, Amersham). Hybridizations were performed according to the protocols supplied by the manufacturer of the membranes.

2.2. Genomic DNA

A λGEM11 genomic library of rat liver DNA (Sprague–Dawley) was obtained from Promega; a λEMBL3 genomic library of rat liver DNA (Wistar) was a kind gift of Dr. Axel Themmen, Department of Endocrinology, Erasmus University Rotterdam, The Netherlands. Isolation of phage λ and plasmid DNA, restriction enzyme analysis and subcloning of fragments were performed according to standard protocols as described in [54].

2.3. Primer extension

For enzymatic procedures total RNA was isolated by the method of Chirgwin et al. [10]. RNA for Northern blots was isolated using RNAzol extraction (Cinna/Biotecx, Friendswood, USA). Poly(A)$^+$ mRNA from 8-day-old rat brain was isolated by either two cycles of oligo(dT) cellulose chromatography [1] or by using the polyA tract kit (Promega). The remaining RNA, not binding to oligo(dT) is referred to as poly(A)$^-$, and does not hybridize with B-50 cDNA. cDNA synthesis was carried out at 37°C for 2 h on 1 μg poly(A)$^+$ mRNA derived from 8-day-old rat brain, with exon 2 primer A+89 (20 pmol) in a final volume of 10 μl containing 50 mM Tris-HCl, pH 7.6, 70 mM KCl, 10 mM $MgCl_2$, 4 mM DTT, 20 U RNAsin, 1 mM dNTPs each, 50 U AMV- or moloney murine leukaemia virus (MMLV)-reverse transcriptase (Pharmacia) and 12.5 μCi [α-^{32}P]dCTP. Prior to the cDNA synthesis the mRNA was heat-denatured at 80°C for 5 min and cooled on ice to reduce its secondary structure. An exon 2 primer was chosen to circumvent amplification of DNA contaminants in the RNA preparation. Primer extension products were separated on a 6% polyacrylamide/7 M urea gel and autoradiographed. cDNA products used in the RT-PCR protocol were synthesized as described above in the absence of radiolabel.

2.4. Polymerase chain reaction (PCR) and sequencing

A typical PCR of 50 μl contained 15 pmol of each primer and 0.05 U SuperTaq polymerase (HT-technology, Cambridge UK) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs each, 0.01% (w/v) gelatin and 0.1% Triton X-100. After denaturation for 5 min at 94°C, amplification was performed in a thermal cycler (Pharmacia, Gene-ATAQ-Controller) for 30 cycles: 1 min at 94°C, 1 min at 62°C, 1 min (cycles 1–15) or 2 min (cycles 16–30) at 72°C and completed by an incubation at 72°C for 10 min. In the PCR reactions performed, the following amounts of template DNA were used: plasmids 1 ng; genomic DNA 0.5 μg; RT-PCR 0.1–5 μl of a 20 μl RT reaction. PCR products used for subcloning were first cloned in either the TA vector (InVitroGen) or pBluescript II KS (Stratagene) and sequenced by the dideoxynucleotide chain termination method [55] with a T7 DNA polymerase sequencing kit (Pharmacia) and Sequagel-6 (National Diagnostics). A summary of the constructs prepared in the various plasmids used in this study is given in Table 2.

2.5. Cell culture and DNA transfection protocol

Since the transfection efficiency both in PC12 cells and P19-EC cells was found to be highly sensitive to changes in cell-culture conditions and the transfection protocol used (data not shown), a detailed description of cell-culturing and transfection conditions is given. All transfections were carried out with a total of 5 μg DNA, i.e. 4 μg Luc vector + 1 μg LacZ vector. Plasmid DNA was purified by two rounds of cesium chloride density centrifugation, the quality of the plasmid preparations was assessed by EtBr staining on agarose gel and the DNA concentration was determined spectrophotometrically.

PC12 cells were grown at 37°C, 7.5% CO_2 in Rosswel Park Memorial Institute medium (RPMI 1640) containing 5% fetal calf serum (FCS), 10% heat-inactivated horse serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml, Flow laboratories) (RPMI-FCS). Transfection of DNA in PC12 cells was essentially performed according to Greene et al. [29]. Two days before transfection the medium was replaced. One day prior to transfection the cells were resuspended and replated at a density of 5×10^5 cells per 35 mm collagen-coated dish. Immediately before the transfection, cells were rinsed twice in RPMI 1640. The transfections were performed in 0.5 ml chemically defined N1 medium (RPMI 1640 supplemented with putrescine, progesterone, selenium, transferrin and insulin [7]) containing 8 μg lipofectin (Gibco-BRL). Five hours after transfection 0.5 ml RPMI-FCS was added. After 16 h, the medium was replaced with fresh RPMI-FCS containing 50 ng/ml βNGF (Boehringer). Cells were harvested 48 h after transfection in 250 μl lysis buffer (25 mM Tris-phosphate, pH 7.8, 15% glycerol, 0.1% Triton X-100). Immediately after harvesting, cell suspensions were frozen in dry-ice/isopropanol and stored at −80°C until further analysis.

Murine P19-EC cells [53] were cultured at 37°C, 7.5% CO_2 in a bicarbonate-buffered 1:1 mixture of Dulbecco's Minimum Essential Medium (DMEM) and Ham's F12 medium (DF-medium) containing 7.5% FCS in gelatin coated culture flasks. The cell line was passaged three times a week by rinsing twice with phosphate-buffered saline (PBS) and dislodging by trypsin-EDTA. In the transfection experiments either undifferentiated P19-EC cells, referred to as P19-EC, or neuroectodermally differentiated P19-EC cells, referred to as RA aggregates, were used.

Twenty-four hours prior to transfection, P19-EC cells were harvested and replated at a density of 1.25×10^5 cells per 35 mm gelatin-coated dish. Cells were transfected in 750 μl DF-medium with 11.25 μl *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl ammonium methylsulphate (DOTAP, Boehringer Mannheim). After 16 h, the medium was renewed, and 48 h after transfection, the cells were harvested and stored as described for the PC12 cells. Neuroectodermal differentiation was accomplished by aggregation of the cells in bacterial grade uncoated 85 mm petri dishes at a density of 10^5 cells/ml and 2×10^6 cells per dish, in the presence of 10^{-6} M RA (Sigma) in DF medium. After 3 days of aggregation the medium was refreshed. Two hours prior to transfection, the aggregates were trypsinized, dispersed through a 22 gauge needle and plated in gelatin-coated 35 mm dishes. Unattached cells were removed by washing; transfection was performed as described for P19-EC cells. Enzyme activity was measured in the cell lysates prepared by three freeze-thaw cycles followed by removal of cell debris by centrifugation at 14,000× *g* at 4°C. Luciferase activity was measured in 10–70 μl lysate using a Lumac/3M Biocounter M2010A [18]. β-Galactosidase activity was determined in 30–60 μl lysate [54].

Several reference and control constructs were included in each transfection series: p19Luc and pLacZ as negative controls, the RSV promoter fused to the luciferase gene (RSVLuc) as positive control and RSVLacZ as an internal reference. Within each experiment transfections were performed in duplicate. The luciferase activity measured was corrected for differences in the size of the various constructs. After correction for both differences in size of the B-50

promoter constructs and differences in transfection efficiency (RSVLacZ was cotransfected with each construct to serve as an internal standard), the activities of the B-50 promoter constructs are expressed as percentages of the activity of the RSV promoter.

3. Results

3.1. Isolation, characterization and sequencing of the rat genomic clones

A rat λGEM-11 genomic library (Sprague–Dawley) was screened with a radiolabelled B-50 cDNA clone pGB_E, ranging from −40 to +1085 [46]. Screening approximately 10^6 phages (duplicate filters, ±4 genome equivalents) yielded 7 B-50-positive clones. These were purified and characterized by restriction fragment analysis. Four clones (λGEM-11.2–λGEM-11.5) hybridized with an exon 1-specific oligonucleotide [40,58] A − 180F (for nomenclature see Materials and methods) based on the most 5′ end of the cDNA described by Rosenthal et al. [52].

Restriction fragment analysis of clones λGEM-11.2–λGEM-11.5 revealed that these clones resulted from amplification of a single B-50 exon 1- positive phage. The insert size of these clones was 11.8 kb of which 8.4 kb was upstream and 3.4 kb downstream of the initiation codon (8.4E3.4). A 2 kb *Hin*dIII fragment of λGEM-11.5 that hybridized on Southern blot with oligonucleotide A − 180F was subcloned in pGEM-3Z ($pG3(1.0E1.0)_{12}$). This insert was sequenced with T7 DNA polymerase using SP6 and T7 primers. Further subclones were generated by digestion of pG3 $(1.0E1.0)_{12}$ with *Bst*X I, *Xba*I, *Ava*I, *Acc*I and *Nhe*I, followed by religation and sequencing to completion using the SP6 primer. The complementary strand was sequenced with oligonucleotides derived from the sequence obtained with T7 and SP6 primers. Comparison of the obtained sequence with that published by Grabczyk et al. [27] revealed the absence of 58 bp of

Fig. 1. Nucleotide sequence of the 5′ UTR and exon 1 of the rat B-50 gene. The sequence, of which the coding strand is shown, is depicted from 1015 nt (*Hin*dIII site) 5′ from the adenine of the translation start codon (+1). The first 60 nt of intron 1 are also shown. Restriction endonuclease recognition sites used in this study are single underlined. The coding region of exon 1 is presented in italics. The forward slash indicates the exon/intron border. The dashed underlined sequences indicate the position of the (GT)-repeat and the (GA)-repeat. Potential binding sites for transcription factors are double underlined (see Results).

the (GA)-repeat in these subclones. In order to obtain a second independent genomic clone containing exon 1, a second rat genomic (Wistar) λEMBL-3 library (duplicate filters, ±10 genome equivalents) was screened with a random-primed labelled insert isolated from clone λGEM-11.3. Two additional independent clones (λEMBL-3.4 and λEMBL-3.6) were characterized by restriction fragment analysis. The insert size of λEMBL-3.4 was 13.8 kb of which 8.8 kb was 5′ of exon1 and 5.0 kb 3′ of exon 1 (8.8E5.0). The insert size of λEMBL-3.6 was and 13.0 kb (8.0E5.0).

In order to determine whether the difference in (GA)-repeat size between our results and the previously published sequences was the result of a cloning artefact or a genuine difference, the length of the (GA) repeat was determined using PCR. The four λGEM-11 clones, rat genomic DNA and $pG3(1.0E1.0)_{12}$ were amplified with oligonucleotides S − 792 and A − 62. The PCR fragment of the genomic subclone was 675 bp, whereas the PCR fragment obtained from rat genomic DNA was 735 bp (data not shown). Both bands were present in the PCR fragments obtained from each of the λGEM-11 clones (data not shown). Sequencing of the longest PCR product showed that our initial genomic subclone missed 58 bp $(GA)_{29}$. These 58 bp have been included in the sequence shown in Fig. 1. Comparison of this sequence with the previously published sequence by Grabczyk et al. [27] showed one mismatch (a G instead of a C at position −501) and an insertion of 2 bp in the (GA)-repeat; comparison with the previously published sequence by Nedivi et al. [42] showed two mismatches (a G instead of an A at position −321 and an A instead of a G at position −309), an insertion of 12 bp in the (GT)-repeat and an insertion of 10 bp in the (GA)-repeat. Genomic Sprague–Dawley and Wistar DNA, genomic clones λGEM-11.2–λGEM-11.5 and $pG3(1.0E1.0)_{12}$ were amplified with primer S − 1036 and A − 445 for the (GT)-repeat and with primers S − 554 and A + 30 for the (GA)-repeat. To circumvent PCR artefacts, multiple clones from independent PCRs were sequenced. The resulting PCR products were subcloned in the TA vector. The (GA)-repeat inserts were sequenced with A − 161, M13-reverse primer was used for sequencing of the (GT)-repeat inserts. The resulting sequences revealed that the length of the (GA)-repeat was variable over different animals, ranging from 36 to 43 repeats, whereas the length of the (GT)-repeat was not variable over different animals or genomic exon 1 clones (23 repeats, Table 3).

The B-50 5′ UTR sequence shown in Fig. 1 was screened for consensus sequences for vertebrate transcription factors according to Faisst and Meyer [22] using the GCG program (Genetics Computer Group Inc. [20]). The consensus binding sites conserved between human [16] and rat B-50, both in position and sequence, are delineated. Conserved sites are AP1 [26], AP2 [11,13], CBP [21], Myb [6], NF-IL6 [38], PEA3 [51,65], PuF [49] and TBP [28,66].

Table 3
Analysis of the (GA)- and (GT)-repeat sizes

DNA source	n in $(GA)_n$	Number of observations
Sprague–Dawley [1]	43	1
	41	2
	40	1
	38	3
	37	1
Wistar	41	1
	38	1
	36	2
λGEM-11 exon 1 clone	41	4
	13	2
DNA source	**n in $(GT)_n$**	**Number of observations**
Sprague–Dawley	23	4
λGEM-11 exon 1 clone	23	4

3.2. Mapping of the B-50 transcription start sites

Primer extension analysis was carried out on poly(A)$^+$ mRNA from 8-day-old rat brain with primer A + 89 (an exon 2-derived primer) using 2 different enzymes (MMLV-RT and AMV-RT) in the presence of [α-^{32}P]dCTP, revealing extension products at −159 and −339/343 (Fig 2, lanes RM and RA respectively). Only those products that could be generated with both enzymes were regarded as specific. S1 nuclease, RNase A, RNase H and rapid amplification of cDNA ends (RACE) was only successful on cRNA and not on poly(A)$^+$ mRNA.

In order to determine the most 5′ located transcription start site, PCRs were performed on cDNA, synthesized as described above in the absence of radiolabel, with primers S − 242, S − 440, S − 488 or S − 509 at the 5′ end and primer A + 30 as nested primer at the 3′ end. A clear EtBr band (280 bp) could be detected with S − 242 and a less intense band with S − 440 (478 bp), but no band was seen with primers S − 488 and S − 509, indicating that the majority of the transcripts initiate within the first 242 bp upstream of the translation start codon and some may start close to −440 (results not shown). In order to determine the most 5′ extended product present in the cDNA a PCR was performed with primers S − 465, S − 440 and S − 410 at the 5′ end and primers A + 89 and A + 30 at the 3′ end. A control PCR was carried out on $pG3(1.0E1.0)_{12}$ (Fig. 3, control panel). The Southern blot (Fig. 3) probed with a radiolabeled PCR product (from −175 to +30) showed that, with both primers at the 3′ end (Fig. 3A,B) and with both enzymes, amplification with S − 440 (lanes 2) as well as with S − 410 (lanes 3) resulted in products of the correct size and

that a weak or no signal was obtained with S − 465 as PCR primer (lanes 1).

To determine the relative contribution of the different transcription start sites to the B-50 mRNA population, Northern blots were hybridized with different 5′ UTR-derived probes and compared to a probe hybridizing within the open reading frame. Triplicate Northern blots (Fig. 4, panel NB) were prepared each with 23 μg total RNA and 2 μg poly(A)$^+$ mRNA obtained from 8-day-old rat brains. A Southern blot with a PCR product from −465 to + 30 was prepared in duplicate (Fig. 4, panel SB). The first Northern blot was combined with one of the Southern blots and hybridized with probe II (−410 to −161). Another pair of blots was hybridized with probe III (−175 to + 30). The third Northern blot was hybridized with probe IV (+585 to + 813). The specific activity of the 3 probes used was equalized by addition of the respective unlabelled PCR products. After exposing all blots to film, the blots were stripped of their respective probes, as verified by autoradiography. The hybridization procedure was repeated two more times so that all Northern blots were hybridized with each of the probes. The result of one round of these hybridizations is shown in Fig. 4. Quantification was performed with a TIM digital analyzing system (DIFA measuring systems, Breda). Multiple exposures were quantified to circumvent non-linearity of the films. Quantification showed that the hybridization signal on the duplicate Southern blots with probe II and probe III is equal (Fig. 4, panel SB). On Northern blot the B-50 signal with probe II was considerably lower in comparison to probe III. Quantification of the poly(A)$^+$ mRNA signal with the three different probes showed that the ratios between the probes are II : III : IV = 9 : 34 : 78, suggesting that the majority of the mRNAs contain a relatively short 5′ UTR. Only a single hybridization signal was detected with the three different probes and no substantial differences in apparent molecular weight were observed.

Fig. 2. Primer extension analysis of rat B-50 mRNA. Primer extension was carried out on 1 μg poly(A)$^+$ mRNA isolated from 8-day-old rat brains with primer A + 89 in the presence of 100 μCi [α-^{32}P]dCTP using AMV- (lane RA) or MMLV-RT (lane RM). The reaction was carried out for 2 h at 37°C. The numbers represent the number of nt the products were extended 5′ of the first base of the startcodon, as calculated from the position to the sequence marker (lane M).

To make a more accurate estimation of the molecular size of the B-50 positive bands, B-50 cRNA markers of 1355, 1606 and 1873 nt in length respectively were prepared. The quality of the in vitro transcripts was assessed on agarose gel by EtBr staining and the amount of RNA was measured spectrophotometrically. The three markers were combined and mixed with 26 μg poly(A)$^-$. The samples were run on duplicate agarose gels and blotted. The resulting Northern blots were hybridized with probe I (−465 to −218) and probe IV (+585 to + 813), Fig. 5, A and B respectively). With both probes the marker lanes containing 160 pg of each in vitro transcript were clearly visible. The B-50 hybridization signal with probe I was much weaker than that with probe IV, confirming the observation of Fig. 4 that the majority of the B-50 mRNAs have a relatively short 5′ UTR. The lanes (f) containing 26 μg poly(A)$^-$ RNA did not give a signal with either probe, showing that the hybridization signals are derived from polyadenylated B-50 mRNAs. The average size of the B-50 bands was estimated from the B-50 markers as 1425 nt with probe I and 1350 nt with probe IV.

3.3. Mapping of B-50 promoter(s)

To identify the B-50 promoter(s), constructs containing various fragments of the B-50 5′ UTR were fused to the firefly luciferase gene in the eukaryotic expression vector p19Luc [62]. The promoter activity of these constructs was determined by transient lipofection into PC12 and P19-EC cells. Transfection efficiency and

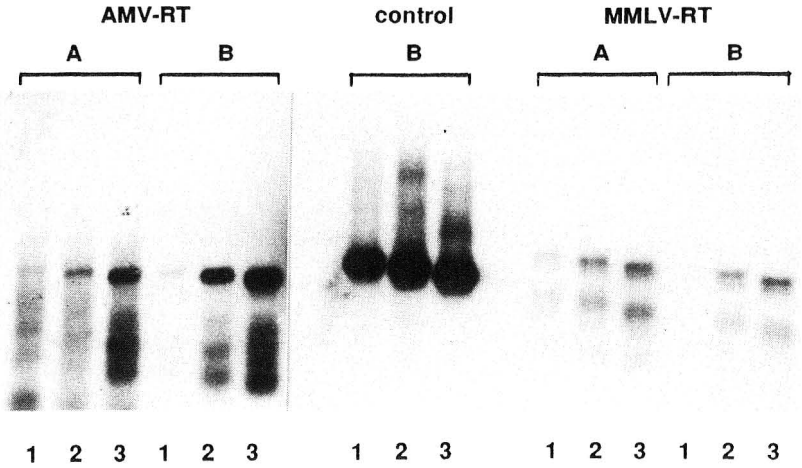


Fig. 3. Reverse transcriptase-PCR. Reverse transcription was carried out as described in the legend of Fig. 2. One percent of the primer extension product synthesized with either AMV- or MMLV-reverse transcriptase was amplified with primers S − 465 (lanes 1), S − 440 (lanes 2) or S − 410 (lanes 3) at the 5′ end and primers A + 89 (A) or A + 30 (B) at the 3′ end. A control PCR was carried out with the above mentioned 5′ primers and A + 30 as the primer at the 3′ end, using pG3(1.0E1.0)$_{12}$ as template. The Southern blot, after separation of the PCR products on a 2% agarose gel, was hybridized with a probe from −175 to + 30 (pG3(1.0E1.0)$_{12}$ amplified with primers S − 175 and A + 30).

cell number were corrected for by co-transfecting all luciferase constructs with RSVLacZ. RSVLuc activity was determined in each transfection round and served as a reference for B-50 promoter activity.

The activity of the B-50 constructs was first tested in NGF differentiated PC12 cells [56]. The constructs tested in PC12 cells were (−1015Luc), (−1015Δ(−321 to −263)Luc), (−1015 to −112Luc) and (−1015 Δ(−321 to −263) to −112Luc). The maximal activity of the (−1015Luc) construct (300 light units) was 1.1% of the activity of the RSV promoter (27,000 light units, Table 4). Deletion of the first 112 bp upstream of the ATG, (−1015 to −112Luc), decreased the activity by 8-fold (0.14% of the RSV promoter). Absence of 58 bp of the GA-repeat only showed an effect in the constructs lacking the first 112 bp upstream of the ATG, (−1015 to −112Luc) vs (−1015 Δ(−321 to −263) to −112Luc), i.e. a 3.8-fold induction (see Table 4).

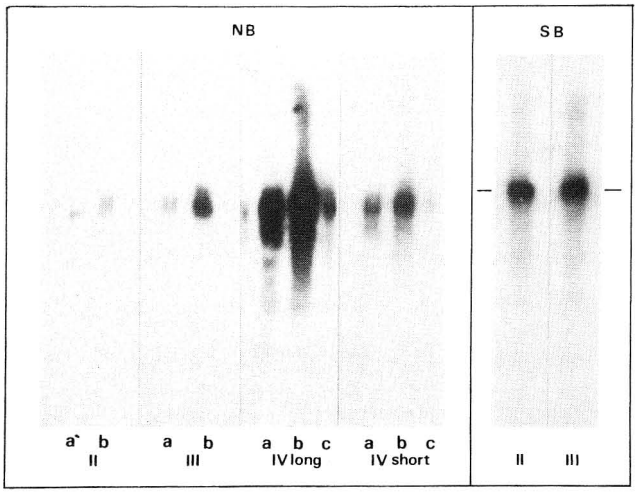


Fig. 4. Differential Northern blotting of B-50 mRNA. An autoradiograph is shown of three Northern blots each containing 23 μg total RNA (lanes a), 2 μg poly(A)$^+$ mRNA (lanes b) and 0.2 μg poly(A)$^+$ mRNA (lanes c) of 8-day-old rat brain RNA. The Northern blots were hybridized with probe II (−410 to −161), probe III (−175 to + 30) and probe IV (+585 to + 813). The specific activities of the probes was equalized for each probe used. In panel SB, an autoradiograph is shown of duplicate Southern blots containing a PCR product from −465 to + 30, probed with probes II and III to control for specific activity. Exposure time was 16 h with intensifying screens for autoradiographs II, III and IV long. The autoradiograph shown in IV short was obtained by exposure for 3 days without intensifying screens.

P19-EC cells (undifferentiated and RA aggregated) were chosen as the next cell line to test the B-50 promoter activity. In order to localize the B-50 promoter(s), a series of deletions of the 8.4 kb luciferase B-50 promoter fusion construct (−8.4kbLuc) were generated and tested for their promoter activity. Deletion of the 5′ end to −1015, (−1015Luc) or to −613, (−613Luc) did not show significant changes in promoter activity in either cell type. A further deletion of 206 bp to −407 (−407Luc) resulted in a 18.2-fold decrease in activity in P19-EC cells and a 12.7-fold decrease in activity in RA aggregates, indicating the presence of an element located between −613 and −407 which contains or modulates promoter activity. Further shortening to −233 (−233Luc) resulted in a

Table 4
Analysis of B-50 promoter constructs in PC12 cells

Construct	Activity as % of RSVLuc
−1015Luc	1.10 ± 0.12 *
−1015 to −112Luc	0.14 ± 0.05
−1015Δ(−321 to −263)Luc	0.98 ± 0.21
−1015Δ(−321 to −263) to −112Luc	0.52 ± 0.03

* Mean ± S.E.M. ($n \geq 6$).

6.8-fold increase in luciferase activity in undifferentiated cells and a 5.9-fold increase in RA aggregates, indicating the presence of an element with promoter activity within the first 233 bp 5′ of the ATG. To map this element further, we subdivided the (−233Luc) construct in 2 fragments, a 121 bp upstream fragment (−233 to −112Luc) and a 112 bp downstream fragment (−112Luc). Neither of these constructs had an activity similar to the 233 bp construct; the upstream part (−233 to −112Luc) was devoid of promoter activity.

To determine whether the two elements located between −613 to −407 and −233 to −1 were promoters or modulated promoter activity, deletions of the 1 kb construct (−1015Luc) from the 3′ end and from both the 5′ and 3′ end were generated. Deletion of the first 112 bp upstream of the translation start (−1015 to −112Luc) and further deletion to −233 bp (−1015 to −233Luc) resulted in less promoter activity in both cell types as compared to the (−1015Luc) construct, confirming the presence of a promoter within the first 233 bp upstream of the ATG. To map the promoter activity within the (−1015 to −233Luc) construct further, this construct was shortened on both ends to give (−750 to −407Luc) and (−613 to −407Luc). The smallest construct with substantial promoter activity was (−750 to −407Luc). Deletion of the BS fragment of the (−1015Luc) construct (−1015Δ(−613 to −407)Luc) reduced its activity 3.0-fold in P19-EC cells and 3.1-fold in RA aggregates resulting in an activity comparable to the activity of the (−233Luc) construct.

The influence of the (GA)-repeat on B-50 promoter activity was assessed in 3 constructs (−1015Δ(−321 to −263)Luc), (−407Δ(−321 to −263)Luc) and (−1015Δ(−321 to −263) to −112Luc). In all constructs tested, deletion of 58 bp of the (GA)-repeat had a moderate effect on the promoter activity (maximal change < 1.6-fold).

To investigate the effect of neuroectodermal differentiation on promoter activity, either stimulating or inhibiting, the ratio of the activity of the promoter constructs in RA aggregates and P19-EC cells is calculated (Fig. 6, ratio RA/EC). The ratio was > 1 for (−8.4kbLuc), (−1015Luc), (−1015Δ(−321 to −263)Luc), (−613Luc), (−407Luc), (−407Δ(−321 to −263)Luc), (−233Luc), (−112Luc), (−1015Δ(−321 to −263) to −233Luc), (−1015Δ(−613 to −407)Luc) and < 1 for (−1015 to −112Luc), (−1015 to −233Luc), (−750 to −407Luc), (−613 to −407Luc). The ratio of the minimal promoter construct at the 5′ end, (−750 to −407Luc), is 0.43 and the ratio of the minimal promoter construct at the 3′ end, (−233Luc) is 1.80, indicating that the latter construct is 4 times more sensitive to neuroectodermal differentiation.

4. Discussion

4.1. Sequence of the B-50 5′ UTR

This paper describes the isolation and characterization of rat B-50 exon 1. The structure of both repetitive sequences (GT- and GA-repeat) present in the sequence 5′ of the translation start site is different from that described previously [27,42]. The size of both repeats was determined in DNA from several sources. The size of the (GT)-repeat was found to be 46 bp in all determinations; this differs 12 bp from that published by Nedivi et al. [42]. The size of the (GA)-repeat differed between the various sources used. The smallest size found was 26 bp and could be attributed to an

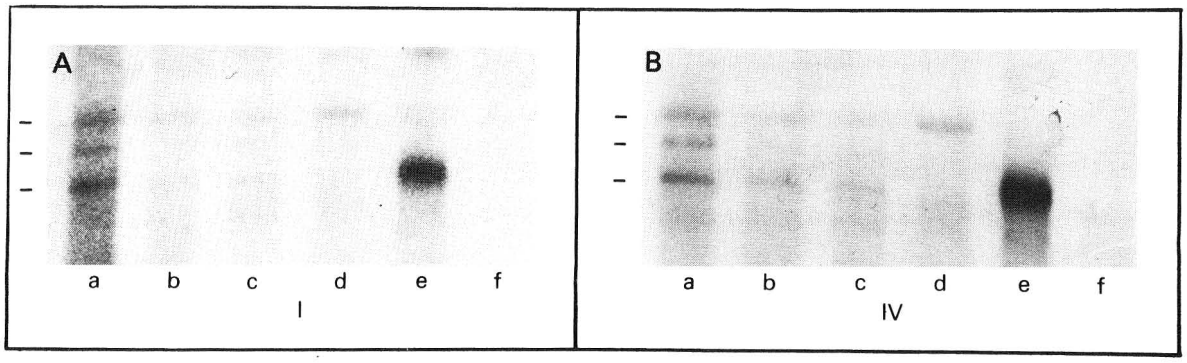
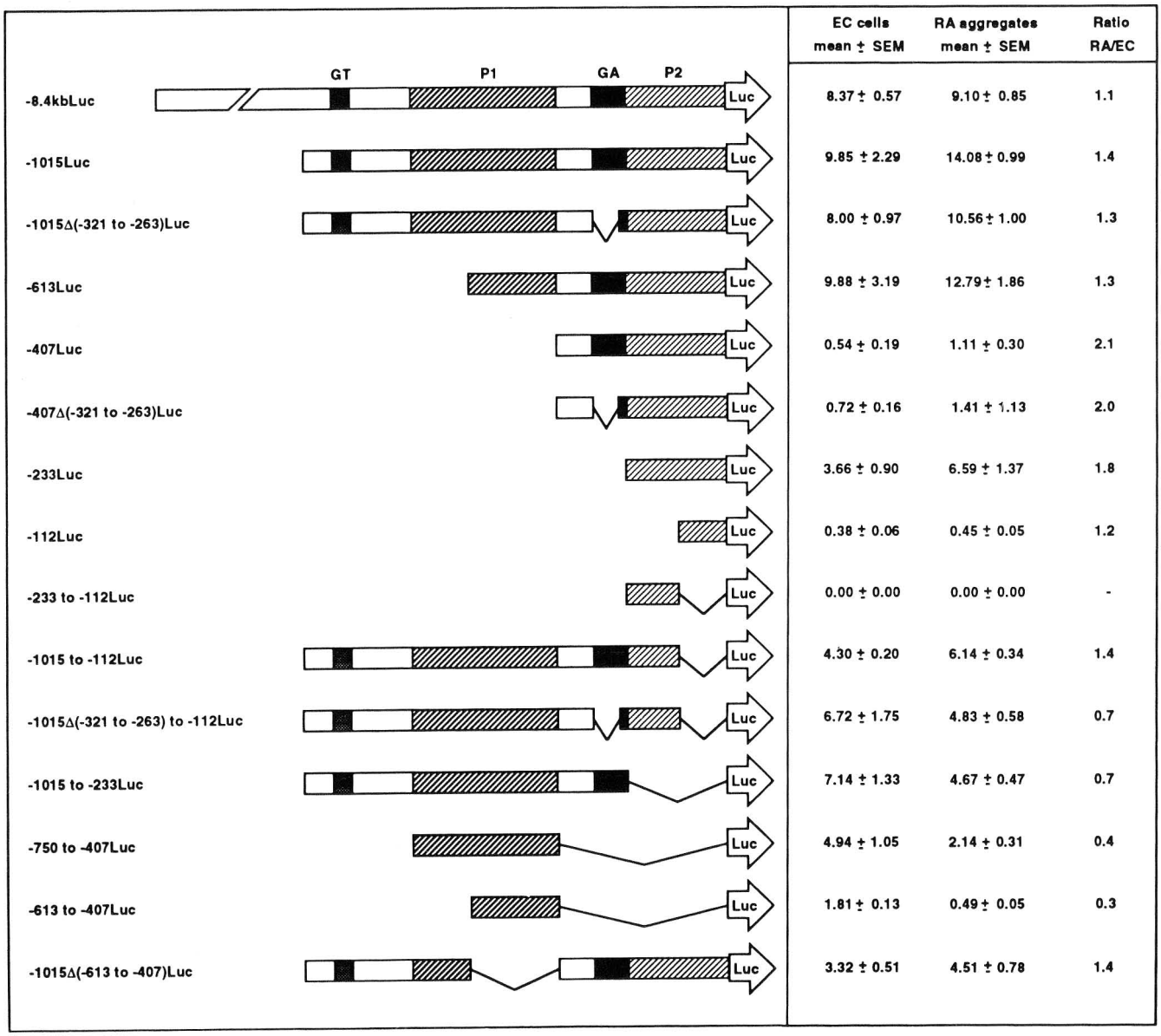


Fig. 5. Determination of the size of 5′ B-50 mRNAs. Three B-50 in vitro transcripts were prepared by transcription of pGB_S after digestion with *Dra*I, *Hin*dIII or *Ear*I yielding products of 1355, 1606 and 1873 nt in length, respectively. These markers were mixed to 160 pg each (lanes a), 40 pg each (lanes b), 20 pg each (lanes c). The other lanes shown contain 80 pg of the 1873 nt marker (lanes d), 26 μg total 8-day-old rat brain RNA (lanes e), 26 μg poly(A)$^-$ RNA (lanes f). To each of the marker lanes containing a B-50 in vitro transcript, 26 μg poly(A)$^-$ RNA was added. Two identical sets of lanes were loaded on an 1.2% agarose glyoxal gel, a Northern blot was prepared and divided in half. One half was hybridized with probe I (panel A: amplification of $pG3(1.0E1.0)_{41}$ with primers S − 465 and A − 218) the other half with probe IV (panel B, amplification of pGB_E with primers S- + 585 and A + 813). The exposure time for the autoradiograph shown in A was 2 days with intensifying screens; the exposure time for the autoradiograph shown in B was 2 days without intensifying screens.

instability of the (GA)-repeat in phage λ in *E. coli*, since at least two different sizes were detectable within phages purified from a single plaque (Table 3). Thus far, we have not noticed deletions in the (GA)-repeat during amplification of any of the plasmid vectors (p19Luc, pBluescript II KS, pGEM-3Z, TA-vector) after transformation in *E. coli*. Amplification of (part of) the insert by thermostable *Taq* DNA polymerase always resulted in the same size of the GA-repeat in the template and in the PCR products (data not shown). The size of the GA-repeat determined by PCR and subsequent sequencing revealed sizes ranging from $(GA)_{37}$ to $(GA)_{43}$ in Sprague–Dawley DNA and from $(GA)_{36}$ to $(GA)_{41}$ in Wistar DNA (Table 3). Apparently, the difference in size of the GA-repeat is the result of genetic variation in the 5′ end of the B-50 gene among individual rats of both strains tested, since we never detected changes in the (GA)-repeat size introduced by PCR.

The (GT)-repeat in the 5′ untranslated region (UTR) is capable of forming right-handed Z-DNA [50] and the (GA)-repeat is capable of forming H-DNA [31]. Both DNA structures have been reported to modulate promoter activity [37,41]. Within the first 350 bp



	EC cells mean ± SEM	RA aggregates mean ± SEM	Ratio RA/EC
-8.4kbLuc	8.37 ± 0.57	9.10 ± 0.85	1.1
-1015Luc	9.85 ± 2.29	14.08 ± 0.99	1.4
-1015Δ(-321 to -263)Luc	8.00 ± 0.97	10.56 ± 1.00	1.3
-613Luc	9.88 ± 3.19	12.79 ± 1.86	1.3
-407Luc	0.54 ± 0.19	1.11 ± 0.30	2.1
-407Δ(-321 to -263)Luc	0.72 ± 0.16	1.41 ± 1.13	2.0
-233Luc	3.66 ± 0.90	6.59 ± 1.37	1.8
-112Luc	0.38 ± 0.06	0.45 ± 0.05	1.2
-233 to -112Luc	0.00 ± 0.00	0.00 ± 0.00	-
-1015 to -112Luc	4.30 ± 0.20	6.14 ± 0.34	1.4
-1015Δ(-321 to -263) to -112Luc	6.72 ± 1.75	4.83 ± 0.58	0.7
-1015 to -233Luc	7.14 ± 1.33	4.67 ± 0.47	0.7
-750 to -407Luc	4.94 ± 1.05	2.14 ± 0.31	0.4
-613 to -407Luc	1.81 ± 0.13	0.49 ± 0.05	0.3
-1015Δ(-613 to -407)Luc	3.32 ± 0.51	4.51 ± 0.78	1.4

Fig. 6. Analysis of B-50 promoter constructs in P19-EC cells. The name of the tested constructs is indicated to the left of the figure. The (GT)- and (GA)-repeat are represented by a grey and a black box respectively. The position of promoters P1 (−750 to −407) and P2 (−233 to −1) are represented by hatched boxes. The activity as a percentage of the activity of the RSV promoter measured in a parallel dish in P19-EC cells and RA aggregates is given with the standard error of the mean ($n \geq 6$). The far right column gives the ratio of promoter activity between differentiated (RA) and undifferentiated (EC) cells.

upstream of the translation start site, the B-50 gene contained none of the classical elements involved in transcriptional regulation, TATA and CCAAT boxes [21] and SP1 binding sites (GC-boxes) [8]. More upstream, two potential binding sites for the TATA binding protein (TBP) could be found and three binding sites for the CAATT binding protein (CBP) on the non-coding strand were detected. Other conserved (between rat and human) consensus potential binding sites on either of the DNA strands were AP1, AP2, Myb, NF-IL6, PEA3, and PuF [22]. None of these putative regulatory elements is restricted to genes expressed exclusively in nervous tissue.

4.2. B-50 transcription start sites

In this study, we tried to identify the 5′ end of the B-50 mRNA and the relative contribution of the different transcription starts to the B-50 mRNA population by Northern blotting and a variety of enzymatic methods, S1 nuclease, RNase A, RNase H and RACE were not successful to determine the transcription start sites (data not shown). Northern blotting showed that the majority of the transcription sites are located close to the translation initiation codon (Fig. 4). The major transcription start site was calculated from the relative intensities of the signals of B-50 mRNAs obtained from Northern blots hybridized with 3 different. The hybridization signal obtained with probes II (−410 to −161) and III (−175 to +30) on duplicate Southern blots was identical (Fig. 4, panel SB). For calculation of the contribution of the activity of P1 and P2 to the B-50 mRNA population four assumptions were made: (1) All probes hybridized equally with the B-50 mRNA, based on the signal obtained with the Southern blots. (2) The 3′ probe IV (+585 to +813) recognized all B-50 mRNAs. (3) The signal from probe II contributed fully to the signal obtained with probe III. (4) The signal obtained with probe III is derived from a single transcription initiation site. The signal obtained with each probe was corrected for its length. Using this method the major transcription initiation site was calculated to be located approximately 50 nt upstream of the adenine of the translation start codon which is in agreement with the results of RNase protection mapping and primer extension analysis by Grabczyk et al. [27] and Nedivi et al. [42]. By comparison of the signals obtained with probes I (−465 to −218) and IV from the Northern blots (Fig. 4) it was concluded that ~ 10% of the B-50 mRNAs initiates 5′ of −161. Primer extension analysis indicated that long 5′ transcripts originate from −159 and −339/343. With RT-PCR the most 5′ transcription start site was found between −465 and −441 nt (Fig. 3), a result similar to that previously reported by Nedivi et al. [42].

Using Northern blotting the length of the longest 5′ transcripts was estimated by comparing the electrophoretic mobility of B-50 mRNAs with that of B-50 cRNAs of discrete sizes. The average length of the B-50 signal obtained with probe IV (+585 to +813) was estimated to be 1350 nt whereas the B-50 signal obtained with probe I (−465 to −218) was estimated to be 1425 nt in length, irrespective of the A-tail length.

4.3. B-50 promoter analysis

Initial promoter analysis of B-50 was carried out in PC12 cells, since the expression of B-50 in this cell line can be modulated by NGF and dexamethasone [12,23,33,34]. Although B-50 promoter construct expression was low in PC12 cells (maximally 1.1% of the RSV promoter, Table 4, and 300 light units, see Results), we determined the activity of 4 of the promoter constructs in NGF-differentiated PC12 cells. The results obtained were similar to those obtained in differentiated P19-EC cells, namely that the deletion of the most 3′ 112 bp diminished the promoter activity [56]. Absence of 60 residues of the (GA)-repeat showed an effect only when the first 112 bp upstream of the translation initiation codon were also deleted, indicating that in PC12 cells promoter activity can be inhibited by the (GA)-repeat, which is in agreement with previous observations by Nedivi et al. [42].

Recently, the expression of B-50 mRNA and protein in the pluripotent P19-EC cells was studied in our group [32]. Neuroectodermal differentiation of these cells by aggregation in the presence of 10^{-6} M RA yielded a mixed culture of neuronal cells, astroglial cells and fibroblasts [53]. This differentiation was accompanied by an increase in B-50 mRNA and protein levels as determined by Northern and Western blotting, and by a B-50-specific ELISA. In these cells, B-50 immunofluorescence always co-localized with the neurofilament 68 kDa subunit, indicating that the expression of B-50 protein, under these conditions, is restricted to neurons [32].

Transfection of undifferentiated and neuroectodermally differentiated cells with B-50 promoter-luciferase fusion constructs revealed that the B-50 gene contains two promoters, P1 localized between −750 and −407 (−750 to −407Luc) and P2 localized within the first 233 bp upstream of the translation initiation codon (−233Luc), Fig. 6. Promoters P1 and P2 are active in both differentiated and undifferentiated P19-EC cells; the activity of P2 is induced at neuronal differentiation of P19-EC cells whereas the activity of P1 is not sensitive to neuronal differentiation. The activity of the RSV promoter is induced at neuroectodermal differentiation (15,000 light units in P19-EC cells vs 100,000 light units in RA aggregates). Since the activity of the B-50 promoter constructs is expressed as a percentage

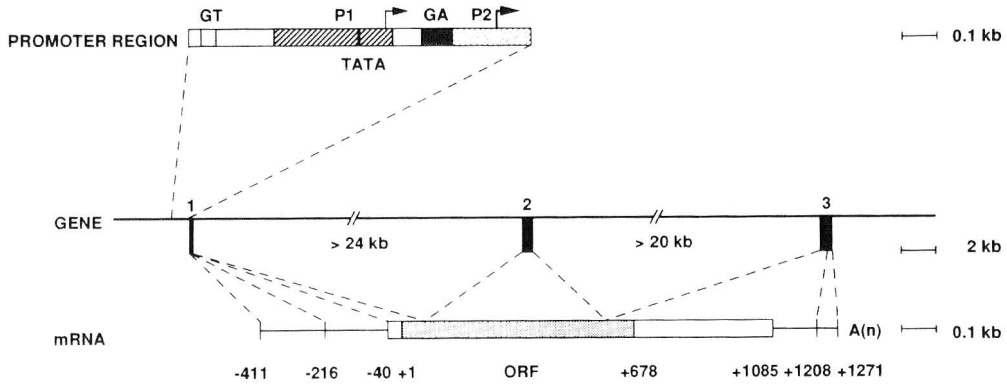


Fig. 7. Organization of the B-50 gene and the 5′ UTR. The B-50 gene contains 3 exons represented by black boxes, numbered 1, 2 and 3 and the minimal size of the introns, derived from the papers of Nedivi et al. [42] Grabczyk et al. [27], is indicated. In the mRNA the open reading frame is represented by a grey box and the 5′ and 3′ UTR by an open box. The dashed lines represent exon borders derived from literature, 5′ ends of the cDNAs were found at: −411 nt [42], −216 nt [52], −40 nt [46]; 3′ ends of the cDNAs were found at: +1085 nt [46,35], +1208 nt [2], +1271 nt [52]. At the left top of the figure the position of promoters P1 and P2 with their respective transcription starts, represented by arrows, as well as the position of the (GT)-repeat, the (GA)-repeat and the putative TATA box are depicted.

of the activity of the RSV promoter, this leads to an underestimation of the induction of the B-50 promoter constructs by neuroectodermal differentiation. Comparison of the ratios of activities in undifferentiated versus differentiated P19-EC cells of constructs (−1015 to −112Luc) and (−233Luc) suggest that potential DNA binding sites for a (protein) factor induced at neuroectodermal differentiation is located between nucleotides −233 and −112. This suggestion is further supported by the ratios of promoter activities in undifferentiated versus differentiated P19-EC cells which is > 1 for all constructs tested containing the sequence between −233 and −112, with the exception of the (−112Luc). The size of the (GA)-repeat had no significant effect on the activity of either P1 or P2 in both phenotypes.

Combining the data from the experiments in which the transcription start sites were mapped and the transfection assays in which the activity and the location of the B-50 promoters was determined, the following conclusions can be drawn. The rat B-50 gene contains 2 distinct promoters both of which act as an initiation site for transcription (Fig. 7). The majority of the B-50 mRNAs isolated from 8 day old rat brain originate from P2. If the stability of P1 and P2 derived transcripts is equal, it is concluded that at this developmental stage, P2 is about 10 times more active than P1 in vivo.

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