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An E-Box Sequence Acts as a Transcriptional Activator for BC1 RNA Expression by RNA Polymerase III in the Brain

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BC1 RNA is a small cytoplasmic RNA that is transcribed by RNA polymerase III (Pol III) in the rodent nervous system. In addition to essential intragenic promoter elements for Pol III, the BC1 RNA gene has five E-box sequences (CANNTG) in its 5′ flanking region. Deletion analysis using an *in vitro* transcription system revealed that the region containing the E2 site (CAATTG) was necessary for effective transcription of BC1 RNA. A construct with point mutations within the E2 site showed reduced transcriptional activity. Furthermore, DNaseT I protection and gel retardation assays demonstrated that the E2 site was recognized specifically by a brain nuclear protein(s). These results suggest that the upstream E-box sequence and its binding protein may be involved in the regulation by Pol III of preferential BC1 RNA expression in the brain.

Rodent BC1 RNA is expressed preferentially in the brain by RNA Polymerase III (Pol III) (1-3) and is distributed into neural dendrites as a ribonucleoprotein particle, BC1 RNP (4-6). The levels of this small nontranslatable RNA increase during the period of synaptogenesis (7, 8) and this RNA is concentrated in synaptodendrosomes with other dendritic mRNAs (9), and the BC1 RNP has been hypothesized to play a role(s) in transport and/or translational regulation of dendritic mRNAs (10, 11).

Because of its neural specificity, the BC1 RNA gene represents an unique class III gene. Recent studies using *in vitro* transcription systems revealed that intragenic promoter elements, A and B boxes (12) and GCAAG/CTTGC motifs (6, 13), are necessary for BC1 RNA transcription. Moreover, Martignetti and Brosius reported a 5′ flanking region containing a TATA-like sequence and a proximal sequence element (PSE) is also necessary for BC1 RNA transcription (12). However, the contribution of other elements in the upstream region to this process has not been analyzed.

We have previously reported that the level of BC1 RNA in skeletal muscle was low, but the level in fetal muscle was high during polyneuronal innervation and decreased progressively after birth (14). Furthermore, BC1 RNA expression in adult skeletal muscle was increased by denervation (14). These expression patterns are similar to those of some muscle-specific genes with the sequence CANNTG, called an E-box, which is the binding site for myogenic factors of MyoD family which contain the basic helix-loop-helix (bHLH) motif, in common (15, 16). The MyoD factors bind to the E-boxes of the acetylcholine receptor promoters and regulate muscle-specific and electrical activity-dependent expression of the gene (17, 18). We noticed that the BC1 RNA gene also has five CANNTG sequences (E1-E5 sites) in its 5′ flanking region. These observations raised the interesting possibility that these E-boxes, together with their binding proteins, may control BC1 RNA expression by Pol III in the brain as well as in the skeletal muscle. Several E-box binding proteins have also been identified in the brain, which may play roles in neural development by regulating neural cell-specific gene expression(19).

In an attempt to elucidate how the preferential production of BC1 RNA by Pol III in the brain is achieved, we focused on E-boxes in 5′ upstream of the BC1 RNA gene, although the E-box is generally known to be an enhancer element for the Pol II system. In this study, we used an in vitro transcription system prepared from rat brain nuclear extract and demonstrated that one of the five E-boxes whose sequence was CAATTG (E2 site) was necessary for effective BC1 RNA transcription

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and that the brain nuclear factor bound specifically to this site.

MATERIALS AND METHODS

Sample preparation. Nuclear protein extracts were prepared from the brains of inbred rats (Wistar), essentially as described by Gorski *et al.* (20). The BC1 RNA gene was isolated from a rat genomic library by hybridization to the oligonucleotide probe for the 3′ portion of BC1 RNA.

Construction of 5′ truncated mutants. Truncated constructs were prepared by polymerase chain reactions (PCRs) using a cloned BC1 RNA gene as a template, the 5′ end primer of each site (K1-K5), which was added to the *Eco* RI site, and the 3′ end primer of the BC1 RNA gene, which was added to the *Bam* HI site. Each PCR product was cloned into the pBR322 vector for *in vitro* transcription. The following primers were used: GTGAGGAATTCACAGGACTTT-GTTTTCCTGAC; TGTCTGAATTCAACAGGCAACCATACTGG-TTG; TATGTGAATTCAACAGGCAACCATACTGGTTG; TGGAAG-AATTCTTTAAACTATATTTTGGAAGG; TTCAGGAATTCTTTAAA-TACTGGCAGCAAG and CCTCGGGATCCTGTCATTTTGTGTAGC-AATCG for clones K1-K5 and the 3′ end, respectively. All the clones were purified by CsCl ultracentrifugation (21).

Mutagenesis. Primers with their *Hind* III sites designed to generate point mutations within the E2 site were synthesized in both orientations (GACAAAAGCTTCTAACAAGTTGATCAGCTTC and TGTTAGAAGCTTTTGTCTAAGGAATTTACTG). An insert of clone K1 was used as a template and PCRs were performed with these primers and the 5′ or 3′ end of the insert described above. The upstream and downstream fragments were linked by *Hind* III site and then cloned into the pBR322 vector. The construct was sequenced by dideoxy-mediated sequencing (22).

In vitro transcription reaction. The transcription reaction was performed in the mixture (20 μ l) containing 40 μ g/ml template DNA and 3mg/ml rat brain nuclear protein, as described previously (13).

DNase I protection assay. The DNase I protection assay was performed essentially as described by Carthew *et al.* (23) with some modification. An end-labeled E2 site containing a 146-bp fragment from −177 to −323 (1×10^4 cpm) was incubated with 8 μ g brain nuclear extract and 1 μ g poly (dI-dC) under the gel retardation assay conditions (5). After incubation for 30 min, $MgCl_2$ and DNase I were added to produce final concentrations of 8 mM and 1.25 g/ml, respectively, and the mixture was incubated at 25 °C for 1 min. The reaction was stopped with 6mM EDTA, the DNA was extracted with phenol-chloroform, precipitated with ethanol, and analyzed electrophoretically using 8 % standard sequencing gel. Free DNA digested with DNase I was used as a control and chemical cleavage at purine residues was performed.

Gel retardation assay. The sequences of the double-stranded oligonucleotides used as probes are shown in Fig. 3. The gel retardation assay was performed as described previously (5).

RESULTS AND DISCUSSION

Sequence analysis of our isolate of the BC1 RNA gene demonstrated that, in addition to the essential promoter elements for Pol III, the BC1 RNA gene contains five E-box sequences in its 5′ flanking region (Fig. 1A and B). In order to examine the contribution of these E-boxes to BC1 RNA transcription, we constructed 5′ truncated mutants of the gene (Fig. 1C). We used brain nuclear extract and these constructs as templates and

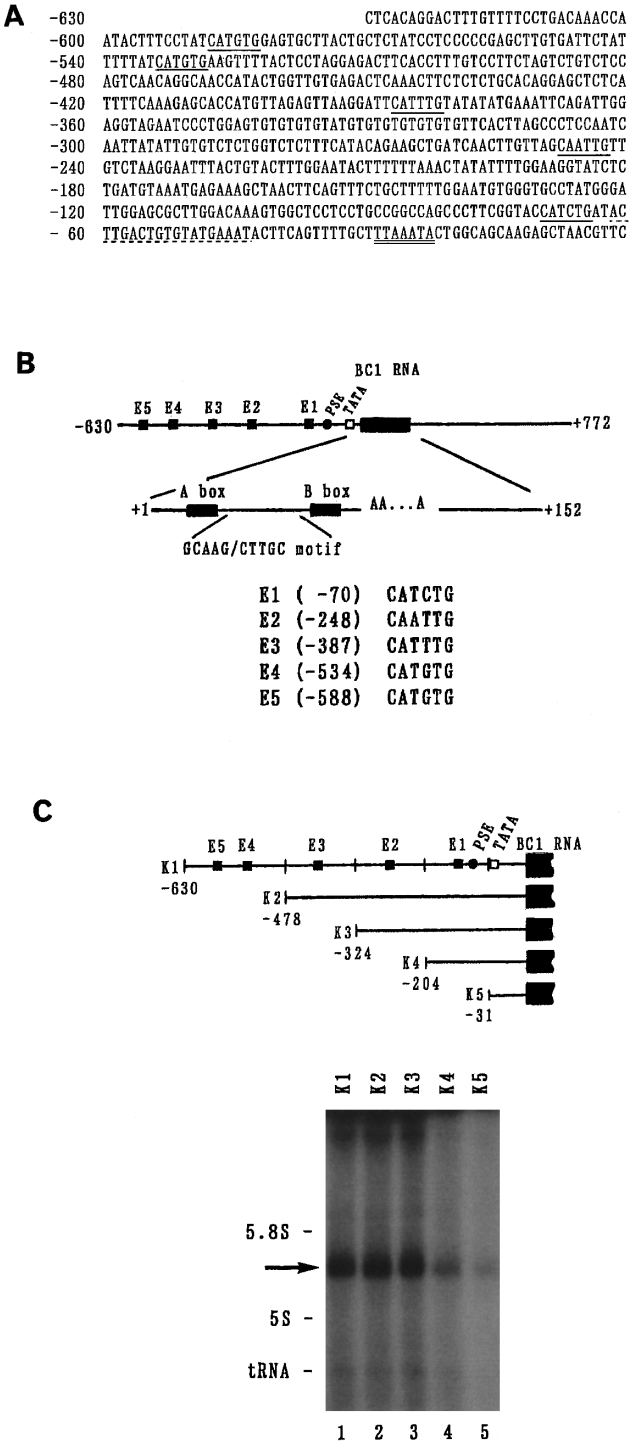


FIG. 1. *In vitro* transcriptional analysis of the 5′ deletion mutants of the BC1 RNA gene. (A) Nucleotide sequence of 5′ flanking region of the BC1 RNA gene. E-box elements (CANNTG) are underlined. PSE and TATA-like sequence are indicated by dotted underline and double underline, respectively. (B) Schematic representation of the BC1 RNA gene and E-box sequence positions. (C) The transcriptional assay was performed using rat brain nuclear extract and deletion mutants as templates (lanes 1-5, clones K1-K5, respectively). The arrow indicates the BC1 RNA transcript.

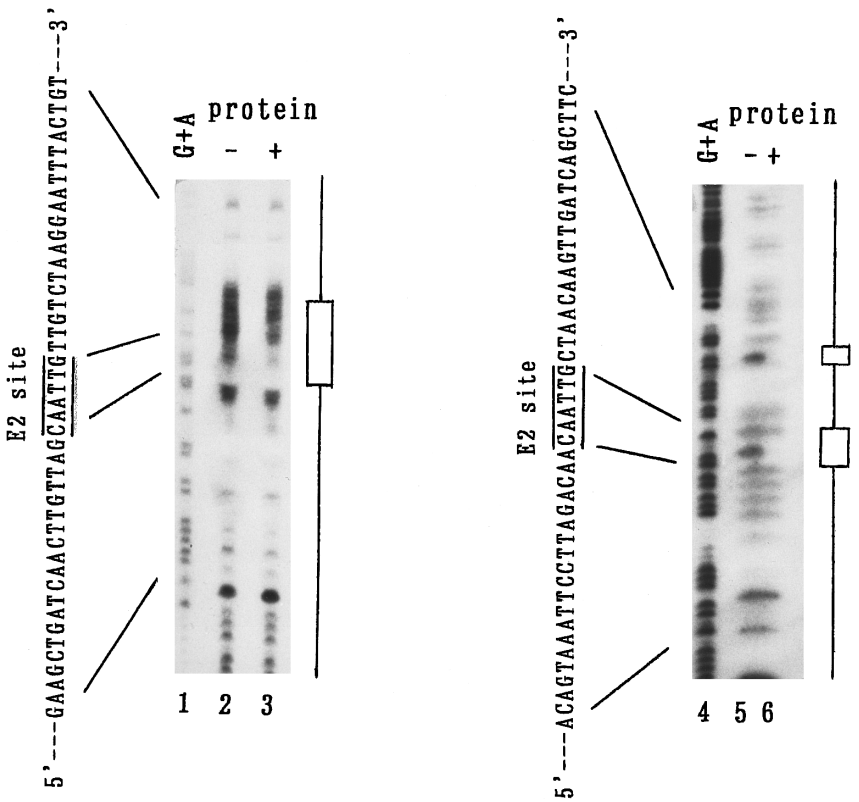
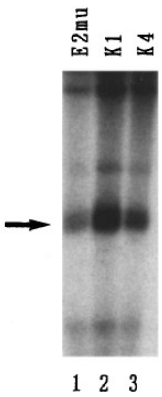


FIG. 2. DNase I protection assay using a ^{32}P-labeled E2 site-containing fragment. The left and right panels indicate the sense and antisense strands, respectively. The autoradiogram shows the digestion patterns with no extract (lanes 2 and 5), with brain nuclear extract (lanes 3 and 6) and after chemical cleavage at the purine residues (lanes 1 and 4).

performed *in vitro* transcription analysis. In our system, clone K1, K2 and K3 were transcribed effectively, but clone K4 showed lower transcription efficiency than those constructs containing further upstream sequences. Expectedly, clone K5, which lacked PSE, significantly decreased the activity. Therefore, we considered that the region between clone K3 and clone K4 contains certain cis-acting elements that enhance BC1 RNA transcription. To detect such elements, we performed the DNase I protection assay of the sense and antisense strands of this region. Fig. 2 shows that the E2 site (CAATTG) on both strands showed DNase I-hypersensitivity and this site was protected from the enzyme action by a brain nuclear protein. Another DNase I-hypersensitive site detected near the E2 site on antisense strand was also protected from the enzyme cut.

In order to determine the functional significance of the E2 site in BC1 RNA expression , a template containing point mutations within the E2 site of clone K1 (clone E2mu) was prepared. The mutant showed reduced transcriptional activity, which was estimated to be about 20 % of that of wild type template K1 (Fig. 3). This indicates that the E2 site plays a crucial role(s) in BC1 RNA expression together with its binding transcriptional activator. In this regard, note that the residual activity of the clone E2mu was even lower than that of the clone K4 that lacked almost all 5′ flanking sequences of the BC1 RNA gene including the E2 site.

Next, we characterized the E2 site binding protein by performing a gel retardation assay with brain nuclear extract. The 46-bp probes used in this assay are shown in Fig. 3. The wild-type E2 site probe formed three complexes, whereas the mutant E2 site probe formed none (Fig. 4A). The complexes observed yielded one main and two comparatively minor bands (solid and open triangles in Fig. 4, respectively). E-box binding bHLH proteins are known to act either as homo- or heterodimers (24) and the appearance of these complexes may be due to different dimerization patterns of the corresponding plural proteins (25). Formation of these complexes was inhibited by a specific competitor, but not by the non-specific double-stranded oligonucleotide 5′ - AGAGCGCTGGTCTAGATCGCTCATGGCCCTG - 3′ (Fig. 4B). Taken together with the DNase T I protection assay results (Fig. 2), these findings indicate that the binding of the brain nuclear factor to the E2 site was specific. We also examined the distribution



E2 site

K1; ---GAAGCTGATCAACTTGTTAGCAATTGTTGTCTAAGGAATTTACTGT---
* ** *
E2mu; ---GAAGCTGATCAACTTGTTAGAAGCTTTTGTCTAAGGAATTTACTGT---

FIG. 3. *In vitro* transcriptional analysis of clone E2mu containing point mutations within the E2 site of clone K1. Transcription reactions were performed using clone E2mu (lane 1), wild-type clone K1 (lane 2) and clone K4 (lane 3). The BC1 RNA transcripts are indicated by arrow and the oligonucleotide sequences corresponding to the wild-type K1 and mutant E2mu are shown. Asterisks indicate point mutations.

of the E2 site binding factor in other tissues (Fig. 4C). The binding patterns of liver and kidney nuclear extracts differed from that of brain nuclear extract, suggesting that the brain is particularly rich in this factor with the ability to form these complexes. Consistently, in our *in vitro* system, BC1 RNA transcription was almost inactive in liver and kidney nuclear extracts (our unpublished observations). It remains to be determined whether the E2 site also mediates BC1 RNA expression in skeletal muscles.

The BC1 RNA gene is a newly discovered type of Pol III gene that requires both external and internal promoter elements for preferential expression in the brain (12, 13). Moreover, we have demonstrated here that an upstream region containing an E-box sequence (CAATTG), the binding site for a brain-specific factor, may play a role in the transcriptional regulation of BC1 RNA. Therefore, it may be possible to increase preferential BC1 RNA production in the brain by increasing the activity of the unique Pol III system in which brain-specific transcription regulatory factors of the Pol II system function as activators. Recently, several E-box binding bHLH transcription factors were detected specifically in developing and adult mammalian neural tissues and these proteins were suggested to play some roles in synaptic plasticity and/or neural activity (26-29). BC1 RNA is also expressed in a developmentally regulated manner and kept high level in adult brain as dendritic BC1 RNP. Moreover, the level of BC1 RNA increased when hyperexcitability was induced by blocking gamma aminobutyric acid receptors with picrotoxin (11). Consequently, it is tempting to speculate that the BC1 RNA expression is activated under the control of such brain-specific bHLH tran-

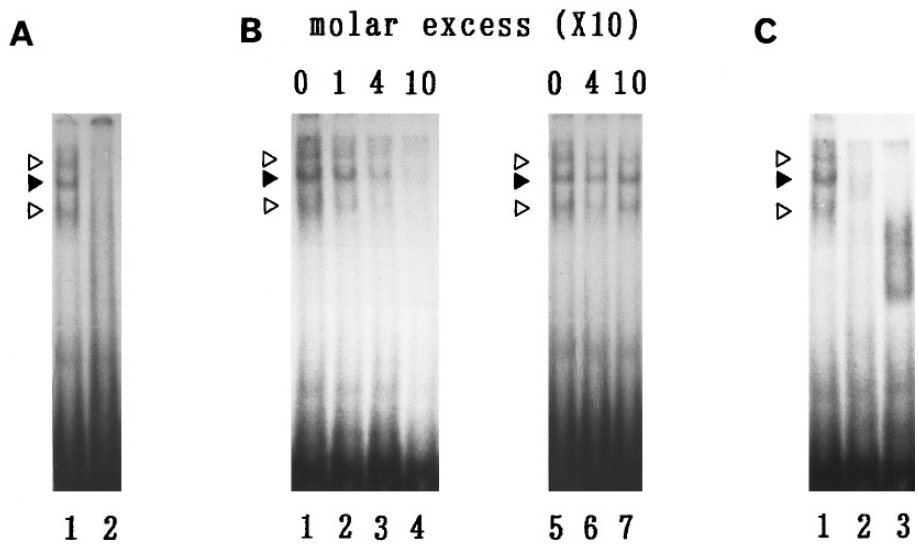


FIG. 4. Analysis of the binding specificity and tissue distribution of the E2 site binding protein. (A) Gel retardation assay of brain nuclear extract using the wild-type (lane 1) and mutant E2mu (lane 2) probes. Each radioactive probe (1×10^4 cpm) was incubated with 3 μ g brain nuclear extract and 1 μ g poly (dI-dC) in a reaction buffer. (B) The reaction mixture containing labeled wild-type probe and brain nuclear extract was incubated in the presence of a molar excess of unlabeled wild-type (lanes 1–4) or non-specific double-stranded oligonucleotide (lanes 5–7) (see text). (C) The reaction described above for (A) was performed using the wild-type probe and 3 μg brain (lane 1), liver (lane 2) or kidney (lane 3) nuclear extract.

scription factors to participate in transport and/or translational regulation of dendritic mRNAs. Identification of the E2 site-binding protein should help elucidate the mechanism(s) responsible for transcriptional regulation of BC1 RNA, the Pol III transcript expressed selectively in the brain.

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