Identification of EphA7 BAC clone containing a long-range dorsal midline-specific enhancer

Yujin Kim & Soochul Park*
Department of Biological Science, Sookmyung Women's University, Seoul 140-742, Korea

Previous studies suggest that EphA7 plays a critical role in neural tube closure or cortical progenitor apoptosis. In this report, enhancer trap assay was used to modify various EphA7 BAC clones and screen a large genomic region spanning 570 kb downstream of the EphA7 gene. We found that the dorsal midline-specific EphA7 enhancer resides on the 457D20 EphA7 BAC clone and is localized to a 35 kb genomic region in between +345.7 kb to +379.8 kb downstream of the EphA7 transcription start site. Identification of the EphA7 BAC clone containing a long-range dorsal midline enhancer may constitute a useful tool for investigating the biological functions of EphA7 in vivo. [BMB reports 2011; 44(2): 113-117]

INTRODUCTION

A recent study revealed that microdeletion of 6q16.1 encompassing EphA7 in a child is correlated with neurological abnormalities and dysmorphic features (1). This clinical case report is somewhat consistent with previous reports that EphA7 plays an important role in the development of the central nervous system. It has been previously confirmed that EphA7 is specifically expressed in various tissues, including the forebrain neural folds, mesencephalon, rhombomere 3, lateral mesoderm, and somites during mouse embryonic development (2-5). Consistent with these developmental expression patterns for EphA7, two studies indicated that EphA7 functions as a key molecule in developmental processes. First, it was shown that EphA7 mRNA is co-expressed with its cognate ligand, ephrin-A5, at the edges of dorsal neural folds, and that EphA7 possibly mediates adhesive interaction with ephrin-A5 during neural tube formation (3). Second, EphA7 null mice display increased cortical size and exencephalic overgrowth of the forebrain, suggesting that ephrin-A/EphA receptor signaling plays a key role in controlling the size of the mouse cerebral cortex by regulating cortical progenitor cell apoptosis (6).

Although EphA7 has been implicated as an adhesion receptor for ephrin-A5 in the neural fold, the possible mechanism is based mostly on in vitro study (3). Moreover, EphA7 null mice do not display neural tube defects resembling ephrin-A5 null mice. In this respect, it remains unclear whether different splice forms of EphA7 mediate cellular adhesion or repulsion in vivo. Nevertheless, the expression pattern of EphA7 detected along the neural fold edge of the diencephalon and mesencephalon suggests that EphA7 plays a role in neural tube formation. In particular, the dynamic expression pattern of EphA7 may provide a clue as to its function in the process of the neural fold fusion in the dorsal midline. To date, little is known about the upstream regulators driving EphA7 gene expression in the dorsal midline of the diencephalon and mesencephalon. Isolation of upstream regulatory elements may provide a useful tool for investigating the biological functions of EphA7 in vivo. However, isolation of EphA7 regulatory elements appears to be challenging since the EphA7 genomic locus spans about 152 kb.

In this study, we employed enhancer trap assay to modify various EphA7 BAC clones and generate LacZ reporter BAC constructs. These modified BAC constructs led us to systematically screen a large genomic region spanning 570 kb downstream of the EphA7 gene. Through this intensive screening procedure, we found that the 457D20 EphA7 BAC clone contains a dorsal midline-specific EphA7 enhancer that directs EphA7 gene expression to the neural fold edge of the diencephalon and mesencephalon.

RESULTS AND DISCUSSION

Construction of recombinant EphA7 bacterial artificial chromosome (BAC) carrying a LacZ reporter by bacterial homologous recombination

Previous studies have indicated that EphA7 is specifically expressed in the dorsal midline of the diencephalon and mesencephalon during embryonic development and is implicated in the formation of the neural tube (3). To identify the dorsal midline specific EphA7 enhancer, we initially selected a BAC clone, 302F2, which spans from approximately 100 kb upstream to 100 kb downstream of the EphA7 transcription initiation site (Fig. 1A). The targeting vector was constructed in

*Corresponding author. Tel: 82-2-710-9330; Fax: 82-2-715-9331; E-mail: scpark@sookmyung.ac.kr
DOI 10.5483/BMBRep.2011.44.2.113

Received 18 December 2010, Accepted 22 December 2010

Keywords: Bacterial artificial chromosome, Enhancer, EphA7, Transgenic mice
such a way that it contained a 0.8 kb region of EphA7 genomic DNA upstream of the transcription start site, a LacZ reporter, a kanamycin cassette as a selection marker, and a 1 kb region of EphA7 genomic DNA downstream of the transcription start site (Fig. 1A). The targeting vector was transformed into bacteria containing both 302F2 EphA7 BAC DNA and pKOBEGA plasmid DNA expressing an inducible lambda phage recombinase system. The first recombinant EphA7 BAC clone was selected in the agar plate containing chloramphenicol and kanamycin and was confirmed by Southern blot analysis as previously described (Fig. 1B-D, lanes 2 and 3). The kanamycin cassette was further eliminated by induction with Flipase, and the second recombinant BAC clone was also confirmed by Southern blot analysis (Fig. 1B-D, lanes 4 and 5). This modified EphA7 BAC DNA was then purified and microinjected into fertilized eggs to generate BAC transgenic embryos as previously described. At embryonic day (E) 10.5, embryos were dissected and analyzed by X-gal staining, which revealed LacZ expression in the branchial arches, heart, and guts but not in the brain (Fig. 1E).

This result suggests that the dorsal midline specific EphA7 enhancer was not located within the genomic region in the 302F2 BAC clone.

Mapping of the dorsal midline-specific EphA7 enhancer by enhancer trap assay
To further explore the dorsal midline-specific EphA7 enhancer, we used enhancer trap assay as previously described. For this experiment, we constructed the reporter vector carrying a minimal β-globin promoter, a LacZ gene, and a kanamycin cassette (Fig. 2A). The entire insert in the vector was flanked by two Tn7 sequences at either end, thus allowing random integration of the insert into BAC through transposase-mediated in vitro transposition. Four overlapping EphA7 BAC clones extending ~570 kb downstream of the EphA7 transcription start site were selected, and each BAC clone was then modified in vitro by incorporation of a single copy of the LacZ expression cassette. Modified BAC DNAs were transformed into DH10B competent cells, and the bacteria carrying the modified BAC DNAs were selected on an agar plate containing kanamycin. The modified BAC clones were further analyzed by Southern blotting with LacZ probe, confirming that a single copy of the reporter cassette was randomly incorporated into the same BAC DNA (Fig. 2B).

One transposition clone was selected from each BAC and then assessed for the insertion of a LacZ reporter cassette by inverse PCR analysis and DNA sequencing (Fig. 3A). Each modified BAC clone was subsequently assayed for reporter activity in transgenic embryos (Fig. 3B). Strikingly, only one of the four
BACs tested directed highly specific and reproducible patterns of reporter expression to the dorsal midline in the developing diencephalon, ventral diencephalon, telencephalon, heart, and guts (Fig. 3B, fourth panels).

The dorsal midline-specific EphA7 enhancer resides in genomic sequences in 457D20 BAC clone

Previous studies using in situ RNA hybridization revealed that EphA7 is expressed in the forebrain neural folds, rhombomere 3, lateral mesoderm, and somites (2-5). In addition, the Allan Developing Mouse Brain Atlas database also shows that EphA7 is highly expressed in the mesencephalon and prosomeres 1 and 2 in the diencephalon at E11.5. Our results for EphA7 expression based on reporter assay using 457D20BAC appeared to match very well with previous results, at least in the developing forebrain neural folds. Our in situ RNA hybridization also demonstrated that EphA7 transcript was highly detectable in the dorsal midline of diencephalon and mesencephalon at E10.5, consistent with the LacZ expression pattern of our 457D20 EphA7 BAC transgenic embryo (Fig. 4A and B).

These results strongly suggest that the cis-acting elements driving EphA7 expression to the dorsal midline of the mesencephalon and diencephalon are located only in the genomic sequences of the 457D20 BAC clone and not in either 425N3 or 461C3BAC. Therefore, the dorsal midline-specific EphA7 regulatory elements could be clearly mapped within an approximately 35 kb genomic sequence located in between +345.7 kb to +379.8 kb downstream of the EphA7 transcription start site (see Fig. 3A). Reporter expression analysis of 457D20BAC transgenic embryo also revealed that EphA7 may be expressed in the caudal telencephalon, ventral diencephalon, heart, and guts, in which EphA7 expression analyses had not been well studied. Therefore, expression analysis using 457D20 BAC clone would provide an opportunity to study the diverse functions of EphA7 in these tissues. The ~35 kb genomic region containing the dorsal midline-specific EphA7 enhancer needs to be further narrowed down in order to identify the specific sequences responsible for interacting with specific transcription factors. In this region, we did not find evolutionary conserved regions (ECRs) among mouse, chick, frog, and fish, suggesting that the putative dorsal midline-specific EphA7 enhancer is likely conserved only in mammals (data not shown). Nevertheless, identification of EphA7 BAC containing this regulatory element would offer many potential applications, including functional analyses for directing the expression of other developmental important genes or EphA dominant-negative mutants to this region.
**MATERIALS AND METHODS**

**Targeting vector for homologous recombination**
Homologous arms A and B flanking the mouse EphA7 transcription start site for EphA7 BAC, 302F2, were synthesized by PCR using the following primer sets: 5'-CAGAACAGAAGTTC AACATTTTGC-3' (forward primer for A arm), 5'-GGTTGAGTGCC GAGGAGTCGTTTGC-3' (reverse primer for A arm), 5'-ATCTG CTGGTCATTGTTGC-3' (forward primer for B arm) and 5'- ATTGCGGAGGAGCCAGCCG-3' (reverse primer for B arm).

The targeting vector was constructed on the backbone of the pGEM11Z vector (Promega) as follows. First, pGEM11Z was digested with XbaI, and then an FRT-Kana-FRT cassette was inserted into this site. Second, the homologous B arm was cloned into the NotI site of pGEM11Z. Then, the homologous arm A and LacZ containing the SV40 polyadenylation site were cloned into the SalI site of pGEM11Z in front of the FRT-Kana-FRT cassette. This targeting vector was digested with NsiI/SfiI, after which the insert was isolated by separation on 0.8% low-melting agarose gel.

**Reporter cassette for transposition**
For modification of several BAC clones, we constructed a transposable vector using pGPS3 vector (NEB). This vector carried two Tn7 transposable elements containing a kanamycin resistance gene between them. The β-globin minimal promoter, LacZ gene, and SV40 poly(A) site were inserted into the NotI and PmlI sites between the Tn7R element and kanamycin resistance cassette.

**Homologous BAC recombination and enhancer trap assay**
Homologous BAC recombination was performed as described previously (7). For the enhancer trap assay, a transposable vector carrying a β-globin minimal promoter, LacZ gene, and SV40 poly(A) site was inserted into NotI and PmlI sites between the Tn7R element and kanamycin resistance cassette.

**Generation of BAC transgenic mice**
Modified BAC DNA was prepared using a large-construct kit (Qiagen). BAC DNA for microinjection was confirmed on a gel and diluted with injection buffer as described previously (7). The BAC DNA was injected into 200 pronuclei of fertilized oocytes from C57BL/6 mice as described previously (7, 10). The 302F2 BAC transgenic embryos were identified by PCR using the primers 5'-ACTTCCGAGCAGCAGCATGCT-3' and 5'-GGAAACCAGGCCAGGCCCAT-3'. The other BAC transgenic embryos were confirmed by PCR using the primers 5'-GGTTCCATATGGGGATGG-3' and 5'-TTACGCTGACTTG ACGGGAC-3'.

**Southern blot analysis and Inverse PCR**
Southern blotting was performed as described previously (7, 10). Inverse PCR was performed to identify an insertion site in the transposable vector as described previously (11). Briefly, the modified BAC DNAs were digested with DpnI restriction enzyme, after which 1 μg/ml of the digested BAC DNA fragments were further ligated with 1-2 Weiss units of T4 DNA ligase for self-ligation. Then, PCR was performed with primers 5'-GAATCTAGATTTCACTTATCTGGTTG-3' and Primer S (NEB). The amplified fragments were further analyzed by DNA sequencing.

**X-gal staining and RNA in situ hybridization**
Embryos to be stained were dissected in phosphate buffered saline (PBS), fixed in 0.2% glutaraldehyde, and subjected to the washing and staining procedure as described previously (12). Whole-mount mRNA in situ hybridization was performed essentially as described previously (13). Single-stranded antisense RNA probe labeled with digoxigenin-UTP was synthesized from linearized EphA7 template DNA as described previously (14). Mouse full-length EphA7 cDNA was used as a template for synthesis of antisense riboprobe (GenBank accession no. BC026153).

**Acknowledgements**
This work was supported by Sookmyung Women’s University in 2008.

**REFERENCES**


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