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

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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

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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 lamda phage recombinase system. The first recombinant *EphA7* BAC clone was selected in the agar plate containing chloroamphenicol 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).

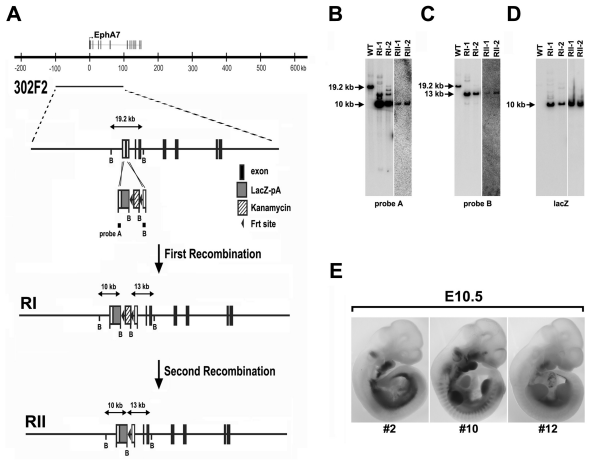

Fig. 1. Strategy for *EphA7* BAC 302F2 modification. (A) The *EphA7* BAC clone, RP23-302F2, contains an approximately 200 kb genomic DNA, which covers an approximately 100 kb genomic region downstream of the EphA7 transcription initiation site. The targeting vector was designed to contain two *EphA7* genomic DNAs flanking the first exon on either side, thus allowing for specific homologous recombination between the targeting vector and 302F2 BAC. RI and RII represent first recombinant and final recombinant BAC clones, respectively. (B) *BamH*I. (B-D) Southern blot analyses of first and second BAC recombinants. The BAC DNAs were digested with *BamH*I and then analyzed by Southern blotting using the indicated probes. WT denotes the original EphA7 BAC clone, RP23-302F2. RI-1 and RI-2 represent two independent recombinant BAC clones induced by RecA, whereas RII-1 and RII-2 indicate two independent recombinant BAC clones induced by Flipase. (E) Analysis of *LacZ* expression pattern for the transgenic embryos harboring the modified 302F2 BAC clone. Whole mount X-gal staining was performed using transgenic embryos at embryonic day (E) 10.5. Note that *LacZ* expression was not detected in the brains of the three independent transgenic embryos.

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

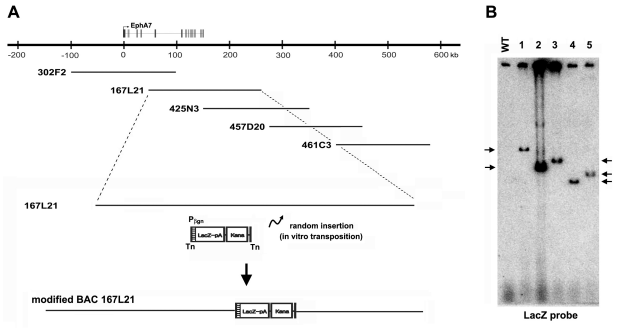

Fig. 2. Strategy for constructing modified *EphA7* BAC clones by enhancer trap assay. (A) Physical map displays the distribution of the *EphA7* gene and BAC clones spanning 0.5 Mb downstream of the *EphA7* gene. The reporter vector contains two Tn7 sequences on either end, a minimal β-globin promoter, a *LacZ* gene cassette, and a kanamycin resistance gene cassette. This vector was introduced into overlapping BACs by random transposon insertion. (B) Five different 167L21 BAC clones were randomly selected, and their DNAs were digested by *Hind*III for Southern blot analysis using *LacZ* as a probe. Note that Southern blotting detects only one *Hind*III fragment with a different size in each BAC clone, indicating that one copy of the reporter cassette was randomly integrated into the 167L21 BAC DNA.

BACs tested directed highly specific and reproducible patterns of reporter expression to the dorsal midline in the developing diencephalon, ventral diencephalons, 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).

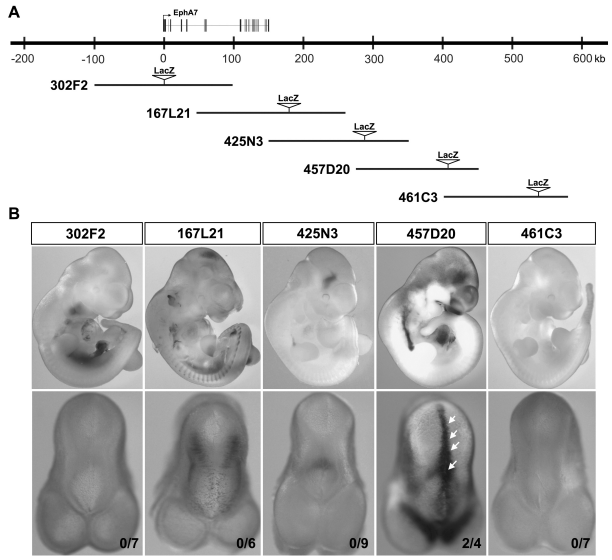

Fig. 3. Screening for long-range acting EphA7 regulatory elements. (A) Schematic representation of the overlapping BAC clones used for generating transgenic embryos. The insertion position of the reporter cassette on each BAC clone was determined by inverse PCR analyses and DNA sequencing. (B) Lateral (top panels) and dorsal views (bottom panels) of the embryos stained for *LacZ* expression in representative BAC transgenic embryos harvested at E10.5. *LacZ* expression was not detected in the brains of 302F2 and 461C3 BAC transgenic embryos. Although 167L21 BAC transgenic embryos revealed *LacZ* expression in the diencephalons and mesencephalon, this expression pattern appears to be ectopic since other transgenic embryos did not show the same *LacZ* expression pattern. The ratio of embryos exhibiting reproducible *EphA7*-specific dorsal midline reporter activity to the total number of transgenic embryos is indicated for each BAC clone.

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 mammalians (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.

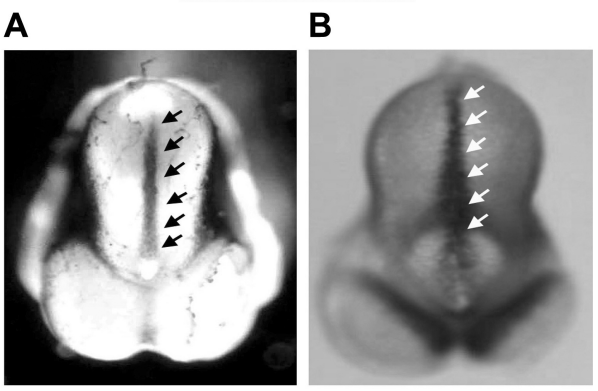

Fig. 4. *EphA7* mRNA expression is consistent with *LacZ* reporter activity in the dorsal midline of the 457D20 BAC transgenic embryo. (A) *EphA7* mRNA was strongly detected in the dorsal midline of the diencephalon and mesencephalon of E10.5 embryos (dorsal view) by *EphA7* anti-sense RNA probes. (B) *LacZ* expression that was similar to the mRNA expression pattern in panel A was also observed in the dorsal midline of the diencephalon and mesencephalon of 457D20 *EphA7* BAC transgenic embryos (dorsal view).

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 AAACTTTTG-3' (forward primer for A arm), 5'-GGTGCATGA GCAGGTTTGATTT-3' (reverse primer for A arm), 5'- ATCTGG CTGCTTGGTTTGC-3' (forward primer for B arm) and 5'-ATTGTTTCGAGCGGCAGCCG-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 *Xba*I, and then an FRT-Kana-FRT cassette was inserted into this site. Second, the homologous B arm was cloned into the *Not*I site of pGEM11Z. Then, the homologous arm A and *LacZ* containing the SV40 polyadenylation site were cloned into the *Sal*I site of pGEM11Z in front of the FRT-Kana-FRT cassette. This targeting vector was digested with *Nsi*I/*Sfi*I, 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 *Not*I and *Pme*I 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 promoter, *LacZ* gene containing SV40 poly(A) site, and a kanamycin resistance gene was introduced into each BAC clone according to the method of Spitz *et al*. (8, 9). Briefly, 40 ng of transposable vector was mixed with 200 ng of BAC DNA, GPS buffer, and TnsABC (NEB), followed by incubation at 37°C for 10 minutes. Start Solution was added, and the reaction was incubated for 1 hour. After heat inactivation at 75°C for 10 minutes, this mixture was transformed into DH10B competent cells and selected on a LB plate containing 20 μg/ml of kanamycin and 12.5 μg/ml of chloramphenicol for overnight at 37°C. Positive colonies were confirmed by Southern blotting using *LacZ* as a probe.

Generation 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'-ACTTCGGAGCAAACAGCATCTA-3' and 5'-CGGAAACCAGGCAAAGCGCCAT-3'. The other BAC transgenic embryos were confirmed by PCR using the primers 5'-GGTTTCCATATGGGGATTGG-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 *Dpn*I 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'-GAACTAGATTTCACTTATCTGGTTG-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).

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