

Possible involvement of SINEs in mammalian-specific brain formation

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Retroposons, such as short interspersed elements (SINEs) and long interspersed elements (LINEs), are the major constituents of higher vertebrate genomes. Although there are many examples of retroposons' acquiring function, none has been implicated in the morphological innovations specific to a certain taxonomic group. We previously characterized a SINE family, AmnSINE1, members of which constitute a part of conserved noncoding elements (CNEs) in mammalian genomes. We proposed that this family acquired genomic functionality or was exapted after retropositioning in a mammalian ancestor. Here we identified 53 new AmnSINE1 loci and refined 124 total loci, two of which were further analyzed. Using a mouse enhancer assay, we demonstrate that one SINE locus, AS071, 178 kbp from the gene *FGF8* (fibroblast growth factor 8), is an enhancer that recapitulates *FGF8* expression in two regions of the developing forebrain, namely the diencephalon and the hypothalamus. Our gain-of-function analysis revealed that *FGF8* expression in the diencephalon controls patterning of thalamic nuclei, which act as a relay center of the neocortex, suggesting a role for *FGF8* in mammalian-specific forebrain patterning. Furthermore, we demonstrated that the locus, AS021, 392 kbp from the gene *SATB2*, controls gene expression in the lateral telencephalon, which is thought to be a signaling center during development. These results suggest important roles for SINEs in the development of the mammalian neuronal network, a part of which was initiated with the exaptation of AmnSINE1 in a common mammalian ancestor.

conserved noncoding element | enhancer | evolution | mouse

Retroposons, including SINEs, LINEs, and long-terminal repeat or LTR retrotransposons, propagate within the host genome via RNA intermediates (1–3). Despite the abundance of retroposons in mammalian genomes (e.g., 42% and 37% of human and mouse genomes, respectively) (4, 5), most are nonfunctional and are regarded as genomic parasites or “junk DNA.” However, in several cases, copies of retroposons have been involved in generating new *cis*-regulatory elements for processes such as alternative splicing, mRNA polyadenylation, and promoter activity (6), all of which are examples of exaptation (7, 8). The idea that retroposons may impact genome evolution and functionality (8–10) has been gradually accepted. However, most examples seem to have only local effects, and no example has been reported where an inserted retroposon plays a pivotal role in higher-order morphological or biological innovations.

On the other hand, recent genome-scale comparative analyses have identified conserved noncoding elements (CNEs) that are suggested to be essential for host survival (11). CNEs constitute 3–4% of the human genome, whereas protein-coding exons constitute only ≈1.5% (4). Recently, it was reported that 16% of eutherian-specific CNEs are derived from transposable elements (retroposons) (12, 13), which supports the importance of retroposons in evolution (14–18). This observation on CNEs is important because retroposons arose only recently in evolution and therefore may have the potential to acquire new functions through

exaptation. We recently identified a new SINE family, AmnSINE1, in the genomes of Amniota (mammals, birds, and reptiles). The ≈100 copies of AmnSINE1s are highly conserved as mammalian-specific CNEs (16), suggesting that these SINEs acquired a function (exapted) during the emergence of mammals (or therians) (Fig. 1). Therefore, these AmnSINE1s are the best available mammalian-specific CNEs to study the evolutionary impact of retroposons on morphological changes among mammals (16).

Studying brain development raises two fundamental questions (19). What characteristics of the brain make mammals distinct from other vertebrates? How have these characteristics evolved? In vertebrates, the anterior neural epithelium undergoes morphological subdivisions during development to generate vesicle-like structures known as the forebrain, midbrain, and hindbrain. The forebrain further divides into the telencephalon and the diencephalon. The telencephalon gives rise to the neocortex, basal ganglia, and hippocampus, whereas the diencephalon develops into the thalamus, prethalamus, and pretectum. Several patterning centers used during development have been well characterized and are generated on boundaries or ridges of each region, such as the anterior neural ridge, the zona limitans intrathalamica, and the midbrain–hindbrain boundary. The patterning centers are thought to establish the regional identities by secreting signaling molecules such as Shh, Wnts, and Fgfs (20, 21). The mammalian-specific brain is identified by three characteristics (19, 22): (i) the six-layered neocortex in the dorsal pallium (the pallial area in birds and reptiles); (ii) generation of the Cajal–Retzius cells, one of whose sources is generated in the pallial–subpallial boundary, and migrate tangentially toward the dorsal telencephalon; and (iii) the absence of the dorsal ventricular ridge, which has been commonly observed in the brains of birds and reptiles. The molecular mechanisms that generate these characteristics are largely unknown. In this study we demonstrate a potential role for SINEs in controlling gene expression and activity during forebrain development. Because the mammalian forebrain is evolutionarily unique, SINEs may have played a role in mammalian-specific evolution.

Results and Discussion

Enhancer Activity of the AS071 Locus for *FGF8* Expression. To elucidate the function of the conserved AmnSINE1 loci, we rechar-

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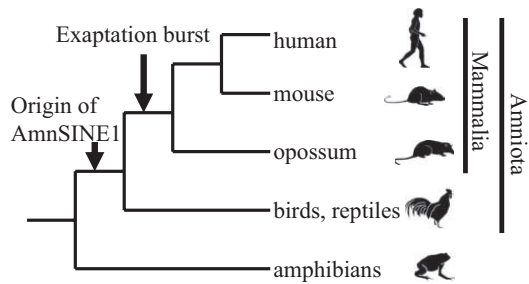


Fig. 1. Exaptation of AmnSINE1s in a common ancestor of mammals. The known phylogeny of tetrapods is shown with the time of exaptation of AmnSINE1 sequences. AmnSINE1 originated in a common ancestor of amniotes, and multiple copies acquired function in a common ancestor of mammals.

acterized conserved AmnSINE1 loci by updating the homology search as described in *Methods*. We refined 124 AmnSINE1 loci and 974 neighboring genes. The 124 loci are scattered among the human chromosomes (except chromosomes 19, 21, 22, and Y) but are not evenly distributed among the chromosomes (Fig. 2).

To test whether the conserved AmnSINE1 loci regulate the expression of adjacent genes, we used a β -galactosidase (*lacZ*) transgenic mouse system. We first focused on locus AS071, located 178 kbp downstream from *FGF8* (Fig. 3*a*). The 570 bp of the putative full-length AmnSINE1 element (red horizontal bar) appears to be conserved among mammals (Fig. 3*b*). Synteny is conserved between human and mouse in this region (data not shown). The mouse sequence of the AS071 conserved region (600 bp) was cloned upstream of a minimal heat-shock protein 68 (*Hsp68*) promoter coupled to the *lacZ* reporter gene [SI Fig. 7 in [supporting information \(SI\) Appendix](#)], and this was injected into a fertilized mouse oocyte. To understand spatial and temporal *lacZ* expression in the mouse embryo, whole-embryo staining for *lacZ* activity was performed at embryonic day 10.5 (E10.5), E11.5, and E13.5 (Fig. 4 *a-h* and SI Table 1 in [SI](#)

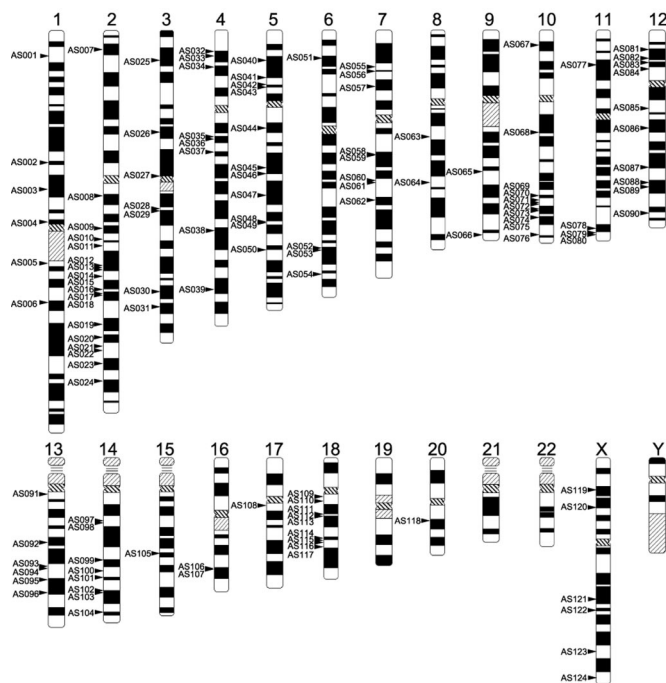


Fig. 2. Positions of the 124 conserved AmnSINE1 loci on the human chromosomes. Each locus is indicated by an arrow.

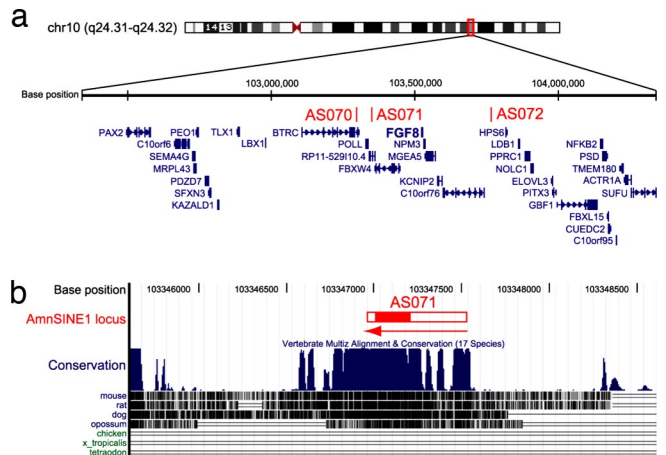


Fig. 3. Characterization of the AS071 locus. (a) The 2-Mb region containing three AmnSINE1 elements in human chromosome 10. Coordinate (*Top*), AmnSINE1-inserted loci (*Middle*), and gene annotations (*Bottom*) are based on the hg18 assembly in the UCSC genome browser (<http://genome.ucsc.edu>). (b) The 3.0-kb region around the AS071 locus and conservation among vertebrate species. The 570-bp region of the putative full-length AmnSINE1 element (red horizontal bar) appears to be conserved among mammals, including opossum. Within the bar, only 200 bp (red fill) can be aligned with the AmnSINE1 consensus sequence. The sequences indicated by the open bar regions of the SINE have decayed and thus cannot be precisely aligned. The arrow indicates the direction of the element (5' to 3').

[Appendix](#)). Consistent expression of *LacZ* is observed in independent transgenic embryos in the lateral wall of the diencephalon, dorsal midline of the caudal telencephalon, and the hypothalamus (Fig. 4 *a-c* and SI Fig. 8 in [SI Appendix](#)) at E10.5 (30 embryos of 50), E11.5 (eight of 18), and E13.5 (eight of 17). *FGF8* is well established as a signaling molecule, and its expression pattern and function during CNS development in mice and chicks have been reported (23–28). Because the expression of AS071 is restricted to the lateral diencephalon and hypothalamus, we compared the expression pattern of AS071 *lacZ* with that of *Fgf8* by *in situ* hybridization at E10.5 and E11.5 (Fig. 4 *i, j, and l*). Interestingly, *lacZ* expression and *Fgf8* expression in the lateral diencephalon (red arrows) as well as in the hypothalamus (white arrowheads) coincided exactly (Fig. 4 *a, b, d, i, j, and l*). Sagittal (Fig. 4 *c* and *k*) and transverse (Fig. 4 *e-h* and *m-p*) sections of brain showed detailed expression of *lacZ* and *Fgf8*, revealing identical expression in the diencephalon (red arrows) and hypothalamus (white arrowheads). These data suggest the possible role of AS071 as a specific enhancer of *Fgf8* in the diencephalon and hypothalamus of the developing forebrain.

Function of *FGF8* Expression in Diencephalon. To elucidate the function of *FGF8* in diencephalon, we used *in utero* electroporation (24) to overexpress *FGF8* in the developing diencephalon at E10.5 (Fig. 5*a*). With the possibility that *FGF8* could be involved in area patterning of the diencephalon through the anterior/posterior axis, we tested the pattern of thalamic nuclei at postnatal day 6 (P6) by cytochrome oxidase staining and Nissl staining (Fig. 5 *b-d*). The somatosensory nucleus in the thalamus, which represents the pattern of whiskers on the animal's snout (barreloid), was easily distinguished in both sides of the thalamus in the control animal (Fig. 5*b*). Unilateral *FGF8* electroporation of the lateral diencephalon caused one side of the barreloid to shift and shrink as compared with the control (Fig. 5*c*, bracket). Nissl staining revealed a shifted mammillothalamic tract, which runs through the boundary of the thalamus and prethalamus. Conversely, the habenulopeduncular tract, which runs through the pretectum and thalamus, was not altered,

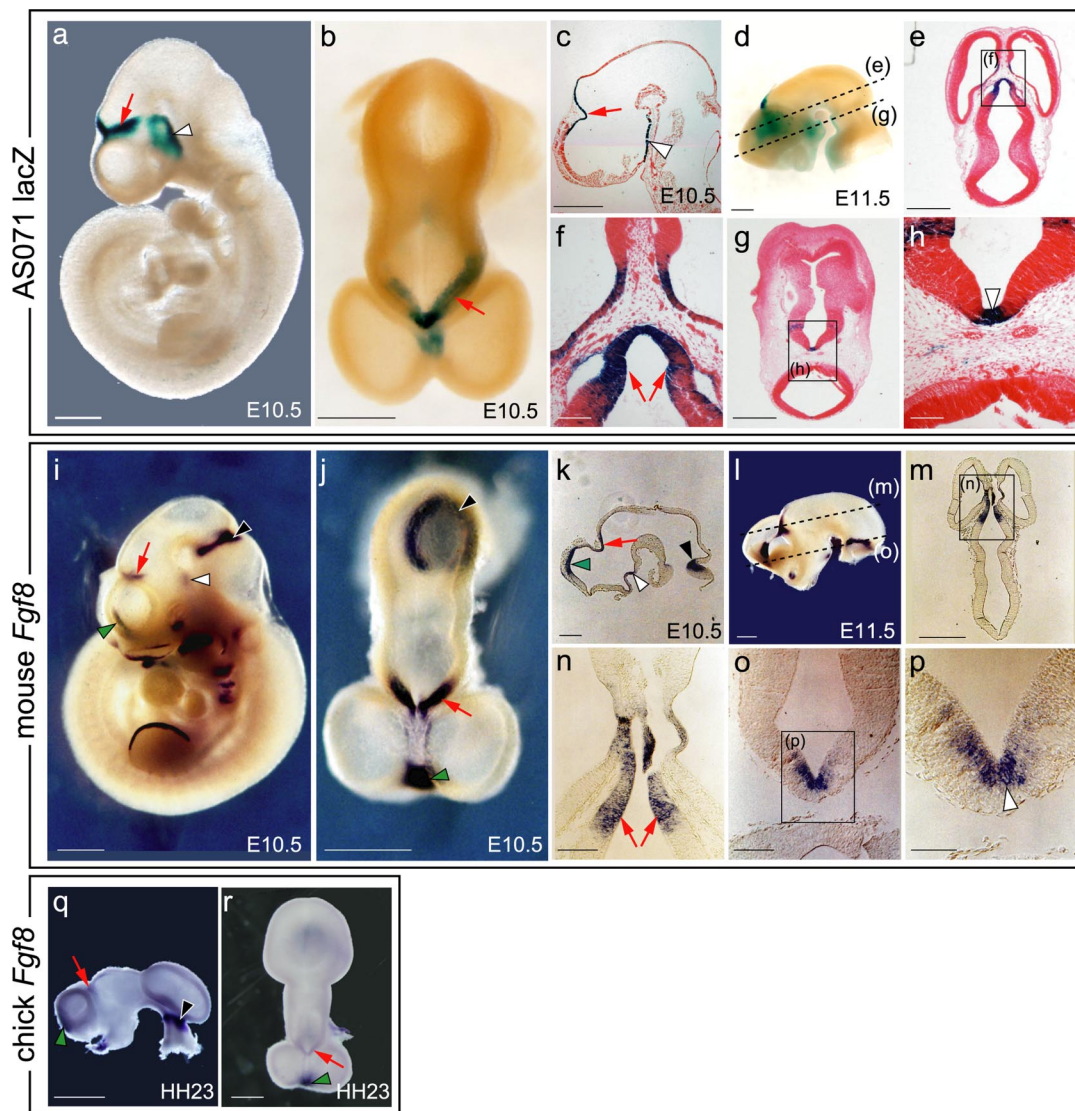


Fig. 4. Comparison of the *lacZ* expression pattern in an AS071 transgenic mouse (*a–h*) with the FGF8 mRNA expression pattern in mouse (*i–p*) or chick (*q* and *r*) embryos. Lateral (*a*, *i*, and *q*) and dorsal (*b*, *j*, and *r*) views of embryos are shown. For hybridization and staining of the head, surface ectoderm and mesenchyme were removed. (*c* and *k*) Sagittal section of the head region. (*d* and *l*) Lateral view of the head. Transverse sections (*e*, *g*, *m*, and *o*) and corresponding close-up views (*f*, *h*, *n*, and *p*) are shown. *lacZ* and FGF8 mRNA expression in the lateral diencephalon and hypothalamus are indicated by red arrows, and those in the ventral diencephalon are indicated by a white arrowhead. FGF8 mRNA expression at the midbrain–hindbrain boundary and the commissural plate are indicated by black and green arrowheads, respectively. The dashed lines indicate where the embryos were sectioned for the staining shown in *e*, *g*, *m*, and *o*. [Scale bars: 500 μm (*a–e*, *g*, *i–m*, *q*, and *r*), 100 μm (*f*, *h*, *n*, and *o*), and 50 μm (*p*).]

suggesting that repatterning upon FGF8 overexpression occurred specific to the thalamus but not to the pretectum (Fig. 5*d*). During CNS development, the diencephalon dives down in between the telencephalon, and its bent longitudinal axis causes its true anterior/posterior axis to rotate 90° (T. Shimogori, unpublished data). Accordingly, the shift of the barreloid upon FGF8 expression is toward the posterior, which is linking to the role of this protein in other parts of the CNS (24, 27). These data demonstrate that the expression of FGF8 in the diencephalon controls patterning of thalamic nuclei.

FGF8 Expression in Diencephalon Is Mammalian-Specific. Because conservation of the AS071 locus is observed only in mammals, the enhancer function of this locus is expected to be mammalian-specific. Based on our results that AS071 is likely to enhance FGF8 expression, we investigated FGF8 expression in chick and performed *in situ* hybridization for *Fgf8* in Hamburger–

Hamilton (HH) stage-23 chick brain to compare with the mouse results (Fig. 4*q* and *r*). Although the chick diencephalon showed expression of *Fgf8*, it was weaker than that of the mouse (compare Fig. 4*i* and *j* with *q* and *r*, red arrows), whereas strong expression in the anterior telencephalon (green arrowheads) was observed in both mouse and chick embryos (Fig. 4*i*, *j*, *q*, and *r*). This result suggests the existence of mammalian-specific enhanced expression of FGF8 in diencephalon, which organizes mammalian thalamic patterning differently from other vertebrates. Although overexpression of FGF8 in chick embryos changes regional identity (29), the different organization of thalamic nuclei between chicks and mice makes direct comparison difficult. However, our findings suggest local gain of function of specific protein by SINE insertion, possibly changing the functional area identity in specific region of the brain. This patterning change may also have the chance to alter the pattern of its wired region of the brain.

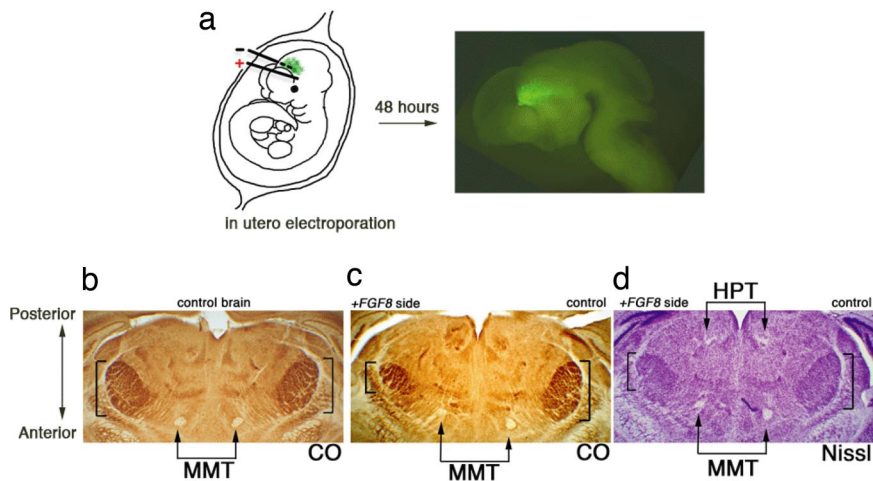


Fig. 5. Gain-of-function analysis of FGF8 in developing diencephalons. (a) Scheme for *in utero* electroporation. Plasmid solution was injected in the third ventricle of E10.5 embryos *in utero*, followed by insertion of needle-type electrodes. A series of three square-wave current pulses (7 V, 100 ms, three times) was delivered, resulting in gene transfection into a restricted region in the unilateral diencephalons (the region is indicated by +, and – indicates the region where gene transfection was not performed). Gene transfer was visualized by GFP after 48 h of electroporation, demonstrating localized expression of the transgene. (b–d) Coronal section of P6 brains processed for cytochrome oxidase (CO) histochemistry (b and c) and Nissl staining (d). The control brain shows the symmetric position of the barreloid (b), whereas the FGF8-electroporated brain shows a posteriorly shifted and shrunken barreloid on the electroporated side (c) as well as mammillothalamic tract (MMT). Nissl staining reveals the habenulopeduncular tract (HPT), which is not altered by FGF8 electroporation (d).

The functional significance of the dorsal forebrain, where FGF8 is expressed, has not been well examined. Based on our results, we propose a role for FGF8 in thalamic pattern formation, and such function may have been introduced by enhancer activity of the AS071 locus. In addition, we confirmed the expression of signaling molecules such as *Bmp4*, *noggin*, and *Wnt* in the dorsal forebrain and observed that *Otx2* (30) expression coincided with that of *Fgf8* (SI Fig. 9 in *SI Appendix*), supporting the assertion that this region constitutes a patterning center for the diencephalon. How the regional identity of the thalamus and thalamic patterning are independently achieved in the diencephalon using signals from this center remains unclear.

Enhancer Activity of the AS021 Locus. Given the above results, we examined other loci, looking for enhancer activities among the 124 conserved AmnSINE1 loci. We found that locus AS021 in human chromosome 2 (Fig. 6a) contains a 600-bp region of AmnSINE1 highly conserved among mammals (Fig. 6b). To test its function as an enhancer of gene expression, we used the same method as above. We assayed the enhancer activity in AS021 locus at E10.5, E11.5, E12.5, and E13.5 (SI Table 1 in *SI Appendix*). At E10.5 we could not observe any consistent lacZ expression patterns. The first consistent lacZ expressions were observed in two of 10 embryos in the dorsolateral side of telencephalon at E11.5 (SI Fig. 10a in *SI Appendix*, arrowhead). At E12.5 the expression patterns in the dorsolateral side of telencephalon became more noticeable (SI Fig. 10b and c in *SI Appendix*). In the later stage, at E13.5, 13 of 23 embryos consistently showed lacZ expression in the neocortex of telencephalon (Fig. 6c–j and SI Fig. 10d–h in *SI Appendix*). Judging from the differences of limb development of embryos, we can discriminate embryos at relatively early phase (Fig. 6c–f) from those at late phase (Fig. 6g–j) even at E13.5. It appears that the lacZ expression patterns in the telencephalon expanded from the restricted area of telencephalon (dorsolateral side, Fig. 6c–f) to the entire of neocortex (Fig. 6g–j) as the developmental stage progressed. We found that locus AS021 is located 390 kbp upstream from the gene *SATB2* (Fig. 6a), which was recently shown to be a multifunctional determinant of craniofacial patterning and osteoblast differentiation (31). *SATB2* is also expressed in the telencephalon at E13.5 (32), suggesting a role in the developing telencephalon. Because

SATB2 is multifunctional and is expressed ubiquitously, including in the brain and skeleton, it is difficult to prove that locus AS021 enhances neocortical subexpression of *SATB2* based on a lacZ expression pattern. Circumstantial evidence, however, suggests that it does. First, *SATB2* is the only gene involved in development in the AS021-neighboring region we searched (2 Mb in total), which includes 10 mouse genes (SI Table 2 in *SI Appendix*). Second, LacZ staining in the neocortex was first observed at E11.5–E12.5. (SI Table 1 in *SI Appendix*). This developmental expression pattern is consistent with that reported for *SATB2* (31) (SI Fig. 10 in *SI Appendix*). Third, a brain section study showed that lacZ staining was first observed in the cortical plate at E13.5 (Fig. 6c–f) and then expanded into other neocortex areas (the intermediate zone; Fig. 6g–j), a developmental expression pattern consistent with that of *SATB2* (32) (SI Fig. 10 in *SI Appendix*). Furthermore, detailed expression of lacZ was evident in sectioned brain tissue, which revealed restricted expression in the lateral telencephalon (Fig. 6e and f).

The cerebral cortex has a laminar organization, which shows a birth date-dependent migration manner (i.e., early-born/inside, late-born/outside). Correct ordering of lamina formation requires Reelin expression and several sources of Reelin-expressing Cajal–Retzius cells (33). One of the sources for a subpopulation of Reelin-positive Cajal–Retzius cells is from the border of the pallium and subpallium (33, 34) (antihem), where cells stained by lacZ (arrows in Fig. 6f) appeared to be first generated. Cells generated from the antihem, as labeled by *Dbx1-LacZ* expression, migrate tangentially toward the dorsal telencephalon, which seems to be lacking in the chick telencephalon (ref. 33 and A. Pierani, personal communication). This result strongly suggests a mammal-specific contribution of locus AS021 activity to the development of a mammal-specific fundamental structure (19). However, further study is required to grasp a total understanding of this phenomenon.

SINES as Distal Enhancers. To date, we have examined 10 AmnSINE1 loci and demonstrated that the two loci, AS071 and AS021, function as distal transcriptional enhancers in developing mouse embryos. It should be noted that both enhancers are specific to the developing forebrain and have functions that are mammalian-specific. Although we speculate that these enhancers contribute to mamma-

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