Loss-of-Function Mutation in a Repressor Module of Human-Specifically Activated Enhancer HACNS1

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Abstract

The cis-regulatory element contributed to gaining humanness is of great interest in human evolutionary studies. A human-accelerated region exceeding neutral evolutionary rates, termed HACNS1, was recently reported as a positively selected sequence acquiring novel TF-binding sites responsible for human-specific gain of limb enhancer function. However, another possibility is loss of function in repressor element in HACNS1. Signature of the human substitutions in the 81-bp region infers that a GC-biased gene conversion (BGC) might create these seemingly excessive substitutions. To evaluate the 81-bp function, we performed transgenic mouse assay of the HACNS1 construct lacking the 81-bp region. The deleted construct showed similar enhancer activity to the intact human HACNS1, suggesting that the function of the human 81-bp region is not an activating enhancer but rather a disrupted repressor. This result infers that loss of function in the HACNS1 81-bp region, possibly via a BGC, played an important role in human-specific evolution.

Key words: HACNS1 cis-regulatory element, loss of function.

Genomic changes responsible for creating humanness after divergence from the human–chimpanzee common ancestor are of great interest to human evolutionary studies (e.g., Saitou 2005). Cis-regulatory mutations in noncoding genomic regions are thought to be responsible for generation of human-specific developmental programs (e.g., Carroll 2003). Pollard, Salama, and king (2006) and Pollard, Salama, and Lambert (2006) found a series of short DNA sequences that are highly conserved in vertebrates but show accelerated evolution only in human and named them "HAR." Prabhakar et al. (2006) and Bird et al. (2007) also conducted similar genome-wide studies. Prabhakar et al. (2008) found one such sequence, HACNS1, 239-bp fragment located neighbor to the *GBX2* limb-expressing gene. HACNS1 includes 119-bp HAR2 and was shown to act as a limb bud enhancer with enhanced limb enhancer activity specifically in human. This change was caused by 13 human-specific substitutions within an 81-bp region and was interpreted that accumulation of these positively selected substitutions created multiple novel transcription factor (TF)–binding sites (gain of function) and that the deposition of those facilitated the human-specific enhanced activity (Prabhakar et al. 2008).

However, loss of function in a repressor element within HACNS1 can be another explanation for it. A GC-biased gene conversion (BGC) may be an alternative explanation for fixation of such mutations without experiencing adaptive evolution (Galtier and Duret 2007; Duret and Galtier 2009; Galtier et al. 2009; Katzman et al. 2010). BGC is consequence of DNA double-strand break repair between homologous chromosomal regions, and the alleles from one chromosome are converted to the other with a bias of A or T to G or C (Strathern et al. 1995). Neutral or even deleterious alleles could be fixed by BGC (Galtier et al. 2001). The observation that all the 13 human-specific substitutions within the 81-bp region are G or C (11 are A or T to G or C, and 2 are G to C) may be consistent with the idea that BGC took place in this region (Duret and Galtier 2009). It is possible that the 13 mutations were caused by BGC and resulted in a disruption of repressor function of the 81-bp region (loss of function). It could eventually enhance the activity of human HACNS1.

To see which process, gain or loss of function, is more plausible, we conducted transgenic mice assay by using two transgene constructs to characterize the 81-bp region in HACNS1 that harbors 13 human-specific GC-biased substitutions. One, HACNS1-full, has an intact human HACNS1 sequence that is combined with heat shock protein 68 promoter (hsp68p) and lacZ reporter gene with SV40 polyA signal sequence. Another construct, HACNS1-Δ81bp, is basically the same as HACNS1-full except a mutation that replaced the 81-bp region that harbors 13 human-specific GC-biased substitutions with 5′-GAATTC-3′ sequence. A total of 367 and 426 fertilized eggs were microinjected with the HACNS1-full construct and the HACNS1-Δ81bp construct, respectively, and they were transferred into oviduct of recipient mice, and then, embryos were dissected out at E11.5. We recovered 72 and 49 E11.5 embryos for HACNS1-full and HACNS1-Δ81bp constructs, respectively. We then obtained eight lacZ-positive embryos for the HACNS1-full construct in which five showed strong to middle-level expression in limb, and three showed weak expression (fig. 1A). This result is consistent with the previously reported pattern (Prabhakar et al. 2008), although the expressions may be slightly less intensive overall in our experiment. As for mice injected HACNS1-Δ81bp construct, we obtained five lacZ-positive embryos. Three of them showed

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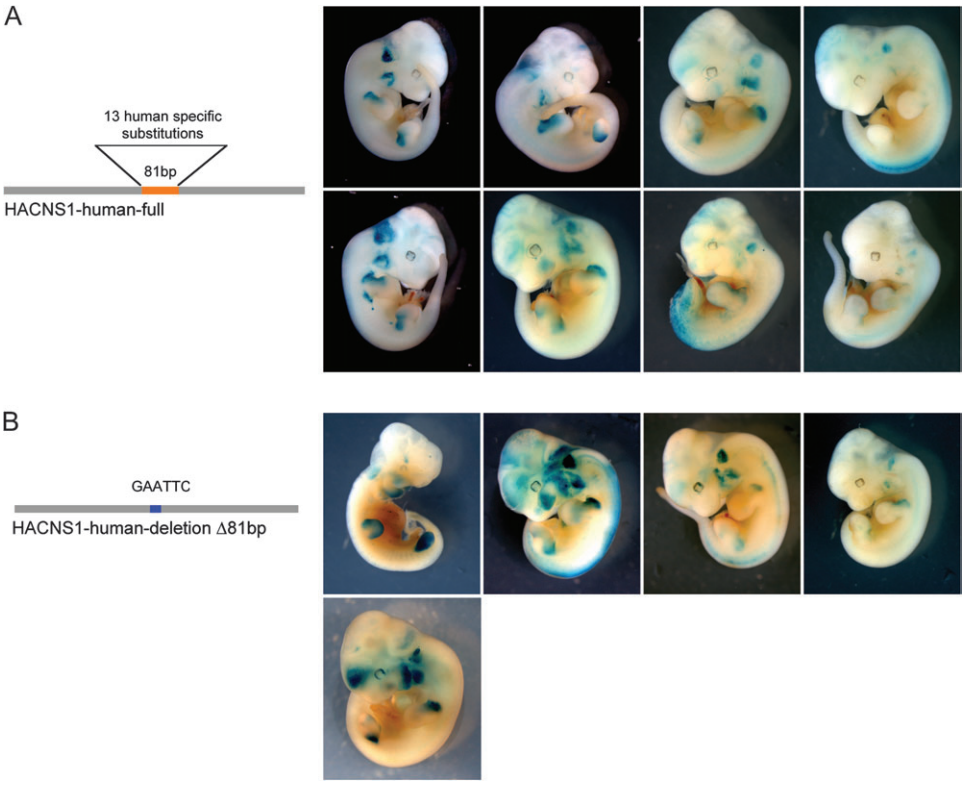

FIG. 1. Transgenic mice assay showed indistinguishable X-gal staining pattern between the two constructs. (*A*) E11.5 transgenic mice embryos with X-gal staining using intact HACNS1 construct (HACNS1-full). All the eight lacZ-positive embryos are shown. (*B*) E11.5 transgenic mice embryos with X-gal staining using the 81-bp region–deleted HACNS1 construct (HACNS1-*Δ*81bp). All the five lacZ-positive embryos are shown.

strong to middle-level expression in limb, whereas the other two showed middle to weak expression (fig. 1*B*). We did not observe apparent difference in expression pattern nor trend in expression intensity between those two constructs.

The purpose of this study is to see if removal of the 81-bp region results in reduction of enhancer activity in limb or unchanged. The positive selection model predicts that accumulation of a series of de novo synthesis of TF-binding sites by adaptive substitutions made the 81-bp region to become an active enhancer module. Therefore, removal of the 81-bp region should decrease HACNS1 enhancer activity significantly to the level of that seen in other primates. Loss-of-function model assumes that the 81-bp region originally functioned as a repressor element in nonhuman primates, and perhaps, a BGC event that occurred in the human lineage disrupted the repressor function of the 81-bp region. The latter model predicts that removal of the 81-bp region from HACNS1 should cause no effect on total enhancer activity.

Our result clearly favors the latter, loss-of-function model (fig. 2). A synthetic human enhancer replaced with the 81-bp region of the chimpanzee and macaque indeed showed reduced expression (Prabhakar et al. 2008), which is consistent with our repressor loss-of-function model. BGC may thus be a major player shaping this human-specific "accelerated" region (HAR2 or HACNS1). Nucleotide substitutions that occurred within accelerated region may not be positively selected due to generation of multiple adaptive TF-binding sites. Therefore, prediction of positively selected de novo TF-binding sites based on excessive accumulation of point mutations assuming a simple substitution model (e.g., Prabhakar et al. 2008) is potentially erroneous. We should pay attention in choosing models predicting genuine positively selected sequences.

The loss-of-function model for the HACNS1-81bp region does not exclude the possibility that the entire HACNS1 is adaptive. We know little about its benefit at individual and population level, or even we do not know if HACNS1 is

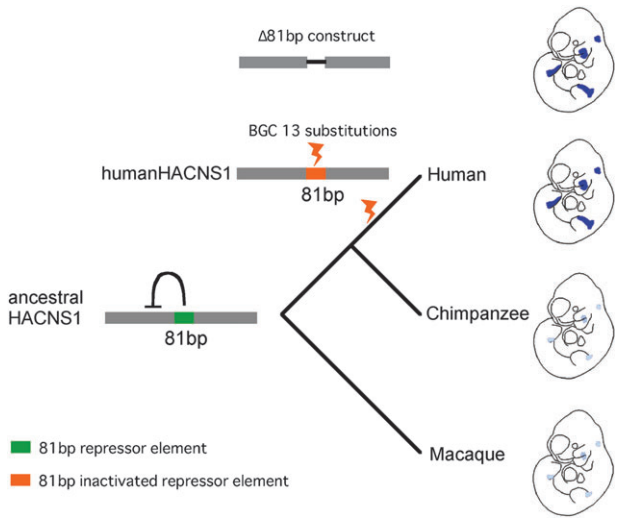

FIG. 2. The proposed model of HACNS1 evolution in primates. The 81-bp element was originally a repressor element within HACNS1, and human-specific 13 substitutions possibly caused by BGC disrupted repressor function and HACNS1 expression expanded. The expression patterns for chimpanzee and macaque were based on Prabhakar et al. (2008).

indeed an enhancer element of the human *GBX2* gene. More extensive functional analysis will be necessary to conclude whether HACNS1 contributed to increasing fitness in the course of human evolution.

Methods

Human HACNS1 was polymerase chain reaction (PCR) amplified by using two primers, HACNS1HSAupHindIII: 5′-GCGAAGCTTACCCCAGCATACAGTAGCTTCA-3′ and HACNS1HSAloHindIII: 5′-GCAAGCTTATCAAGTGTGGCAAAAATGAC-3′. The 81-bp deletion construct was made using an oligomer (5′-TCGTATAAAGCCCCGATTGAATTCGCCACACACAGGTCAG-3′) together with PCR primers listed above. Those PCR products were digested with *Hin*dIII and cloned into minimal general promoter-reporter system vector HSF51 containing hsp68 heat shock protein promoter and lacZ gene. ScaI digested–linearized constructs were adjusted to 10 ng/μl and injected into pronuclei of one-cell mouse embryos. Transgenesis and X-gal staining of transgenic embryos were performed according to Sumiyama and Ruddle (2003).

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