Letter to the Editor

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Transactivation Function of an ~800-bp Evolutionarily Conserved Sequence at the *SHOX* 3′ Region: Implication for the Downstream Enhancer

Table 1

Primer Sequences and PCR Conditions Used for Genotyping

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

To the Editor:

In the October 2005 issue of the *Journal,* Benito-Sanz et al. (2005) reported an association of Léri-Weill dyschondrosteosis (LWD [MIM 127300]) with a novel class of heterozygous pseudoautosomal region 1 (PAR1) deletions downstream of *SHOX* (short-stature homeobox-containing gene [MIM 312865]) in 12 patients with two copies of intact *SHOX* coding sequences. The deletions were variable in size, with the smallest region of overlapping deletion (SRO) of ~30 kb between *DXYS10086* and *rs7067102*. The results—in conjunction with the report of Flanagan et al. (2002) describing a monoallelic *SHOX* expression in the bone marrow fibroblasts taken from the distal radius of a patient with LWD with two copies of normal *SHOX* coding exons and hemizygosity for a region around *DXYS233* downstream of *SHOX*—suggest the presence of a downstream enhancer for *SHOX* transcription around the ~30-kb SRO. Consistent with this, Fukami et al. (2005) found that (1) a 240–350-kb deletion including *DXYS233* is present in a heterozygous status in a mother with LWD and two copies of intact *SHOX* coding exons and (2) the same deletion is present in a hemizygous status in her daughter with Langer mesomelic dysplasia and a mosaic-ring X chromosome missing the PAR1. Here, we report that the putative *SHOX* enhancer may reside on an ~800-bp evolutionarily conserved sequence (ECS).

First, we analyzed the *SHOX* 3′ region in five Japanese families in which the proband and one of the parents had variable degrees of LWD and stature from short to low normal in the presence of two copies of intact *SHOX*-coding exons. The study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. Genotyping analysis was performed with primers and methods shown in table 1; results indicate that a deletion between *SHOX-SNP792* on the 3′ UTR and *DXYS85* was shared by the LWD-affected mother and daughter in family A and that a deletion between *rs5946324* and *rs4504827* was common to the LWD-affected father and daughter in family B (table 2 and fig. 1*A*). Furthermore, FISH analysis was performed with an RP13-167H21 BAC probe defining a region from *rs5946324* to *DXYS233* (Ensembl Genome Browser); results show only a single signal in the mother and the daughter of family A and an obviously different signal intensity in the father and the daughter of family B (fig. 2). The results, together with our previous data (Fukami et al. 2005), imply that an ~40-kb region between *rs5946326* and *rs4504827* is the SRO in the Japanese patients (SRO-J) (fig. 1*A*). The SRO-J is in a close agreement with the ~30-kb SRO in the white patients (SRO-W) (Benito-Sanz et al. 2005), and a region between *rs5946326* and *rs7067102* is shared by all the patients with *SHOX* 3′ deletions (fig. 1*A*).

Next, we searched the UCSC Genome Browser for the ECSs within the SROs. Seven ECSs (ECS1–ECS7) were present within the SRO-J, whereas ECS6 and ECS7 were found to reside outside the SRO-W (fig. 1*B*). ECS3–ECS7 were well conserved in chicken and dog, which preserve *Shox,* and were absent in mouse and rat, which lack *Shox* (Clement-Jones et al. 2000; Ensembl Genome Browser). ECS4 was also conserved in *Fugu* and zebrafish, which preserve *Shox*. By contrast, ECS1 was absent in chicken, and ECS2 was conserved in mouse and rat. Whereas ECS3 and ECS4 were not described in chimpanzee, the sequence analysis remains poor for the *Shox* 3′ region in chimpanzee, in contrast to the detailed analysis of that region in chicken and dog. These findings suggest that ECS3–ECS5 can be regarded as candidate regions harboring the putative downstream enhancer. In this regard, since ECS3–ECS5 reside between *rs5988437* and *rs5946533* (UCSC Genome Browser), they should be deleted from the patients in the three families defining the SRO-J (fig. 1*A*).

Thus, we examined the transcription activity of ECS3–ECS5 as well as ECS6 with the dual-luciferase reporter assay system (Promega). Luciferase reporter constructs containing each ECS (ECS3, 861 bp; ECS4, 824 bp; ECS5,

Table 2

Summary of Polymorphism Analyses

Locus[a]	Polymorphism[b]	Allele or Polymorphism Position				
		Family A		Family B		
		Daughter LWD (+)	Mother LWD (+)	Daughter LWD (+)	Father LWD (+)	Mother LWD (−)
SHOX $(CA)_n$[c]	MS $(CA)_n$	141/153	141/149	149	149/153	149/153
SHOXa/b +3/1239[d]	SNP (C/G)	C	C	G	C/G	NE
SHOXa/b +3/1248[d]	SNP (G/A)	G	G	A	A/G	NE
SHOX-SNP657[e]	SNP (G/A)	A	A/G	NE	NE	NE
SHOX-SNP792[f]	SNP (T/G)	T/G	T	T	T	T
rs5988407	SNP (C/T)	***C***	***T***	NE	NE	NE
rs7055778	SNP (G/A)	A	A	NE	NE	NE
rs5946324[g]	SNP (C/G)	G	G	G	G/C	G/C
rs5946325[g]	SNP (C/T)	T	T	T	T	NE
rs5946326[g]	SNP (G/A)	G	G	***A***	***G***	NE
rs5988281[g]	SNP (C/G)	C	C	***G***	***C***	NE
rs5946329[g]	SNP (C/T)	C	C	***T***	***C***	NE
rs5988432[g]	SNP (C/T)	C	C	C	C	NE
rs5946331[g]	SNP (G/A)	G	G	***A***	***G***	NE
rs5988437[g]	SNP (C/G)	***C***	***G***	***G***	***C***	NE
rs6644384[g]	SNP (G/A)	A	A	NE	NE	NE
rs5946336[g]	SNP (G/A)	A	A	***G***	***A***	NE
rs7067102[g]	SNP (G/A)	NE	NE	A	A	NE
rs5946533[g]	SNP (C/T)	NE	NE	***T***	***C***	NE
rs4504827[g]	SNP (T/A)	T	T	T/A	T/A	NE
rs5988299[g]	SNP (C/T)	NE	NE	C	C	NE
rs5988300[g]	SNP (G/A)	NE	NE	G	A/G	NE
rs5988301[g]	SNP (C/G)	NE	NE	G	G/C	NE
rs5988494[g]	SNP (C/G)	NE	NE	C	C	NE
DXYS233[g]	MS $(CA)_n$	***273***	***279***	273	273/283	273/279
rs4468091	SNP (G/A)	G	G	G	G	A/G
rs5946712	SNP (G/A)	G	G	G	G	G
DXYS85	4-bp ins/del	82	78/82	78/82	82	78/82

Note.—The loci present in two copies are underlined, and those not shared by the LWD-affected proband and parent are in bold italics. A plus sign (+) = affected; a minus sign (−) = not affected; NE = not examined.

[a] *SHOXa/b +3/1239* and *SHOXa/b +3/1248* are based on the Polymorphisms around *SHOX* Database, *SHOX-SNP657* and *SHOX-SNP792* are based on the study by Flanagan et al. (2002), and the remaining loci are based on dbSNP and dbSTS databases.

[b] MS = microsatellite.

[c] Located in the *SHOX* 5′ UTR.

[d] Located between exon 6a inherent to *SHOXa* and exon 6b specific to *SHOXb*.

[e] Silent polymorphism on exon 6b.

[f] Located in the *SHOX* 3′ UTR.

[g] Loci included in the RP13-167H21 BAC probe used for FISH analysis.

441 bp; ECS6, 634 bp) inserted into the 3′ region of the luciferase gene were created using the pGL3 vector with the SV40 promoter or the human *SHOX* promoter on exon 2 (−432 to +5 bp) (Blaschke et al. 2003) (fig. 1*B*). The U2OS osteosarcoma cell line expressing *SHOX* (Rao et al. 2001) was transfected using lipofectamine (Invitrogen) with each reporter vector together with the pRL-CMV vector used as an internal control for the transfection, and luciferase assays were performed 36 h later. After the experiments were performed five times, the normalized luciferase activity was found to be significantly increased only when the reporter vector with ECS4 and the *SHOX* promoter was transfected to U2OS cells (empty vs. ECS4; $P = .011$ by t test) (fig. 1*B*). This implies that the putative *SHOX* enhancer resides in ECS4 and interacts with the *SHOX* promoter on exon 2.

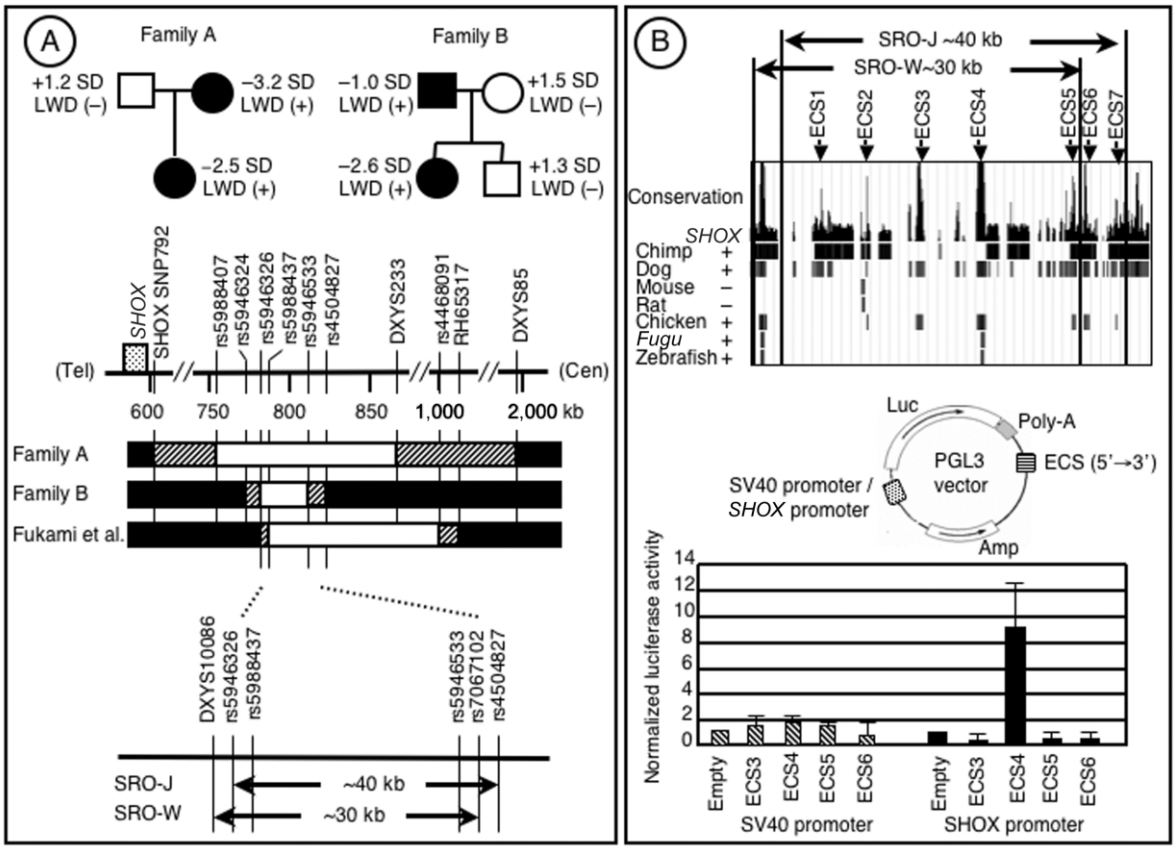

Figure 1 *A*, PAR1 deletions in the *SHOX* downstream region. *Top*, Pedigrees of families A and B. LWD is exhibited by the mother and the daughter in family A and by the father and the daughter in family B. The height of each subject is expressed as an SD score. *Bottom*, Deletion maps of the *SHOX* 3′ region. In families A and B, the blackened segments represent the disomic regions, the unblackened segments depict the monosomic regions, and the striped segments indicate the dosage-unknown region in which the breakpoints should exist. The physical distance (kb) from the Xp/Yp telomere ("Tel") is shown below the horizontal line. The results of the present study and those reported by Fukami et al. (2005) indicate that the SRO-J spans ~40 kb in physical length and is largely similar to the SRO-W reported by Benito-Sanz et al. (2005). *B*, Functional analysis of the ECSs. *Top*, Seven ECSs (ECS1–ECS7) are identified in the SRO-J (UCSC Genome Browser). *Bottom*, Transcription analysis of ECS3–ECS6. A luciferase reporter construct has been created with the SV40 or the human *SHOX* promoter, and each ECS inserted into the 3′ region of the luciferase gene ("Luc"). Only the combination of the *SHOX* promoter and ECS4 has significantly increased the luciferase activity.

Finally, we searched ECS4 for potential binding sites for transcription factors relevant to bone development, using the MATINSPECTOR, TESS, and TFSEARCH programs. The putative binding sites with the maximum core similarity of 1.0 and a matrix similarity >0.75 were identified for HOXA9, HOXB9, PBX1, and MEIS1, as well as for PBX1-HOXA9 and MEIS1-HOXA9 heterodimers, which are known to be involved in limb development (Mercader et al. 1999; Shanmugam et al. 1999; Zákány and Duboule 1999) (fig. 3). Thus, the sequence-specific DNA binding of such a factor(s) might mediate the enhancer activity for *SHOX*. However, other potential binding sites were also detected for various transcription factors, and the relevance to skeletal development has not been studied or excluded in most of the transcription factors. In addition, the binding sites remain unidentified for many transcription factors. Thus, further studies are necessary to define the enhancer sequence.

In summary, the results suggest that the ~800-bp ECS4 harbors the putative downstream enhancer for *SHOX* transcription. This information will provide a useful clue

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 2 Results of the FISH analysis. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

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Figure 3 Nucleotide sequence of ECS4 and putative binding sites for several transcription factors relevant to skeletal development. The human sequence is aligned with the chicken sequence.

for the clarification of the molecular network involved in *SHOX*-dependent skeletal development.

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Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
dbSTS, http://www.ncbi.nlm.nih.gov/dbSTS/
Ensembl Genome Browser, http://www.ensembl.org/
MATINSPECTOR, http://www.genomatix.de/products/MatInspector/MatInspector3.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for LWD and *SHOX*)
Polymorphisms around SHOX Database, http://www.le.ac.uk/genetics/ajj/SHOX/mapdetails.html
TESS: Transcription Element Search System, http://www.cbil.upenn.edu/tess/
TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH.html
UCSC Genome Browser, http://genome.ucsc.edu/

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