Transcriptional and Translational Regulation of the Léri-Weill and Turner Syndrome Homeobox Gene SHOX*

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The human pseudoautosomal homeobox gene SHOX has recently been shown to encode a cell type-specific transcription factor involved in cell cycle and growth regulation (1).1 Haploinsufficient loss of the SHOX gene causes short stature and has been correlated with variable skeletal phenotypes frequently observed in Léri-Weill and Turner syndrome patients (2–6). SHOX-deficient individuals exhibit a considerable phenotypic heterogeneity ranging from mild, barely detectable skeletal malformations to severe dysplasia adversely affecting the life of these patients (7, 8). This phenotypic heterogeneity is clinically defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: UTR, untranslated region; IRES, internal ribosomal entry sites.

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**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The complete 5′-UTR of the SHOX mRNA was assembled from two genomic PCR fragments representing exon 1 and the SHOX non-coding portion of exon 2. PCR products were carried out with the HotProof polymerase system (Qiagen) using the following primers: Ex1for(SpeI), 5′-GGAGTACGTCGGCCCTTGGCCGTCGGAAG-3′; Ex2rev, 5′-CAAGCTTGAGCGAGGCAGCGGCAGG-3′; Ex2for, 5′-GAAACTCGAGTTGCTTCTTTCGCG-3′; Ex2rev(EcoRI), 5′-CGGAAAGTTCATGCTGTTGGCCGGC-3′. Both PCR fragments were digested with SpeI and EcoRI, respectively, cloned into a SpeI restricted Bluescript plasmid (Stratagene), and sequence verified. All UTR-containing clones were derived from this plasmid (pBSK/SHOX-UTR). The expression constructs pHPL/UTR and pFRF/UTR were generated by subcloning the complete 5′-UTR fragment from pBSK/SHOX-UTR via SpeI/Ncol into the respective parental vectors (19).

Nested deletions of the SHOX 5′-UTR were obtained from pBSK/SHOX-UTR by PCR using the following forward primers: Ex2for(SpeI), 5′-GGAGTACGTTACGGTGCTTTGGCG-3′; Ex2for(EcoRI), 5′-GGAGTACGTTACGGTGCTTTGGCG-3′; and Ex23forSpeI, 5′-GGAGTACGTTACGGTGCTTTGGCG-3′ in combination with the Ex2rev(EcoRI) oligo. Resulting PCR fragments were digested with SpeI/EcoRI and cloned into pbblueScript. The plasmid constructs pUTR-Luc, pEx2-Luc, pEx21-Luc, and pEx23-Luc were generated by introducing an Ncol/SalI fragment containing the firefly luciferase coding cassette from the commercially available pGL3 vector (Promega) into the appropriately digested pBSK/UTR containing vectors.

The plasmids pBSK/UTR-Xnoggin, pBSK/Ex23-Xnoggin, and pBSK/UTR-AUGm1–4, Xnoggin were generated by substituting the firefly coding sequence constructs pGAS-Luc and pBSK/UTR-aUG-m1–4 by a noggin PCR fragment generated with the following primers: Xnoggin-for, 5′-GGCAATGGGATGCATCCATTCTAGTGCTTG-3′ and Xnoggin-rev, 5′-GGCTCTGACTAATCTGATGAGTTGATGATGCAGGACTGATGCTGCCGAGATT-3′. The final construct was cloned into Ncol/BamHI.

**Structural Features of the SHOX 5′-UTR**—Upstream AUUGs within the SHOX 5′-UTR were mutated using the QuiChangep Multi site-directed mutagenesis kit according to the procedure recommended by the supplier (Stratagene). Individual mutations were introduced into pBSK/SHOX-UTR with the following primers: uAUG-mut, 5′-CTACCTGCGAACAAGAATTTGGAGGAGGCGAGCGG-3′; uAUG-mut, 5′-GACCCGGACGCGATGGAACCTCCCGGGG-3′; uAUG-mut, 5′-CCCTTCTAAAATTTGCGTTGCTTCCGCGC-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′; uAUG-mut, 5′-GGGCAATGCTGCTTTGGCG-3′. The plasmids pBSK/UTR-Xnoggin, pBSK/Ex23-Xnoggin, and pBSK/UTR-AUGm1–4 were generated by mutating the UAG codon of pBSK/UTR-AUGm1–4, by a noggin PCR fragment generated with the following primers: Xnoggin-for, 5′-GGCAATGGGATGCATCCATTCTAGTGCTTG-3′ and Xnoggin-rev, 5′-GGCTCTGACTAATCTGATGAGTTGATGATGCAGGACTGATGCTGCCGAGATT-3′. The final construct was cloned into Ncol/BamHI.

**RNA Transfection and Quantitation of Translation Efficiency**—Synthetic mRNAs generated with the mMESSAGE mMACHINE™ or MEGaScript™ reaction systems (Ambion) were directly transfected into different cell lines using 2 μg RNA and 8 μl of TransMessenger™ Transfection Reagent (Qiagen) per well of a 6-well plate. Transfection procedure was carried out following the recommendations of the supplier. Transfected cells were trypsinized, washed once with phosphate-buffered saline, and the cell pellet was resuspended in 200 μl phosphate-buffered saline. 40 μl of this cell suspension was mixed with 10 μl of 5X Passive Lysis Buffer (Promega) and directly used for Luciferase assays. The remaining 160 μl of the cell suspension were used for isolation of total RNA by standard procedures (Qiagen). 1 μg aliquots of total RNA were reverse transcribed and analyzed by quantitative real time RT-PCR amplification with the FastStart reaction system (Roche Applied Science) in a LightCycler® instrument (Roche Applied Science) using the following primers: Luc1for, 5′-GGGAGGACCACTGATAAACATCG-3′, and Luc1rev, 5′-ACTCTGATGACCTCCCTTTG3′. The reactions contained 60 mmol MgSO4 in a final volume of 20 μl and were carried out under the following conditions: 95 °C/10 min; (95 °C/15 s, 60 °C/15 s, 72°C/15 s) 40 cycles. Luciferase activities were corrected for RNA transfection efficiencies using the results from real-time RT-PCR.
to $-24.2$ kcal/mol. The capacity to form extraordinary stable secondary structures, the terminal oligopyrimidine tract, and the presence of multiple uAUGs strongly suggest regulatory properties of this 5'-UTR related to the control of SHOX gene expression on a translational level.

Exon 2 of the SHOX Gene Harbors Alternative Promotor Activities—The calculated high degree of folding is indicative for IRES (15) and has prompted us to first analyze the SHOX 5'-UTR translational activity by transfection assays using the reporter constructs pHPL/UTR and pRF/UTR (Fig. 2A). In pHPL a stable hairpin at the 5'-end of the SV40-driven transcription unit represses translation of a firefly luciferase encoding mRNA generated from the parental vector (19). As shown in Fig. 2B, the SHOX 5'-UTR is able to rescue this reporter gene expression if inserted between the 5' hairpin structure and the firefly luciferase encoding sequence. Within different cell lines, the observed up-regulation varies between 25-fold (HEK293), 72-fold (COS7), and 95-fold in U2Os cells over wild type pHPL activity (data not shown). Also, the SHOX 5'-UTR up-regulates protein expression from the downstream reporter gene if inserted between the 5' hairpin structure and the firefly luciferase encoding sequence. Within different cell lines, the observed up-regulation varies between 25-fold (HEK293), 72-fold (COS7), and 95-fold in U2Os cells over wild type pHPL activity (data not shown).

In conclusion, these results demonstrate that the SHOX gene is transcribed from at least two alternative promoters ($P_1$ and $P_2$) generating distinct mRNAs that encode identical proteins, but vary in their 5'-UTR sequences. These transcripts are hereafter referred to as type 1 and type 2 transcripts, respectively.

The SHOX 5'-UTRs Differentially Regulate Translation Efficiency—As the promoter activity within exon 2 of the SHOX gene interferes with DNA transfection experiments, all subsequent analyses were directly based on in vitro generated mRNAs. To address the mechanism by which the two SHOX 5'-UTR variants are translated, we generated in vitro transcripts from pBSK/UTR-Luc (type 1 mRNA) and pBSK/EX2-Luc (type 2 mRNA) in the presence of either a generic CAP analog (m7G(5')ppp(5')G; A-CAP) or an unmethylated variant (A(5')ppp(5')G; A-CAP) interfering with CAP-dependent translation initiation. The results from in vitro translation assays primed with these transcripts are shown in Fig. 3B. Although type 1 transcripts are translated at very low levels independently of the CAP structure, the unmethylated A-CAP substantially interferes with the high translation efficiency of type 2 variant. These results suggest that both type 1 and 2 transcripts do not support internal ribosomal entry, but require a CAP-dependent mechanism of translation initiation. Furthermore, they uncover considerable differences in translation competence between the 5'-UTRs of type 1 and type 2 transcripts. We next analyzed nested deletions of the type 1 SHOX 5'-UTR (Fig. 3A) by in vitro translation assays. As shown in Fig. 3C, translation efficiencies are indeed inversely related to the length of the 5'-UTR with an 85-fold difference between type 1 and type 2 variants. To confirm this in vitro-derived data in living cells, we transfected these mRNAs and quantitated their translational efficiency. As expected, we observed the same inverse correlation between length and translation activities in the osteogenic cell line U2Os (Fig. 3D). We next transfected the same in vitro generated mRNAs into different cell lines and determined their translational competence (Fig. 3E). Interestingly, this analysis not only confirms the translation-attenuating properties of the long 5'-UTR variant but also suggests some cell line-dependent responsiveness to the translation inhibitory elements presented by the different forms of the SHOX 5'-UTR (Fig. 3E). Taken together, these results provide compelling evidence that type 1 and type 2 transcripts generated from the alternative promoters $P_1$ and $P_2$ exhibit differential
translation efficiencies due to inhibitory elements within the long SHOX 5′-UTR variant.

*Upstream AUG Codons within the SHOX 5′-UTR Inhibit Translation In Vitro and in Vivo*—Because the use of nested 5′ deletions does not allow to discriminate structural effects from the influence of uAUGs within the SHOX 5′-UTR, we generated constructs harboring mutations of individual uAUGs or different combinations thereof. *In vitro* transcription of these constructs yield mRNAs identical in size and with similar overall structural features of their 5′-UTRs (Fig. 4, A and B).
These *in vitro* transcripts were directly transfected into U2Os cells and their translation efficiency determined. As shown in Fig. 4B, mutation of all seven uAUGs within *type 1* transcripts yields translational activities comparable with the high efficiency of *type 2* mRNAs. Therefore, we can exclude length and overall structure, but rather define the uAUGs as critical determinants for the observed translational down-regulation. We next investigated if this effect can be attributed to individual uAUGs by transfection of reporter RNAs harboring individual uAUG mutations. We can show that none of the individual mutations is able to confer substantial activity increase, whereas combined mutations of uAUG$_{3-7}$ yield high translation efficiencies. These results argue for a concerted function of the uAUGs with a major contribution of uAUG$_{3-7}$. To confirm these results *in vivo*, we fused the wild type and mutated type 1 and the type 2 SHOX 5'-UTRs to the coding sequence of *Xenopus* noggin. Expression of noggin is well documented to cause dorsalization (loss of trunk and tail structures) during early embryogenesis (26). Injection of these constructs into early *Xenopus* embryos yields three distinct phenotypes that can be classified as wild type, mild, and severely dorsalized embryos (Fig. 4D). In agreement with cell culture-derived data, type 2 mRNAs induce the strongest effects, whereas type 1 mRNAs cause milder phenotypes only at significantly higher RNA levels. In contrast, reporter mRNAs harboring mutations in all uAUGs cause a phenotype comparable with type 2 mRNAs with respect to both severity and dose response (Fig. 4D).

**DISCUSSION**

Haploinsufficiency of the human pseudoautosomal gene SHOX causes short stature and skeletal malformations associated with the Léri-Weill and Turner syndromes with a remarkable phenotypic heterogeneity observed among SHOX-deficient patients. Besides other possible factors, this heterogeneity might be caused by variations in functional protein levels. Here we describe the concatenation of transcriptional and translational mechanisms regulating SHOX expression. We have identified an alternative, intragenic promoter residing within exon 2 of the SHOX gene. Using several reporter constructs, we were able to narrow down this promoter to a region of 300 bp upstream of the AUG start codon. Interestingly, this region contains a canonical TATA box and a CAAT box at positions −137 and −257, respectively. Deletion of the CAAT box containing fragment (pEX2Δ2) dramatically reduces reporter activity, indicating its relevance for the function of the identified P$_2$ promoter. In addition to these core promoter characteristics, our analysis reveals regulatory elements between P$_2$ and P$_0$ that negatively control the activity of this intragenic promoter. Although the physiological function of the dual promoter structure remains to be elucidated, it is tempting to speculate that the two SHOX promoters are utilized in a developmental- and/or differentiation-specific manner. Recent studies (27–29) in *Drosophila* have led to the concept of “core promoter competition” within single or among neighboring genes. This model suggests that different core promoters selectively interact with each other by competing not only for basic transcription factors but also for tissuespecific enhancer elements. This competition leads to a mutually
exclusive utilization of individual core promoters. Application of such a competition model will be particularly interesting, because alternative utilization of the SHOX promoters yields distinct mRNAs with diverse 5′-UTRs but identical coding capacities.

Although the identified intragenic promoter P2 generates mRNAs with a short 5′-UTR (type 2), utilization of the upstream promoter P1 yields mRNAs with a long and highly structured 5′-UTR (type 1). Interference of such complex 5′-UTRs with the translational scanning mechanism has led to the concept of alternative, CAP-independent translation mechanisms mediated by internal ribosomal entry sites. IRES-regulated translation, initially described for viral RNAs (30–32), was more recently suggested as the underlying mechanism controlling the expression of several cellular transscripts (15, 33–35). We have therefore investigated the possibility of internal ribosomal entry into type 1 SHOX mRNAs by transient transfection of mono- and bicistronic vectors, in vitro TNTa s-UTRs, and direct mRNA transfection into different cell lines. From these analyses, we conclude that the 5′-UTR of type 1 SHOX mRNAs does not contain significant IRES activities and that both SHOX transcripts are translated by the canonical CAP-dependent pathway.

We next analyzed nested deletions of the long SHOX 5′-UTR by mRNA transfection assays and observed an inverse correlation between length and translation activities of the SHOX 5′-UTRs in different cell lines. However, because nested deletions of the SHOX 5′-UTR increasingly disturb the overall secondary structure and sequentially remove important sequence characteristics, these results do not automatically substantiate length itself as the critical determinant for the low translation efficiency of type 1 mRNAs.

After having established its translation attenuating effects, we focused on the seven uAUGs within the type 1 SHOX 5′-UTR. The presence of such uAUGs has been reported to regulate protein expression from the major open reading frame by interfering with ribosomal scanning of the 5′ leader sequence (36–38). In agreement with these reports, we could demonstrate that mutations of these uAUGs lead to a markedly up-regulation of translation efficiency after direct mRNA transfection. In fact, type 1 mRNAs harboring mutations in all seven uAUGs resemble or even augment the activity of type 2 SHOX mRNAs in the osteogenic cell line U2Os. This observation excludes that the overall secondary structure or length itself interferes with the CAP-dependent scanning but rather attributes the low translation efficiency of type 1 SHOX mRNAs to the presence of uAUGs within its 5′ leader. Similar effects of uAUGs have been reported for the translational control of several mRNAs encoding proto-oncogenes, growth factors, transcription factors, and other highly regulated proteins (13). For some of these cases, it could be demonstrated that translation initiates at one or more uAUGs and scanning is reinitiated after translation of the respective uORF. Such selective reinitiation after uORF translation was described for the GCN4 mRNA of Saccharomyces cerevisiae (39) and the murine mRNA for the activating transcription factor 4 (40). Upstream open reading frames within the 5′-UTR of both mRNAs promote an up-regulation of translation in response to the stress-dependent phosphorylation of the eukaryotic initiation factor eIF2. It will be particularly interesting to find out if type 1 SHOX transcripts generated from the upstream promoter P1 are regulated in a similar manner, because SHOX expression has been observed in hypertrophic chondrocytes and may be related to cell cycle regulation and apoptosis in osteoblastic and chondrocytic cells.1

In summary, the results reported here can be integrated into a combinatorial model of SHOX gene expression as depicted in

Fig. 5. According to this model, SHOX expression is regulated on the transcriptional level by alternative promoters generating type 1 or type 2 transcripts with identical coding capacities but distinct translation efficiencies. This unique situation suggests that P2 is utilized in situations with immediate needs of high SHOX amounts, whereas P1 allows the generation of transcripts that facilitate fine tuning of protein levels by translational control mechanisms possibly related to cellular stress situations. Therefore, this model certainly merits further investigation to identify the molecular details determining alternative promoter utilization and developmental- and/or differentiation-dependent translational regulation of the SHOX gene.

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