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Translational regulation by the 5′-UTR of thyroid hormone receptor α mRNA

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Morihiro Okada, Keisuke Nakajima and Yoshio Yaoita*

Division of Embryology and Genetics, Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Higashihiroshima 739-8526, Japan

*Yoshio Yaoita, Division of Embryology and Genetics, Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Higashihiroshima 739-8526, Japan.
Tel: +81-82-424-7481, Fax: +81-82-424-0739,
email: yaoita@hiroshima-u.ac.jp

The nucleotide and deduced amino acid sequences reported in this article are available in the DDBJ/EMBL/GenBank databases under accession numbers AB669465, AB669466, AB669467, AB669468, AB669469, AB669470 and AB669471.

Thyroid hormone (TH) regulates gene transcription by binding to the thyroid hormone receptor (TR) and plays a critical role in the regulation of development, growth and metabolism. The ligated TR activates many effector genes, which contributes to the orchestrated remodelling of the amphibian metamorphosis. However, the mechanisms regulating TRα protein level remain unknown. We determined the nucleotide sequences of the 5′-untranslated regions (5′-UTRs) in amphibian TRα mRNAs. The TRα 5′-UTR contains evolutionarily conserved regions. We demonstrated that a 161-nt stretch of the *Xenopus* TRα 5′-UTR strongly represses the translation of the downstream open reading frame in both frog and human cell lines, as well as in a cell-free translation system. An analysis using successive deletions of the TRα 5′-UTR revealed five elements possessing translational repressive activity. We analysed two elements, the 14-nt GC-rich region and the 15-nt upstream open reading frame (uORF), by introducing point mutations. This analysis showed that the GC-rich region, which shares its nucleotide sequence with the Sp1-binding site, requires stringent sequence specificity at a nucleotide level for translational repression to take place, whereas under our study conditions, the uORF does not. This study provides an example of complex translational regulation by multiple elements.

Keywords: thyroid hormone receptor/translation/5′-UTR/uORF/*Xenopus*.

Abbreviations: Fluc, firefly luciferase; GFP, green fluorescent protein; 5′-RACE, 5′-rapid amplification of cDNA ends; Rluc, *Renilla* luciferase; 5′-UTR, 5′-untranslated region; RT-PCR, real-time reverse transcription–polymerase chain reaction; RXR, 9-*cis*-retinoic acid receptor; TH, thyroid hormone; TR, thyroid hormone receptor; uORF, upstream open reading frame; *X.*, *Xenopus*.

Thyroid hormone (TH) and the thyroid hormone receptor (TR) act on nearly every cell in the body and control many diverse biological processes, including development, growth and metabolism. TRs are ligand-regulated transcription factors and are encoded by two homologous genes, TRα and TRβ, which belong to the superfamily of nuclear hormone receptors (*1*, *2*) and function as heterodimers with the 9-*cis*-retinoic acid receptor (RXR) (*3*). The TR represses and activates the transcription of target genes in the absence and presence of TH, respectively (*4*).

Amphibian metamorphosis is a complex developmental process (*5*) that shares many similarities with mammalian postembryonic development (*6*). This entire process is controlled by TH and TR. In *Xenopus laevis* (*X. laevis*), several lines of evidence indicate that the onset of TRα synthesis correlates with the ability to respond to exogenously applied TH (*7*, *8*). In addition, some desert frogs exhibit increased responsiveness to TH and have short larval periods. Accordingly, increasing levels of TRα expression have been suggested to contribute to the faster metamorphic rates in these frogs (*9*).

TRα protein level remains constant in both head and tail extracts throughout amphibian metamorphosis despite a progressive rise in TRα mRNA, which implies strong post-transcriptional regulation of TRα protein level (*8*). The rate of TRα protein synthesis is suggested to be regulated at the translational level by the upstream open reading frame (uORF) in the 5′-untranslated region (5′-UTR) preceding the translation start codon, which is conserved in mammalian, chicken, *X. laevis* and bony fish TRα mRNAs. In support of this suggestion, the uORF is known to be an efficient inhibitor of translational initiation (*10*). In mammals, there is little or no correlation between TRα mRNA levels and nuclear T3-binding activities in various tissues and cell lines, although TRβ mRNA levels correlate reasonably well with measurements of nuclear T3-binding sites, raising the possibility of a lower translational efficiency of TRα mRNA (*11*). The regulatory mechanisms governing TRα protein level remain largely unknown.

Many capped, polyadenylated eukaryotic mRNAs contain *cis*-acting sequences in their UTRs that control interactions between the mRNA and the translational apparatus. Transcript-specific translational regulation usually requires elements within the RNA, such as secondary structures, positioning of the AUG within the Kozak sequence, uORFs, upstream initiation codons, internal ribosome entry sites and motifs that *trans*-acting factors interact with (*12*–*14*). The presence of uORFs can disrupt the efficient translation of the cistron located downstream because the ribosome

translates the uORF and then dissociates or stalls at the stop codon (*15*). In addition, *cis*-acting elements have been shown to interact with *trans*-acting factors required for accurate translation in many mRNAs. For example, the iron-regulatory proteins repress their translation by binding the iron-responsive elements located in the 5′- and 3′-UTRs (*16*).

In this study, we describe our analysis of the role of the 5′-UTR of TRα mRNA in the translation of TRα *in vivo* and *in vitro*. We found that the TRα 5′-UTR acts as a *cis*-regulatory region that significantly represses downstream translation in *X. laevis* tadpole tail cells and frog and human cultured cells. In addition, our analysis using successive deletions of the TRα 5′-UTR revealed five sequence elements that repress downstream translation. We characterized two of these elements using expression constructs with point mutations and demonstrated that one, a GC-rich element, requires stringent sequence specificity to achieve translational repression, but the other, a uORF, requires only moderate sequence specificity.

Materials and Methods

Animals

Anurans (*Xenopus laevis*, *Hyla japonica*, *Rana catesbeiana* and *Bombina orientalis*) and urodeles (*Ambystoma mexicanum*) were generous gifts of the subdivision of Breeding and Maintenance of Frogs, Institute for Amphibian Biology, Hiroshima University. *Xenopus tropicalis* was provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan. Caecilians (*Typhlonectes compressicauda* and *Ichthyophis kohtaoensis*) were obtained from local breeders. *Xenopus laevis* tadpoles were reared in dechlorinated tap water (24°C) and fed a mixture of boiled and mashed spinach and Sera Micron (Sera GmbH). Nieuwkoop and Faber (NF)-stage 51 or 52 tadpoles were treated for >2 weeks before DNA microinjection in 1 mM methimazole (Sigma), which inhibits iodination of thyroglobulin in the thyroid gland. All surgical procedures were performed after animals were anaesthetized with 0.02% MS-222 (Sigma).

Polymerase chain reaction cloning of the 5′-ends of TRα mRNAs

DNA fragments from the TRα 5′-UTRs were cloned by 5′-rapid amplification of cDNA ends (5′-RACE) (*17*). Reverse transcription was performed with PowerScript (Clontech) using 1 μg of total RNA from *H. japonica*, *R. catesbeiana*, *B. orientalis*, *A. mexicanum*, *T. compressicauda* or *I. kohtaoensis*. Polymerase chain reaction (PCR) products were inserted into the pGEM-T-easy vector (Promega). Individual clones were isolated and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3130*xl* Genetic Analyzer (Applied Biosystems). DNA fragments were amplified using primers corresponding to the most upstream sequence of the 5′-UTR and the sequence of the TRα coding region to confirm the result of 5′-RACE.

Expression constructs

The position of a nucleotide in the 5′-UTR is denoted as the distance in nucleotides from the first nucleotide A of the initiation codon in the open reading frame (ORF). The TRα 5′-UTR from −161 to −1 was inserted 0 and 35 bp upstream of the firefly luciferase (Fluc) initiation codon in the PGV-C vector (Tokyo Ink Co.) to construct (−161/−1)Fluc and (−161/−1)-Fluc, respectively. DNA fragments from −161 to −1 and from −813 to −653 of the TRβ-A 5′-UTR were introduced 36 bp upstream of the Fluc initiation codon in the PGV-C vector to construct TRβ(−161/−1)-Fluc and TRβ(−813/−653)-Fluc, respectively (*18*). Point-mutated Element V and DNA fragments made by deletions from the 5′-end of the TRα 5′-UTR were placed directly in front of the Fluc gene. DNA fragments made by deletions from the 3′-end of the TRα 5′-UTR were inserted 29 bp upstream of the Fluc gene. Each element and point-mutated Element IV were placed 35 bp upstream of the Fluc gene. DNA fragments made by deletions from the 3′-end of −56/−1 or from the 5′-end of −161/−82 were introduced 30 bp upstream of the Fluc gene. The TRα 5′-UTR −161/−1 was inserted 10 bp upstream of the *Renilla* luciferase (Rluc) gene of pRL-CMV (Promega) to construct (−161/−1)-Rluc. Each element was placed 16 bp upstream of the Rluc gene. A sequence similar to the TH response element was eliminated from the CMV promoter of pEGFP-N2 (CLONTECH) and pCMV-Script EX (Stratagene) by PCR using the primers 5′dTRE and 3′dTRE with TaKaRa LA Taq Hot Start Version (Takara). The conditions for amplification were as follows: pre-denaturation (94°C for 60 s), followed by amplification (94°C for 30 s, 50°C for 30 s and 72°C for 5 min) × 30 cycles and a final extension at 72°C for 10 min. The PCR product was blunted, ligated and used for transformation. The constructed vectors were named pEGFP-N2 (dTRE) and pCMV-Script EX (dTRE). The cDNAs encoding the full-length *X. laevis* TRα containing 6, 161 or 528 bp of the 5′-UTR, RXRα (a gift from Dr Y.-B. Shi) (*19*) and the dominant negative TR-α (DNTRα) were cloned into the expression vector pCMV-Script EX and pCMV-Script EX (dTRE). The primer sequences are listed in Supplementary Table S1.

Transfection and luciferase reporter assays

A6 and XLT-15-9 cells were maintained at 20°C as described previously (*20*). A6 and XLT-15-9 cells plated in 6-cm dishes were transfected with 1 μg of the TRα or luciferase expression constructs using FuGENE6 transfection reagent (Roche) according to the manufacturer's instructions and lysed 5 or 6 days later to prepare RNA for the real-time reverse transcription–PCR (RT–PCR) analysis. In addition, total cellular lysates were used for western blot analysis and the luciferase reporter assay. The cultured cells in 24-well tissue culture plates were transfected with 100 ng of PGV-C vector containing the TR 5′-UTR and 10 ng of pRL-CMV as an internal control and lysed 5 days after transfection. The activities in the lysates of transfected cells were determined for Fluc and Rluc. Luciferase activities were measured using the Dual-Luciferase Assay System (Promega) and a TD 20/20 Luminometer (Promega). HEK293 cells were cultured in Minimum Essential Medium Eagle (Sigma) supplemented with 10% foetal calf serum (FCS), non-essential amino acids and penicillin/streptomycin in a humidified atmosphere containing 5% CO_2. HEK293 cells in 24-well tissue culture plates were transfected with 100 ng of pRL-CMV containing the TRα 5′-UTR and 10 ng of pGL3-Control Vector (Promega) as an internal control using FuGENE6 transfection reagent and lysed 2 days after transfection to determine the luciferase activities.

Preparation of a polyclonal antibody recognising TRα protein by western blotting analyses

The cDNA corresponding to the entire coding region of *X. laevis* TRαA (*18*) was amplified by PCR and cloned into pCold I (Takara) as an EcoRI–BamHI fragment. The resulting pCold I-TRα was transformed into *Escherichia coli* BL21 cells. TRα protein with an N-terminal six-residue histidine tag was expressed as recommended by the manufacturer, purified by nickel column HisTrap FF (GE Healthcare) and used to prepare polyclonal antibodies in rabbits. The transfected cells were solubilized in buffer (60 mM Tris–HCl, pH 7.4, 2% SDS, 10% glycerol, 1 mM DTT and 1 mM phenylmethylsulphonyl fluoride) and sonicated. Protein content was determined by the BCA protein assay (PIERCE), and SDS gel electrophoresis was performed using a 10% polyacrylamide gel. Gels were blotted onto Immobilon-P polyvinylidene difluoride membrane (Millipore). The membranes were incubated with rabbit anti-TRα and anti-actin (Sigma-Aldrich) antibodies.

RT–PCR

Total RNA was isolated from fresh A6 cells or XLT-15-9 cells using the SV Total RNA Isolation System kit (Promega), which includes a DNase I treatment step. The RNA was quantified by measuring absorbance at 260 nm, and the *A*260/*A*280 ratio was confirmed to be between 1.8 and 2.1. The total RNA was resolved by electrophoresis through a denaturing formaldehyde agarose gel to visualize the ratio of 28S to 18S ribosomal RNAs. Samples of 1 μg of total RNA were denatured at 65°C for 5 min and reverse transcribed with random 12-mer oligos and oligo-dT primers using the ReverTra Ace qPCR RT Kit (Toyobo) at 37°C for 15 min followed by an inactivation step at 98°C for 5 min. Diluted products (2 μl) were subjected to quantitative RT–PCR using a SYBR Premix Ex Taq

kit (Takara) in 20 μl of reaction solution. The quantitative RT–PCR was performed using a Thermal Cycler Dice Real-Time System (Takara) according to the manufacturer's protocol. The reaction conditions were as follows: pre-denaturation (95°C for 30 s) followed by amplification (95°C for 5 s, 60°C for 10 s and 72°C for 30 s) × 45 cycles. The results were analysed using a Thermal Cycler Dice Real-Time System Ver. 4.00 (Takara). The primer sequences used for the amplifications are reported in Supplementary Table S1. A control reaction, without reverse transcriptase enzyme, was performed in parallel to exclude the effect by the contamination of transfected DNA.

Microinjection of DNA into tadpole tails

Microinjection of DNA and electroporation were performed as described previously (*21*, *22*). DNA-injected tadpoles were maintained in 1 mM methimazole (Sigma) for 1 week with food. Subsequently, they were treated with 1 mM methimazole without food in the presence of 1 nM thyroxine (Sigma). The water was changed for every 2 or 3 days.

Quantification of green fluorescent protein (GFP) fluorescence intensity in tadpole tails

Tadpoles were anaesthetized, and the GFP expression pattern was photographed under a fluorescent dissecting microscope equipped with a colour CCD camera DP70 (Olympus). The fluorescence signal of each photograph over the threshold value was extracted and quantified as the sum of the brightness of extracted pixels.

In vitro *translation*

The luciferase-coding region, along with either the TRα 5′-UTR −161/−1 or one of the five elements, was amplified by PCR using primers that contain the T3 promoter or poly-T. The corresponding mRNA was synthesized with a T3 *in vitro* transcription kit (mMESSAGE mMACHINE; Ambion) and resolved by electrophoresis with a denaturing formaldehyde agarose gel to determine the integrity of the mRNA. *In vitro* translation was carried out using nuclease-treated wheat germ extract (Promega) according to the manufacturer's protocol.

Statistical analysis

Data are presented as means ± SE. The significance of differences between groups was evaluated by one-way ANOVA or Kruskal–Wallis followed by Dunnett's or Tukey–Kramer's test.

Results

The TRα 5′-UTR contains evolutionarily conserved regions

The 5′-UTR of *X. laevis* TRα mRNA has a 4 amino acid ORF, which adjoins the translational start site of the main ORF in almost all TRα mRNAs (*8*). The uORF is believed to decrease the translational efficiency of the downstream ORF. To delineate the conserved regions in the TRα 5′-UTR among amphibians, we performed 5′-RACE using crude RNAs isolated from several amphibians, including anurans (*H. japonica*, *R. catesbeiana* and *B. orientalis*), urodeles (*A. mexicanum*) and caecilians (*T. compressicauda* and *I. kohtaoensis*). Comparison of the TRα 5′-UTR nucleotide sequences revealed ∼160-, 130- and 100-nt regions that are conserved among *Xenopus* frogs, anurans and amphibians, respectively. The uORF immediately upstream of the start site encodes the same 4 amino acid sequence, Met–Glu–Leu–Arg, in all amphibians (Fig. 1). However, the 3′-UTRs of TRα genes are conserved for only 30 nt downstream of the termination codon among *X. laevis*, *X. tropicalis* and *R. catesbeiana*.

The TRα 5′-UTR possesses translational repressive activity

Xenopus laevis TRα mRNA contains 528 nt of the 5′-UTR. To investigate the regulatory effect of the 5′-UTR on TRα protein level, TRα expression constructs with −6/−1, −161/−1 and −528/−1 of the TRα 5′-UTR, denoted as (−6/−1)TRα, (−161/−1)TRα and (−528/−1)TRα, respectively (Fig. 2A), were transiently transfected into A6 cells, the *X. laevis* adult kidney cell line. Western blot analysis was carried out 6 days after transfection to assess the levels of TRα protein in cell extracts (Fig. 2B). TRα protein expression was scarcely detected in the cultured cells that had been transfected with (−161/−1)TRα or (−528/−1)TRα. When TRα protein levels were normalized to actin levels, TRα protein level was decreased to 1/25 and 1/64 in the cells transfected with (−161/−1)TRα and (−528/−1)TRα, respectively, compared with levels in (−6/−1)TRα-transfected cells (Fig. 2C). In contrast, 161 nt of the 5′-UTR had little effect on the mRNA levels, suggesting that this stretch of the 5′-UTR changed neither the TRα transcription rate nor the mRNA stability (Fig. 2D). When TRα protein levels were normalized to TRα mRNA levels, the transfection with (−161/−1)TRα and (−528/−1)TRα reduced TRα protein levels to 1/20 of the level in cells transfected with (−6/−1)TRα (Fig. 2E), which shows that 528 and 161 nt of the 5′-UTR have the same translational repressive activity. Similar repression of TRα protein level by the 5′-UTR was observed in a transfection assay using a tadpole tail myoblast cell line of *X. laevis*, XLT-15-9 (Fig. 2F–H), demonstrating that the translational repression by the TRα 5′-UTR is not cell type specific. Thereafter, we focused on the analysis using 161 nt of the TRα 5′-UTR.

To examine whether the 161-nt fragment of the TRα 5′-UTR represses translation of a heterologous ORF, the TRα 5′-UTR was inserted directly in front of a firefly luciferase (Fluc) reporter gene in PGV-C to obtain the expression construct (−161/−1)Fluc (Fig. 3A), which was introduced into the cultured cells. The luciferase activity was reduced to 1/15 and 1/31 in (−161/−1)Fluc-transfected A6 and XLT-15-9 cells (Fig. 3B and C), respectively, compared to vector-transfected cells, while the luciferase mRNA levels were not conspicuously changed (Fig. 3D). Furthermore, the same experiment was conducted instead of Fluc reporters using *Renilla* luciferase (Rluc) reporter constructs, which were transfected into A6 cells, XLT-15-9 and a human cell line HEK293 (Fig. 3E–H). Similar results were obtained using TRα, Fluc and Rluc as the main ORF. These results corroborate the idea that the TRα 5′-UTR suppresses translation in frog and human cells when it is placed upstream of an ORF.

Strong translational regulation is implicated in TRα protein expression but not in TRβ protein level because the TRα protein level remains relatively constant in spite of a gradual rise in TRα mRNA during development, whereas both TRβ mRNA and protein levels rise along with the increase in endogenous TH (*8*). To test whether the TRβ 5′-UTR has translational

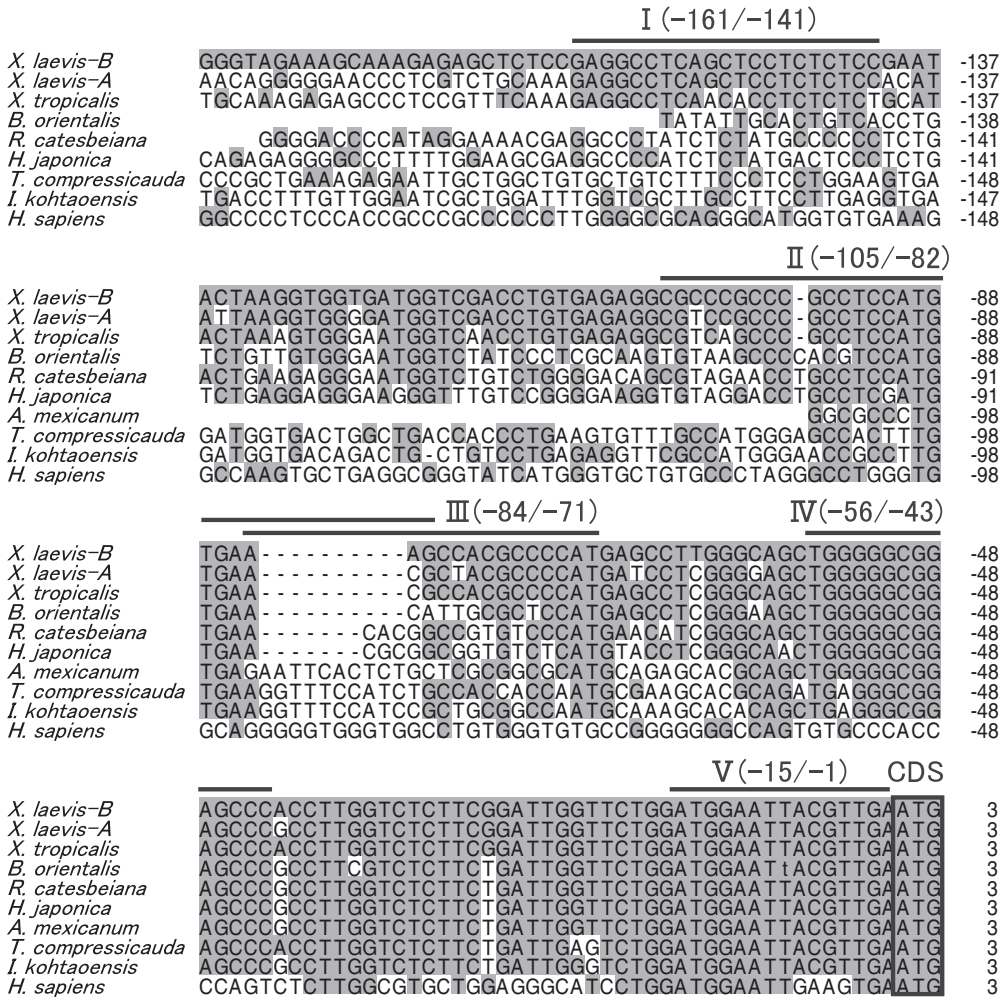

Fig. 1 Sequence comparison of the 5′-UTRs of TRα genes. Nucleotide sequences of the 5′-UTRs of *X. laevis* TRα-A and -B genes are aligned with those of anurans (*X. tropicalis*, *B. orientalis*, *R. catesbeiana* and *H. japonica*), urodeles (*Ambystoma mexicanum*) and caecilians (*Typhlonectes compressicauda* and *Ichthyophis kohtaoensis*), as well as *Homo sapiens* TRα. In *X. laevis*, there are two TRα (A and B) genes due to the pseudo-tetraploidy. Shaded nucleotide sequences indicate identity with *X. laevis* TRα-B sequence. CDS means the coding sequence of TRα. The horizontal lines above the sequences indicate the elements from I to V that are described in 'Results' section.

repressive activity, we made Fluc constructs containing TRβ-A 5′-UTR exons a and h, called TRβ (−813/−653)-Fluc and TRβ(−161/−1)-Fluc, respectively (*18*). Transfection of these constructs into A6 and XLT-15-9 cells yielded no translational regulation by the TRβ 5′-UTR (Fig. 4).

The TRα 5′-UTR reduces the TH-sensitivity of TRα-injected tail myomeres

To examine whether the TRα 5′-UTR has a function in tadpole tail muscle cells, the GFP marker and TRα expression construct with or without the 5′-UTR were injected into the tails of tadpoles that had been treated with an inhibitor of TH synthesis, methimazole, for >2 weeks beginning at NF-stages 51–52. This treatment decreases the endogenous levels of TH so that subsequent responses to TH can be specifically attributed to exogenous TH. A DNTRα expression construct was injected, along with a GFP marker gene, into a different myomere as an internal control (Fig. 5A). Normalizing fluorescence using this internal control permits excluding factors other than TH-induced cell death that can cause reductions in fluorescence, *e.g.* diminishing UV light and nutritional status because overexpression of DNTRα fully protects the tail muscle from TH-induced cell death until tail shortening during the peak of metamorphosis (*22*). Treatment of tadpoles with 1 nM thyroxine (TH) was initiated 1 week after the DNA injection. GFP fluorescence was observed after 0, 3, 6 and 10 days of TH treatment.

The GFP fluorescence in the myomeres that had been injected with the TRα expression construct containing the long 5′-UTR (−161/−1) was reduced to <40% of the original intensity after 10 days of TH treatment (Fig. 5B and C), which means that >60% of the GFP-positive muscle cells died and disappeared during the 10-day treatment (*22*). A similar level of GFP fluorescence was attained only after 6 days of treatment in the myomeres that had been injected with the TRα expression construct including the short 5′-UTR (−6/−1). It has been reported that

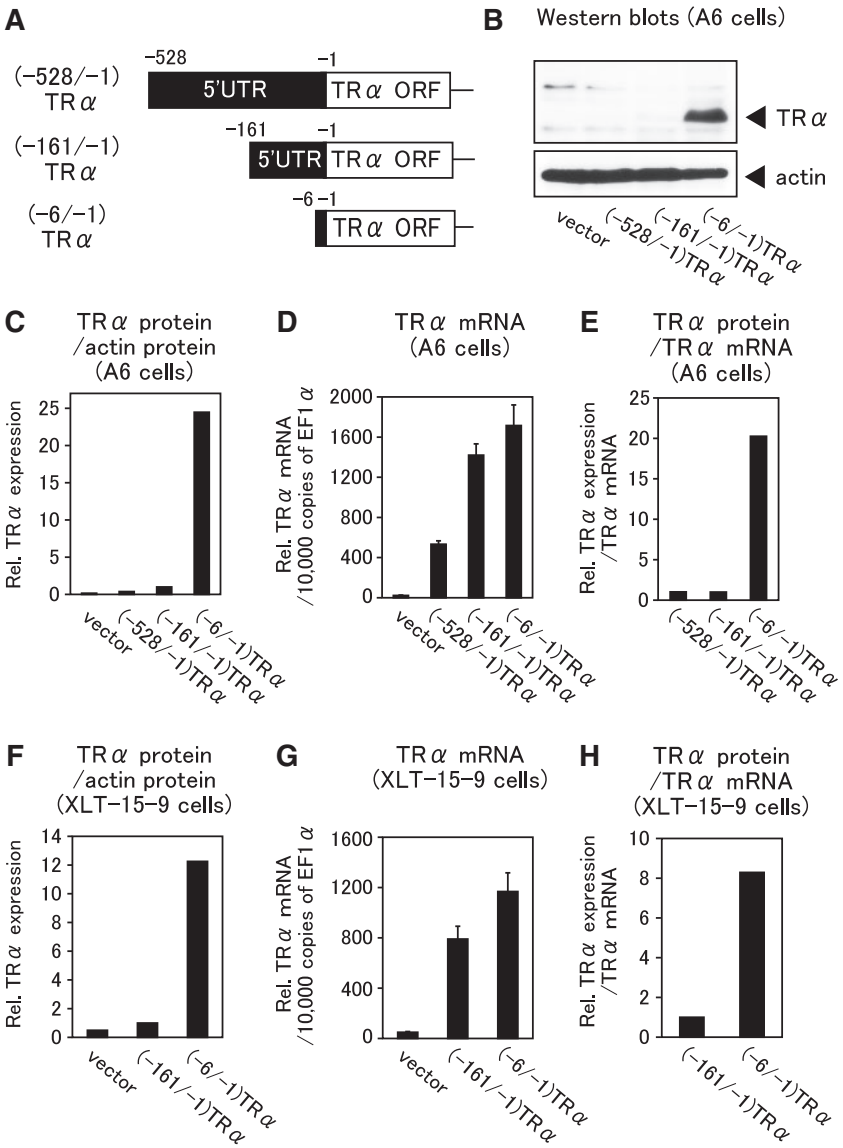

Fig. 2 Translational repressive activity of the TRα 5′-UTR. (A) A schematic representation of the analysed constructs. Black and white boxes represent the TRα 5′-UTRs and ORF sequences, respectively. (B–E) A TRα expression construct with 528, 161 or 6 nt of the 5′-UTR was transiently transfected into A6 cells. (B) Western blot analysis of 20 μg of the total cellular protein isolated from A6 cells transfected with TRα expression constructs probed with anti-TRα and anti-actin antibodies. The positions of proteins recognized by the specific antibodies are indicated (arrow heads). (C) Quantification of western blots shown in (B). TRα protein was normalized to the actin level (a loading control). The signal for the lysate of (−161/−1) TRα-transfected cells was set to 1. (D) Quantification of TRα mRNA from cells shown in (B) by RT–PCR. Levels of TRα mRNA are indicated as copy numbers relative to 10,000 copies of EF1α mRNA. Data are shown as means ± SE ($n = 3$). (E) The TRα protein was normalized to the TRα mRNA level, and the signal for the lysate of (−161/−1) TRα-transfected cells was set to 1. (F–H) The translational inhibition of TRα mRNA by the 5′-UTR is not cell type specific. A TRα expression construct with 6 or 161 nt of the 5′-UTR was transiently transfected into XLT-15-9 cells. (F) Quantification of TRα protein signals from XLT-15-9 cells transfected with TRα expression constructs by western blot analysis. TRα protein was normalized to the actin level. The signal for the lysate of (−161/−1) TRα-transfected cells was set to 1. (G) Quantification of TRα mRNA from XLT-15-9 cells shown in (F) by RT–PCR. Levels of TRα mRNA are indicated as copy numbers relative to 10,000 copies of EF1α mRNA. Data are shown as means ± SE ($n = 3$). (H) The TRα protein was normalized to the TRα mRNA level, and the signal for the lysate of (−161/−1) TRα-transfected cells was set to 1.

high levels of TRα expression enhance metamorphic rates (*9*), so this result indicates that 161 nt of the TRα 5′-UTR suppressed TRα expression in tadpole tail myomeres.

Five elements with translational repressive activity identified by an analysis using deletion mutants of the TRα 5′-UTR

To localize the regions with translational repressive activity within the 161-nt sequence from the TRα 5′-UTR, we constructed a series of reporter genes containing sequential deletion mutants from the 5′-end and/or the 3′-end of the TRα 5′-UTR upstream of the Fluc ORF, transfected them into XLT-15-9 and measured luciferase activities (Fig. 6). Transfection using the luciferase constructs with deletions of the first 111 and 155 nt of the TRα 5′-UTR, (−50/−1)Fluc and (−6/−1)Fluc, yielded 9- and 16-fold higher Fluc activities, respectively, compared to a construct with 161 nt of the 5′-UTR,

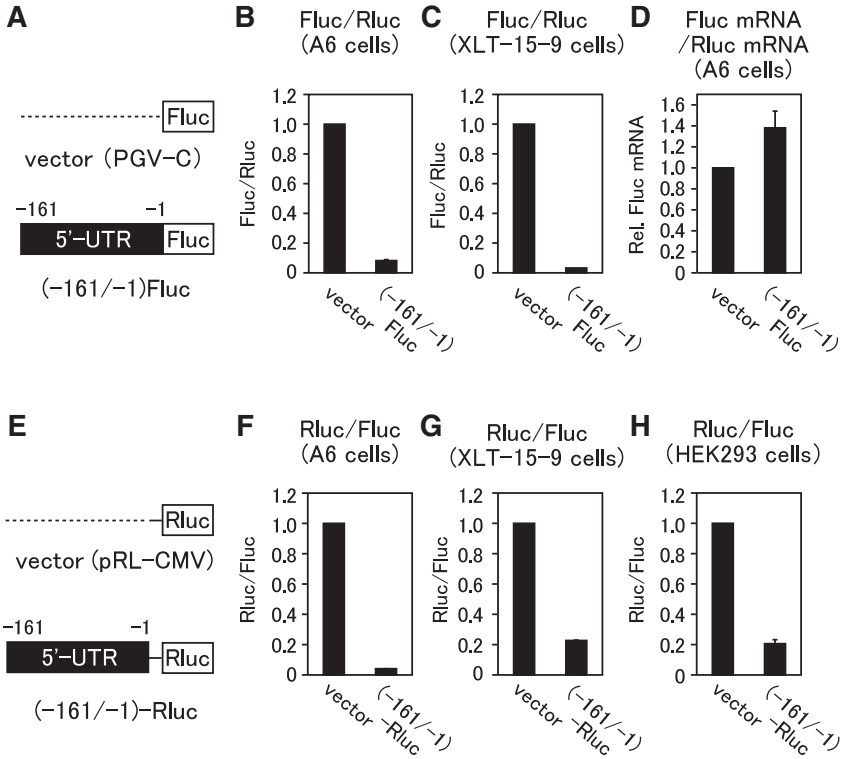

Fig. 3 Translational repressive activity of the TRα 5′-UTR for heterologous ORFs. (A) A schematic representation of the analysed constructs used in (B–D). The TRα 5′-UTR was inserted directly in front of the firefly luciferase (Fluc) ORF in PGV-C to construct (−161/−1)Fluc. (B and C) (−161/−1)Fluc or PGV-C was transfected into A6 (B) and XLT-15-9 cells (C) together with the *Renilla* luciferase (Rluc) reporter gene pRL-CMV as a transfection control. The Fluc activity was normalized to the Rluc activity, and the signal for the lysate of PGV-C-transfected cells was set to 1. (D) Quantification of Fluc mRNA from A6 cells shown in (B) by RT–PCR. Fluc mRNA was normalized to Rluc mRNA level, and the signal for RNA of PGV-C-transfected cells was set to 1. (E) A schematic representation of the analysed constructs used in (F–H). The TRα 5′-UTR was inserted upstream of an Rluc ORF to construct (−161/−1)-Rluc. (F–H) (−161/−1)-Rluc or pRL-CMV was transfected into A6 (F), XLT-15-9 (G) and HEK293 cells (H) together with PGV-C as a transfection control. The Rluc activity was normalized to the Fluc activity, and the signal for the lysate of pRL-CMV-transfected cells was set to 1. Data are shown as means ± SE ($n = 3$).

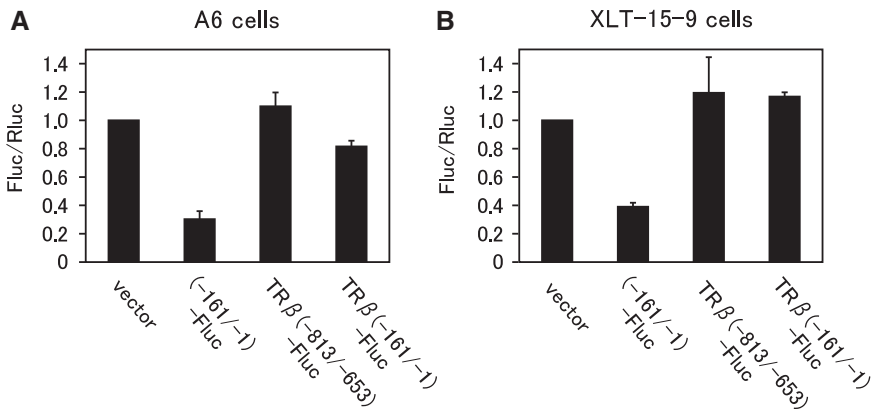

Fig. 4 No translational repressive activity in the TRβ 5′-UTR. DNA fragments from the TRβ 5′-UTR were inserted upstream of the Fluc ORF to construct TRβ(-161/-1)-Fluc and TRβ(-813/-653)-Fluc, which were transfected into A6 (A) and XLT-15-9 cells (B), together with pRL-CMV as a transfection control. The signal for the lysate of PGV-C-transfected cells was set to 1. Data are shown as means ±SE ($n = 3$).

(−161/−1)Fluc (Fig. 6A), implying the existence of two translational inhibitory RNA elements in the vicinities of −56/−50 and −20/−6. The latter corresponds to the short uORF of the TRα 5′-UTR. Deletion analysis starting from the 3′-end of the TRα 5′-UTR revealed two possible translational repressive regions in the vicinities of −94/−82 and −161/−141 of the 5′-UTR because the transfection using a luciferase construct with a DNA fragment from −161 to −94, (−161/−94)-Fluc, induced 5-fold greater luciferase activity compared to (−161/−1)-Fluc, and transfection with the deletion mutant (−161/−141)-Fluc still produced a lower level of luciferase activity compared to the PGV-C vector (Fig. 6B). In total, four regions

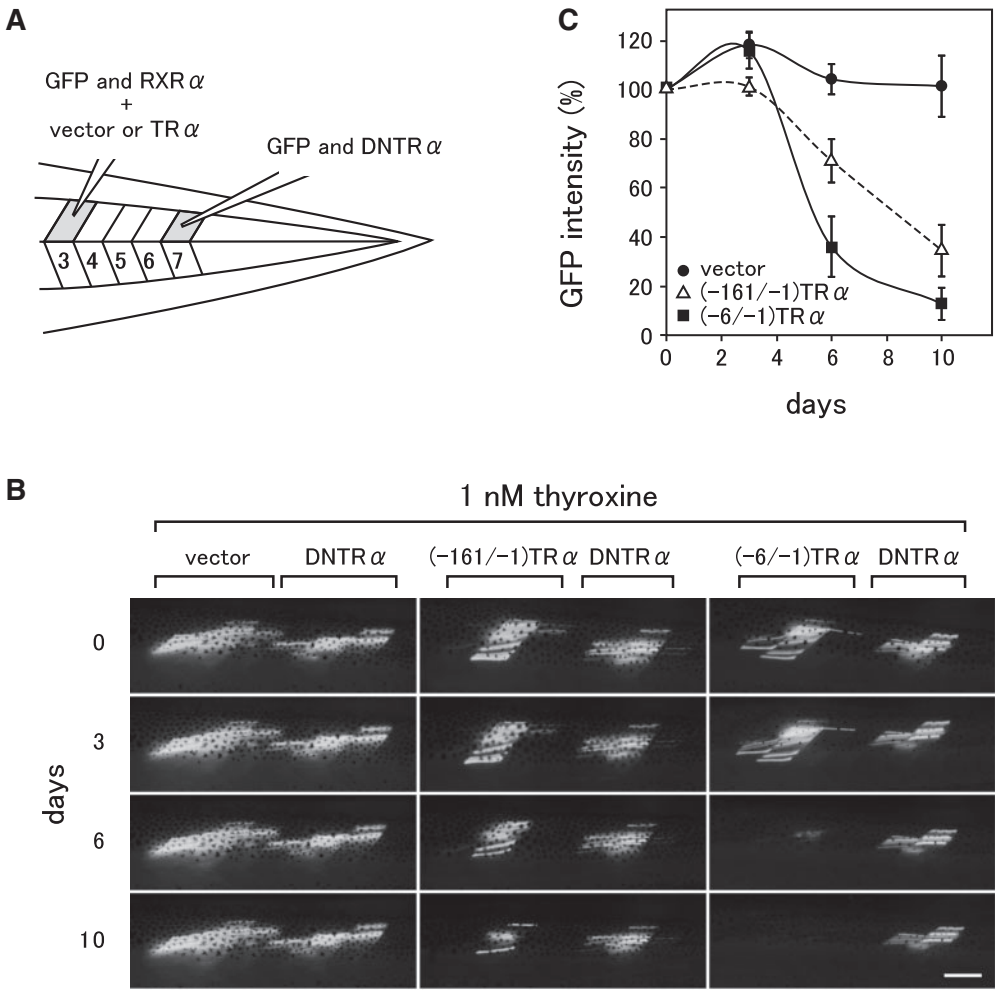

Fig. 5 TH-sensitivity reduction by the TRα 5′-UTR in the TRα-injected tail myomeres. (A) The scheme of the injection protocol. The numbers of myomeres are indicated. (B and C) Empty vector, (−161/−1)TRα or (−6/−1)TRα expression construct was injected with an RXRα expression construct and a GFP reporter gene (25 ng each) into the third tail myomere of NF-stage 53–54 methimazole-treated tadpoles, and GFP and DNTRα genes were injected into the seventh myomere as an internal marker. (B) Representative photographs of GFP fluorescence of tadpole tails injected with a vector or TRα expression construct. Treatment with 1 nM thyroxine induced the reduction of GFP fluorescence in TRα-injected myomeres. Scale bar: 1 mm. (C) GFP expression in myomeres injected with a vector (closed circle), (−161/−1)TRα (open triangle) or (−6/−1)TRα (closed square) in the presence of 1 nM thyroxine. The ratio of the fluorescence intensity of the third myomere to that of the DNTRα-injected myomere was calculated. The value at the starting point was set to 100%. Data are expressed as means ± SE ($n = 6$–12).

were identified as potential *cis*-acting translational repressors by this deletion assay.

More deletion mutants were constructed to delineate the translational repressive elements precisely. The translational repressive elements were localized to two regions, −56/−43 and −105/−82, based on an analysis using luciferase constructs with deletion mutants from the 3′-end of the DNA fragment −56/−1 (Fig. 6C) and from the 5′-end of the DNA fragment −161/−82 (Fig. 6D), respectively.

The sequence from −56 to −43 of the TRα 5′-UTR contains the Sp1-binding site 5′-(G/T)GGGCGG (G/A)(G/A)(C/T)-3′ (*23*, *24*), in which the complementary nucleotide sequence is also found as a fifth repressor candidate in the −84/−71 region of the TRα 5′-UTR. Each of these five elements, −161/−141 (I), −105/−82 (II), −84/−71 (III), −56/−43 (IV) and −15/−1 (V), was inserted upstream of the luciferase ORF to examine whether it has translational repressive activity in A6, XLT-15-9 and HEK293 cells. A transient expression assay using these expression constructs demonstrated that the presence of each element upstream of a luciferase ORF repressed luciferase activity in those cultured cells, although Elements I and II showed weak activity in the human and frog cells, respectively (Fig. 7).

The experiment using the sequential deletion mutants from the 5′- and 3′-ends of the TRα 5′-UTR could identify the downstream Elements IV and V and the upstream Elements I and II, respectively, but could not identify Element III. Since the combinations of these two elements showed additive repressive activity, our experimental system could not discriminate the stronger activity of three elements (I–III or III–V) from activities by these combinations of two elements, I–II or IV–V.

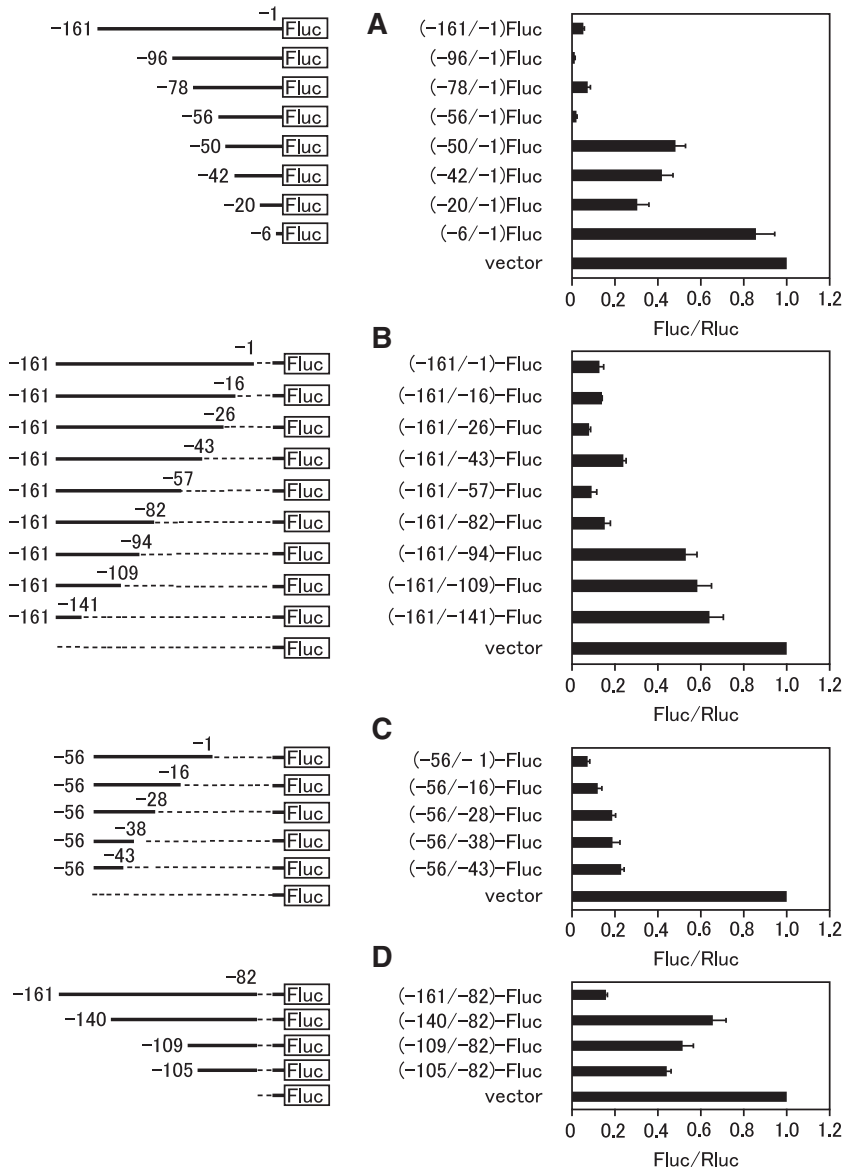

Fig. 6 The identification of the translational regulatory elements in the TRα 5′-UTR. XLT-15-9 cells were transfected with pRL-CMV as a transfection control and the Fluc expression constructs containing sequential deletion mutants from the 5′-end (A) or 3′-end (B) of 161 nt of the TRα 5′-UTR, from the 3′-end of a DNA fragment (−56/−1) (C) or from 5′-end of a fragment (−161/−82) (D). The Fluc activity was normalized to the Rluc activity. The signal for the lysate of PGV-C-transfected cells was set to 1. The DNA fragments of TRα 5′-UTR that are included in expression constructs are shown on the left side. Data are shown as means ± SE ($n = 3$).

Characterization of Element V by point mutational analysis

Element V is a uORF with a nucleotide sequence that is completely conserved among amphibians. We examined the effect of extensive point mutations on translational repressive activity in the transient expression assay using A6 cultured cells. The deletion of the initiation or termination codon in the uORF completely eliminated any reduction in luciferase activity. To test the coding sequence specificity of the uORF, we changed the nucleotide sequences and transfected the mutated constructs into the frog cells (Fig. 8). Translational repression by the uORF was not affected by the base substitutions, which included a 7 nt alteration that did not change the encoded amino acid sequence (synonymous mutations) and missense mutations that changed the second codon from glutamic acid to alanine, the third codon from leucine to serine and/or the fourth codon from arginine to cysteine. The luciferase activity was partially, although significantly, restored by alteration of 8 or 9 nt to change three amino acids. The translational repressive activity was weakened, when the nucleotides in the vicinity of the Element V initiation codon were changed to the more divergent Kozak sequence (Supplementary Fig. S1). These results indicate that under our working conditions, stringent sequence specificity is not required except at the start and stop codons for translational suppression by Element V, but some degree of specificity might be needed.

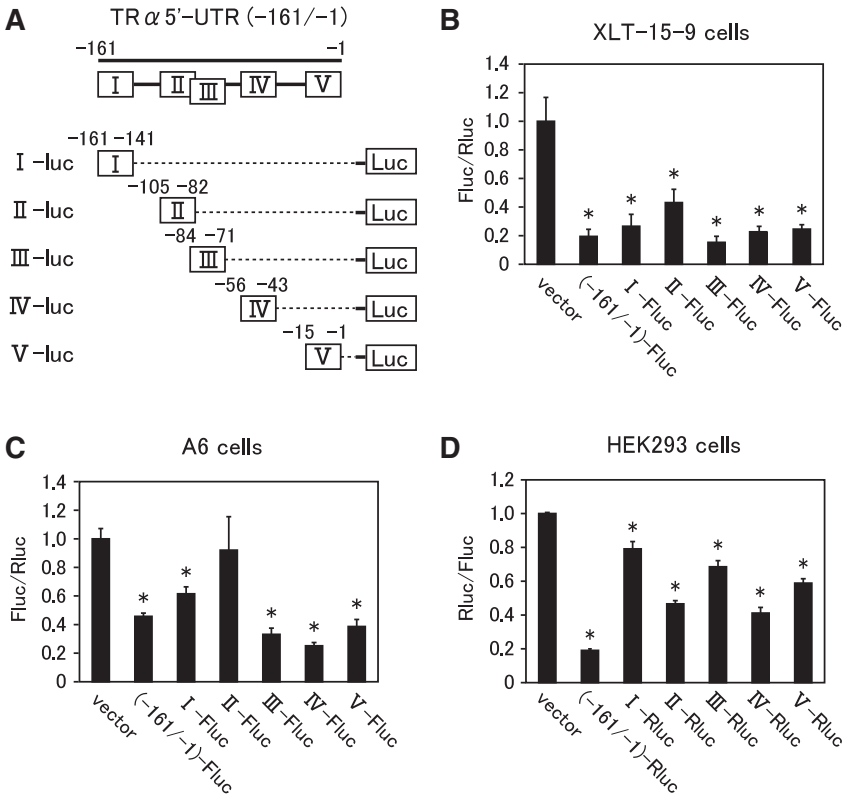

Fig. 7 Translational repressive activities of the regulatory elements in a transient expression assay. (A) A schematic representation of the position of five regulatory elements in the TRα 5′-UTR, I (−161/−141), II (−105/−82), III (−84/−71), IV (−56/−43) and V (−15/−1). (B–D) Each of five elements was inserted upstream of the Fluc (B and C) and Rluc ORFs (D) to confirm translational repressive activity in XLT-15-9 (B), A6 (C) and HEK293 cells (D). The Fluc activity was normalized to the Rluc activity, and the signal for the lysate of PGV-C-transfected cells was set to 1 (B and C). The Rluc activity was normalized to the Fluc activity, and the signal for the lysate of pRL-CMV-transfected cells was set to 1 (D). Data are shown as means ±SE ($n = 3$–6). Statistical significance by comparison with vector-transfected cells was assessed using one-way ANOVA (B) or Kruskal–Wallis (C and D) followed by Dunnett's test. $^*P < 0.01$.

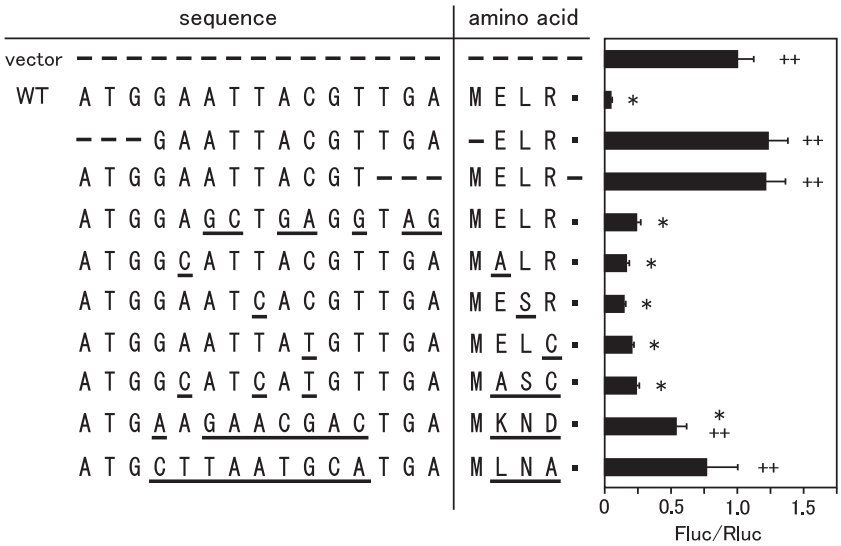

Fig. 8 Characterization of Element V by point mutational analysis. The nucleotide sequences of the wild type and mutants of Element V and the corresponding amino acid sequences are shown. Nucleotide and amino acid alterations are denoted by being underlined. The deleted initiation and termination codons are indicated by a dash in the nucleotide and the amino acid sequences. A6 cells were transfected with Fluc expression constructs containing the indicated mutations of Element V and pRL-CMV as a transfection control. The Fluc activity was normalized to the Rluc activity, and the signal for the lysate of PGV-C-transfected cells was set to 1. WT indicates the wild-type version of Element V. Data are shown as means ± SE ($n = 3$–9). Significantly different values are indicated by asterisks and double plus symbols ($P < 0.05$; Kruskal–Wallis followed by Tukey–Kramer's test) compared to the vector and wild-type-transfected cells, respectively.

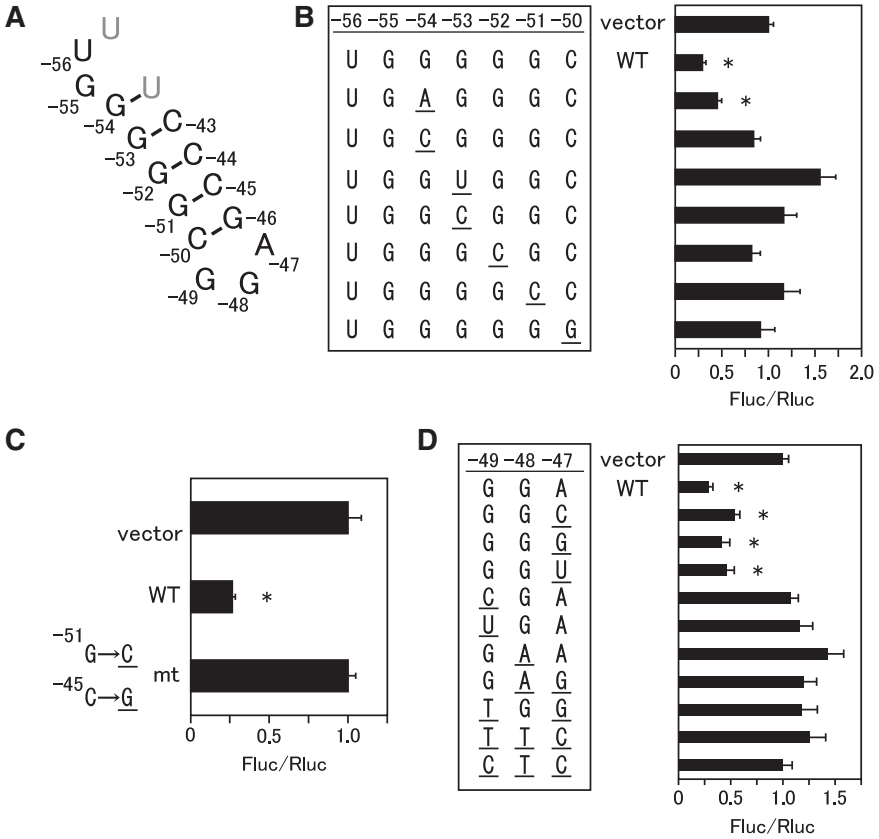

Fig. 9 Characterization of Element IV by point mutational analysis. (A) The secondary structure of Element IV predicted by the Centroid fold program. Grey nucleotides are derived from the PGV-C vector. (B–D) A6 cells were transfected with Fluc expression constructs containing the indicated mutations of Element IV and pRL-CMV as a transfection control. The Fluc activity was normalized to the Rluc activity, and the signal for the lysate of PGV-C-transfected cells was set to 1. The sequences of the wild-type and mutants of Element IV are shown, and the nucleotide alterations are denoted by being underlined. (B) The luciferase constructs with single point mutations in a double-helix stem formed by −53/−50 and −46/−43, which should destabilize hairpin–loop secondary structure of Element IV. (C) The luciferase construct (mt) with 2 nt exchanges, from G to C at position −51 and from C to G at position −45, which should have no effect on the hairpin–loop structure of Element IV. (D) Random mutations were introduced into the loop region (−49/−47) of the Element IV. WT indicates the wild-type version of Element IV. The Fluc activity was normalized to the Rluc activity, and the signal for the lysate of PGV-C-transfected cells was set to 1. Data are shown as means ± SE ($n = 6$–24). Statistical significance by comparison with vector-transfected cells was assessed using Kruskal–Wallis followed by Dunnett's test. *$P < 0.01$.

Characterization of Element IV by point mutational analysis

The nucleotide sequence of Element IV is conserved among amphibians except for a change from G to A at position −54 in caecilians. The Fluc reporter with the caecilian version of Element IV was constructed and transfected into A6 cells. The caecilian Element IV also showed translational repressive activity (Fig. 9B).

The Centroid fold program (http://www.ncrna.org/software/centroidfold/) predicted that the secondary structure of Element IV would have a hairpin–loop structure with a double-helix stem formed by −53/−50 and −46/43 (Fig. 9A). We made luciferase constructs with single point mutations in the stem to destabilize the secondary structure of the element (Fig. 9B). The single-base mutations in the −54/−50 region abolished translational repressive activity, suggesting that Element IV suppresses the translation of the downstream cistron in a manner that is dependent on the sequence and/or the RNA secondary structure. The translational suppression was completely relieved by a mutation with 2 nt exchanges, from G to C at position −51 and from C to G at position −45, which should not affect the hairpin–loop structure of the element (Fig. 9C). Random mutations were introduced into the loop region of Element IV at −49, −48 and −47 positions, which should have no effect on the secondary structure. All constructs with a mutation at position −49 or −48 had a similar level of luciferase activity to that of PGV-C vector, but the constructs with a base substitution at position −47 retained translational repressive activity (Fig. 9D). These data testify that translational repression by Element IV requires strict sequence specificity at a nucleotide level, except for the A at position −47, and that merely preserving the hairpin–loop structure is not sufficient.

Each of the five elements has translational repressive activity in a cell-free translation system

To confirm the *cis*-regulatory translational repressive activity of Elements I–V, an *in vitro* translation assay was performed using *in vitro*-transcribed luciferase

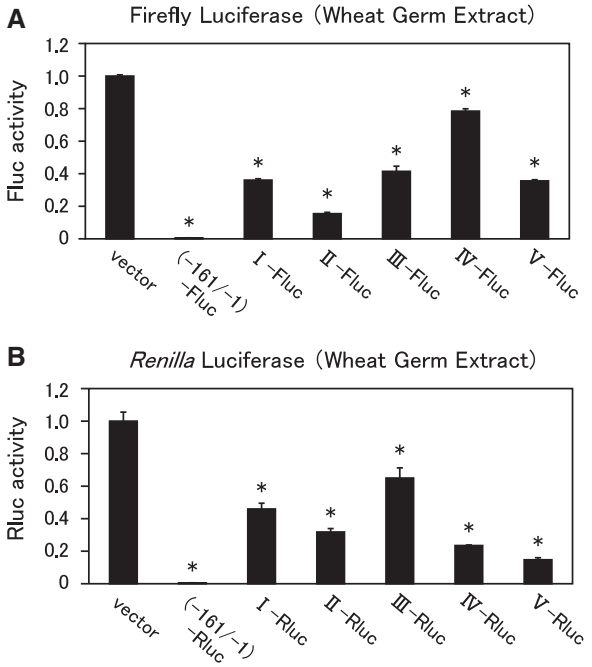

Fig. 10 Translational repressive activities of the regulatory elements in a cell-free translation system. The *in vitro* translation assay was performed using an equal amount of *in vitro*-transcribed Fluc mRNA (A) or Rluc mRNA (B). The indicated single element was inserted upstream of the Fluc (A) and Rluc ORFs (B). The mRNAs were translated in wheat germ extract, and the luciferase activities of the translated products were measured. (A) The signal for the translated product by a control Fluc mRNA synthesized using a vector as a template was set to 1. (B) The signal for the translated product by a control Rluc mRNA synthesized using a vector as a template was set to 1. Data are shown as means ± SE ($n = 3$). Statistical significance by comparison with the luciferase activity of translated product using vector-driven Fluc mRNA was assessed using Kruskal–Wallis followed by Dunnett's test. $^*P < 0.01$.

mRNAs. Moderately reduced luciferase activities were observed in reaction mixtures containing equal amounts of mRNAs with one of the five elements upstream of the Fluc or Rluc ORF, while the full 161-nt sequence of the TRα 5′-UTR showed potent repressive activity (Fig. 10A and B). These results demonstrate that each of the five elements in the TRα 5′-UTR is a *cis*-regulatory translational repressor not only in transient expression assays using cultured cells but also in *in vitro* translation systems.

Discussion

We demonstrated that a 161-nt section of the TRα 5′-UTR strongly represses the translation of the downstream ORF in both frog and human cell lines as well as in a cell-free translation system using wheat germ extract, which implies that the molecular mechanism of this translational repression is conserved from plants to human and is mediated by the basal translational machinery. The translational repressive activity of the TRα 5′-UTR is supported by the observation that overexpression of a TRα expression construct with its 5′-UTR made frog tail muscle cells less sensitive to TH relative to overexpression of a TRα construct without its 5′-UTR.

Several lines of evidence suggest that cells expressing more TRα are capable of responding to lower levels of TH and that the differential sensitivity of tissues to TH depends on TRα expression levels. In this paradigm, strong translational regulation by the TRα 5′-UTR may play a critical role in the ordering of important and orchestrated tissue remodelling that occurs as TH levels increase gradually during amphibian metamorphosis. A genome-wide study showed that the cellular abundance of proteins is predominantly controlled at the level of translation (*25*). It is possible that the translational down-regulation by the TRα 5′-UTR is compromised in the first tissues to transform, including the hind limbs, allowing them to express high levels of TRα protein early during metamorphosis, whereas the TRα 5′-UTR reduces the translation of its mRNA in tissues that transform later, such as the gills and tail. As hind limbs express several fold more TRα mRNA than tails, both transcriptional and translational regulations might control the TRα protein level in many tissues to co-ordinate the systematic transformation.

Our analysis using deletion mutants of the TRα 5′-UTR revealed five *cis*-acting translational repressive elements, including Element IV, in which the nucleotide sequence is conserved among amphibians except for one base change in caecilians. This change has no significant effect on the element's translational regulatory activity. The nucleotide sequence of Element IV (5′-TGGGGGCGGAGCCC-3′) contains a high-affinity Sp1-binding site, 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ (*23*, *24*). A nucleotide sequence complementary to the Sp1-binding site is also found in Element III (5′-AAGCCACGCCCCAT-3′) with 1 nt difference at the sixth position, although Element III is not conserved well in amphibians. These tentative Sp1-binding sites in Elements III and IV do not appear to be involved in the transcriptional activation mediated by Sp1 because the insertion of the TRα 5′-UTR upstream of ORF did not stimulate mRNA level. Our point mutational analysis showed that the regulatory activity of Element IV has a stringent sequence specificity similar to that of the Sp1-binding site (*23*, *26*). However, the TRα 5′-UTR did not significantly affect the TRα mRNA accumulation in the frog cultured cells, and transfection of *Xenopus* Sp1 had no effects on the translational regulation of the TRα 5′-UTR in cultured cells and an oocyte system (data not shown).

Recently, a variety of *cis*-acting elements for accurate translation have been found in many mRNAs. For example, guanine-rich nucleic acid sequences can fold into a non-canonical tetrahelical structure called a G-quadruplex. Many researchers have demonstrated that G-quadruplex-forming sequences within the 5′-UTRs of mammalian mRNAs can modulate translation efficiency both in cell-free systems and in mammalian cultured cells (*27–29*). It is noteworthy that the 5′-UTR of human TRα mRNA contains a G-quadruplex motif (*27*). Although the 5′-UTR of amphibian TRα mRNA represses downstream translation in vertebrate cultured cells, no G-quadruplex motifs were found in it. These findings imply that the 5′-UTRs of human and amphibian TRα mRNAs both

repress their translation although by different molecular mechanisms.

The uORFs reduce the protein level by 30–80% with a modest impact on mRNA levels. The uORF-derived protein is functional in several cases, but the mere presence of the uORF is sufficient to reduce expression of the downstream ORF in most cases (*30*). In some genes (*31*, *32*), particular missense mutations of codons in the uORF abolish its inhibitory activity, whereas modifications that retain the wild-type amino acid coding information preserve the translational suppressive activity by the uORF. However, the translational inhibitory activity of Element V (−15/−1) did not appear to depend on the amino acid sequence of the encoded peptide, even though it is conserved among amphibians. It is possible that our experimental system cannot perfectly reproduce the physiological translational regulation by Element V and that we overlooked a peptide-sequence dependency in the translational repressive activity of Element V.

All elements except Element V are GC-rich regions. The GC contents of Elements I, II, III, IV and V are 67, 76, 57, 86 and 33%, respectively. The GC richness of the 5′-UTR significantly influences the rate of translation of mRNAs along with uORF and internal ribosome entry sites (*15*). It is reported that the mRNAs of scarcely expressed eukaryotic proteins have higher GC content in the 5′-UTR than mRNAs of highly abundant proteins (*33*). However, the GC content is quite high, ~60%, for the 5′-UTRs in mammals (*34*). Although several mutants of Element IV had the same or higher GC content when compared with wild-type Element IV, they failed to repress translation. There is no correlation between GC content and repressive activity in Element IV.

Tadpole cells that express higher levels of TRα protein are suggested to respond earlier to the gradually rising levels of TH during the amphibian metamorphosis. We demonstrated translational repressive activity of the TRα 5′-UTR, which is supported by an *in vivo* functional assay in a tadpole tail. This translational regulatory activity of the TRα 5′-UTR may determine the timing of cellular differentiation. This study points out the possibility of contribution by the spatiotemporal regulation of the 5′-UTR to the systemically coordinated remodelling in amphibian metamorphosis.

Supplementary Data

Supplementary Data are available at JB Online.

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Conflict of interest
None declared.

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