Translational Control of the *Xenopus laevis* Connexin-41 5′-Untranslated Region by Three Upstream Open Reading Frames*

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The *Xenopus laevis* Connexin-41 (Cx41) mRNA contains three upstream open reading frames (uORFs) in the 5′-untranslated region (UTR). We analyzed the translation efficiency of constructs containing the Cx41 5′-UTR linked to the green fluorescent protein reporter after injection of transcripts into one-cell stage *Xenopus* embryos. The translational efficiency of the wild-type Cx41 5′-UTR was only 2% compared with that of the β-globin 5′-UTR. Mutation of each of the three uAUGs into AAG codons enhanced translation 82-, 9-, and 4-fold compared with the wild-type Cx41 5′-UTR. Based on these increased translation efficiencies, the percentages of ribosomes that recognized the uAUGs were calculated. Only 0.03% of the ribosomes that entered at the cap structure scanned the entire 5′-UTR and translated the main ORF. The results indicate that all uAUGs are recognized by the majority of the scanning ribosomes and that the three uAUGs strongly modulate translation efficiency in *Xenopus laevis* embryos. Based on these data, a model of ribosomal flow along the mRNA is postulated. We conclude that the three uORFs may play an important role in the regulation of Cx41 expression.

Gap junctions are cell-to-cell channels that enable adjacent cells to share ions, second messengers, and small metabolites up to a molecular mass of about 1500 Da. They play an important role in many cellular processes, e.g. in contraction of muscles, exocrine and endocrine secretion by the pancreas, and in transmission of neuronal signals in synapses. Besides a role of gap junctional communication in fully differentiated cells, gap junctional communication is also essential in early developmental signaling and pattern formation (1).

A complete gap junction channel spans the plasma membranes of two adjacent cells and is the result of the association of two half-channels or connexons. Each connexon is a multimeric assembly of six proteins, the connexins. A connexin (Cx) contains four transmembrane domains and cytoplasmic N- and C-tails (2). Connexins form a multigene family comprised of at least 17 members in mouse, with orthologues in other vertebrate species (3). It has been suggested that the different family members are created by gene duplications (4). Connexins are highly related, i.e. 50–80% identical at amino acid level, with the most conserved sequences located in the transmembrane domains (5). Nearly all connexins studied so far have a common gene structure. Each gene consists of two exons, with a 5′-untranslated region (UTR) in the first exon and an uninterrupted open reading frame (ORF) and the 3′-UTR in the second exon (6, 7). The only exception known is the Cx35/36 subgroup that contains two introns (8, 9).

Cell-cell communication via gap junctions is dynamically regulated at different levels as follows: transcription, translation, intracellular trafficking, oligomerization, docking, and gating (10). The best studied mechanism is phosphorylation, which has an effect on the latter four levels. Regulation of gap junctional communication by phosphorylation is kinase- and connexin-specific, which makes it a very complex phenomenon (10). The turnover rate of Cx proteins is rather high, 1–5 h (11, 12). This enables regulation at the transcriptional and translational levels.

Several lines of evidence indicate that at least some connexins can be regulated at the mRNA level. (i) Cx43 expression is transcriptionally as well as translationally regulated in PC12 cells by Wnt1 (13). (ii) Translational control of rat Cx32 and Cx43 expression was suggested after discovery of an internal ribosome entry site enabling translation of these mRNAs under conditions when cap binding is compromised (14). (iii) The stability of the rat Cx43 messenger is mediated by the binding of neuronal-specific proteins to its 3′-UTR (15). (iv) The existence of different mRNAs encoding mouse Cx26 (6) and Cx32 (17), different in the 5′-UTR only, suggests translational control.

The ribosomal scanning model can explain translational control of most cellular messengers (18). The 43 S preinitiation complex binds at the 5′-terminal cap structure and scans the 5′-UTR until the complex recognizes an AUG codon. Subsequently, the 60 S subunit joins and protein synthesis starts. Recognition of the AUG codon is dependent on the surrounding sequences and on the secondary structure of the mRNA (19). Usually, translation starts at the most 5′ AUG codon. When the 5′-UTR contains upstream AUGs (uAUGs), the main ORF can be translated by (i) ribosomes entering on an internal ribosome entry site, (ii) by termination at the upstream ORF and reinitiation at the next AUG, (iii) by leaky scanning, i.e. uAUGs are not recognized by all scanning ribosomal complexes, or (iv) by a combination of these processes (20).

Four *Xenopus laevis* connexins are known as follows: Cx30, Cx38, Cx41, and Cx43 (21–23). The latter three connexins are expressed in the ovary. Only maternal Cx38 mRNA remains during early embryonic development (until stage 11). The first embryonic messenger is Cx30 (from stage 11 onward), while

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF238222.

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¹ The abbreviations used are: Cx, Connexin; ORF, open reading frame; uORF, upstream open reading frame; UTR, untranslated region; GFP, green fluorescent protein; eIF4A, eukaryotic initiation factor 4A; XH3, *Xenopus* histone 3; RACE, rapid amplification of cDNA ends; WT, wild type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; PCR, polymerase chain reaction; nt, nucleotides.
Embryonic Cx41 and Cx43 transcripts appear at stage 25. The Cx41 transcript is expressed at a very low level (23, 24).

This paper describes the study on the translational control of Xenopus Cx41. The Cx41 5'-UTR contains three small open reading frames (uORFs) that might be involved in translational regulation of Cx41 expression. The effect of the three individual upstream AUG codons on translation efficiency was analyzed by mutating each of them into an AAG triplet. The presence of the three uAUGs strongly decreased the flow of ribosomes towards the initiation codon of the ORF of the Cx41 mRNA. The effects of the single mutations of the uAUGs on reporter gene expression enabled us to calculate the efficiency of uAUG recognition by the ribosome.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The Cx41 5'-UTR was amplified with a 5'/3'-RACE Kit (Roche Molecular Biochemicals). Ovaries of adult X. laevis were dissected out of the animals after a 20-min anesthesia on ice, and total RNA was isolated and used for the synthesis of cDNA. The Cx41-specific reverse primers (Table I) Cx41-OR2, Cx41-OR1, and Cx41-5R1 were designed based on the published sequence (23). The latter primer mutates the sequence around the ATG into an NcoI restriction site. The PCR cycling conditions were dependent on the primers used. In the first PCR, using Cx41-OR2, after the initial denaturation (5 min, 95 °C), 40 cycles were performed for denaturation (1 min, 95 °C), annealing (2 min, 55 °C), and elongation (2 min, 72 °C), followed by an extra elongation period of 10 min (72 °C). In the PCR with primer Cx41-OR1 the conditions were the same, except for the annealing temperature (5 cycles at 40 °C and 35 cycles at 45 °C). The PCR product was cloned into pBluescript SK+ provided with a Ncol site (kindly provided by A. Buchel, Leiden University, The Netherlands). After confirmation of the sequence (T7 Sequencing Kit, Amersham Pharmacia Biotech) a forward primer (Cx41–5F1) was designed, consisting of a HindIII restriction site and nucleotides 1–18. A PCR was set up with this primer to facilitate the cloning of the PCR product into pT7TS. This vector was a gift of P. A. Krieg (University of Texas) and contains the T7 transcriptional part was incubated with rat antibody raised against eIF4A (1:5000 in Tris-buffered saline (0.15% NaCl, 0.05% Tween, 0.05% BSA)). After Ponceau Red staining (0.2% in 1% acetic acid) the blot was cut into two parts just below the 40-kDa marker band. A PhosphorImager and ImageQuant software (Molecular Dynamics) was used to analyze the autoradiograms. Small peptides were visualized after incorporation of labeled [35S]methionine/cysteine and separation on a Tris-Tricine acrylamide gel (26).

**Embryo Injections**—X. laevis frogs were reared as described (27). Female animals were injected with 500 IU human chorionic gonadotropin (Pregnyl, Organon) 16 h before use. The female animals were kept at 14 °C after injection. Males were injected twice with 400 IU human chorionic gonadotropin, once in the week prior to the experiment and once 16 h before use. Males were anesthetized on ice for at least 20 min. After decerebration, the testes were dissected, rinsed, and stored at 4 °C in 100 mM MMR (100 mM NaCl, 2 mM KCl, 2 mM CaCl2 and 5 mM Hepes, 1 mM EDTA, pH 7.4). Immediately after squeezing the female frogs, the eggs were rinsed with tap water and fertilized with sperm squeezed out of a small piece of testis. Twenty minutes after fertilization the embryos were dejellied in 2% cysteine-HCl in tap water, pH 7.9. Embryos were reared in 3% Ficoll in 25% MMR at 18 °C and staged according to the normal table for X. laevis (28).

**Injection needles** (GC150-TF10, Clark Electromedical Instruments) were pulled (micropipette puller, Sutter Instruments) and broken to a diameter of ~10 μm. Needles were calibrated after injection of water into oil (PV830 Pneumatic Pico Pump, World Precision Instruments), measurement of the diameter of the droplet, and adjustment of the pressure. One-cell stage embryos were injected with 0.8–1 ng of RNA in 8–10 nl of water. Twenty four hours after fertilization the embryos were pooled, rapidly frozen in liquid nitrogen, and stored at −80 °C for RNA or protein isolation.

**Analysis of Injected Embryos**—RNA was isolated with RNAzol B (Campro Scientific B.V.) according to the manufacturer’s protocol with an extra centrifugation step after homogenization of the embryos. Northern blots were performed with 5 μg of total RNA per lane. RNA samples were glyoxylated to melt any secondary structure and analyzed on a sodium phosphate-buffered 1.5% agarose gel (29). RNA was blotted onto Hybond membranes (Amersham Pharmacia Biotech) and probed with [32P]dCTP (Amersham Pharmacia Biotech)-labeled GFP probe (T7 Quick Prime kit, Amersham Pharmacia Biotech) was added, and hybridization was performed overnight at 65 °C. The probe was purified by Sephadex G-50 fine filtration (Amersham Pharmacia Biotech). Excess probe was removed by washing in 0.1× SSC, 0.1% SDS, 100 mg of dextran sulfate, and 50 μg of salmon sperm DNA per ml, a [32P]dCTP (Amersham Pharmacia Biotech)-labeled GFP probe (T7 Quick Prime kit, Amersham Pharmacia Biotech) was added, and hybridization was performed overnight at 65 °C. The probe was purified by Sephadex G-50 fine filtration (Amersham Pharmacia Biotech). Excess probe was removed by washing in 0.1× SSC, 0.1% SDS at room temperature. Signal was detected by exposure of the blot to Hyperfilm (Amersham Pharmacia Biotech) followed by phosphorimaging and quantification using the ImageQuant program (Molecular Dynamics). Prior to hybridization a Xenopus histone 3 (XIII) probe was stripped by pouring boiling 1% SDS directly onto the blot.

Proteins were isolated by resuspending the embryos in 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 10% glycerol, and 0.1 mM EDTA (10 μl/embryo). After repeated freezing and thawing, the mixture was centrifuged at 13,000 rpm at 4 °C for 10 min. Fat-free supernatant was transferred to a clean Eppendorf tube. This was repeated once to obtain a clear supernatant. Protein equivalent to one or three embryos was separated on a 12.5% acrylamide gel and blotted onto Hybond-P (Amersham Pharmacia Biotech). After Ponceau Red staining (0.2% in 1% acetic acid) the blot was cut into two parts just below the 40-kDa marker band. Both parts of the blot were blocked in 5% fat-free milk powder in PBS, 0.2% Tween for 1 h. The lower part was incubated with GFP antibody (CLONTECH, 1:100 in blocking solution), and the upper part was incubated with rat antibody raised against eIF4A (1:5000 in blocking solution). Amounts of transcript were translated in a reticulocyte lysate system in the presence of [35S]methionine (25). Labeled proteins were separated on a 12.5% acrylamide gel and detected by exposure to Hyperfilm MP (Amersham Pharmacia Biotech). Signals were quantified after overnight exposure to a PhosphorScreen and subsequently analyzed using a PhosphorImager and ImageQuant software (Molecular Dynamics). Small peptides were visualized after incorporation of labeled [35S]methionine/cysteine and separation on a Tris-Tricine acrylamide gel (26).

**Translational Control of Xenopus Cx41**

**TABLE I**

| Name | Sequence (/5′–3′/) | Position | Remarks |
|------|-------------------|----------|---------|
| Cx41-5F1 | CGGCCAAGCTTGCTCTTCCTATCACCACAG | 1–18 | HindIII |
| Cx41-5F2 | GCCAGAATGGAATTTATAAGGGTTTACAGGAAG | 23–58 | Mutagenesis uATG1 |
| Cx41-5F3 | CTACACATAATTTGGCGCGGAGCGGATGTGCTCG | 78–118 | Mutagenesis uATG2 + 3 |
| Cx41-5R1 | CAGCTCCCACTGGTGCCTGTTATCTTAG | 185–159 | 5′-RACE NcoI |
| Cx41-OR1 | AGCACCATAGAAGCCAGATCTTCCC | 260–239 | 5′-RACE |
| Cx41-OR2 | CCAGGGTGTCTGCTGTTATACAG | 353–332 | cDNA synthesis |

* Restriction sites are underlined.
**RESULTS**

Cx41 5′-Untranslated Region—The partial sequence of Cx41 cloned earlier (23) contains a 154-nucleotide 5′-UTR with three potential uORFs, the complete coding region (1146 nt), and a 3′-UTR of 2317 nucleotides. The potential uORFs in the 5′-UTR and the extremely long 3′-UTR suggest translational control of this messenger. A 5′-RACE PCR was performed to obtain the complete Cx41 5′-UTR. Eight clones were sequenced, and all clones contained a 5′-UTR of 174 nucleotides (Fig. 1A), slightly longer than the sequence reported before (23). Presumably, the cDNA library clone resulted from incomplete reverse transcription of the mRNA (23).

The stability of the 5′-UTR and of the strongest hairpin within the leader were calculated for 18 °C, the breeding temperature of Xenopus laevis (30). The free energy (ΔG) of the complete 5′-UTR was −62 kcal/mol and that of the strongest hairpin −38 kcal/mol, corresponding to −34 and −21 kcal/mol at 37 °C. Structures with a free energy more than −50 kcal/mol (at 37 °C) are not a severe problem for the eukaryotic translational machinery (31, 32). Therefore, the stability of the hairpins within this 5′-UTR is expected not to cause substantial translational repression. The 5′-UTR contains three upstream start codons, all followed by in frame stop codons (Fig. 1B). The upstream open reading frames potentially encode three small peptides of 28, 8, and 6 amino acids (Fig. 1C). These uORFs might have a function in translational control. None of the sequences around the uAUGs conform to the Xenopus consensus sequence (A/C)/A/C/A/C/A/C/AUG/A/G (33). In contrast, the region around the start codon of the Cx41 open reading frame contains the best conserved nucleotides, i.e., the A at −3 and the G at +1 relative to the AUG (34).

Translation Efficiency of the Cx41 5′-UTR—To investigate the translational capacity of the Cx41 5′-UTR, constructs containing the 5′-UTR of either Cx41 or Xenopus β-globin, the ORF of the GFP reporter, and the 3′-UTR of β-globin were made (Fig. 2A). DNA was cut after the A30C30 sequence and used to make transcripts for in vivo and in vitro translation. Addition of the A30C30 tail supports stability and thereby translation of the downstream ORF (35). The construct with the β-globin 5′-UTR was used as a positive control since this 5′-UTR enables very efficient translation of the downstream ORF (35). The in vitro translation of these transcripts in the reticulocyte lysate system showed that the Cx41 5′-UTR was about 3-fold less efficient than the globin 5′-UTR construct (Fig. 2, B and C). This indicates that, despite the three uORFs, the Cx41 5′-UTR does not cause strong translational repression in vitro. Apparently, the reticulocyte system is relatively insensitive to the presence of three potential upstream initiation codons.

The same constructs were injected into one-cell stage X. laevis embryos. In each embryo 0.8–1 ng mRNA was injected. This is the smallest amount of transcript resulting in a detectable amount of GFP with the wild-type Cx41 5′-UTR (data not shown). Injected embryos were pooled 24 h after fertilization (stage 13–14) for protein and RNA isolation. GFP protein production was visualized by Western blotting. The amount of GFP protein (Fig. 3A) was corrected for the amount of injected GFP mRNA (Fig. 3C), whereas both signals were corrected for the loading controls (eIF4A and Xenopus Histone 3, Fig. 3, B and D). In vivo translation efficiency of the Cx41 5′-UTR was only 2%, compared with the β-globin 5′-UTR. Apparently, the replacement of the efficient β-globin 5′-UTR for the Cx41 5′-UTR strongly reduces translation. The relative translation efficiencies were also analyzed 8 h after fertilization (stage 8–9). They

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* A. W. van der Velden, unpublished results.
appeared to be similar compared with 24 h after fertilization (data not shown).

Peptide uORF1—Since the uORFs were expected to be the cause of the translational repression, the uAUGs were mutated into AAG. To facilitate the analysis of the contribution of each individual start codon, four different constructs were made with either one or with all uAUGs mutated (Fig. 4A). The translation efficiency of the transcripts with single mutations (Δ1, Δ2, and Δ3) and compared with the wild-type Cx41 5′-UTR (WT) and the triple mutant (Δ123) in reticulocyte lysate. In this assay, no remarkable differences were found (Fig. 4B).

The in vitro translation assay was also performed with a mixture of [35S]methionine and [35S]cysteine and analyzed on Tris-Tricine gels to enable the visualization of small peptides (Fig. 5). Three transcripts were analyzed as follows: the wild-type (WT), the single mutant Δ1, and the triple mutant (Δ123). Translation of the wild-type 5′-UTR produced a small peptide that migrated slightly slower than a 20-amino acid residue control peptide (2.3 kDa). No peptides were detected with the Δ1 and the Δ123 mutant transcripts. Therefore, the peptide produced was due to the presence of uAUG1. Note that the exposure times for GFP (16 h) and the peptide (2 weeks) were very different (Fig. 5). Even taking into account the number of cysteines and methionines in GFP and the 28-amino acid peptide, the amount of peptide formed was very low. The results are in agreement with the moderate translational repression in vitro of the WT Cx41 5′-UTR compared with the globin 5′-UTR and with the lack of differences in in vitro translational capacities between the Cx41 mutants.

Effect of the Three Upstream AUG Triplets on Translation Efficiency—Wild-type or mutant Cx41 transcripts were injected into one-cell stage Xenopus embryos. The embryos were pooled 24 h after fertilization. Extracts were made and analyzed for the amount of GFP protein and mRNA present at the time of harvesting. The amount of produced GFP protein was corrected for the amount of injected GFP mRNA and for loading differences (Fig. 6).

The translation efficiency of the triple mutant (Δ123) was remarkably high compared with the wild-type transcript. The efficiency of Δ123 was set at 100%. Only 1% of GFP was formed by the wild-type transcript. The mutation of the three uAUGs enhanced translation efficiency 82- (Δ1), 9- (Δ2), and 4-fold (Δ3) compared with the wild-type Cx41 5′-UTR. Calculation of the efficiency of translational repression by each AUG will be discussed in detail later. When the embryos were collected 8 h after fertilization the results were similar (data not shown). The results show that the presence of the uAUGs in the leader of Cx41 strongly represses translation of the downstream GFP ORF in young Xenopus embryos.

One of the differences between the analyses of translation efficiency in reticulocyte lysate and analyses after injection of *X. laevis* embryos is the incubation temperature. The in vitro results was the result of difference in temperature at which the analyses were done, the in vitro translation was performed at 30 °C, whereas the injected embryos were cultured at 18 °C. The results might reflect a difference in a temperature-dependent mechanism. For example, the secondary structure of an mRNA is dependent on temperature, and alteration in the secondary structure may influence recognition of uAUGs. To test whether the difference between the in vitro and in vivo results was the result of difference in temperature at which the analyses were done, the in vitro translation was performed at 18, 24, and 30 °C. The Cx41 WT and the Δ1 transcripts as well as the globin-GFP mRNA were analyzed (Fig. 7). Translation efficiency in reticulocyte lysate was strongly dependent on the temperature. However, at each temperature tested, the ratio of the translation efficiency of Cx41 WT, Δ1, and the globin 5′-UTR transcripts was similar (see also Fig. 2). The differences between in vitro and in vivo translation efficiencies were apparently not caused by a temperature-dependent recognition of the uAUGs. Therefore, the scanning rate and translation initiation complex formation were not different at the measured temperatures with regard
to the relative translation efficiencies of the three RNAs. Probably, regulation of translation by cofactors modulating uAUG recognition is different in young Xenopus embryos and in reticulocyte lysate. The striking difference in upstream AUG recognition between in vitro and in vivo assays was noticed before (36).

**DISCUSSION**

After mutation of the uAUGs in the Cx41 5′-UTR the translation efficiency increased. Based on this increased translation the percentage of Xenopus ribosomes that recognized the uAUGs after RNA injection was calculated (Fig. 8). Mutation of uAUG3 into an AAG codon caused a 4-fold increase in translation efficiency. According to the leaky scanning model, this implies that for each ribosome that reached the main ORF four times more scanning ribosomal complexes must be present on the 5′-UTR upstream of uAUG3. When there are four scanning complexes present upstream of uAUG3, the 9-fold increase due to mutation of uAUG2 means the presence of 36 (9 × 4) scanning complexes upstream of uAUG2. Mutation of uAUG1 enhanced the translation efficiency 82-fold, so for each ribosome that reached the main ORF, about 3000 (82 × 36) ribosomal complexes should enter the mRNA at the cap end. The number of cap-binding ribosomal complexes was set at 100% which means that only 0.03% of these complexes scan the entire 5′-UTR and translate the main ORF.

The model in Fig. 8 accounts for all observations and is fully consistent with the ribosomal scanning model (18). The presented data do not suggest any involvement of the secondary or higher order structure of the mRNA. Furthermore, the minor amino acid changes due to the AUG to AAG mutations are most likely not responsible for the increase in translation efficiency. We have made a mutant construct in which approximately 40% of the amino acid residues was changed. Preliminary data indicate that ablation of uAUG1 had the same effect in this severely mutated 5′-UTR as in a wild-type 5′-UTR (data not shown).

The number of ribosomes leaving the mRNA is a subtraction of the number of ribosomes upstream and downstream of the
corresponding uAUG (Fig. 8). By dividing the number of ribosomes leaving the mRNA after recognition of an uAUG by the number of ribosomes scanning the 5'-UTR upstream of the same uAUG, the percentage of ribosomes leaving the mRNA before arrival at the GFP initiation codon was calculated. This calculation shows that all uAUGs were recognized by the majority of the scanning complexes. Therefore, only 0.03% of the ribosomes, entering the transcript, scan the entire 5'-UTR and translate the main ORF.

Simultaneous mutation of all uAUGs increased translation 100-fold, whereas the proposed model (Fig. 8) predicts a 3000-fold (82 x 9 x 4) increase. The replacement of the wild-type Cx41 5'-UTR by the 5'-UTR of β-globin increased translation 50-fold. The β-globin is among the most efficiently translated messengers (35). We suggest that the β-globin mRNA as well as the Δ123 transcript are maximally loaded with translating ribosomes and that therefore a more than 100-fold increase in translation due to mutation of each one of the uAUGs the percentage of ribosomes leaving the mRNA after recognition of an uAUG was calculated as described in the text. Note that despite the differences in translation increase due to the mutation of the different uAUGs, all uAUGs were recognized by the majority of the scanning complexes. Therefore, only 0.03% of the ribosomes, entering the transcript, scan the entire 5'-UTR and translate the main ORF.

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