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Analysis of a Charcot-Marie-Tooth Disease Mutation Reveals an Essential Internal Ribosome Entry Site Element in the Connexin-32 Gene*

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A mutation located in the 5′-untranslated region (5′-UTR) of the nerve-specific connexin-32 mRNA, previously found in a family with Charcot-Marie-Tooth disease (CMTX), was analyzed for its effect on the expression of a reporter gene (luciferase) in transgenic mice and in transfected cells. Whereas both mutant and wild-type genes appeared to be transcribed and spliced efficiently, no luciferase was detected from the mutant in either system, suggesting that the mutation affects translation of the mRNA. When the 5′-UTR of nerve-specific connexin-32 mRNA was inserted between the two genes of a bicistronic vector and transfected into various cell lines, expression of the second gene was significantly increased. Because the mutant did not facilitate translation of the second gene in the bicistronic mRNA system, this result suggested that the CMTX mutation abolished function of an internal ribosome entry site (IRES) in the 5′-UTR of the wild-type connexin-32 mRNA. The CMTX phenotype of the mutant 5′-UTR further suggested that the wild-type IRES was essential for the translation of the connexin-32 mRNA in nerve cells. In addition, other sequence elements of the connexin-32 IRES were characterized by mutation analysis. A mutation in either of the first two elements investigated showed loss of IRES function, whereas mutation of a third element showed gain of function.

Translation of most eukaryotic mRNAs is initiated from the cap structure that is commonly found at the 5′ end of eukaryotic mRNAs. As a consequence eukaryotic mRNAs are usually monocistronic. This is in contrast to prokaryotic mRNAs that often are polycistronic, containing multiple internal ribosome binding sites (Shine-Dalgarno sequence). Eukaryotic RNA sequence elements that can function as internal ribosome entry sites (IRES)[1] were first discovered in picornavirus and later also in some other viral RNAs (1, 2). More recently several cellular mRNAs were also found to contain potential IRES elements (3–7). Whereas uncapped viral mRNAs by necessity utilize this cap-independent method of translation initiation, it is less clear if cellular mRNAs, presumably capped, would as well. Subsequently, it was demonstrated that cellular IRES elements, in their natural capped mRNAs, could mediate translation in poliovirus-infected cells (8); but it remained unknown whether translation of any cellular gene is IRES-dependent under normal conditions. Recently, there have been examples of IRES-containing cellular mRNAs that are translated in cells under normal physiological conditions in which cap-dependent translation is suppressed (9). During mitosis, cap-dependent translation is shut down, but translation of some proteins important for cell cycle progression continues (10, 11). Similarly, during cell stress and apoptosis, cap-mediated translation is impaired. Nevertheless, certain mRNAs such as XIAP, the apoptosis inhibiting factor, continue to be translated under these conditions (12). It it not clear why or under what conditions other IRES-containing mRNAs, such as those that code for the transcription factor MYT2 (13) or the ion channel Kv1.4 (14), need a cap-independent mode of translation initiation. Possibly, IRES-mediated translation allows for fine-tuned regulation of expression of these genes.

Connexin-32 is a member of the family of gap junction proteins that form cell-cell channels. The protein is expressed in liver and other exocrine glands as well as in the nervous system, *e.g.* in Schwann cells. The connexin-32 gene is transcribed from two tissue-specific promoters producing mRNAs with different 5′-untranslated regions (5′-UTRs) but identical coding regions (15) (see Fig. 1*A*). In the liver, connexin-32 mRNA is made from promoter 1 (P1) and in the nervous system from promoter 2 (P2).

Mutations in the connexin-32 gene have been linked to one form of Charcot-Marie-Tooth disease (CMTX), a neurodegenerative disorder (16, 17). Whereas most of the mutations that were found in patients with CMTX are located in the coding region of the connexin-32 gene, a few non-coding mutations were also described. One of these mutations, a C→T transition found at position −458 in relation to the start codon, called *mut-2* throughout this paper, was found in a family of CMTX patients (18). It resides in the 5′-UTR of the connexin-32 mRNA. The study of this non-coding mutation led to the discovery of an IRES element in the 5′-UTR of the connexin-32 mRNA.

No consensus sequences have been determined as universal IRES recognition elements in eukaryotes. A sequence element is functionally defined as an IRES if it can mediate translation of a downstream cistron in a bicistronic mRNA (2). The nerve-specific 5′-UTR of connexin-32 mRNA fits this definition of an

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[1] The abbreviations used are: IRES, internal ribosome entry site(s); UTR, untranslated region; P, promoter; CMTX, Charcot-Marie-Tooth disease; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; EMCV, encephalomyocarditis virus; uAUGs, upstream AUGs; rLUC, *Renilla* luciferase; fLUC, firefly luciferase; nt, nucleotides; PPT, pyrimidine tract.

IRES. The *mut-2* mutation abolishes IRES function. The fact that a mutation in this sequence abolishes translation of the mRNA, leading to a CMTX phenotype, suggests that the connexin-32 IRES element is essential for the expression of the connexin-32 gene in the nervous system.

EXPERIMENTAL PROCEDURES

Transgenic Animals—The Quick Change site-directed mutagenesis kit (Stratagene) was used to mutate the pgCx32–1.8 reporter construct (15) to make the *mut-2* construct. Sequences were confirmed by automated DNA sequence analysis at the University of Miami DNA Core Facility. The *mut-2* construct was used for injection into the pronuclei of fertilized mouse eggs (strain B6S-JL) at the University of Miami Transgene Facility. Screening of animals was done by PCR amplification of genomic DNA and Southern blot analysis using luciferase-specific primers and probe. All animal care and use was in accordance with University of Miami guidelines.

Animals were anesthetized with ether and sacrificed by cervical dislocation. Brain, spinal cord, and sciatic nerve were homogenized in TRI Reagent™ (Molecular Research Center, Inc.), and total RNA was extracted according to the manufacturer's protocol. 1 μg of total RNA was used for RT-PCR. For amplification of the luciferase message the following primers were used: forward primer, ATATGACTCTCCAGCACCG; reverse primer (antisense luciferase coding region), CCAGAGGACTTCATTATCAG. For amplification of the endogenous connexin-32 mRNA the same forward primer and a reverse primer complementary to the Cx32 coding region (CCCTCAAGCCGTAGCATTTT) were used. For sequencing of the remainder of the mRNA other primer pairs were used (data not shown). RT-PCR products were run on 1% agarose gels, and the bands were excised, purified (Qiaex II, Qiagen), and subjected to automated DNA sequence analysis.

In Vitro Transcription and Translation—The complete cDNAs of connexin-32 were cloned into pGEM3zf(+) (Promega) between the *Hin*dIII and *Eco*RI sites of the multiple cloning region to create the P1-cx32 and P2-cx32 constructs. Deletion mutagenesis was performed to remove the nucleotides between the Sp6 transcription start site and that of the natural connexin-32 mRNA. *In vitro* transcription from this construct yields an mRNA that is identical to the mRNA made *in vivo*. Plasmids were linearized with *Ssp*I. *In vitro* transcription was performed with Sp6 RNA polymerase in the presence of cap structure analog ($m^7G(5')ppp(5')G$). RNA was extracted with phenol/chloroform and precipitated by ethanol. *In vitro* translation was performed in a rabbit reticulocyte lysate system supplemented with canine pancreatic microsomes and [^{35}S]methionine. Translation products were layered onto 3% sucrose and centrifuged for 1 h at 4 °C. The pellets were resuspended in 2× SDS loading buffer, and the whole sample was loaded onto a 10% SDS-polyacrylamide gel. Autoradiography was performed overnight. RNA was analyzed in a 1% agarose gel stained with ethidium bromide. RNA and protein levels were determined digitally on a Kodak Image Station 440CF.

Bicistronic Constructs—The firefly luciferase coding region from pGL3-promoter (Promega) was PCR-amplified with primers containing *Spe*I restriction sites in their 5′ ends. In addition, the forward primer used in this reaction contained *Eco*RI and *Xho*I sites to facilitate subsequent cloning. The pGL3 PCR product was then cloned into the *Xba*I site of pRL-CMV (Promega), containing the *Renilla* luciferase gene, resulting in a bicistronic vector. The P2, *mut-2*, and EMCV (from pIRES-EGFP, CLONTECH, Palo Alto, CA) elements were all PCR-amplified with primers containing *Eco*RI and *Xho*I sites, 5′ and 3′, respectively, and inserted between the two luciferase genes of the bicistronic vector. All sequences were verified by DNA sequence analysis after cloning. A stable stem-loop structure ($\Delta G = -41$ kcal/mol) similar to that used by others (14) was created by synthesizing two complementary oligonucleotides, which upon annealing produced *Pst*I sites at both ends (oligo 1a, GAAAAGCGCAGGTCGCGACCGCGCATGCGCGGTCGCGACCTGCGCTAAACTGCA; oligo 1b, GTTTAGCGCAGGTCGCGACCGCGCATGCGCGGTCGCGACCTGCGCTTTTCTGCA). This stem-loop structure was inserted into the *Pst*I site in the 5′-UTR of the *Renilla* gene to reduce the high level of expression due to cap-dependent translation. Another stem-loop ($\Delta G = -40$ kcal/mol) was created in a similar way, with *Eco*RI sites at both ends, and inserted into the *Eco*RI site to reduce the background level of firefly luciferase due to ribosomal scanning and translational reinitiation (oligo 2a, AATTCAAAAGGCGAGGTCGCGAGCGCACATGTGCGCTCGCGACCT CGCCTAAAG; oligo 2b, AATTCTTTAGGCGAGGTCGCGAGCGCACATGTGCGCTCGCGACCTCGCCTTTTG).

Cell Culture and Transfection—Neuro-2a cells were maintained in Eagle's minimal essential medium with 2 mM l-glutamine, Earle's balanced salt solution, 0.1 mM non-essential amino acids, 0.1 mM sodium pyruvate, and 10% fetal bovine serum. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium with 0.1 mM sodium pyruvate and 10% fetal bovine serum. HeLa cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 15 mM HEPES and 10% fetal bovine serum. All cell lines were grown at 37 °C in an atmosphere of 5% CO_2. Transient transfections were performed on cells that were 50–60% confluent in 60-mm dishes with 3 μg of plasmid DNA by the method of calcium phosphate precipitation (19), with the exception of HeLa cells. HeLa cells were grown in 6-well plates and were 80% confluent at the time of transfection. Cells were incubated for 6 h with 6 μl of LipofectAMINE (Life Technologies, Inc.) mixed with 1 μg of plasmid DNA in Opti-MEM (Life Technologies, Inc.). After 48 h, cells were lysed with passive lysis buffer, and dual luciferase assays were performed following the manufacturer's protocol. The two luciferase enzymes utilize distinct substrates. 10 μl of cell lysate was mixed with 50 μl of Luciferase Assay Reagent II (Promega, Madison, WI) and measured for firefly luciferase activity for 10 s after a 2-second delay on a Turner Luminometer TD 20/20. Stop and Glo reagent (Promega) was added to quench the firefly luciferase activity, and the activity of *Renilla* luciferase was measured for 10 s following a 2-second delay. All readings were greater than 100-fold above background.

RESULTS

mut-2, a C→T transition found at position −458 in patients with CMTX, is located in the 5′-UTR of the nerve-specific connexin-32 mRNA. To determine the molecular events that might be responsible for the CMTX phenotype caused by *mut-2*, we used a reporter assay system. The wild-type construct, pgCx32–1.8, contains the rat connexin-32 promoter P2 and its entire 5′-UTR, including the intron, fused to the coding region of the firefly luciferase gene (Fig. 1*B*). We had previously shown that expression of luciferase from this construct was restricted to cells of the nervous system when expressed in transgenic mice (15). We introduced the *mut-2* mutation into this construct to simulate the situation found in CMTX patients carrying this mutation. It should be noted that the human, rat, and mouse sequences are 100% identical in this region of the gene.

The *mut-2* construct was tested for expression of the reporter in NIH 3T3 cells. After transfection, luciferase-specific mRNA levels were measured by Northern blot analysis, and luciferase activity was measured in a scintillation counter set to single photon mode. The results of this experiment are shown in Fig. 1*C*. Whereas both wild-type and mutant mRNA were made at significant levels, no luciferase activity could be seen in cells transfected with the *mut-2* construct.

Because the *mut-2* mutation creates a potential donor splice site, it could be argued that the connexin-32 mRNA is not spliced correctly and thus cannot be translated. To use a system that more closely resembles the *in vivo* situation, we generated transgenic mice with the mutant construct. We had previously shown that expression of the wild-type construct in transgenic mice is limited to the nervous system (brain, spinal cord, and peripheral nerve) (15). Fig. 1*D* shows the results of RT-PCR of RNA isolated from the nervous tissues of these transgenic mice. As a control, RT-PCR was performed on the same samples with primers specific for the endogenous Cx32 mRNA. Although only semi-quantitative, the RT-PCRs revealed no gross differences in the level of expression of the transgenic and endogenous mRNAs (Fig. 1*D*). The size of the RT-PCR product indicated that the mRNA was spliced correctly, suggesting that the potential splice site that was created by the mutation was not used. This was confirmed by sequence analysis of the RT-PCR products. No luciferase activity, however, was detected in these tissues. The luciferase assay is extremely sensitive. Less than one femtogram (10^{-20} moles) of firefly luciferase can be detected. Thus, the mutant reporter

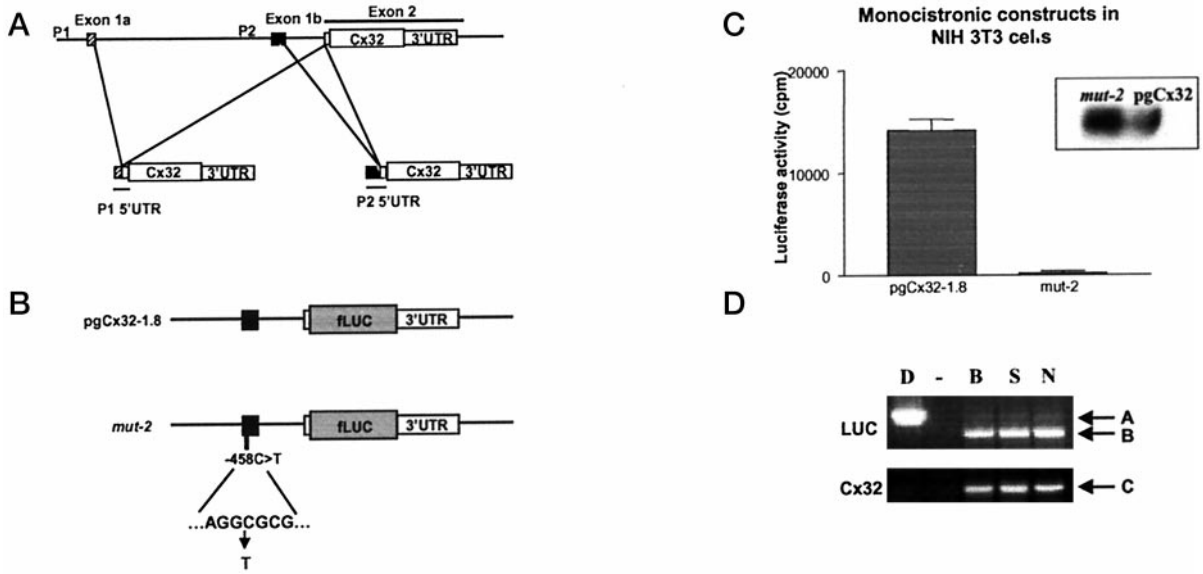

FIG. 1. *A*, connexin-32 gene structure. The connexin-32 gene is under the control of two tissue-specific promoters. Promoter 1 is active in exocrine glands such as liver and pancreas, whereas promoter 2 is active exclusively in the nervous system. Alternative use of the promoters results in two transcripts, differing only in their 5′-UTRs. *B*, pgCx32–1.8 reporter construct with the *mut-2* mutation. The construct contains the rat connexin-32 promoter 2, exon1b, the small intron, the luciferase-coding region, and the connexin-32 3′-UTR. Please note that the rat, mouse, and human connexin-32 gene sequences are all identical in this region. *C*, transient transfections of reporter constructs. NIH 3T3 cells were transfected with either the pgCx32–1.8 or the *mut-2* construct. Poly(A) RNA was purified from transfected cells and subjected to Northern blot analysis. The blot was probed with a fragment of the luciferase-coding region (see *inset*). Cell lysates were assayed for luciferase activity in a scintillation counter set to single photon mode and measured as counts per minute (cpm). *D*, RT-PCR of total RNA from *mut-2* transgenic mice. RNA was extracted from brain, spinal cord, and peripheral nerve. The *upper panel* shows the following: *D*, PCR from *mut-2* plasmid; **−**, brain RNA from negative mouse; *B*, brain RNA from *mut-2* mouse; *S*, spinal cord RNA from *mut-2* mouse; *N*, sciatic nerve RNA from *mut-2* mouse. The *lower panel* shows RT-PCR of endogenous Cx32 from the same RNA preparations from *mut-2* mouse. All mRNA sequences were confirmed by DNA sequence analysis of the PCR products. *Arrow A* indicates the expected size of the luciferase PCR product from DNA or unspliced RNA; *arrow B* indicates spliced luciferase mRNA; and *arrow C* indicates spliced Cx32 mRNA.

gene was properly transcribed and processed but not translated. This was in contrast to the wild-type connexin-32 reporter construct that showed high levels of luciferase activity in the brain, spinal cord, and peripheral nerves of transgenic mice (15).

How can a single nucleotide change in an untranslated region completely shut down translation? To answer this question we looked at the role of the wild-type connexin-32 5′-UTR on gene expression. Use of alternative promoters of the connexin-32 gene results in two mRNAs differing only in their 5′-UTR sequences. The effects of the two different 5′-UTRs, one from promoter P1 and the other from promoter P2, on the translational efficiency of the connexin-32 messages were examined in an *in vitro* translation system. The complete P1 and P2 mRNAs were transcribed *in vitro* (Fig. 2*A*) and then translated in a rabbit reticulocyte lysate system. The results showed that, whereas the P1-derived connexin-32 message was translated efficiently, little protein was made from the mRNA carrying the wild-type P2-derived 5′-UTR (Fig. 2*B*). Comparison of the two 5′-UTRs revealed that the P2-derived 5′-UTR contains additional AUG codons upstream of the connexin-32 initiation site (Fig. 2*C*). It has been shown that upstream AUGs (uAUGs) can inhibit cap-dependent translation (20–22). Two of these uAUGs, labeled A1 and A2, have flanking sequences that are favorable for initiator codons (23). As expected, when these two uAUGs in the P2 message were mutated to AAGs, more connexin protein was produced (Fig. 2*B*). These results suggest that the ribosomes in the reticulocyte lysate system cannot bypass the uAUGs in the P2 5′-UTR. Because nerve cells apparently have no problem translating this mRNA *in vivo*, they must have a mechanism that allows the ribosomes to bypass the uAUGs to allow initiation of protein synthesis from the correct AUG codon. One such mechanism would be the use of an IRES.

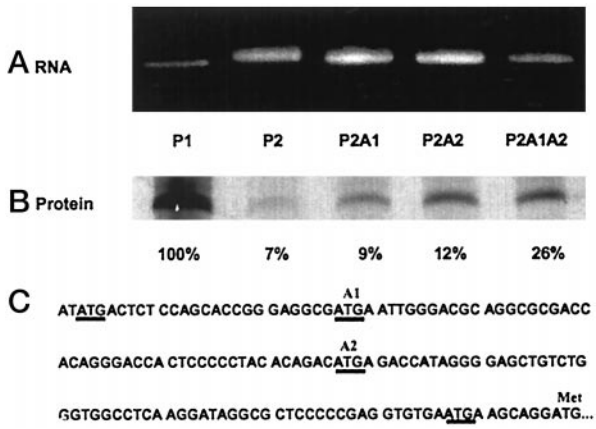

FIG. 2. ***In vitro* transcription/translation.** Plasmids P1-cx32 and P2-cx32 containing the connexin-32 cDNAs were linearized and transcribed *in vitro* with Sp6 RNA polymerase. This RNA was used for *in vitro* translation in rabbit reticulocyte lysates supplemented with canine pancreatic microsomes and [^{35}S]methionine. *A*, agarose gel electrophoresis of RNAs used for translation. *B*, autoradiograph of connexin-32 protein analyzed by 10% SDS-polyacrylamide gel electrophoresis. The bands in *A* and *B* were digitally analyzed for net intensity. The ratio of protein to RNA of P1-cx32 was set to 100% efficiency. The ratios of the other samples were calculated and are expressed as percent efficiency relative to that of P1-cx32. *C*, sequence of the P2 5′-UTR, showing upstream ATGs (*underlined*). The ATGs labeled A1 and A2 were mutated to AAGs.

To investigate this possibility we tested the 5′-UTRs of nerve cell-derived connexin-32 mRNA in a bicistronic assay system that is commonly used to demonstrate IRES activity. We constructed a bicistronic vector, SL2, with two luciferase open reading frames (24). The cytomegalovirus promoter drives transcription of the mRNA for these two genes. The first gene,

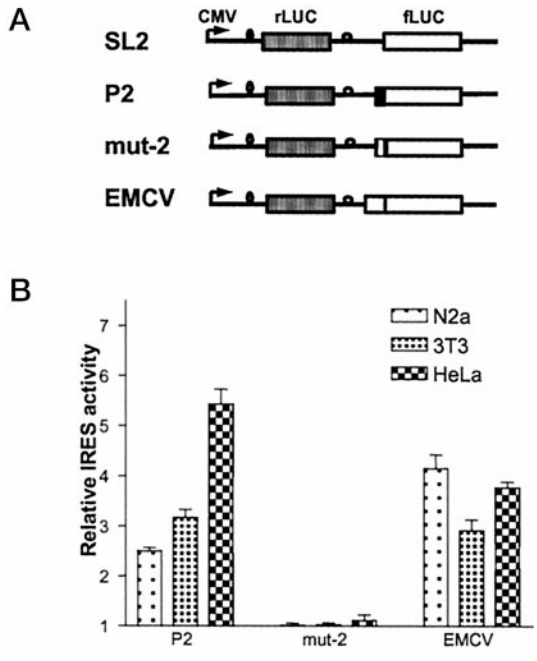

FIG. 3. **Bicistronic assay for IRES function.** *A*, bicistronic constructs containing the *Renilla* luciferase gene upstream of the firefly luciferase gene under the control of the cytomegalovirus promoter. The control construct, SL2, does not contain any IRES in the intercistronic space. The 5′-UTRs of P2 wild type and the *mut-2* connexin were cloned into the intercistronic space. The IRES of EMCV was used as a positive control for IRES function in this system. *B*, the constructs were transiently transfected into the cells indicated in triplicate sets. IRES activities are represented as ratios of firefly luciferase activity to *Renilla* luciferase activity. Relative IRES activities were calculated and normalized to those of the control vector, SL2, in each of the cell lines. *Error bars* represent S.E. *N2a*, Neuro-2a; 3T3, NIH-3T3.

encoding *Renilla* luciferase (rLUC), should be translated by a cap-dependent mechanism, but the second gene, encoding firefly luciferase (fLUC), should be translated efficiently only if preceded by an internal ribosome entry site. The two luciferase activities can be assayed independently.

The wild-type P2 or mutant P2 5′-UTR was inserted between the two luciferase genes, downstream of the second stem-loop structure. Insertion of a known IRES element from EMCV (25) was used as a positive control. The parent vector, SL2, without any IRES, served as negative control. The vectors were transiently transfected into Neuro-2a, NIH 3T3, and HeLa cells, and *Renilla* (rLUC) and firefly (fLUC) luciferase activities were measured independently. IRES function is determined by the ratio of the two luciferase activities, fLUC/rLUC. The results obtained with the three types of cells are shown in Fig. 3. The fLUC/rLUC ratio obtained with the SL2 control construct, containing no IRES, was normalized to the value 1 so that any ratio above this value was due to IRES function. For each cell line tested, the ratio of the two luciferase activities was calculated and normalized to the ratio of the negative control, SL2, in the same cell line. The EMCV construct showed an increase in the fLUC/rLUC ratio of 3–4 over the control. Insertion of the nerve-specific connexin-32 5′-UTR sequence into SL2 raised the fLUC/rLUC ratio by 2.5–5.5, depending on the cell type. In contrast, the 5′-UTR bearing the −458C→T point mutation showed no change in the ratio relative to the control, indicating loss of IRES function. This result, observed in all three cell types, suggested that the region of the P2 5′-UTR where the *mut-2* mutation resides was essential for IRES function.

Because some IRES-containing 5′-UTRs have been shown to have similar predicted structures, it has been suggested that the ribosome might recognize the secondary structure of the mRNA rather than a specific sequence. The secondary structures of the wild-type and mutant 5′-UTR sequences were predicted by using the Zuker algorithm (26, 27) (Fig. 4). The point mutation did not affect the predicted structure. In fact, the nucleotide at that position is in an open loop structure. This suggests that some of the nucleotides in the loop may be involved in recognition of the IRES by the ribosome.

Certain sequence elements have been shown to have a role in IRES function. For example, the sequence GNRA (where N is any nucleotide, and R is a purine) is highly conserved in picornaviruses (28) and important for function of the EMCV IRES (29). The *mut-2* mutation is directly adjacent to this sequence and may form part of this recognition element. Additional common sequences found in IRES elements are polypyrimidine tracts, which have been shown to bind polypyrimidine tract-binding protein, and in some cases this binding was shown to be essential for IRES function. Other IRES elements contain polypyrimidine tracts consisting mainly of poly(rC) sequences. This sequence has been shown to bind the poly(rC)-binding protein, also known as heterogeneous nuclear ribonucleo protein (hnRNP) E, which modulates IRES activity. Another sequence element shown to contribute to IRES function is the sequence AGACA (7). Many of the mammalian IRES elements (*e.g.* c-*myc*, *VEGF*, *eIF4G*, *Kv1.4*) contain this sequence (6–8, 14). The newly identified connexin-32 P2 IRES element contains two polypyrimidine tracts located at positions −442 and −381 (nt 61–68 and 121–127, respectively, in Fig. 4) as well as the AGACA sequence element, located at position −435 (nt 73–77 in Fig. 4). To further characterize the P2 IRES, site-directed mutagenesis was performed on these three elements. If the P2 IRES behaves like other IRES elements, then mutagenesis of at least one of the polypyrimidine tracts or the AGACA sequence would be expected to inhibit IRES function. Bicistronic constructs were made containing the mutated IRES elements, and these mutant constructs were assayed for luciferase activity in NIH 3T3 cells (Fig. 5). The pyrimidine tract 1 (PPT1) mutant and the AGACA mutant abolished IRES function. Surprisingly, mutating the PPT2 sequence led to an increase in IRES activity.

DISCUSSION

The effects of the *mut-2* mutation on the expression of a reporter gene were tested by transfection experiments with the *mut-2* construct. The results of this experiment clearly showed that the mutant mRNA was made at levels similar to those observed with wild-type mRNA, but no luciferase activity was detected from the *mut-2* construct, indicating that translation of the mutant mRNA did not occur. Identical results were obtained when the *mut-2* construct was expressed in transgenic mice. These results suggested that the *mut-2* mutation did not affect transcription or splicing but rather prevented translation of the mutant mRNA. In a recent publication it was reported that RT-PCR of RNA from sural nerve biopsies of two heterozygous female CMTX patients revealed only wild-type connexin-32 mRNA (30). Because of X-chromosome inactivation only one of the two alleles was expressed in a particular cell. It is possible that those cells that expressed the mutant X-chromosome were eliminated, or at least reduced in number, during development. Because of this, the presence of the mutant mRNA may have been missed by the RT-PCR.

The question of how a point mutation in the 5′-UTR of an mRNA can have such a profound effect on translation is intriguing. To address this question, we studied the translation of nerve-specific connexin-32 mRNA in an *in vitro* reticulocyte lysate system and found that it was much less efficient than that of the liver-specific connexin-32 mRNA. This observation led us to look for the presence of an internal ribosome entry site

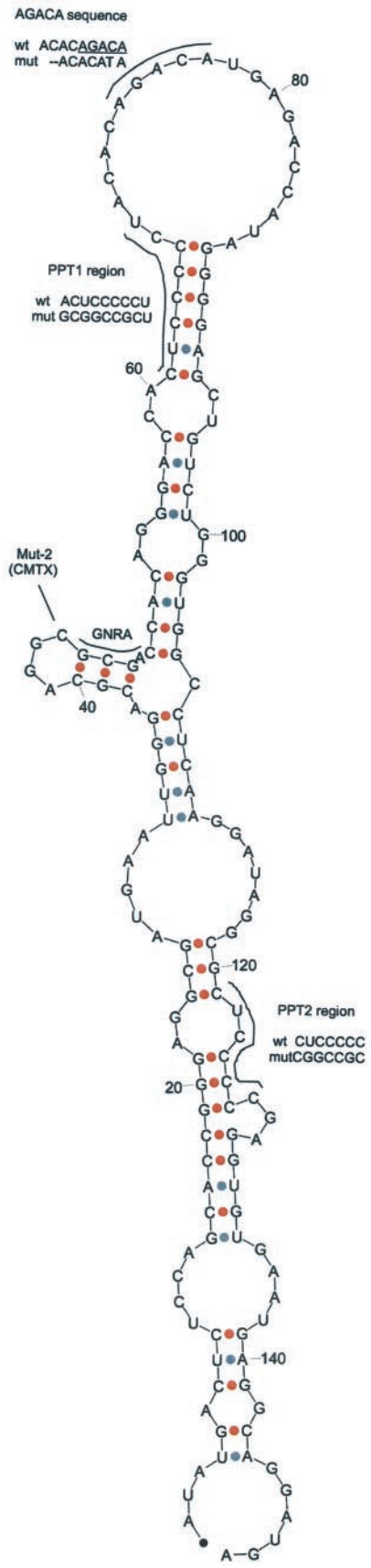

FIG. 4. **Predicted structure of P2 IRES.** The most thermodynamically stable structure predicted by the Zuker algorithm on the mFOLD server is shown (the default parameters of the program were used). The location of the C→T mutation found in CMTX patients is at nucleotide 44 in the figure. This mutation does not affect the predicted structure of the IRES. Features common to other IRES elements are the GNRA sequence (nt 45–48), polypyrimidine tracts PPT1 and PPT2 (nt 61–68 and 121–127), and the AGACA sequence (nt 73–77). The sequences were mutated as indicated in the figure. *wt*, wild type; *mut*, mutant.

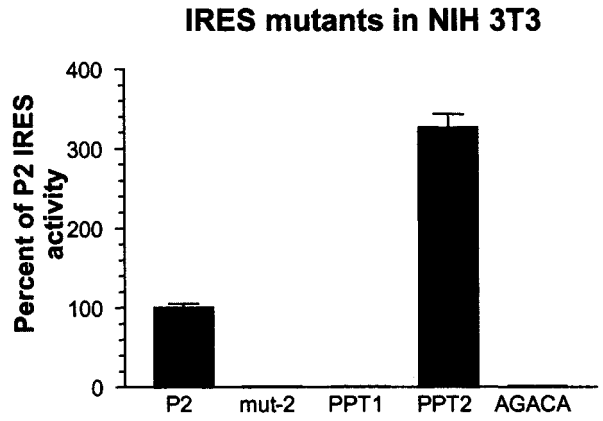

FIG. 5. **Effect of other mutations on the activity of the P2 IRES.** The PPT1, PPT2, and AGACA sequences were mutated and tested for their effects on IRES activity in the bicistronic system. The mutants were transiently transfected into NIH 3T3 cells. IRES activities are expressed as a percentage of the activity of the P2 IRES. *Error bars* represent S.E.

in the 5′-UTR of the nerve-specific mRNA. Our results with a bicistronic vector system showed that the nerve cell-specific connexin-32 mRNA did indeed contain a functional IRES element. The activity of the connexin-32 IRES is similar to that of the known IRES element from EMCV. Different cell lines exhibit different degrees of IRES activity, possibly because of varying availabilities of protein factors that activate the IRES. This cell-specific behavior has also been observed with other IRES elements (31).

Further evidence that the 5′-UTR of connexin-32 mRNA represents a true IRES element was provided by our mutation studies. Several short sequence segments of the connexin-32 IRES were also found in other IRES elements. Some of these sequences had been shown to be essential for IRES activity. When these segments were mutated, IRES activity was abolished. Our site-directed mutagenesis experiments showed similar inactivation of the IRES. One mutation, however, located in PPT2, increased IRES activity severalfold. A similar gain-of-function mutation has been reported in a pyrimidine tract of another IRES element (32). Taken together, these results support our conclusion that the 5′-UTR of the nerve-specific connexin-32 mRNA contains a *bona fide* IRES element.

Three lines of evidence support our conclusion that this IRES element is essential for expression of connexin-32 in Schwann cells. First, the 5′-UTR of connexin-32 mRNA functions in a bicistronic vector, and the function is abolished by the *mut-2* mutation. Second, even though the reporter construct containing the point mutation in the connexin-32 5′-UTR is transcribed in transfected cells and in transgenic mice, it is not translated, whereas the reporter with the wild-type connexin-32 5′-UTR is translated. Third, the *mut-2* mutation in humans produces a CMTX phenotype. This last point suggests that the connexin-32 protein cannot be synthesized in Schwann cells if the 5′-UTR contains the *mut-2* mutation.

We recently reported that the mRNA of another connexin, connexin-43, also contains an IRES element. We can only speculate about the need for connexin mRNAs to use IRES elements rather than cap structures for initiation of translation. There are many reports of connexins being detected at the mRNA level by RT-PCR in tissues where protein expression is undetectable. Perhaps the accumulation of translationally in-

active mRNA allows the cell to switch on connexin protein synthesis in a matter of seconds. Such an instantaneous turning on of connexin-43 synthesis might be required, for example, in the myometrium, where gap junctions must appear very quickly at parturition to allow the onset of labor. Because the precise role that connexin-32 plays in Schwann cells is unknown, we can only speculate about the need for such fine regulation of protein synthesis. Schwann cells synthesize vast amounts of myelin and form a very distinct highly ordered structure that wraps around axons. Expression of connexin-32 in the wrong place in the membrane could be disruptive to compact myelination. Connexin-32 and myelin proteins must be mutually exclusive in the Schwann cell membrane. Indeed, connexin-32 is only found exclusively in the Schmidt-Lanterman incisures and nodes of Ranvier (16), where myelin is absent. Perhaps this is the reason why expression of connexin-32 must be tightly regulated at both the translational and transcriptional level.

A more definitive answer to this question may have to wait until more is known about the factors that affect IRES activity. A more general argument for the necessity of IRES-mediated translation of connexins might be that cell-to-cell communication is so vital for a cell that the continued synthesis of connexins must be ensured even under conditions where cap-mediated translation is impeded, *e.g.* heat shock or stress. Many of the cellular IRES elements described thus far are in genes that code for proteins that are involved in growth control (c-Myc, FGF2, TGF-β, platelet-derived growth factor, etc.) or cell survival (Bip, Hsp 70, eIF4G, Apaf1, XIAP). The discovery of an active IRES element in the 5′-UTR of the connexin-32 gene suggests that connexin-32 may belong to one of those classes of proteins that are regulated in the same manner.

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