FEBS Letters 464 (1999) 118–122 FEBS 23127

Connexin43 mRNA contains a functional internal ribosome entry site

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Received 12 November 1999; received in revised form 2 December 1999

Edited by Ned Mantei

Abstract A reporter gene construct was used to study the regulation of connexin43 (Cx43) expression, the major gap junction protein found in heart and uterus, in transfected cell lines. The construct had the firefly luciferase gene under the control of the Cx43 promoter. Inclusion of the 5′-untranslated region (UTR) of the mRNA in the construct increased luciferase expression by 70%. A bicistronic vector assay demonstrated that the Cx43 5′-UTR contains a strong internal ribosome entry site (IRES). Deletion analysis localized the IRES element to the upstream portion of the 5′-UTR.

Key words: Connexin43; mRNA; Translation control; Internal ribosome entry site

1. Introduction

Connexin43 (Cx43) is the major gap junction (cell-cell channel) protein expressed in the ventricle of the heart. It is expressed constitutively and is responsible for the anisotropic propagation of action potentials in the heart [1]. In the uterus, Cx43 gap junctions are necessary for the synchronous contraction of the myometrium during labor. Cx43 expression is normally sparse in the myometrium but is upregulated by ovarian hormones and mechanical stretch [2]. This upregulation appears to occur at both the transcriptional and translational level, since Cx43 mRNA accumulates before the rapid appearance of Cx43 protein just prior to parturition [3–6].

The Cx43 gene, like most connexin genes, consists of two exons separated by a large intron. Exon 1 encodes most of the 5′-untranslated region (5′-UTR) of the mRNA. Exon 2 contains the remaining 13 bases of the 5′-UTR followed by the entire coding region and the 3′-UTR. The unusually long 5′-UTR of Cx43 suggested to us that it might be involved in translational regulation. Thus, to study the regulation of the Cx43 gene at both transcriptional and translational levels, we created a reporter construct in which the basal Cx43 promoter, including the entire 5′-UTR, drives the expression of the reporter gene for firefly luciferase. The results of these studies led to the discovery of an internal ribosome entry site (IRES) within the 5′-UTR of Cx43 mRNA.

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2. Materials and methods

2.1. Cell Culture

HeLa and RL Clone9 (normal rat liver) cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (Life Technologies, Gaithersburg, MD, USA) medium supplemented with 15 mM HEPES and 10% (w/v) fetal bovine serum (FBS), Neuro-2a cells were maintained in Eagle's minimum essential medium supplemented with 2 mM L-glutamine, Earle's balanced salt solution, 0.1 mM minimum essential amino acids, 0.1 mM sodium pyruvate and 10% FBS. NIH 3T3 cells were maintained in DMEM supplemented with 0.1% sodium pyruvate and 10% FBS. All cell types were grown in an atmosphere of 5% CO_2 at 37°C.

2.2. Plasmid constructs

pE0 was derived from a modified reporter vector [7] into which 145 bases of the rat Cx43 promoter, exon 1 and the 13 bases from the 5′-terminus of exon 2, encoding the remainder of the 5′-UTR [4], were inserted upstream of the luciferase coding region. pE0Xdel has most of exon 1 deleted except for the first seven bases that are found at the 5′-end of the mRNA. Thus, pE0Xdel retains the natural Cx43 transcription and translation start sites. pSL2 was constructed by PCR amplification of the firefly luciferase coding region in the pGL3 promoter vector (Promega, Madison, WI, USA) with a forward primer containing 5′ *Spe*I, *Eco*RI and *Xho*I restriction sites and a reverse primer containing a 5′ *Spe*I site. This PCR product was cloned into the *Xba*I site of pRL-CMV (Promega, Madison, WI, USA). 43SL2 and 43SL2XM1 were made by PCR amplification of bases +1 to +195 and +1 to +163, respectively, of Cx43 exon 1 with a forward primer containing a 5′ *Eco*RI site and a reverse primer containing a 5′ *Xho*I site. The product was inserted between the two luciferase genes of pSL2. A stable stem loop structure ($\Delta G = -41$ kcal/mol) (annealed, synthetic oligonucleotides: (a) GAAAAGCGCAGGTCGCGACCGCGCATGCGCGGTCGCGACCTGCGCTAAACTGCA and (b) GTTTAGCGCAGGTCGCGACCGCGCATGCGCGGTCGCGACCTGCGTTTTCTGCA) was cloned into the *Pst*I site in the *Renilla* gene to reduce cap-dependent translation [20]. Another stem loop ($\Delta G = -40$ kcal/mol) (AATTCAAAAGGCGAGGTCGCGAGCGCACATGTGCGCTCGCGACCTCGCCTAAAG and AATTCTTTAGGCGAGGTCGCGAGCGCACATGGCGCTCGCGACCTCGCCTTTTG) was cloned into an *Eco*RI site just downstream of the *Renilla* luciferase coding sequence to reduce ribosomal scanning and re-initiation. All sequences were verified by DNA sequence analysis.

2.3. Transient transfections and analysis

HeLa cells and RL Clone9 cells were seeded for transfection in six well plates 24 h prior to transfection. Cells (50–80% confluent) were incubated for 6 h with 6 μl of lipofectamine (Life Technologies, Gaithersburg, MD, USA) mixed with 1.5 μg of plasmid DNA (1 μg reporter/0.5 μg pCMVβ; Clonetech, Palo Alto, CA, USA) or 1 μg of bicistronic plasmid DNA in Opti-MEM (Life Technologies, Gaithersburg, MD, USA), and rescued in normal media containing 10% FBS. Neuro-2a and NIH 3T3 cells were transfected in 60 mm dishes at 50–60% confluence with 1 μg of plasmid DNA using calcium phosphate precipitation. For luciferase assays, cells were lysed after 48 h in reporter lysis buffer and assayed for luciferase in a liquid scintillation counter set on single photon mode. β-Galactosidase activity was determined in a spectrophotometer measuring absorbance at 420 nm as described in the Promega Luciferase Reporter Assay System (LRAS). Dual luciferase assays were performed on cells lysed after 48 h with passive lysis buffer and measured for *Renilla* and firefly luciferase activity as described in the Promega Dual-LRAS and measured on a Turner luminometer.

PII: S0014-5793(99)01699-3

3. Results

3.1. Exon 1 contains an element that influences expression of luciferase from a reporter construct

To determine what role, if any, exon 1 might have in the regulation of the Cx43 gene, two reporter gene constructs, pE0 and pE0Xdel, were created. The only difference between the two constructs is that most of exon 1 is deleted in pE0Xdel, save the first 7 bp, so that the natural transcription start is maintained (Fig. 1A). Each of these constructs was transfected into HeLa or RL Clone9 cells. All cells were cotransfected with pCMVβ, in which β-galactosidase is under control of the CMV promoter. Luciferase and β-galactosidase activities were measured 48 h later, and luciferase values were corrected for transfection efficiency as determined from the β-galactosidase values. In both cell types, luciferase expression from pE0 was found to be about three times higher than from pE0Xdel (Fig. 1B,C). These results suggest that there is an element in exon 1 that enhances the expression of the Cx43 gene. This element could be an effector of transcription, translation or mRNA stability.

3.2. The 5′-UTR of Cx43 has strong IRES activity

Intrigued by our recent discovery of an IRES element in the 5′-UTR of the nerve-specific mRNA of connexin32 [8], we suspected that the 5′-UTR of the Cx43 gene contained a similar element. We constructed a bicistronic reporter vector, pSL2, which contains two reporter genes under the control of the CMV promoter (Fig. 2A). The upstream cistron, the *Renilla* luciferase (*rluc*) gene, will be translated by normal cap-dependent translation. The second cistron, the firefly luciferase (*fluc*) gene, however, is normally not translated except for a small amount of illegitimate ribosomal scanning and re-initiation. Two stable stem loop structures were inserted into pSL2, one upstream of the *rluc* gene to reduce cap-mediated translation initiation, and another one between the two cistrons to minimize ribosomal re-initiation. The *fluc* coding sequence will be translated efficiently only if there is an IRES element inserted between the two cistrons. The ratio of firefly luciferase to *Renilla* luciferase is a measurement of IRES activity of any sequence inserted between the two cistrons.

To test if translation of the Cx43 gene can originate from an IRES element, we inserted the entire 5′-UTR of Cx43 between the two luciferase cistrons. The bicistronic vector was transiently transfected into four cell lines of different origins to discount possible cell line-specific effects. The fluc/rluc ratio was expressed as a multiple of the fluc/rluc ratio observed with the empty vector pSL2, which was set to one. All four cell lines exhibited a striking increase in the fluc/rluc ratio upon insertion of the 5′-UTR of Cx43 (Fig. 2B–E). HeLa cells showed the highest relative fluc/rluc ratio of 46 relative to that of the empty pSL2 vector. The change in the fluc/rluc ratio was solely due to an increase in IRES-mediated translation of firefly luciferase because the level of cap-dependent translation of *Renilla* luciferase was unaffected by insertion of the Cx43 IRES element. The EMCV IRES, used here as a positive control, had a fluc/rluc value of only 2.5, making the Cx43 IRES activity 18 times greater than that of EMCV.

To rule out the possibility that translation of the *fluc* gene originated from an mRNA that contained only the *fluc* cistron, either because of fragmentation of the bicistronic

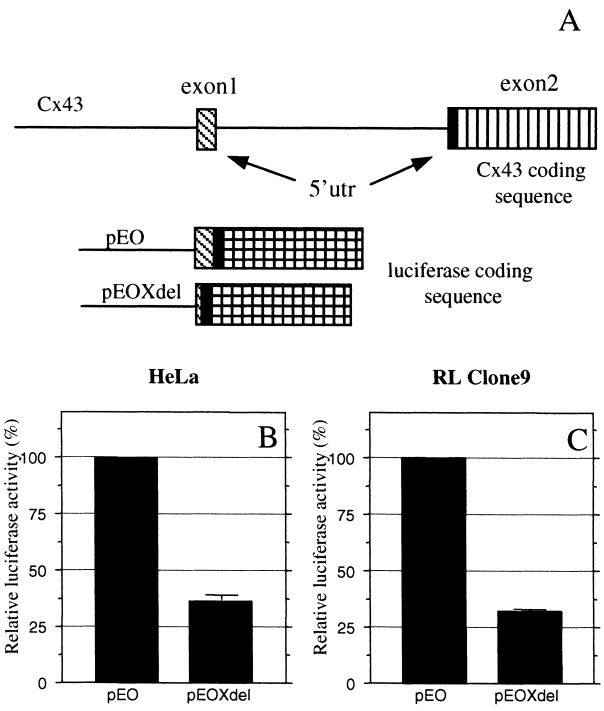

Fig. 1. The Cx43 5′-UTR stimulates luciferase expression from a Cx43 promoter-luciferase reporter construct. Construct pE0 includes exon 1, whereas pE0Xdel has all but the first 7 bp of exon 1 deleted (A). Both constructs retain the natural transcription and translation start sites of Cx43. HeLa cells (B) and RL Clone9 normal rat liver endothelial cells (C) were transiently transfected with the fusion constructs. Luciferase and β-galactosidase activities were measured 24 h later. Luciferase values are normalized for β-galactosidase activity to correct for transfection efficiency. Luciferase values from pE0Xdel are expressed in percentages of the pE0 values, arbitrarily set to 100%. S.E.M.s were determined from two independent experiments, each employing duplicate transfections.

mRNA or because of transcription from a promoter located in exon 1 of the Cx43 gene, Northern blot analysis was performed on total RNA extracted from HeLa cells transfected with p43SL2 or pSL2. The *fluc* coding sequence was used as hybridization probe. A single band of approximately 3 kb was detected in both cells transfected with p43SL2 or pSL2, and the bands from both transfections were of equal intensity (data not shown). The length of the bicistronic mRNA is 3.1 kb, whereas a fragment encoding only the fluc protein would have a maximum size of 1.7 kb. These data further support the notion that the increase of fluc activity from cells expressing 43SL2 is not caused by mRNA fragmentation but is due to IRES activity.

3.3. The 5′-UTR of Cx43 forms an extended stem loop structure

One of the hallmarks of RNA sequences that contain IRES elements is that they have a high degree of predicted secondary structure when analyzed by computer-aided folding models. While there does not seem to be any easily recognizable structure or sequence that is indicative of an IRES element, there are some consistencies. For example, it has been shown by computer modeling of several viral and cellular IRES elements that a Y-type structure followed by a stem loop is important for a functional IRES [9]. A poly-pyrimi-

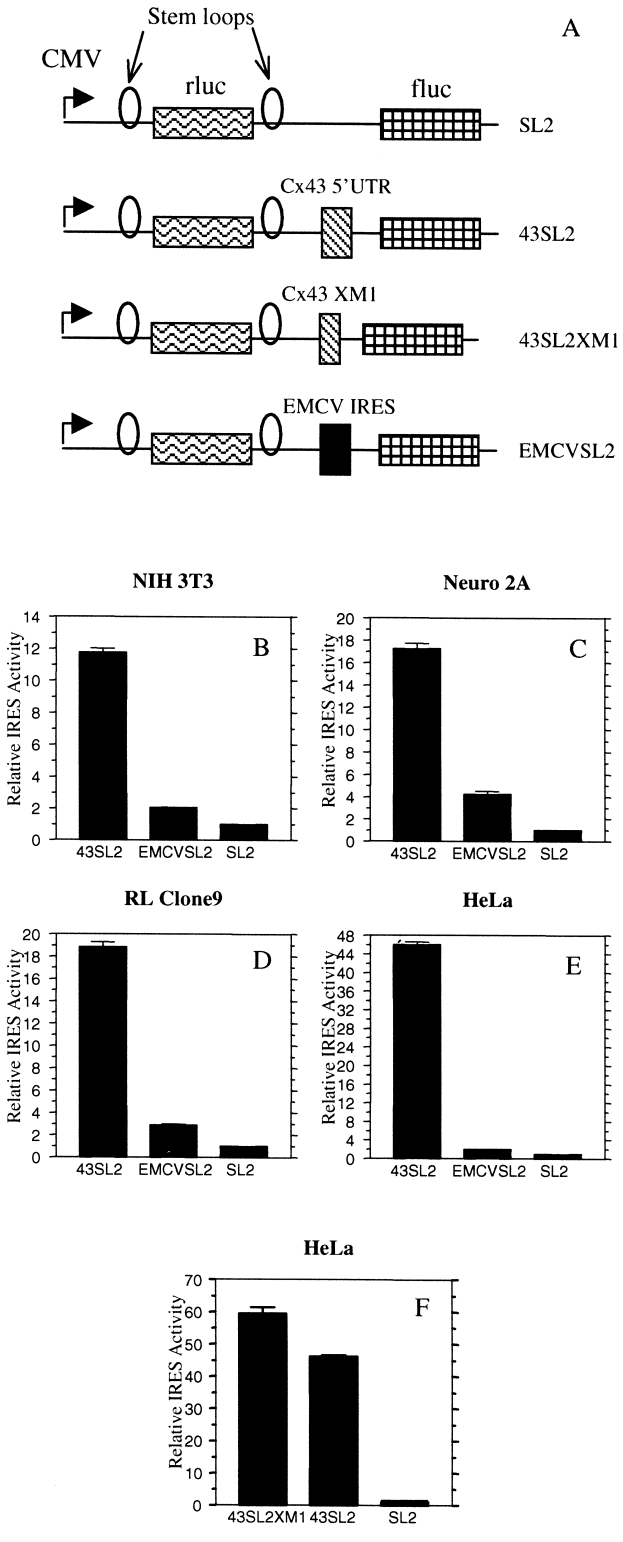

Fig. 2. The Cx43 5′-UTR is capable of mediating translational initiation from an IRES as defined by activity in a bicistronic vector assay. Bicistronic vectors were constructed with *Renilla* luciferase as the upstream cistron and firefly luciferase as the downstream cistron. Stable stem loops were inserted upstream and downstream of the rluc cistron to lower background levels (SL2). The Cx43 5′-UTR (p43SL2), Cx43 5′-UTR with 44 bp deleted off the 3′-end (p43SL2XM1), or the EMCV IRES element (pEMCVSL2) were inserted downstream of the second stem loop, between the two cistrons (A). NIH 3T3, Neuro-2a, RL Clone9 and HeLa cells were transiently transfected with the bicistronic vectors, and cells were prepared for measurement of rluc and fluc activity with the Promega Dual-LRAS 48 h later (B–F). Deletion of the 3′-end of the Cx43 5′-UTR does not abolish IRES activity (F). The bicistronic vector containing the 5′-UTR of Cx43 (43SL2) was mutated by deleting 44 bp off of the 3′-end of exon 1 to make 43SL2XM1. IRES activity is shown by an increase in the ratio of the activity of firefly luciferase to *Renilla* luciferase with respect to the control SL2. Error bars represent S.E.M. of three independent experiments, each employing triplicate transfections.
←

dine tract of varying lengths has also been suggested as being important in IRES function [10]. We used the MFOLD algorithm of Zuker [11] to model the full Cx43 5′-UTR (Fig. 3A). There is a notable Y-type structure from nucleotides (nt) 70 to 123 at the end of a long stem loop. Furthermore, two polypyrimidine tracts are present from nt 28 to 35 and nt 121 to 130. These consistencies with other described IRES elements, most notably the IRES located in the 5′-UTR of the nerve-specific connexin32 mRNA [8], provide further support for the existence of an IRES in the 5′-UTR of Cx43.

Various viral IRES elements have been characterized by deletion analysis. In many, 3′-deletions were found to abolish internal ribosomal entry [12–14]. This was interpreted as suggesting that important sequences or structures for function of an IRES are located close to the AUG. The 50 3′-terminal nt of the Cx43 5′-UTR form a large stem loop and may contribute to the function of the IRES (Fig. 3A). To determine if the 3′-sequences of the IRES, including this stem loop, are required for IRES function, we deleted 44 bases from the 3′-end of the 5′-UTR and inserted this 43SL2XM1 construct into the pSL2 bicistronic vector. The folding model for this sequence retains the Y-like structure but deletes the 3′-stem loop (Fig. 3B). Surprisingly, p43SL2XM1 had stronger IRES activity than p43SL2, suggesting that the 3′-stem loop and/or the sequences of the 3′-end of the IRES are not required for Cx43 IRES activity (Fig. 2F).

4. Discussion

It is widely accepted that initiation of protein synthesis in eukaryotes begins with the binding of the small ribosomal subunit to the 5′-cap structure. The 40S ribosome then scans the mRNA until it encounters an AUG codon where the 60S ribosomal subunit joins, and translation begins. Most cellular mRNAs contain fewer than 50 nt between the cap structure and the first AUG codon. The 5′-UTR of Cx43 mRNA, however, contains 208 nt and, in addition, has a stable secondary structure. Such structures can be inhibitory to the scanning of the 40S ribosome. In fact, we had previously reported that removal of most of its 5′-UTR made Cx43 mRNA translationally much more efficient when injected into *Xenopus* oocytes [15]. The present study, however, demonstrates that the 5′-UTR of Cx43 mRNA is required for efficient translation in transfected mammalian cells. Reporter gene constructs that retain the 5′-UTR have a significantly higher level of luciferase expression than constructs in which the exon is deleted. This is similar to the observation that the 5′-UTR of *c-myc* inhibits translation in a reticulocyte lysate in vitro translation system [16]. Like Cx43, *c-myc* 5′-UTR has no upstream AUGs (uAUGs) but contains extensive secondary structure [17,18]. In cultured cells, however, the 5′-UTR of *c-myc*

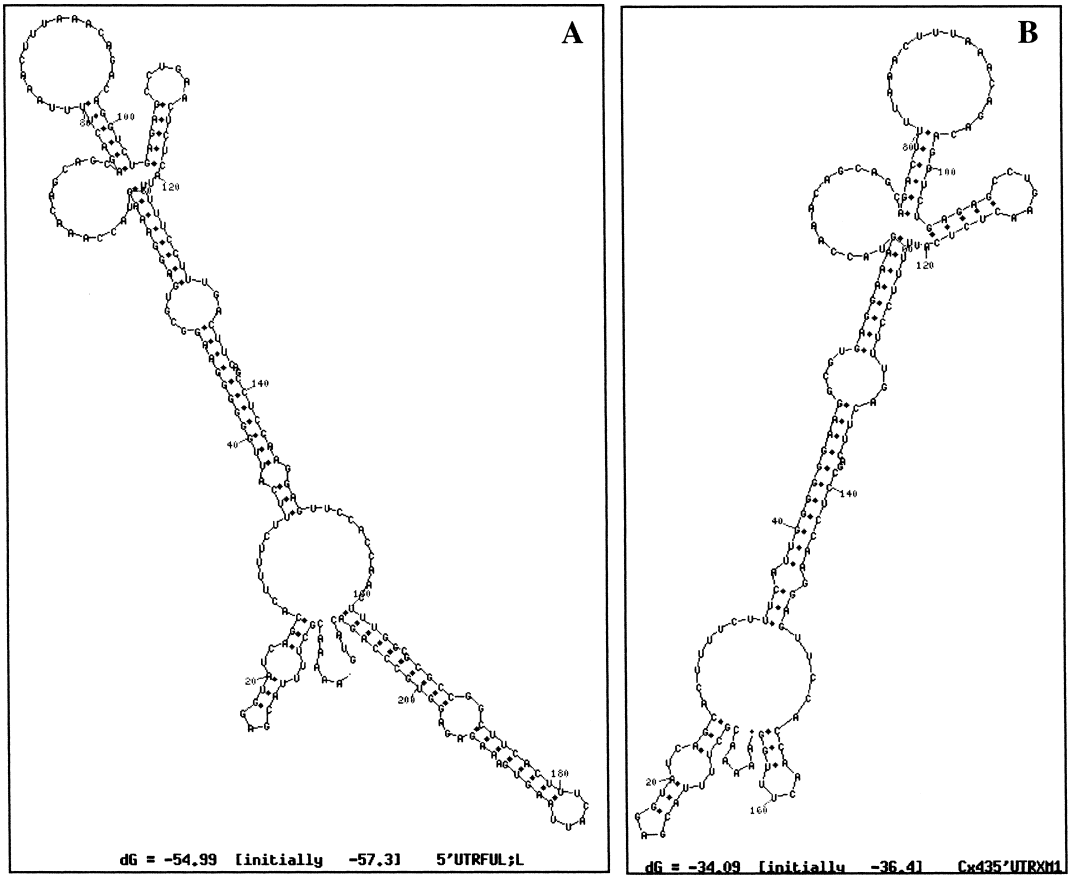

Fig. 3. The predicted secondary structure of the 5′-UTR of Cx43 forms an extended stable stem loop with a semi-conserved Y structure. A 44 base deletion from the 3′-end of the UTR retains function and maintains this structure. RNA folding patterns are predicted by the 'MFOLD' package by M. Zuker [11].

mRNA was shown to stimulate expression of a reporter gene. This was attributed to the presence of a functional IRES in the 5′-UTR which allowed additional translational initiation independently of a cap structure [19].

Analysis of the 5′-UTR of Cx43 mRNA in a bicistronic vector revealed that it also contains an active IRES element. One might ask whether this IRES is functional under normal cellular conditions. Our results suggest it is, because removal of exon 1 from the pE0 construct also reduces luciferase expression. Although we cannot rule out 5′-cap-mediated translation initiation, it seems likely that a large part of the translation of Cx43 mRNA occurs from the IRES because of the observed inefficient translation of Cx43 mRNA in oocytes. The factors that are required for IRES-mediated initiation of translation are likely to be present in cultured cells but absent from *Xenopus* oocytes. Northern blot analysis revealed that mRNA levels were identical in cells transfected with pSL2 or p43SL2. This makes it unlikely that exon 1 harbors a transcriptional enhancer that acts on the CMV promoter and thus might be responsible for the increased luciferase levels.

The predicted secondary structure of the Cx43 IRES element resembles that of other described IRES elements. It contains the semi-conserved Y-like structure described for other IRES elements, which is retained upon deletion of 44 bases from the 3′-end of exon 1 without loss of function. The fact that the IRES is still functional with its 3′-end deleted indicates that the element is located in the 5′-terminal 163 nt and that there are no sequences directly upstream of the AUG that are required for function of the IRES. While this is consistent with the *c-myc* IRES [19], it is in contrast with other IRES elements such as in the cardiac voltage-gated potassium channel Kv1.4 where the IRES element is located in the 3′-end of the 5′-UTR [20] and in various viral IRES elements where 3′-deletions ablate IRES function. In picornoviruses, the poly-pyrimidine tract binding protein (PTB) has been shown to be required for IRES function by binding to pyrimidine tracts in the IRES and that this element is proximal to the AUG [21]. Although the pyrimidine tracts of the Cx43 IRES and the picornoviruses do not share similar spacing, it does not rule out the possibility that PTB can act on this IRES as it does in the VEGF IRES [10].

Stress seems to be a common theme in the functioning of IRES elements in eukaryotic cells. One of the cellular responses to stress is inhibition of cap-dependent translation [22]. This would allow cells to continue synthesizing proteins essential for survival while stopping synthesis of non-essential proteins. There are examples of this. For instance, the chaperone proteins Bip [23] and hsp70 are translated under conditions of cellular stress in response to misfolded and degraded proteins, VEGF is translated in response to hypoxia [24], and FGF-2, a factor which functions in wound healing, is involved in the salvage of cells in the infarcted myocardium [25]. All of these genes exhibit the ability to be translated

under stress conditions, and IRES elements have been found in all of them. There is likely to be a need to maintain intercellular communication via Cx43 channels under certain stressful conditions. For example, in the hypoxic heart, gap junctional remodeling occurs [26] which requires the synthesis of new Cx43. In the uterus, it has been reported recently that mechanical stretch, in addition to estrogen, is required to upregulate expression of Cx43 at the onset of labor [2]. During the end phase of pregnancy, the fetus grows faster than the uterus causing physical stretch in the myometrium. Cx43 must be rapidly upregulated during this time, and the IRES may offer a mechanism by which a high level of translation can be achieved during this stress.

Acknowledgements: This study was supported by a Grant from the National Institute of Health (HD34152). DNA synthesis and sequence analysis was subsidized by the Sylvester Comprehensive Cancer Center through their DNA Core Facility.

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