Structurally Distinct Elements Mediate Internal Ribosome Entry within the 5' Noncoding Region of a Voltage-gated Potassium Channel mRNA

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SUMMARY

The ~1.2 kb 5′ noncoding region of mRNA species encoding mouse Kv1.4, a member of the Shaker-related subfamily of voltage-gated potassium channels, was shown to mediate internal ribosome entry in cells derived from brain, heart, and skeletal muscle, tissues known to express Kv1.4 mRNA species. We also showed that the upstream ~1.0 kb and the downstream ~0.2 kb of the Kv1.4 5′ NCR independently mediate internal ribosome entry; however, separately, these sequences were less efficient in mediating internal ribosome entry than when together in the complete (and contiguous) 5′ NCR. Using enzymatic structure probing, the 3′-most ~0.2 kb was predicted to form three distinct stem-loop structures (stem-loops X, Y, and Z) and two defined single-stranded regions (Loops Ψ and Ω) in the presence and absence of the upstream ~1.0 kb. Although the systematic deletion of sequences within the 3′-most ~0.2 kb resulted in distinct changes in expression, enzymatic structure probing indicated that local RNA folding was not completely altered. Structure probing analysis strongly suggested an interaction between stem-loop X and a downstream polypyrimidine tract; however, opposing changes in activity were observed when sequences within these two regions were independently deleted. Moreover, deletions correlating with positive as well as negative changes in expression altered RNase cleavage within stem-loop X, indicating that this structure may be an integral element. Therefore, these findings indicate that Kv1.4 expression is mediated through a complex interplay between many distinct RNA regions.
INTRODUCTION

Potassium channels are ubiquitously expressed in nature, facilitating diverse cellular processes in response to intra- and extra-cellular changes (1). Voltage-gated potassium (Kv) channels modulate action potentials in electrically excitable cells and facilitate cell proliferation, cell volume regulation, and secretion in non-electrically excitable cells. Kv channel genes encode a single α-subunit which forms potassium-selective pores through the assembly of tetramers. Physiological stimulation during stress regulates transcription through the release of hormones and neurotransmitters (2). Similar changes are also observed in response to pharmacological agents and with the onset of pathological conditions. Post-translational modifications, interactions with auxiliary β-subunits, and heterotetramerization between subfamily members may alter channel properties, promote cell surface expression, and/or modify channel localization (1,3).

Many mammalian Kv channel mRNA species contain extensive 5′ and 3′ noncoding regions (NCRs) (4). The noncoding regions of many eukaryotic mRNAs have been shown to regulate gene expression by affecting mRNA localization, mRNA stability, and translation. Although the roles of the 5′ and 3′ NCRs in Kv channel expression have yet to be investigated in detail, the presence of unusually long 5′ NCRs in this gene family raised the possibility that such sequences may mediate and, perhaps, modulate translation.

Kv1.4, a member of the Shaker-related subfamily of voltage-gated potassium channels, forms rapidly activating and inactivating A-type channels (5-7) and has been determined to have cell-type and species-specific patterns of expression (8-10). Two distinct Kv1.4 mRNA species, ~3.5 and ~4.5 kb in length, are expressed in mouse brain and cardiac tissue (11). The ~1.2 kb 5′ NCR, common to both mRNA species, contains eighteen upstream AUGs and a distinct
polypyrmidine tract (4). Dicistronic assays in cell culture and in vitro indicated that the 5’ NCR of mKv1.4 mRNAs mediates internal ribosome entry (4).

Translation initiation of most eukaryotic mRNAs is mediated through the cap- and end-dependent mechanism of ribosome scanning (12). However, atypical features within the 5’ NCR, including extensive length, stable secondary structure, and multiple upstream initiation codons, have been shown to inhibit ribosome scanning (13). Additionally, cellular conditions unfavorable to conventional cellular protein synthesis necessitate the existence of alternative mechanisms of translation initiation.

Internal ribosome entry, initially described for two small positive-strand RNA viruses (14,15), has since been identified in other positive-strand RNA viruses (16) as well as gammaherpesviruses (17-19). Since the identification of the first cellular internal ribosome entry site (IRES) (20), cellular IRES elements have been shown to facilitate protein expression when cap-dependent translation is down-regulated during picornaviral infection (21,22), mitosis (G2/M phase) (23,24), apoptosis (25,26), differentiation (27), and cellular stress (28-34). The study of cellular IRES elements has revealed the significance of internal ribosome entry in cellular gene expression and has underscored important aspects in the analysis of IRES elements (35).

In this paper, two different subsets of mKv1.4 5’ NCR sequences are shown to independently mediate internal ribosome entry. These sequences demonstrated cell-type specific activities distinct from those mediated by a viral IRES. RNA structure probing indicates that the 3’-most ~ 0.2 kb forms defined structural elements in the presence and absence of the upstream ~ 1.0 kb. Deletion of sequences within the 3’ ~ 0.2 kb resulted in distinct changes in 0.2- and 1.2-mediated expression, initially indicating that specific RNA elements may positively or negatively
affect expression. Secondary structure probing suggested that local RNA folding is partially conserved with the introduction of various deletions within the 3′ ~ 0.2 kb. Therefore, these findings suggest that interactions between functionally/structurally distinct RNA elements collectively promote internal ribosome entry.
Materials and Methods

Cell culture

HeLa cells were cultured in minimal essential media (MEM) supplemented with 8% newborn calf serum, 1% nonessential amino acids, 2 mM glutamine, and 1% antibiotics/antimycotics. Human neuroblastoma cell lines, SK-N-SH and NLF, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% antibiotics/antimycotics. African green monkey kidney cell lines, CV1 and COS, and a mouse muscle myoblast cell line, C2F3, were cultured in DMEM supplemented with 10% FBS and antibiotics/antimycotics. Primary rat cardiac myocytes (PrCM) (36) were cultured in DMEM supplemented with 10-20% FBS and 1% antibiotics/antimycotics. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. All cell culture reagents were obtained from GibcoBRL.

Cloning of plasmid constructs and PCR deletion mutagenesis

The monocistronic firefly luciferase reporter construct, p1209luc, was constructed by inserting mKv1.4 5′ NCR cDNA sequences into pGEMluc (Leong and Semler, unpublished). mKv1.4 5′ NCR cDNA sequences, excised from the CAT/LUC dicistronic construct (4) with Hind III and Nco I, lack the 5′ terminal thirteen nucleotides. p229luc was subsequently generated by removing the 5′ ~ 1.0 kb from p1209luc using Hind III and Xba I, filling in 5′ overhangs with Klenow fragment, and ligating the resultant blunt-ended plasmid vector with T4 DNA ligase. The 3′ ~ 0.2 kb 5′ NCR cDNA sequence within p1209luc and p229luc differed from the original published sequence by two nucleotides [nucleotide 1196 (C) was deleted and nucleotide 1197 was an A instead of an C].
Deletions within the 3′-most ~ 0.2 kb of mKv1.4 5′ NCR were created using a PCR-based deletion mutagenesis scheme previously described by Imai et al. (37). Briefly, divergent, non-overlapping oligonucleotides flanking targeted sequences were used to PCR amplify p229luc or p1209luc with thermophilic DNA polymerases, Deep Vent® or PfuTurbo (Stratagene). Plasmid template was removed by Dpn I digestion, leaving PCR-amplified DNA fragments which were gel purified, phosphorylated with T4 polynucleotide kinase, and transformed into competent XL1-Blue bacteria. Deletions were verified by restriction digest and sequence analysis.

The following oligonucleotides were utilized for deletion mutagenesis: 1703N (5′-ACCACCATGGAAGACGCCAA-3′), 1732N (5′-ACAACGTGGAAGCAGCCATT-3′), 1740N (5′-CTTCTCTTACAACTGGAAGC-3′), 1880N (5′-TAAGGCTTCCAAAATCTACCT-3′), 1840N (5′-GTTGGACTGAAATATCCCA-3′), 1880N (5′-GGAGCATAGGCTGTGCTGAT-3′), 1740X (5′-AAGAAAGAAAAATAGGGCAG-3′), 1758X (5′-CAGCTTATTTTCTTACCAAA-3′), 1780X (5′-AAGGTAAGTTTGGAAGCCTT-3′), 1830X (5′-TTCAGTCCAACTTGCATTT-3′), 1880X (5′-CCTCTTCTCAGAGACTCGGC-3′), 1910X (5′-AGAGCTTGCCTCGTCTAGAG-3′), 1920X (5′-TCGTCTAGAGCTTGTCTCCC-3′).

Deletions at the 3′ end of 0.2 (3′∆), Δ1′, ΔpY, Δ1, Δ2, and Δ3, were generated using oligonucleotide pairs 1703N/1740X, 1732N/1758X, 1703N/1780X, 1740N/1780X, and 1740N/1830X, respectively. Deletions at the 5′ end of 0.2 (5′∆), Δ4, Δ5, Δ6, Δ7, Δ8, and Δ9, were generated using oligonucleotide pairs 1800N/1830X, 1800N/1880X, 1840N/1880X, 1840N/1910X, 1880N/1910X, and 1880N/1910X, respectively. Δ10 and Δ11 were generated with oligonucleotide pairs 1740N/1880X and 1740N/1910X, respectively.

**Dual luciferase dicistronic constructs**
A dual luciferase plasmid construct, p2luci [a gift from Dr. R. F. Gesteland, University of Utah; (38)], was used to generate dicistronic constructs. Firefly luciferase coding region sequences were excised from pGL3 (Promega) using Nco I and Xba I and ligated with the vector fragment of p2luci, which was digested with Bam HI, treated with Klenow fragment, and digested with Xba I, resulting in p2luci-fx. p2luci-fx was linearized with Sal I, treated with Klenow fragment, and religated to generate pR2TAAF, which encodes two tandem stop codons in-frame with the Renilla luciferase coding region. pRstF was subsequently generated by inserting an inverted repeat between Renilla and firefly luciferase coding region sequences within pR2TAAF at the Bgl II restriction site using two ~ 50 bp fragments excised from the multiple cloning site of pGEM-4z with Bam HI to Sph I (Promega). Plasmids were examined by restriction digestion.

The full ~ 1.2 kb mKv1.4 5′ NCR was excised from p1209luc with Hind III and Nco I, while the 3′ ~ 0.2 kb and the 5′ ~ 1.0 kb were excised with Xba I and Nco I and Hind III and Xba I, respectively, and treated with Klenow fragment. Similarly, 3′ ~ 0.2 kb fragments containing deletions (0.2Δ) were excised from appropriate p229Δluc or p1209Δluc constructs using Xba I and Nco I. Fragments were treated with Klenow fragment or T4 DNA Polymerase and inserted into Eco47 III- or Afe I-linearized pRstF plasmid which had been treated with calf intestinal alkaline phosphatase (CIAP). 1.2 kb 5′ NCR deletion sequences (1.2Δ) were similarly inserted into pRstF using a three-fragment ligation. The 5′ ~ 1.0 kb was excised from p1209luc with Hind III and Xba I, while 3′ ~ 0.2 kb fragments containing deletions were excised from appropriate p229Δluc constructs using Xba I and Nco I. Only the Hind III and Nco I overhangs were filled in with T4 DNA polymerase, allowing cohesive Xba I ends to preferentially ligate. Plasmids were verified by restriction digest and sequence analysis.
pRstCVB3F was generated by inserting the 5′ NCR of coxsackievirus B3 into p2luci-fxnk1, a dicistronic construct containing a hairpin and additional intercistronic sequences. p2luci-fx was linearized with Not I, treated with Klenow fragment, and subsequently religated with T4 DNA ligase to generate p2luci-fxnk. p2luci-fxnk1 was generated by inserting a 148 bp fragment, excised from pTrap +1 (Leong and Semler, unpublished) with Stu I and Apa I, between the blunted Sal I and Apa I sites of p2luci-fxnk, introducing a hairpin previously described by Vagner et al. (39) and additional restriction sites, including Pac I and Not I. pT7CVB+1 was generated by inserting the 5′ NCR of CVB3 into pTrap +1 with restriction sites Pac I and Not I. The 5′ NCR of CVB3 was PCR amplified from pCVB3-0 (40) with CVB3 Pac I (+) (5′-CCTTAATTAATTTAAACAGCCTGTGTTGTTGA-3′) and CVB3 Not I (-) (5′-ATAAGATATGCAGCCGCTCCCATTTTGCCTGATTCAACTTA-3′). CVB3 5′ NCR sequences were removed from pT7CVB+1 using Pac I and Not I and inserted into p2luci-fxnk to generate p2luci-fxnkC1. Finally, p2luci-fxnkC1 was linearized with Bam HI, treated with Klenow fragment, and religated to generate pRstCVB3F.

Dicistronic constructs lacking the SV40 promoter, the chimeric intron, and the T7 promoter were generated by two- or three-fragment ligations. The vector backbone of pRstF was isolated by digesting the plasmid with Nco I and Not I to remove sequences encompassing the SV40 promoter to the end of firefly luciferase. The vector was ligated with the Nhe I-Not I fragment isolated from pRstF, pRst1.2F, pRst0.2F, and pRst1.0F or two fragments from pRstCVB3F, generated by digestion with Nhe I, Pac I, and Xba I. Additionally, following restriction digestion with Nco I and Nhe I, reactions were incubated with T4 DNA polymerase to fill-in 3′ overhangs.

Unless noted, enzymes were obtained from New England BioLabs.
**In vitro transcription**

Dicistronic constructs were digested with Not I or Xba I and p229/229Δluc constructs were digested with Bst BI. Linearized plasmids were subjected to phenol/chloroform extraction and ethanol precipitation. In vitro transcription reactions were performed using 1.0 µg of linearized template and incubated at 37°C for four hours (Ambion). Reactions were subsequently treated with DNase I at 37°C for 30 minutes, subjected to phenol/chloroform extraction, and isolated using RNeasy columns (Qiagen).

**Transient transfection**

Cells were seeded 24 to 48 hours before transfection, generating monolayers with 50-80% confluency. 35-mm or 22-mm well plates and 100-mm dishes were used for luciferase assays and total RNA isolation, respectively. Primary neonatal rat cardiac myocytes (PrCM), seeded into 35-mm or 22-mm well plates, were allowed to grow for 3 days before transfection. A 1:3 ratio of plasmid (µg) to FuGENE 6 (µl) [Roche Molecular Biochemicals] was complexed in Opti-MEM (Invitrogen) at room temperature for ≥ 15 minutes. Monolayers were initially incubated with transfection complexes for 5 hours, rinsed with media, and incubated for an additional 19 hours after the addition of fresh media. The CMV β-Gal reporter construct used in experiments presented in Figure 2A was generously provided by Judy Jimenez and Dr. Marian L. Waterman (University of California, Irvine).

For transient RNA transfections, cells were seeded into 35-mm well plates ≥ 24 hours prior to transfection, generating monolayers with 80-90% confluency. Two micrograms of uncapped, in vitro transcribed RNA was transfected per well. RNA was initially incubated with Buffer EC-R and Enhancer R [1:2 ratio of RNA (µg) to Enhancer R (µl)] at room temperature for 5 minutes. A 6:1 ratio of Transmessenger transfection reagent (µl) (Qiagen) to
RNA (µg) was added and transfection complexes were incubated at room temperature for 10 minutes and combined with Opti-MEM. Monolayers were rinsed twice with 2.0 ml Opti-MEM prior to the addition of transfection complexes and incubated for 1 hour at 37°C. Cells were subsequently rinsed with DMEM and incubated with serum containing media for an additional 14 hours.

**Dual luciferase assays**

Monolayers were washed with 1X phosphate buffer saline and lysed with 1X Passive Lysis Buffer (Promega) ~ 24 hours post-transfection. Cell lysates were subjected to two freeze/thaw cycles at -70°C and 30°C, respectively, and assayed for luciferase activities using the Dual Luciferase Reporter Assay System (Promega). Firefly and *Renilla* luciferase activities were sequentially assessed by initially adding lysate to 100 µl firefly luciferase substrate, measuring luminescence for 10 seconds, adding 100 µl *Renilla* luciferase substrate to the same sample, and measuring luminescence for an additional 10 seconds. Assays were performed using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) or a SIRIUS luminometer (Berthold Detection Systems). Transfections were performed in triplicate. Fluc/Rluc or Fluc/β-Gal ratios were calculated for each sample and the average value is represented as fold over background, expression measured in the absence of insert. Dicistronic assays were independently performed at least twice in all cells/cell lines used.
Isolation of total RNA and oligo-dT selection

Total RNA was isolated with TRI Reagent (Molecular Research Center, Inc.) and subjected to oligo-dT selection using the Micro-FastTrack mRNA isolation kit (Invitrogen) and DNase I digestion (Worthington Biochemical Corporation). Following DNase I digestion, oligo-dT selected RNAs were subjected to phenol/chloroform extraction, ethanol precipitation, and subsequently resuspended in DEPC-treated H₂O.

Northern blot analysis

Two micrograms oligo-dT selected RNA were denatured with glyoxal/DMSO (1 M deionized glyoxal, 50% v/v deionized DMSO, 25 mM sodium phosphate) at 50°C for 60 minutes and separated on a 1.0% 10 mM sodium phosphate agarose gel with continuous buffer circulation at 5 V/cm (~100V). RNA was transferred to a GeneScreen Plus hybridization transfer membrane (NEN Life Science Products) by upward capillary transfer with 20X SSC for 20-24 hours. Membranes were briefly rinsed with 2X SSC, UV-irradiated, incubated in 20 mM Tris, pH 8.0 for ~30 min at room temperature, prehybridized (10% w/v dextran sulfate, 0.3 M NaCl, 1% SDS) at 65°C for 3 hours, and hybridized with random hexamer primed ³²P-labeled probes against firefly luciferase coding region sequences in prehybridization buffer at 55°C, overnight. Following hybridization, membranes were initially washed with 2X SSC, 0.1% SDS at ambient temperature and subsequently washed with 0.2X SSC, 0.1% SDS at ambient temperature, 37°C, and 42°C and exposed to a phosphorimager screen. The membrane was subsequently reprobed for β-actin to determine relative RNA loading. Images were quantified using Quantity One version 4.3.0 (BioRad).
Random primer-labeling of DNA fragments and 5’ end-labeling of oligonucleotides

Random hexamer priming reactions were performed based on methods previously described (41,42). A ~ 1.7 kb fragment encoding the firefly luciferase coding region was excised from pRstF using Nco I and Not I and a ~ 1.8 kb fragment excised from a plasmid containing human β-actin cDNA sequences (43). Gel purified DNA fragments were denatured and incubated with random hexamer primer (New England BioLabs), [α-32P]dATP, Klenow fragment at 37°C for 30 minutes. Unincorporated [α-32P]dATP was removed using CHROMA SPIN-30 DEPC-H2O columns (Clontech).

Nine pmoles (~ 60 ng) of oligonucleotide [Fluc (5’-TCCATCTTTCCAGCGGATAGA-3’), 1740X, or 1780X] were incubated in 70 mM Tris-HCl (pH 7.6), 10 mM MgCl2, and 5 mM DTT with 50 µCi 6000 Ci/mmol [γ-32P]ATP and 20 units T4 polynucleotide kinase in a final volume of 50 µl for 60 minutes at 37°C. Unincorporated [γ-32P]ATP was removed using CHROMA SPIN-10 TE columns (Clontech).

Enzymatic structure probing by primer extension

RNA structure probing was carried out as a modification of methods described previously (44-46). In vitro transcribed RNA (0.5 µg) was combined with 40 µg yeast tRNA in 0.7x TMK buffer (30 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 270 mM KCl) under reducing conditions (18 mM 2-mercaptoethanol) in a final volume of 40 µl. RNAs were sequentially incubated at 68°C and 37°C for 5 minutes each, and then at room temperature for 5-10 minutes before the addition of RNase V1 (Pierce) or RNases T1, PhyM, or CL3 (Industrial Research Limited) at room temperature for 5 to 20 minutes (as described in figure legends). Reactions were halted by the addition of 160 µl stop solution (final concentrations: 0.3 M NaOAc, 10 mM EDTA, 0.3% SDS), subjected to phenol/chloroform extraction, and ethanol precipitated in the presence of glycogen.
RNA pellets were initially resuspended in a final volume of 15.5 µl with 10^6 cpm 32P-5'-end labeled oligonucleotide and sequentially incubated at 70°C and on ice for 10 minutes each. RNAs were incubated at 42°C for 40-60 minutes with 10 units AMV reverse transcriptase (Life Sciences) in 25 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM DTT, 5 mM MgCl₂, 1 mM each dNTP, and 40 units RNasin (Promega). After the addition of 180 µl stop solution (final concentration 0.3 M NaOAc, 10 mM EDTA, 0.3% SDS), reactions were subjected to phenol/chloroform extraction and ethanol precipitation with glycogen. Samples were resuspended in 7.2 µl Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 µg/µl RNase A and incubated at 37°C for 30 minutes. Finally, 4.8 µl formamide loading buffer was added for a final volume of 12 µl. 2.5-3.0 µl of each sample was resolved on an 8% polyacrylamide-7 M urea gel with appropriate sequencing ladders.

**Secondary structure predictions**

Secondary structure predictions were generated for mKv1.4 5' NCR sequences using *mfold* (version 3.1) by Zuker and Turner with the latest energy parameters under default conditions (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) (47,48).
Results

The 5′ NCR of mKv1.4 mRNA species mediates internal ribosome entry in HeLa cells.

The ~1.2 kb mKv1.4 5′ NCR (1.2), previously reported to mediate internal ribosome entry (4), the 3′ most ~0.2 kb (0.2), and the 5′ most ~1.0 kb (1.0) were examined for IRES activity in HeLa cells utilizing a dual luciferase dicistronic construct (Figure 1A). The 3′ ~0.2 kb sequence described in this paper contains the 3′ most 208 nucleotides originally examined (4) as well as an additional upstream twenty two nucleotides. The 5′ NCR of coxsackievirus B3 (CVB3), encoding a Type 1 IRES element (49,50), was employed as a positive control. Following transient DNA transfection, the full mKv1.4 5′ NCR, 1.2, mediated the highest level of expression, ~174-fold above background, while 0.2 and 1.0 independently exhibited substantial (albeit lower) levels of expression, ~40-fold over background (Figure 1B). The CVB3 5′ NCR mediated intermediate levels of expression, ~90-fold above background (Figure 1C). The ability of the 3′-most ~0.2 kb as well as the 5′-most ~1.0 kb to separately promote internal initiation indicates a substantial level of complexity involved in IRES-mediated translation for this transcript.

Full-length dicistronic RNAs were detected 14 hours post transfection in transiently transfected HeLa cells using Northern blot analysis (Figure 1D, lanes 2-6). However, a smaller species, ~2.0 kb (**), was detected when 1.2 or 0.2 were encoded within the intercistronic sequence (ICS) (Figure 1D, lanes 4 and 5), while two additional species, ~3.0 kb (*) and ~2.2 kb (**), were detected with CVB3 (Figure 1D, lane 2). The intensities of the smaller RNA species were ~10-20% of their respective dicistronic RNAs for CVB3 and 1.2 and 60-75% for 0.2 (Figure 1D, lanes 2, 4, and 5). The presence of smaller RNA species suggested the involvement of cryptic promoter activity or RNA splicing. When the SV40 promoter was
removed from dicistronic constructs, firefly luciferase (Fluc) expression (normalized to β-Gal activity from a co-transfected reporter plasmid) was similar to background in HeLa cells (Figure 2A) as well as CV1 and SK-N-SH cells (data not shown), indicating that mKv1.4 5′ NCR cDNA sequences do not mediate cryptic promoter activity. The smallest RNA species observed (Figure 1D, lanes 2, 4, and 5) lacked the majority of Renilla luciferase (Rluc) sequences (data not shown) and may possibly represent a splicing product between the donor site of the chimeric intron and a cryptic acceptor site within the 3′ ~ 0.2 kb of mKv1.4 5′ NCR. When the chimeric intron was removed from dicistronic constructs, Fluc expression, although decreased overall, was still 8- to 24-fold over background for 1.2, 0.2, and 1.0 (data not shown).

To eliminate possible contributions to Fluc expression by splicing products, in vitro transcribed dicistronic RNAs were transfected directly into HeLa cells (Figure 2B). Both 1.2 and 1.0 demonstrated expression 20- and 14-fold over background, unequivocally demonstrating IRES activity. The 0.2 IRES construct generated lower levels of Fluc activity compared with dicistronic RNAs encoding 1.2 or 1.0, ~ 2.5-fold above background (Figure 2B). Similar results were observed in CV1 and COS cells (data not shown). Dicistronic transcripts were detected 5 and 15 hours post transfection (Figure 2C). Although the levels of RNA decreased over time, smaller RNA species were not detected, indicating that these RNAs are not subject to specific cleavage.

The 5′ NCR of mKv1.4 mRNA differentially mediates IRES activity in cell culture.

mKv1.4 mRNA species are natively expressed in brain, heart, and skeletal muscle (11,51); therefore, it was of particular interest to examine IRES activity in cells/cell lines derived from these tissues. IRES activity was assessed in two human neuroblastoma cells lines, SK-N-SH and NLF, primary neonatal rat cardiac myocytes (PrCM), a mouse muscle myoblast cell line, C2F3,
as well as two African green monkey kidney cell lines, CV1 and COS. The results parallel those observed in HeLa cells with 1.2 mediating the highest level of expression and 0.2 and 1.0 independently mediating lower levels of expression (Figure 1B). In NLF, C2F3, and COS cells, the levels of expression mediated by 0.2 or 1.0 were $\leq 2$-fold over background, indicating the lack of significant IRES activity (Figure 1B). mKv1.4 5' NCR sequences, 1.2, 0.2, and 1.0, mediated higher levels of expression in HeLa and CV1 compared with the human neuroblastoma cells lines (Figure 1B), while CVB3 promoted similar levels of expression in all four cell lines (Figure 1B). In transient RNA transfections, 1.2-mediated expression was ~ 20-fold above background in HeLa and CV1 cells, but only ~ 13-fold in COS cells (Figure 2B and data not shown). In contrast, CVB3 promoted expression > 200-fold over background in these three cell lines (Figure 2B and data not shown). This suggests that mKv1.4 5' NCR sequences mediate cell-type dependent IRES activities distinct from CVB3 5' NCR sequences.

**Deletion analysis of the 3' most ~ 0.2 kb encoded within mKv1.4 5' NCR.**

To investigate the importance of specific RNA elements, overlapping deletions within the 3' ~ 0.2 kb were generated in the context of 0.2 (0.2$\Delta$) and 1.2 (1.2$\Delta$) (Figure 3A). Deletion-containing sequences (0.2/1.2$\Delta 1'$-$\Delta 11$) were examined for IRES activity in HeLa cells (Figure 3B) and subsequently in PrCM and SK-N-SH cells (Figure 3C and data not shown).

3' deletions ($\Delta 1'$, $\Delta pY$, $\Delta 1$, $\Delta 2$, and $\Delta 3$) which partially or completely remove a twenty nucleotide polypyrimidine tract (pY) decreased 0.2 expression 12.5- to 50-fold (Figure 3B, left panel) and 1.2 expression 11- to 100-fold in HeLa cells. (Figure 3B, right panel). The effect observed in 1.2 is surprising since 1.0 independently demonstrated IRES activity (Figure 1B and 2B). In contrast, 5' deletions ($\Delta 4$, $\Delta 5$, $\Delta 6$, $\Delta 7$, and $\Delta 8$) increased 0.2 expression $\geq 2.3$- to 3.9-fold (Figure 3B, left panel), but had little effect on 1.2 expression in HeLa cells (Figure 3B, right panel).
Interestingly, \( \Delta 9 \), which differs from \( \Delta 8 \) only by the deletion of an additional 10 upstream nucleotides, slightly decreased 0.2 expression. The most extreme deletions, \( \Delta 10 \) and \( \Delta 11 \), containing only \( \sim 98 \) and \( \sim 68 \) nucleotides of the original \( 3' \sim 0.2 \) kb, decreased the signal generated by 0.2 to background levels of expression (Figure 3B, left panel), while 1.2, closer to the \( 5' \sim 1.0 \) kb construct in sequence with these deletions, mediated levels of expression similar to 1.0 (Figures 3B, right panel). In PrCM, \( 5' \) deletions increased 0.2 expression \( \geq 3 \) to 4-fold, equal to or greater than wild type 1.2 (Figure 3C, left panel), while deletions introduced into 1.2 minimally affected expression (Figure 3C, right panel). Deletion of the polypyrimidine tract also decreased 0.2- and 1.2-mediated expression to background levels in PrCM (Figure 3C). Similar results were observed in SK-N-SH cells (data not shown).

**Secondary structure analysis of mKv1.4 5' NCR sequences**

To investigate the relationship between RNA structure and IRES function, enzymatic structure probing and the RNA folding algorithm *mfold* were used to examine mKv1.4 5' NCR sequences. *In vitro* transcribed RNAs were subjected to partial digestion using RNases with specificities for paired (RNase V1) and unpaired (RNases T1, PhyM, and CL3) nucleotides. Nucleotide-specific cleavage was detected by primer extension and subsequent analysis on a sequencing gel. Since RNA cleavage occurs 3' of the nucleotide, reverse transcribed products are one nucleotide shorter than the corresponding fragment on the DNA sequencing ladder.

The 3' terminal 200 nucleotides of mKv1.4 5' NCR had previously been predicted to form an extended helix flanked by three distinct stem-loop structures (4). Using constraints determined by enzymatic structuring probing (Figure 4), 0.2 was predicted to form the structure shown in Figure 5A. Although encompassing additional upstream nucleotides, the structure for
0.2 includes elements in common with those previously predicted for the 3′ terminal 200 nucleotides.

The polypyrimidine tract (nucleotides 1154-1173) had been predicted to participate in the formation of an extended helical structural (4). Nucleotides 1162, 1165, 1167, 1168, and 1170 were susceptible to RNase V₁ cleavage (Figure 4D, lanes 4 and 5), correlating with base pair formation; however, nucleotides 1165-1170 were positioned within a stem-loop, not an extended helix as previously predicted (Figure 5A). Strong RNase PhyM and/or CL3 cleavage at nucleotides 1173 and 1175 indicated the presence of unpaired residues (Figure 4D, lanes 7-10). These data are consistent with the formation of a stem-loop (designated stem-loop Z, nucleotides 1165-1186) within the 3′ end of the polypyrimidine tract (Figure 5A).

Nucleotides 1155-1157 within the 5′ end of the polypyrimidine tract were sensitive to RNase PhyM digestion (Figure 4D, lanes 7 and 8). Surrounding nucleotides, 1150 and 1162, were sensitive to RNase V₁, while nucleotide 1158 was susceptible to both RNase V₁ and PhyM (Figure 4C-D, lanes 4, 5, 7, and 8). Upstream nucleotides 989 and 990, predicted to base pair with nucleotides 1147 and 1148, were susceptible to RNase V₁ (Figure 4A, lanes 4 and 5). The data suggest the presence of a single-stranded U-rich loop (nucleotides 1151-1157) linking two regions of helical structure.

Of the three stem-loop structures which had been previously predicted by Negulescu et al. to form within the 3′ 200 nucleotides (4), the two stem-loop structures positioned upstream of the polypyrimidine tract were corroborated by structure probing: the proximal stem-loop (nucleotides 1119-1137) and the distal stem-loop structure (nucleotides 1048-1106); however, the latter structure was only partially corroborated. Strong RNase V1 cleavage at nucleotides 1119 and 1120 and weaker cleavage at adjacent nucleotides (1121-1123) and on the
complementary strand (nucleotides 1133-1135) were consistent with stem formation of the proximal stem-loop structure (Figure 4C, lanes 4 and 5), while strong RNase PhyM cleavage at nucleotides 1125-1129 was indicative of a highly exposed single-stranded region, consistent with the loop of the proximal stem-loop structure (Figure 4C, lanes 7 and 8). The intensity of the bands suggested that this stem-loop (Figure 5A, now designated as stem-loop Y) is highly exposed, perhaps forming a putative protein binding site. Identical RNase cleavage patterns were also observed for 1.2 as well as 1.2Δ(pY and 4) and 0.2Δ(pY, 4, 6, 8, and 9) (data not shown) and stem-loop Y was predicted to form in 0.2Δ(pY, 4, 6, 8, and 9) (Figures 5B, 5C-E and data not shown).

Nucleotides (1072-1088) predicted to form the loop of the distal stem-loop structure (nucleotides 1048-1106) were sensitive to single-strand-specific RNases. Specifically, nucleotide 1074 was sensitive to RNase T1/PhyM, while nucleotides 1075-1081 were susceptible to RNase PhyM/CL3 (Figure 4B, lanes 2-3 and 7-10). Upstream nucleotides, 1071 and 1072, were susceptible to RNase V1, while nucleotide 1073 was susceptible to both RNase V1 and PhyM cleavage (Figure 4B, lanes 4, 5, 7, and 8). Downstream nucleotides, 1092 and 1093, were also sensitive to RNase V1 cleavage (Figure 4B, lanes 4 and 5). These data corroborate the formation of a large loop (Figure 5A, now designated as Loop Ω); however, although adjacent nucleotides were paired, structure probing did not clearly support the formation of an extended helix as previously predicted.

Nucleotides 1002-1039 were predicted to form an additional stem-loop structure (Figure 5A, designated stem-loop X). RNase V1 cleavage at nucleotides 1035-1038 and 1024-1026 were consistent with helix formation at the base of the structure and within the terminal stem-loop, respectively; however, only low level RNase CL3 cleavage at nucleotide 1030 was
consistent with the presence of an internal loop. Nucleotides 1019-1020 within the loop were equally susceptible to low level cleavage by RNase V1, T1, and PhyM. Although a loop was not directly supported by structure probing, susceptibility to RNase V1, T1, and PhyM may indicate the formation of transient higher order RNA interactions or multiple RNA conformations within this domain.

Nucleotides 991-1001 preceding stem-loop X were predicted to form an internal loop (Figure 5A, designated Loop Ψ). Nucleotides 997-998 were highly sensitive to RNase T1/PhyM cleavage, while flanking nucleotides 995, 996, and 999 were susceptible to RNase PhyM cleavage (Figure 4A, lanes 2, 3, 7, and 8). The intensity of these bands, especially 997 and 998, suggests that this loop is highly exposed.

**Local RNA structures are conserved in the absence of upstream and adjoining sequences.**

Enzymatic structure probing of 1.2 demonstrated that RNase cleavage within nucleotides 978-1207 was fundamentally conserved with those observed for 0.2 (compare Figures 4A, 4B, and 4E; data not shown) and indicated that the upstream ~ 1.0 kb does not significantly contribute to the formation of RNA interactions within the 3' ~ 0.2 kb. The conservation of local RNA structure also suggested the potential modular character of the mKv1.4 IRES. This idea is underscored by the ability of non-overlapping regions to independently mediate internal ribosome entry (Figures 1B and 2B). Furthermore, RNase cleavage within mKv1.4 5' NCR sequences was partially maintained in the presence of various deletions within the 3' ~ 0.2 kb (data not shown) and 0.2Δ(pY, 2, 4, 6, 8, and 9) were predicted to form structures similar or identical to 0.2 (Figure 5 and data not shown).

**Alterations in secondary structure formation**
The systematic deletion of sequences within 0.2 resulted in distinct changes in IRES activity (Figure 3). To investigate the impact of the deletions on local RNA interactions and folding, a subset of RNA sequences harboring deletions within the 3′ ~ 0.2 kb was examined by enzymatic structure probing. The general pattern of RNase cleavage was not drastically altered with the introduction of these deletions (data not shown), suggesting that RNA folding is partially conserved. Indeed, defined RNase cleavage patterns were consistently observed, substantiating the formation of specific structural elements, including stem-loop Y and Loop Ψ (Figure 7, 8, and data not shown). As expected, sequences adjacent to the deletion displayed variable differences in RNase cleavage (data not shown); however, defined regions distal to the deletion were also notably altered by the loss of sequences.

Nucleotide-specific cleavage within stem-loop X was altered by the deletion of downstream sequences (Figure 6). 0.2ΔpY, 0.2Δ2, and 0.2Δ4 demonstrated increased RNase PhyM cleavage at nucleotides 1018-1020 (Figure 6A-D, compare lanes 7 and 8). Increased PhyM cleavage was also observed at nucleotides 1023 and 1025-1027 in 0.2ΔpY and, to a lesser extent, in 0.2Δ2 (Figure 6B and 6C, lanes 7 and 8). Accordingly, nucleotides 1024-1027 were no longer significantly susceptible to RNase V₁ cleavage in 0.2ΔpY and 0.2Δ2, while only nucleotide 1024 remained distinctly susceptible to RNase V₁ in 0.2Δ4 (Figure 6A-D, lanes 4 and 5). The data suggest that stem-loop X may directly interact with downstream sequences. Alternatively, stem-loop formation may be influenced by distal sequences as indicated by the prediction of alternative stem-loop X structures in 0.2ΔpY, 0.2Δ2 and 0.2Δ4 (Figure 5B-D, denoted stem-loop X'). Interestingly, nucleotides 1013-1026 within 0.2Δ4 were predicted to form a GNRA tetraloop (Figure 5D). In 0.2Δ6, juxtaposition of stem-loop X and Loop Ψ sequences was predicted to generate a novel stem-loop structure (data not shown).
1020 was sensitive to RNase V₁ cleavage, while nucleotides 1023, 1025, and 1026 were cleaved by RNase PhyM (Figure 6E, lanes 4, 5, 7, and 8).

While deletion of sequences within the polypyrimidine tract affected stem-loop X, conversely, disruption of stem-loop X altered RNase cleavage within the polypyrimidine tract. Deletions (Δ6 and Δ8/Δ9) which extend into stem-loop X altered RNase cleavage within the polypyrimidine tract (Figure 7D and data not shown). In 0.2Δ8, nucleotides 1158, 1161, 1162, and 1165 were equally susceptible to RNase V₁ and PhyM cleavage (Figure 7D), indicating the possible formation of more than one structure in this region of the RNA. Nucleotides 1168 and 1170 remained susceptible only to RNase V₁ cleavage while adjacent nucleotides, 1169 and 1171, displayed weak RNase V₁ and CL3 cleavage. Only nucleotides 1156 and 1157 were distinctly unpaired, as indicated by RNase PhyM cleavage (Figure 7D, lanes 7 and 8). 0.2Δ6 and 0.2Δ9 demonstrated similar patterns of RNase cleavage (data not shown). In contrast, although 0.2Δ4 displayed altered cleavage within stem-loop X, RNase cleavage within the polypyrimidine tract remained similar to 0.2 (compare Figure 7A and 7C). Similarly, when the upstream portion of the polypyrimidine tract (nucleotides 1154-1162) was removed in 0.2Δ2, remaining downstream nucleotides retained a pattern of RNase cleavage comparable to 0.2 (compare Figure 8A and 8B). Collectively, these data suggest that stem-loop X and the polypyrimidine tract interact; however, mfold predictions indicated that these two regions contribute to the formation of discrete structures in 0.2 (Figure 5A). Alternatively, the formation of stem-loop X may indirectly stabilize RNA interactions within the polypyrimidine tract.

Although displaying identical cleavage patterns throughout most of the sequence (data not shown), 0.2Δ8 and 0.2Δ9, which differ by ten nucleotides (989-998), demonstrated opposing changes in IRES activity (Figure 3). These ten nucleotides contribute to the formation of Loop
Ψ in 0.2 (Figure 5A). Within Loop Ψ, strong RNase T₁/PhyM cleavage at nucleotides 997 and 998 was apparent in 0.2 (Figure 8A, lanes 2, 3, 7, and 8) and highly conserved in 1.2, 1.2Δ(pY, 2, and 4), and 0.2Δ(pY, 2, 4, and Δ6) (data not shown). However, in 0.2Δ8, nucleotides 997 and 998 border the deletion and were not cleaved by RNase T₁/PhyM. Instead, two upstream guanosine residues (nucleotides 987 and 988) were strongly susceptible to RNase T₁/PhyM (Figure 8B, lanes 2, 3, 7, and 8). In 0.2Δ9, nucleotides 987 and 988, which border the deletion, were less susceptible to RNase T₁/PhyM cleavage than corresponding nucleotides in 0.2 and 0.2Δ8 (Figure 8C, lanes 2, 3, 7, and 8). Although a comparable single-stranded region was predicted to form within 0.2Δ9 (Figure 5F), cleavage of adjacent nucleotides suggested that RNA folding within this region differs from corresponding structures form by 0.2 and 0.2Δ8 (Figure 8C). Therefore, differences in 0.2Δ8- and 0.2Δ9-mediated expression may be attributed to the ability of sequences within 0.2Δ8 to form a structurally/functionally equivalent element that compensates for the disruption of Loop Ψ (Figure 5E).
Discussion

In this study, we used dual luciferase dicistronic constructs to investigate internal ribosome entry mediated by mKv1.4 5’ NCR sequences in several mammalian cell lines (Figure 1B). Although the expression of dicistronic RNAs was confirmed by Northern blot analysis, submolar amounts of smaller RNA species, possibly generated through cryptic promoter activity or RNA splicing, were also detected (Figures 1D). In the absence of the SV40 promoter, Fluc expression was similar to background (Figure 2A), demonstrating that mKv1.4 5′ NCR cDNA sequences do not mediate promoter activity. Moreover, transfection of in vitro transcribed dicistronic RNAs directly into cultured cells clearly demonstrated that 1.2 and 1.0 constructs, and to a lesser extent, 0.2, mediate internal ribosome entry (Figure 2B). In vitro translation using HeLa S10 cytoplasmic extract as well as rabbit reticulocyte lysate also demonstrated that 1.2 and 1.0 mediate IRES activity (data not shown); however, when compared with CVB3, mKv1.4 IRES activity is much lower in vitro than in transfected cells in culture.

IRES activity had previously been attributed to the 3′-most 208 nucleotides (4). In the present study, we have shown that the upstream ~ 1.0 kb can independently mediate internal ribosome entry (Figure 1B and 2B). The significance of functionally independent IRES elements within mKv1.4 5′ NCR sequences is not known, but their presence in the 5′ NCR of this mRNA may provide an additional level of translational regulation via RNA-RNA interactions between distal elements or RNA-protein interactions. Non-contiguous, non-overlapping sequences have also been demonstrated to mediate internal ribosome entry within other cellular IRES elements (52-54). Chappell et al. proposed that IRES elements are composed of distinct functional modules individually capable of promoting internal ribosome entry (33). While mKv1.4 5′ NCR sequences have not been determined to be composed of many discrete IRES elements, deletion
analysis supports the idea that functionally/structurally distinct regions interact to mediate IRES activity.

The presence of two non-overlapping IRES elements within the 5′ NCR of vascular endothelial growth factor mRNA had been correlated with the expression of distinct protein isoforms (53,55). Although only a single polypeptide is known to be expressed from mKv1.4 mRNA species, the authentic start codon is preceded by eighteen upstream AUGs. Several are in good Kozak consensus (56) and may potentially initiate the expression of upstream open reading frames (ORFs); however, their significance has yet to be examined. In the proto-oncogene c-myc, expression of c-Myc proteins and an upstream ORF (MYCHEX1) is mediated through independent IRES elements (57-59). The significance of the MYCHEX1 protein is unknown; however, Nanbru et al. speculated that translation of the MYCHEX1 ORF, which overlaps the c-Myc IRES, may alter RNA structure and affect IRES-mediated expression of c-Myc proteins (59). Similarly, during amino acid deprivation, translation of an upstream ORF was hypothesized to up-regulate IRES-mediated expression of the cationic amino acid transporter Cat-1 by altering RNA structure (60).

The 3′ ~ 0.2 kb was predicted to form three distinct stem-loop structures (stem-loops X, Y, and Z) and two single-stranded regions (Loop Ψ and Ω) (Figure 5A). The formation of stem-loop Y and Loop Ω was consistent with the structure previously predicted for the 3′ 200 nucleotides (4). Furthermore, the full-length 5′ NCR (1.2) demonstrated similar patterns of RNase cleavage within the 3′ ~ 0.2 kb, indicating that RNA folding within 0.2 is independent of upstream sequences. Strong cleavage within stem-loop Y and Loop Ψ suggests that these regions are exposed and may potentially facilitate RNA-protein interactions.
Structure-function analyses of mKv1.4 5′ NCR sequences indicated that the formation of complex RNA interactions contribute to IRES activity. Significant decreases in 0.2- and 1.2-mediated expression in the absence of the complete polypyrimidine tract initially indicated the importance of this motif (Figure 3). Deletion of polypyrimidine tract sequences correlated with changes in RNase cleavage within sequences forming stem-loop X in 0.2 (Figure 6). Conversely, when stem-loop X was directly disrupted, as in 0.2Δ6, 0.2Δ8, and 0.2Δ9, nucleotides within the polypyrimidine tract no longer clearly displayed strand-specific RNase cleavage (Figure 7D and data not shown). However, in contrast to 0.2ΔpY and 0.2Δ2, 0.2Δ6 and 0.2Δ8 demonstrated increased expression, while 0.2Δ9 was slightly decreased (Figure 3). The data strongly suggest an interaction between stem-loop X and the polypyrimidine tract. Purine-rich sequences within stem-loop X may mediate this interaction (Figure 5A).

Complementary sequences, nucleotides 1151-1157 within the polypyrimidine tract and upstream nucleotides (1138-1143), were predicted to form single-stranded bulges in 0.2 and may potentially form a pseudoknot (Figure 5A, denoted PK). Deletion of these sequences in 0.2ΔpY and 0.2Δ2 correlate with the loss of IRES activity. Although these sequences are present in 0.2Δ4, 0.2Δ6, 0.2Δ8, and 0.2Δ9, they were not necessarily predicted to be in the same context as 0.2 (Figures 5D-F).

When sequences between stem-loop X and the polypyrimidine tract were deleted, as in 0.2Δ4, expression increased (Figure 3); however, RNase cleavage within stem-loop X, but not the polypyrimidine tract, changed (Figure 6D and 7C). An alternative stem-loop structure corresponding to stem-loop X in 0.2 was predicted to include a GNRA tetraloop, a highly stable RNA motif (Figure 5D, denoted stem-loop X'). Therefore, stem-loop X is likely to be a significant structural element, forming a focal point for higher order RNA interactions.
The significance of the polypyrimidine tract is less clear in the context of 1.2 considering that the data reported here demonstrate that the 1.0 construct can independently mediate internal ribosome entry (Figure 1B and 2B). When additional sequences upstream of the polypyrimidine tract were also removed, as with 1.2Δ10 and 1.2Δ11, expression levels were similar to 1.0 (Figure 3B). Therefore, in the absence of a complete polypyrimidine tract, putative interacting sequences located upstream of the polypyrimidine tract may potentially facilitate the repression of 1.0-mediated IRES activity. Conversely, when these sequences were deleted, 0.2-mediated expression increased, while 1.2-mediated expression was minimally affected (Figure 3).

Our data showed that the mKv1.4 IRES is able to mediate translation initiation in cells derived from brain, heart, and skeletal muscle. These cell types are derived from tissues where Kv1.4 mRNA expression has been shown to occur, suggesting that the IRES elements derived from such mRNAs are active in the “appropriate” cell types. Dicistronic assays in transfected cells in culture also demonstrated that mKv1.4 and CVB3 5′ NCR sequences display distinct cell-type specific IRES activities (Figure 1B). Moreover, although the deletion of sequences within 0.2 similarly affected 0.2- and 1.2-mediated expression in three cell types, increases in expression were greater in PrCM and SK-N-SH cells than in HeLa cells (Figure 3 and data not shown). Differences in the levels of mKv1.4 5′ NCR-mediated IRES activity may be attributed to the expression of cell-specific trans-acting factors.

Preliminary experiments suggested that 0.2 and 1.0 interact with a unique set of proteins (Leong, Negulescu, Gutman, and Semler, unpublished data). The 3′ ~ 0.2 kb specifically interacts with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Leong, Gutman, and Semler, unpublished data); however, the functional significance of this interaction has yet to be determined. In addition to its role in glycolysis, GAPDH has been linked to many other cellular functions.
processes (61). GAPDH has been reported to destabilize RNA duplex formation within the hepatitis A virus (HAV) IRES and may modulate IRES activity in conjunction with PTB (62,63), a cellular protein required for other IRES elements (64-66). Secondary structure analysis supports the formation of single-stranded AU-rich regions within the polypyrimidine tract, stem-loop Y, and Loop Ω (Figure 5A). GAPDH has also been reported to selectively bind AU- and U-rich RNA sequences (67); however, specific sites of interaction between GAPDH and mKv1.4 5′ NCR sequences have yet to be determined. Ongoing studies are aimed at identifying sites of functional RNA-protein complexes within the mKv1.4 5′ NCR. Such structures may form nucleation sites for formation of IRES-dependent translation initiation complexes, perhaps in a cell-specific manner.
Acknowledgements

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Reference List


Figure Legends

Figure 1. **mKv1.4 5′ NCR sequences mediate internal ribosome entry in the context of dicistronic RNAs.** (A) Dicistronic constructs encoding *Renilla* and firefly luciferase as the upstream and downstream cistrons, respectively, were employed to assess IRES function within the 5′ NCR from Kv1.4 mRNA species expressed in mouse brain tissue. Plasmid constructs contain the SV40 promoter (SV40pm), a chimeric intron to promote nuclear export, and the T7 promoter (T7pm) upstream of *Renilla* luciferase and the SV40 polyadenylation signal (SV40pA) downstream of firefly luciferase. An inverted repeat or hairpin was inserted within the intercistronic sequence (ICS). (B) Dicistronic constructs encoding mKv1.4 5′ NCR sequences, the full ~1.2 kb (1.2), the 3′ most ~0.2 kb (0.2), or the 5′ most ~1.0 kb (1.0), within the ICS were transiently transfected into a human cervical carcinoma cell line (HeLa), two human neuroblastoma cell lines (SK-N-SH and NLF), a mouse muscle myoblast cell line (C2F3), primary rat cardiac myocytes (PrCM), and two African green monkey kidney cell lines (CV1 and COS). (C) A dicistronic construct encoding the CVB3 5′ NCR was also transfected in parallel as a positive control for IRES activity. Constructs were transfected in triplicate and cell lysates were isolated 24 hours post-transfection and assayed for luciferase activity. Fluc/Rluc ratios were calculated for each sample and the average value is represented as fold over background, expression in the absence of insert. Standard deviations were calculated for Fluc/Rluc ratios obtained from triplicate assays. (D) Total RNA was isolated from HeLa cells transiently transfected with dicistronic constructs 14 hours post-transfection and subjected to oligo-dT selection and DNase I digestion. Two micrograms of oligo-dT selected RNA was initially analyzed by Northern blot analysis using random hexamer primed $^{32}$P-labeled probe against the Fluc coding sequences and subsequently reprobed for β-actin to determine relative RNA loading.
RNA was isolated from untransfected HeLa cells (NT, lane 1) and cells transiently transfected with dicistronic constructs containing no insert (lane 3) or mKv1.4 (lanes 4–6) or CVB3 (lane 2) 5′ NCR sequences. Smaller RNA species are denoted by asterisks (∗ or ∗∗). Slower migrating bands observed in lanes 3–6 may be attributed to incomplete DNase I digestion of transiently transfected plasmid DNA.

Figure 2. (A) Dicistronic constructs +/- the SV40 promoter were transiently cotransfected into HeLa cells with a CMV β-Gal reporter construct for 24 hours and assayed for firefly luciferase and β-Gal activities. (B) In vitro transcribed dicistronic RNAs synthesized from plasmid constructs described in Figure 1A were transiently transfected into HeLa cells for 15 hours and assayed for luciferase activities. All transfections were performed in triplicate and Fluc/Rluc or Fluc/β-Gal ratios were calculated for each sample. The average value is represented as fold over background, expression measured in the absence of insert. Standard deviations were calculated from calculated ratios obtained from triplicate samples. (C) Northern blot analysis of total RNA from transiently transfected HeLa cells 5 and 15 hours post-transfection. Dicistronic RNAs were detected using random hexamer primed 32P-labeled probe against Fluc coding region sequences and the blot was subsequently reprobed for β-actin to determine relative RNA loading.

Figure 3. Deletion analysis of mKv1.4 5′ NCR. (A) Schematic representation of deletions within the 3′ ~ 0.2 kb of mKv1.4 5′ NCR. Deletions spanning the 3′ ~ 0.2 kb of mKv1.4 5′ NCR were introduced into the 3′ ~ 0.2 kb (0.2Δ) and the full ~ 1.2 kb (1.2Δ) 5′ NCRs and inserted into the ICS of the dual luciferase dicistronic construct (Figure 1A). In addition to removing contiguous overlapping sequences (Δ1′-Δ11), a 25 nucleotide sequence containing a polypyrimidine tract (pY, nucleotides 1153-1173) upstream of the start codon was specifically
deleted (ΔpY). Deletions are roughly grouped as 3′ (Δ1′, ΔpY, Δ1, Δ2, and Δ3) and 5′ (Δ4, Δ5, Δ6, Δ7, and Δ8) deletions, based on position and expression compared to wild type sequences. Δ9 differs from Δ8 by the deletion of an additional upstream 10 nucleotides and Δ10 and Δ11 represent the most extreme deletions, removing 139 and 169 nucleotides from the original 234 nucleotides, respectively. Dicistronic constructs containing mKv1.4 5′ NCR deletion-containing sequences within the ICS were transiently transfected into (B) HeLa and (C) PrCM, for 24 hours and assayed for luciferase activity. The top four columns represent identical results from dicistronic constructs encoding no insert or wild type mKv1.4 5′ NCR sequences, 1.2, 0.2, and 1.0 (Figure 1A) and are set relative to appropriate wild type sequences, 0.2 and 1.2 (indicated by asterisks) on the left and the right panels, respectively. Expression mediated by deletion sequences, 0.2Δ (left) and 1.2Δ (right), are represented as fold relative to corresponding wild type sequences, 0.2 or 1.2.

Figure 4. **Enzymatic structure probing of mKv1.4 5′ NCR sequences using primer extension.** 0.5 µg of *in vitro* transcribed RNA was subjected to mock digestion for 10 and 20 minutes (lanes 1 and 6) or partial RNase digestion with 18.2 units RNase V₁ for 10 and 5 minutes (lanes 4 and 5), 0.025 and 0.017 units RNase T₁ (lanes 2 and 3) for 20 minutes, 1.0 unit RNase PhyM for 20 and 15 minutes (lanes 7 and 8), and 0.04 and 0.02 units RNase CL3 (lanes 9 and 10) for 20 minutes at room temperature in the presence of 40 µg tRNA. Primer extension reactions were performed using 32P-5'-end-labeled oligonucleotides (~ 10^6 cpm). Samples were subsequently resolved on an 8% acrylamide-7 M urea sequencing gel. (A-D) The 3′-most 229 nucleotides (0.2) were analyzed using two oligonucleotides, 1740X (A and B, 2 hours and 30 minutes, 3 hours and 40 minutes) and Fluc (C and D, 2 hours 5 minutes, 3 hours and 40 minutes). (E) The full ~ 1.2 kb 5′ NCR was subjected to partial RNase digestion and subsequent
primer extension with $^{32}\text{P}$-5′-end-labeled oligonucleotide (1740X). mKv1.4 5′ NCR sequences were preceded by ten additional nucleotides and followed by 169 nucleotides of the Fluc coding region.

Figure 5. **mfold secondary structure predictions for 0.2 and 0.2Δ.** Using the RNA folding algorithm *mfold* (version 3.1) and constraints determined from enzymatic structure probing, mKv1.4 5′ NCR sequences, (A) 0.2, (B) 0.2ΔpY, (C) 0.2Δ2, (D) 0.2Δ4, (E) 0.2Δ8, and (F) 0.2Δ9, were predicted to form the structures shown above. Triangles denote cleavage by single-strand specific RNases T₁, PhyM, and/or CL3, while arrows indicate cleavage by RNase V₁, the double-strand specific RNase. The intensity of RNase cleavage was subjectively assigned as greater or lesser and is denoted by filled and unfilled triangles or arrowheads, respectively. Nucleotides comprising the polypyrrimidine tract (nucleotides 1149-1173) are indicated by shading for 0.2. Altered structures predicted to form within 0.2Δ sequences were denoted with a prime. In 0.2, a potential pseudoknot interaction between neighboring single-stranded regions (nucleotides 1138-1143 and 1151-1157) is indicated by arrows and denoted by PK. The numbering of the nucleotides is relative to the full 1.2 kb mKv1.4 5′ NCR.

Figure 6. **Enzymatic structure probing of sequences forming stem-loop X.** (A) 0.2, (B) 0.2ΔpY, (C) 0.2Δ2, (D) 0.2Δ4, and (E) 0.2Δ6. RNAs were subjected to partial RNase digestion (as described in the legend to Figure 4) and primer extension using $^{32}\text{P}$-5′-end labeled oligonucleotides (1740X for 0.2, 0.2Δ4, and 0.2Δ6 or 1780X for 0.2ΔY and 0.2Δ2).

Figure 7. **Enzymatic structure probing within the polypyrrimidine tract.** (A) 0.2, (B) 0.2Δ2, (C) 0.2Δ4, and (D) 0.2Δ8. RNAs were subjected to partial RNase digestion (as described in the
legend to Figure 4) and primer extension using $^{32}$P-5$'$-end labeled oligonucleotides (1740X for 0.2, 0.2Δ4, and 0.2Δ8 or 1780X for 0.2Δ2).

Figure 8. **Enzymatic structure probing of sequences forming Loop Ψ.** (A) 0.2, (B) 0.2Δ8, and (C) 0.2Δ8. RNAs were subjected to partial RNase digestion (as described in the legend to Figure 4) and primer extension using $^{32}$P-5$'$-end labeled oligonucleotides (1740X).
Figure 1

A

pRstF

\[ SV40pm \quad T7pm \quad \text{Inverted Repeat} \quad SV40pA \]

\[ \text{chimeric intron} \quad \text{ICS} \]

\[ \text{Hind III} \quad (14) \quad \text{Xba I} \quad (978) \quad \text{Nco I} \quad (1207) \quad 5' NCR \]

pRst1.2F

pRst0.2F

pRst1.0F

pRstCVB3F

CVB3

B

mKv1.4 5' NCR

\[ 1.2 \text{ } 0.2 \text{ } 1.0 \text{ } \checkmark \text{CVB3} \]

FOLD

HeLa CV1

SK-N-SH NLF C2F3 PrCM COS

C

CVB3

FOLD

HeLa CV1 SK-N-SH NLF C2F3 PrCM COS

D

mKv1.4 5' NCR

\[ 1.2 \text{ } 0.2 \text{ } 1.0 \]

\[ \text{kb} \]

\[ 9.5 \quad 7.5 \quad 5.5 \quad 4.5 \quad 2.5 \quad 1.5 \quad 1.0 \]

\[ \beta\text{-Actin} \]
Figure 2

A

B

C
Structurally distinct elements mediate internal ribosome entry within the 5' noncoding region of a voltage-gated potassium channel mRNA
Gwendolyn M. Jang, Louis E.-C. Leong, Lily T. Hoang, Ping H. Wang, George A. Gutman and Bert L. Semler

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