Translation Initiation of a Cardiac Voltage-gated Potassium Channel by Internal Ribosome Entry*

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The mammalian Kv1.4 voltage-gated potassium channel mRNA contains an unusually long (1.2 kilobases) 5'-untranslated region (UTR) and includes 18 AUG codons upstream of the authentic site of translation initiation. Computer-predicted secondary structures of this region reveal complex stem-loop structures that would serve as barriers to 5' → 3' ribosomal scanning. These features suggested that translation initiation in Kv1.4 might occur by the mechanism of internal ribosome entry, a mode of initiation employed by a variety of RNA viruses but only a limited number of vertebrate genes. To test this possibility we introduced the 5'-UTR of mouse Kv1.4 mRNA into the intercistronic region of a bicistronic vector containing two tandem reporter genes, chloramphenicol acetyltransferase and luciferase. The control construct translated only the upstream chloramphenicol cistron in transiently transfected mammalian cells. In contrast, the construct containing the mKv1.4 UTR efficiently translated the luciferase cistron as well, demonstrating the presence of an internal ribosome entry segment. Progressive 5' → 3' deletions localized the activity to a 3'-proximal 200-nucleotide fragment. Suppression of cap-dependent translation by extracts from poliovirus-infected HeLa cells in an in vitro translation assay eliminated translation of the upstream cistron while allowing translation of the downstream cistron. Our results indicate that the 5'-untranslated region of mKv1.4 contains a functional internal ribosome entry segment that may contribute to unusual and physiologically important modes of translation regulation for this and other potassium channel genes.

Voltage-gated potassium (Kv) channels play integral roles in regulating ion balance, membrane potential, secretion, and cell excitability in many different cells (1). Mammalian Kv channels are encoded by a family of 19 known genes, each with its own unique profile of pharmacological and physiological properties and tissue distribution (2). Kv channel expression is known to be regulated in complex ways in both embryonic and adult cells. One member of this family, Kv1.4, is expressed in brain, heart, and skeletal muscle (3, 4). The functional channel consists of four identical subunits and has properties resembling a typical rapidly inactivating "A" current channel (5). Within neurons, Kv1.4 channels are found in nerve terminals where they are thought to underlie the rapid after-hyperpolarization during action potentials (6). This channel is sensitive to external 4-aminopyridine and Ba2+ (7) and is potently blocked by pandinus toxin (8), as well as by the dihydroquinoline compound CP339818 (9).

The mKv1.4 gene is known to generate at least three transcripts, 2.4, 3.5, and 4.5 kb in size (3), that are under the control of a single GC-rich promoter (4). The 2.4-kb transcript has been found only in skeletal muscle and in the mouse myoblast cell line C2C12 where it is up-regulated during myotube formation (3). The 3.5- and 4.5-kb transcripts, which are expressed in heart and brain, begin at a single transcription initiation site and differ only by their use of alternate polyadenylation sites in their 3'-untranslated regions (UTRs) (4). The 5'-UTRs of typical mammalian genes are relatively short (average length, ~150 nt) and lack both AUGs stable secondary structure (10). By contrast, the 5'-UTR of mKv1.4 (mouse Kv1.4) is long (~12 kb), contains 18 upstream AUG codons, and has a predicted complex secondary structure with numerous stable hairpin structures (Fig. 1). Several of the upstream AUG codons are contained within a favorable context for initiation (11) and are followed by open reading frames as long as 40 amino acids, none of which is contiguous with the authentic coding region. At least three mechanisms are known for eukaryotic translation initiation, namely 5' → 3' scanning, "shunting," and internal ribosome entry. In the scanning model, the 40 S ribosomal subunit (bound to the ternary complex) binds to the 5' cap complex and travels in a 5' → 3' direction until it encounters an initiator AUG codon in a favorable context; the 60 S ribosomal subunit then joins the complex, and peptide synthesis begins (11). Translation initiation of most vertebrate genes is thought to occur by this mechanism. The shunting mechanism is also 5' cap-dependent but differs from scanning in that the 40 S subunit bypasses the majority of the 5'-UTR by shunting or "jumping" to a region at or near the authentic site of translation initiation, avoiding having to scan through most of the 5'-UTR (12, 13). In contrast, translation initiation by internal ribosome entry involves the binding of the 40 S ribosomal subunit to an internal ribosome entry site (IRES) at or near the authentic AUG, thereby eliminating the requirement both for the presence of a 5' cap structure and for scanning through the greater part of the 5'-UTR (for review see Refs. 14–16).

The presence of upstream AUG codons and stable secondary...
these features in the 5'-UTR of mKv1.4 thus suggests the possibility that translation initiation for this gene may occur by some mechanism other than 5' cap-dependent scanning. Here we report that the 5'-UTR of mKv1.4 contains an IRES that can initiate efficient translation of a reporter protein in a 5' cap-independent fashion. We propose that translation initiation by internal ribosome entry may represent a general and important mechanism for regulation of expression of this gene as well as other members of the extended family of potassium-selective channels.

MATERIALS AND METHODS

Constructs—Bicistronic constructs (20) containing the reporter genes chloramphenicol acetyltransferase (CAT) and luciferase (LUC) were gifts from Peter Sarnow. The SV40 promoter and the T7 promoter control transcription of both reporter genes in the SV construct and the T7 vector, respectively. The intercistronic spacer of both vectors contains a unique SalI site for cloning.

The cDNA for mKv1.4 was isolated from mouse brain by reverse transcription-PCR using the following primer pair: sense, 5'-AGCTAGAGGAAGACTGGG-3', and antisense, 5'-ATAAGGCAATGTCGATGTTGC-3'. The 5'-UTR was amplified by PCR to generate SalI restriction sites on both the 5' and 3' ends using the following primer pair: sense, 5'-AGCTAGAGGAAGACTGGG-3', and antisense, 5'-ATTCACCTGCGACGAGGCTTG-3'.

The resulting PCR product was inserted into the intercistronic spacers of both constructs (SV and T7) at the unique SalI site, and its orientation was determined by restriction digestes and DNA sequencing. This procedure removed 13 nucleotides from the 5' end of the full-length 5'-UTR.

Constructs containing a hairpin loop were generated by annealing the complementary oligonucleotides shown below, which are flanked by SalI sites at each end of the insert:

Constructs—

**A**

**B**

**FIG. 1. Sequence and predicted secondary structure of mKv1.4 5'-UTR.** A, sequence of the 5'-UTR of mouse Kv1.4. Eighteen abortive AUGs are shown in **bold** and **underlined**, and the authentic initiating AUG is shown as **bold** only. A polypyrimidine tract near the 3' end is **underlined**. The 1.2-kb fragment used in the bicistronic constructs begins at nt 14 of this sequence, indicated by an **arrow**. This sequence can be found in the GenBank™ data base under accession numbers U03722 and U03723. B, secondary structure of the 200-nt 3'-proximal fragment of the mKv1.4 5'-UTR predicted by MFOLD; the polypyrimidine tract (see Fig. 1A) is shown in **bold**. This structure, which has a calculated free energy of ~38 kcal/mol, was an element in several of the most stable structures predicted by MFOLD using both the entire 1.2-kb 5'-UTR and the 200-nt fragment alone.

**Sequences 1 and 27**

The underlined regions form 19-nt RNA stems with a calculated free energy of ~41 kcal/mol, and a SauI site is restored at only one of the two ends (the right-hand end above) after insertion. The orientation of this insert was determined by DNA sequencing, and clones were chosen that had the restored SauI site at the 3' end of the insert.

The 200-nt 3'-proximal region of the 5'-UTR was amplified by PCR using the following primers (which generated SauI sites at their termini): sense, 5'-AGCTAGAGGAAGACTGGG-3', and antisense, 5'-AGCTAGAGGAAGACTGGG-3'. After digestion with SauI, the PCR-amplified fragment was cloned into the SauI site of the intercistronic spacer. The orientation of this fragment was determined by DNA sequencing.

**Cell Culture, Transfection of Constructs, and CAT and LUC Assays—**NIH-3T3 cells were maintained in minimum essential medium containing 10% fetal calf serum. 24 h prior to transfection cells were plated at a density of 5 x 10^5 cells/90-mm plate. On the day of transfection, cells were washed twice with 1× phosphate-buffered saline and then covered with 8 ml of fresh medium. 20 μg of DNA (isolated either from cesium chloride density gradients or by use of the Qiagen Maxi-prep (Qiagen Inc., Chatsworth, CA)) was combined with 50 μl of 2× HBS (275 mM NaCl, 10 mM KCl, 42 mM HEPES, 12 mM dextrose) by vortexing for 15 s. The solution was held at room temperature for 20 min and gently pipetted onto the cells, and the cells were washed and resuspended as described above. 64–72 h after transfection, cells were washed with 1× phosphate-buffered saline, harvested, and extracted on lysis buffer. Cell lysates were pelleted, and the supernatants were assayed for LUC activity using a luciferase assay kit (Promega, Madison, WI) with a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). CAT activity was measured with the Promega CAT assay kit.

**In Vitro Transcription of Translation Templates—**DNA templates for in vitro transcriptions were linearized with HpaI, and transcriptions using bacteriophage T7 RNA polymerase were performed as described by Roehl and Semler (21), with the modification that all transcription reactions were performed in the presence of 1 mM 7GpppG cap analog. All transcripts were purified by phenol/chloroform extraction, twice precip-
reduces translational read-through to negligible values (Fig. 2, line 2). All subsequent studies of the IRES were carried out using constructs containing this stem-loop. In the presence of the 1.2-kb 5'-UTR of mKv1.4 (Fig. 2, line 3), LUC/CAT activity is increased to nearly 400, indicating that this sequence possesses IRES activity. A construct containing only the 3'-most 200 nt of the 5'-UTR (Fig. 2, line 4) also shows activity comparable with that of the 1.2-kb fragment, suggesting that IRES activity is largely contained within this region.

Neither the 1.2-kb nor the 200-nt fragments exhibit substantial IRES activity when cloned into the SVs element in the antisense direction (Fig. 2, lines 3 and 4), confirming the sequence specificity of this activity. Similar results were obtained when transfections were carried out in COS-1 cells (data not shown).

**RESULTS**

The 5'-UTR of mKv1.4 Mediates IRES-dependent Translation Initiation in Mammalian Cells—To test for the presence of an IRES in the 5'-UTR of mKv1.4, we introduced this sequence into the intercistronic spacer of a bicistronic vector (20) containing two reporter proteins, CAT and LUC, present on a single transcript under the control of the SV40 promoter. Translation of CAT occurs by the 5' cap-dependent ribosomal scanning mechanism, whereas efficient translation of LUC, on the other hand, occurs only if the intercistronic spacer contains a sequence with IRES activity that allows internal binding of ribosomes. These constructs were transfected into NIH-3T3 or COS-1 cells, and CAT and LUC activities were measured 64–72 h post-transfection. CAT activity served as an indicator of transfection efficiency, and results (determined in triplicate) for each experimental condition are displayed as the ratio of LUC to CAT activity.

Representative results of such transfections are shown in Fig. 2. The control vector lacking the mKv1.4 5'-UTR sequence (Fig. 2, line 1) shows some LUC activity in NIH-3T3 cells, possibly because of leaky translation termination at the CAT stop codon and ribosomal read-through (11). To reduce this background activity, we inserted a stable hairpin structure downstream of the CAT stop codon, as indicated in line 2 of Fig. 2. This structure has a predicted free energy of -41 kcal/mol and reduces translational read-through to negligible values (Fig. 2, line 2). All subsequent studies of the IRES were carried out using constructs containing this stem-loop. In the presence of the 1.2-kb 5'-UTR of mKv1.4 (Fig. 2, line 3), LUC/CAT activity is increased to nearly 400, indicating that this sequence possesses IRES activity. A construct containing only the 3'-most 200 nt of the 5'-UTR (Fig. 2, line 4) also shows activity comparable with that of the 1.2-kb fragment, suggesting that IRES activity is largely contained within this region.

Neither the 1.2-kb nor the 200-nt fragments exhibit substantial IRES activity when cloned into the SVs element in the antisense direction (Fig. 2, lines 3 and 4), confirming the sequence specificity of this activity. Similar results were obtained when transfections were carried out in COS-1 cells (data not shown).

**IRES Activity in mKv1.4 Is 5' Cap-independent—IRES-dependent translation initiation is expected to be 5' cap-independent, and extracts from poliovirus-infected cells have been used to suppress cap-dependent translation while not affecting IRES activity in a bicistronic expression vector (23). In poliovirus-infected cells, viral mRNA is translated efficiently, whereas translation of host mRNA is severely inhibited due to the proteolytic cleavage of the eIF-4G (formerly called p220 or eIF-4-s) subunit of the cap binding complex eIF-4F (24) by viral protease 2A. The only cellular mRNAs translated in polivirus-infected cells are those that use a 5' cap-independent internal ribosome entry mechanism. If the mKv1.4 5'-UTR does indeed encode an IRES, its translation should be 5' cap-independent, and translation initiation should be unaffected by the presence of extracts from poliovirus-infected cells.

We inserted the 1.2-kb fragment of the 5'-UTR of mKv1.4 into the intercistronic spacer of a bicistronic construct similar to the one used in the transfection experiments, but with transcription directed by the bacteriophage T7 promoter. The stem-loop sequence described above was inserted downstream of the CAT stop codon to reduce leaky translation termination and ribosomal read-through. Bicistronic mRNAs were synthesized in vitro using T7 RNA polymerase in the presence of the m7GpppG 5' cap analog. RNAs were then added to a rabbit reticulocyte lysate containing 40% ribosomal salt wash from either poliovirus-infected or uninfected HeLa cells. In the presence of extracts from uninfected HeLa cells, the control vector RNA lacking the 1.2-kb mKv1.4 sequence showed detectable CAT activity but little or no LUC activity, leading to a low LUC/CAT ratio (Fig. 3A). Insertion of the 1.2-kb mKv1.4 5'-UTR resulted in a significant increase in the LUC/CAT ratio, suggesting that the mKv1.4 5'-UTR was able to efficiently direct translation of the downstream cistron via an internal ribosome entry site.
ribosome entry mechanism. Insertion of the mKv1.4 5′-UTR into the intercistronic spacer in the antisense orientation displayed considerably reduced levels of IRES-like activity (Fig. 3A) compared with those detected in the sense orientation.

When extracts from poliovirus-infected HeLa cells were added to the in vitro translation reactions, dramatic effects were seen on the translation of the upstream cistron. Because CAT activity was reduced to nearly background levels in the presence of extracts from poliovirus-infected HeLa cells, the ratio of LUC/CAT is not meaningful in evaluating IRES activity. Thus, the data shown in Fig. 3B were expressed as CAT or LUC activity in extracts from infected cells relative to the maximum values obtained when translations were carried out in the presence of extracts from uninfected cells (1.9 × 10^3 units for CAT, and 1.8 × 10^3 for LUC). The data reveal a dramatic suppression of cap-dependent translation (CAT activity) compared with cap-independent translation (LUC activity) when translations were carried out in the presence of extracts from poliovirus-infected cells. Although ~20% of maximum LUC expression is retained, CAT activity is reduced to ~0.3% of its maximum. Fig. 3B also shows that this remaining LUC activity is dependent on the presence of the mKv1.4 5′-UTR and that the LUC signal with the insert in the antisense direction is very low. These data show that the IRES activity in the mKv1.4 5′-UTR is 5′ cap-independent.

**DISCUSSION**

Translation initiation by internal ribosome entry was first demonstrated for picornaviruses (23, 25) and has since been shown for other RNA viruses (26, 27). In addition, although the precise mechanism for internal ribosome loading is not yet understood, at least one picornavirus IRES has been shown to initiate translation in a completely 5′ end-independent manner (28). However, only a few cellular genes are known to be translated in this manner, including Antennapedia in Drosophila (29), and Bip (30), fibroblast growth factor 2 (31), and c-myc (32) in mammalian cells. Thus, mKv1.4 represents one of a limited number of mammalian genes known to contain a functional IRES. The 200-nt stretch in mKv1.4 containing IRES activity shares two important characteristics with known IRES elements from RNA viruses, namely a polypyrimidine tract (Fig. 1A) and extensive computer-predicted RNA secondary structures (Fig. 1B) that might be targeted by trans-acting cellular factors necessary for the formation of the 40 S pre-initiation complex. Indeed, trans-acting cellular factors corresponding to known cellular RNA binding proteins have been shown to have functional interactions with IRES elements of picornavirus and hepatitis C virus (33–40). Known IRES elements have also been reported to have complex secondary structures (41), and stem-loop elements within these regions are thought to contain motifs necessary for IRES activity (42, 43). Computer-predicted models of the 200-nt mKv1.4 IRES-containing region predict a similarly complex structure with multiple hairpin loops (Fig. 1B). Because the 5′-UTR of mKv1.4 is significantly longer (~1.2 kb) than that of the average mammalian gene, and contains multiple (eighteen) abortive upstream AUGs, the IRES present in the 200-nt stretch might bind cellular factors that ultimately enable the ribosome to efficiently translate the channel protein from the authentic translation initiation site. In support of this possibility, we have obtained preliminary data from electrophoretic mobility shift experiments showing specific binding of cellular proteins to the mKv1.4 IRES.2

**IRES Control of Translation May Be a Common Mechanism**

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**Fig. 4.** Lengths of 5′- and 3′-UTRs of mammalian voltage-gated channel transcripts, showing the presence of 5′ AUGs and 3′ AUUUA motifs. The ruler at the top indicates the lengths of the UTRs represented below, and the two downward arrowheads on this ruler mark the mean length of vertebrate UTRs (see text), ~150 nt for the 5′ and ~500 nt for the 3′-UTRs. Short vertical bars on each UTR mark the location of AUG (in the 5′-UTRs) or AUUUA (in the 3′-UTRs), and upward arrowheads indicated alternate end points due to alternate transcription start sites (5′ ends) or use of alternate polyadenylation signals (3′ end). The termini of those transcripts for which the ends of the mRNA have been experimentally determined are indicated by solid circles; UTRs lacking this symbol represent minimum estimates of their true length. The dotted line in the 5′-UTR of Kv1.5 represents a sequence that was not available for analysis. The dotted line in the 5′-UTR of Kv3.1 represents a region that is removed by alternate splicing. CDS represents the coding region for these mRNAs (not drawn to scale).

**Utilized by Kv Channel Genes—**Our demonstration of IRES activity in the 5′-UTR of mKv1.4 may account for its normal translation despite the presence of several unusual features (length, abortive AUGs, and stable secondary structure) that are known to inhibit 5′ cap-dependent ribosome scanning. We have also previously shown (4) that AUUUA motifs present in the long 3′-UTR of Kv1.4 can significantly inhibit translation, presumably by affecting translation initiation, and that alternate use of polyadenylation sites plays a role in regulating this process.

The UTRs of many other mammalian Kv channel transcripts also show strikingly different structures from the typical vertebrate pattern. Inspection of Fig. 4 shows that there is no Kv transcript known to have either of its UTRs as short as the typical mean values, and most are considerably longer. It should be noted that lengths of these UTRs represent only lower limits, because the ends of many of these transcripts have not yet been mapped (including, for example, both of the long UTRs of Kv1.1), and many longer transcripts have yet to be characterized at all (e.g. >9-kb transcripts of Kv1.1 and Kv1.3). Fig. 4 also illustrates the presence of multiple upstream AUGs in the 5′-UTRs and of AUUUA motifs in the 3′-UTRs of many Kv transcripts. The UTRs of Kv genes are not only strikingly unusual with respect to their lengths and their containing these unusual motifs, but as has been pointed out by us (see Ref. 2), their sequences are considerably more highly conserved than in most other mammalian genes, lending further support to the notion that they have been conserved over evolutionary time for some important function(s).

Thus, translational control by internal ribosome entry may be important not only for Kv1.4 but may represent a mechanism more generally utilized for regulation of gene expression by the extended family of Kv genes. In fact, despite the more limited data available for the UTRs of other families of potassium selective channels, it is not difficult to find examples of transcripts whose UTRs share the unusual features we have described in Kv1.4. The mouse calcium-activated K+ channel slopoke (44), for example, has at least three abortive AUGs in its 820-nt 5′-UTR and three AUUUA motifs in its 627-nt 3′-UTR. Likewise, the recently described transcript of the hu-
man inward rectifier hGIRK1 (45) has no fewer than 12 AUGs in its >1360-nt 5′-UTR and five AUUUU motifs in its 314-nt 3′-UTR. Translation initiation through the use of internal ribosome entry and its regulation by 3′ elements may therefore be a significant factor in the regulation of expression not only of Kv1.4 and its voltage-gated cousins but also of other members of the extended family of K+-selective ion channels.

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