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Alternative polyadenylation signals in the 3' noncoding region of a voltage-gated potassium channel gene are major determinants of mRNA isoform expression

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Abstract

We investigated the role of the 3' noncoding region of a mouse voltage-gated potassium channel mRNA (mKv1.4 mRNA) in post-transcriptional regulation of gene expression. In contrast to an earlier report from studies carried out in *Xenopus* oocytes, we found that 3' noncoding region sequences of mKv1.4 mRNAs did not significantly affect expression of a heterologous reporter RNA *in vitro* or in mammalian cells/cell lines. Instead, our data revealed a possible role for alternative polyadenylation mediated by distinct determinants ~ 0.2 kb and ~ 1.2 kb downstream of the Kv1.4 coding region. The use of the downstream polyadenylation signal correlated with the synthesis of a larger Kv1.4 mRNA isoform that was more abundantly expressed than the smaller mRNA species, whose expression was regulated by the upstream polyadenylation signal. Our results suggest that the relative strengths of the polyadenylation signals are major determinants of overall Kv1.4 mRNA abundance in cells.

1. Introduction

Elements within the 3' noncoding region (NCR) of eukaryotic mRNAs contribute to co- and post-transcriptional regulation of gene expression. In the nucleus, distinct signals within the 3' NCR mediate endonucleolytic cleavage and polyadenylation of newly synthesized pre-mRNAs, while *cis*-acting elements within the 3' NCR of mature mRNA species may affect mRNA stability, RNA localization, or translation in the cytoplasm. Although biochemically distinct, 3' end processing is coupled to other nuclear processes, including transcription, splicing, and RNA export (Zhao et al., 1999). During transcription initiation, cleavage and polyadenylation factors associate with the C-terminal domain of RNA polymerase II (Calvo and Manley, 2003). Recruitment of such factors may be influenced by the promoter, while transcription termination requires a functional polyadenylation site (Proudfoot, 1989). Splicing factors bound to splice site determinants may compete with proximal polyadenylation signals to alter 3' end processing and mRNA expression patterns (Berget, 1995; Zhao et al., 1999). Finally, during splicing and 3' end processing, transport factors required for mRNA export are recruited to the mRNA (Zhao et al., 1999).

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Alternative polyadenylation sites with different efficiencies for 3' end processing or tissue-specific utilization have been shown to affect the abundance of specific mRNA species (Edwards-Gilbert et al., 1997; Zhao et al., 1999). The use of alternative polyadenylation signals located on separate exons combined with the presence of alternative splice sites alters mRNA coding capacity and may result in protein isoforms with distinct C-terminal domains. Tandem polyadenylation signals within the 3' terminal exon can generate mRNA species with different 3' NCRs (Edwards-Gilbert et al., 1997). In addition, 3' NCR sequences may contain regulatory determinants, such as AU-rich elements (AREs), that affect mRNA stability and/or translation. AREs have been demonstrated to mediate the destabilization of mRNAs encoding cytokines, proto-oncogenes, and growth factors (Bakheet et al., 2001; Bakheet et al., 2003; Chen and Shyu, 1995).

For the mouse voltage-gated potassium channel (mKv1.4), two distinct mRNA species, ~ 3.5 kb and ~ 4.5 kb in length, had previously been determined to be expressed in mouse brain and cardiac tissues (Wymore et al., 1996). Northern blot analysis indicated that these two mRNA species differ only within the 3' NCR. Analysis of genomic sequences revealed the presence of hexameric A(A/U)UAAA sequences, potential polyadenylation determinants, located ~ 0.2 kb and ~ 1.2 kb downstream of the coding region (Wymore et al., 1994; Wymore et al., 1996). This correlates with the observed expression of mRNA species differing by ~ 1.0 kb. To determine the significance of the different 3' NCRs, expression from mRNAs containing 3' NCR sequences of mKv1.4 was initially examined in *Xenopus* oocytes. In the presence of the complete ~ 1.2 kb 3' NCR, 4–5 fold lower levels of current and ~ 11 fold lower levels of protein were observed compared with RNA containing the upstream ~ 0.2 kb alone (Wymore et al., 1996). The ~ 1.2 kb 3' NCR was reported to contain five AUUUA pentamers (a form of AREs) dispersed within the distal ~ 1.0 kb. Removal of the two distal AUUUA pentamers increased protein expression ~ 3-fold compared to RNA containing the intact ~ 1.2 kb 3' NCR, while additional mutagenesis of the proximal three AUUUA pentamers to AGUGA produced expression levels equivalent to those detected for constructs containing just the upstream ~ 0.2 kb sequences (Wymore et al., 1996). The levels of RNA did not vary significantly between wild type, truncated, or mutated 3' NCR sequences, suggesting that RNA stability could not account for differences in expression. Collectively, data from this previous study suggested that the presence of AREs in the downstream ~ 1.0 kb inhibited translation in *Xenopus* oocytes. Therefore, mKv1.4 mRNA 3' NCR sequences were hypothesized to have a similar effect in mammalian cells (Wymore et al., 1996).

In the study described in this communication, the effects of mKv1.4 mRNA 3' NCR sequences were examined in mammalian cell lines and *Xenopus* oocytes. mKv1.4 mRNA 3' NCR sequences did not specifically affect expression of a heterologous reporter RNA. Results from our experiments performed in mammalian cell culture, *in vitro*, and in *Xenopus* oocytes were markedly different from the previous results observed in *Xenopus* oocytes. However, the presence of putative mKv1.4 polyadenylation signals located ~ 0.2 kb and ~ 1.2 kb downstream of the coding region correlated with the expression of two distinct mRNA species. The larger mRNA species was more abundant than the smaller mRNA species, suggesting that its expression is the result of more efficient recognition of cleavage and polyadenylation determinants by post-transcriptional activities that mediate 3' end processing of mKv1.4 mRNAs.

2. Materials and Methods

2.1. Cell culture

HeLa (human cervical carcinoma), SK-N-SH (human neuroblastoma), and C2F3 (mouse muscle myoblast) cell lines and primary rat cardiac myocytes (PrCM) were maintained as previously described (Jang et al., 2004).

2.2. Sequencing the genomic clone

The mouse KCNA4 gene (Wymore et al., 1994) was sequenced using the following oligonucleotides:

0-Kv1.4 (+): 5' ACGCAGTCAGTTGTCCATAC 3'

1-Kv1.4 (+): 5' TGGAGAAGAGGGAGGCATTA 3'

2-Kv1.4 (+): 5' CTGCGAGGAAATGTAACAAG 3'

3-Kv1.4 (+): 5' GCACAGAGGAATTCCTGGAG 3'

4-Kv1.4 (+): 5' CCCGAGAGAGACTTCATACT 3'

5-Kv1.4 (+): 5' TCCAGCTCAGAACACTCAGT 3'

1-Kv1.4 (−): 5' CACACATACACAAAAGGGAC 3'

3-Kv1.4 (−): 5' CTCCAAGAATTCCTCTGTGC 3'

−21 M13 forward primer: 5' TGTAACGACGGCCAGT 3'

2.3. Luciferase constructs

p2luci-fxnk0 was generated by excising a 147 bp fragment from pTrap +0 (Leong and Semler, *unpublished*) with *Stu* I and *Apa* I and inserting into the blunted *Sal* I and *Apa* I sites of p2luci-fxnk (Jang et al., 2004). pRluc was generated by digesting p2luci-fxnk0 with *Avr* II alone or *Avr* II and *Xba* I and ligating the *Avr* II-*Avr* II fragment containing *Renilla* luciferase with the *Avr* II-*Xba* I vector fragment. pFluc was generated by excising *Renilla* luciferase from p2luci-fxnk0 with *Nhe* I and *Bam* HI, treating with Klenow fragment, and re-ligating the vector. p5Fluc was generated by digesting pRst1.2F (Jang et al., 2004) with *Nhe* I, treating with Klenow fragment, digesting with *Not* I, and treating with calf intestinal alkaline phosphatase (CIAP) to generate the vector. pRst1.2F was also digested with *Hind* III, treated with Klenow fragment, and digested with *Not* I to generate the insert.

pFluc and p5Fluc constructs containing 0.2, 1.2, 1.0, 1.2wt 3' NCR sequences, and variations with mutated polyadenylation signals were generated by inserting 3' NCR sequences excised from p1224luc *Btr* I *Bsr* BI² or different forms of pT7Kv1.4. p1224luc *Btr* I *Bsr* BI² was generated by digesting p1224luc with *Btr* I and *Sma* I and treating with CIAP to generate vector which was ligated with the *Bsr* BI-*Bsr* BI fragment from the genomic clone (Wymore et al., 1994). pT7Kv1.4 was derived from p1224luc *Btr* I *Bsr* BI² by excising the *Xba* I-*Xba* I fragment and inserting the *Xba* I-*Xba* I fragment from the genomic clone. 0.2 and 0.2mA1,2 were excised from p1224luc *Btr* I *Bsr* BI² and pT7Kv1.4 pA1,2, respectively, using *Xba* I and *Hinc* II, while 1.2, 1.2wt, and 1.2mA3 were excised from pT7Kv1.4 pA1,2, pT7Kv1.4, and pT7Kv1.4 pA3, respectively, using *Xba* I and *Kpn* I and treated with T4 DNA polymerase. The 1.0 insert was excised from p1224luc *Btr* I *Bsr* BI² in two fragments using *Bgl* II or *Kpn* I, treated with T4 DNA polymerase, and digested with *Pac* I. mKv1.4 mRNA 3' NCR sequences were inserted into pFluc and p5Fluc which had been digested with *Nae* I or *Not* I and *Pme* I and treated with T4 DNA polymerase and CIAP.

pFluc(0.2) containing the SV40 polyadenylation signal was generated by linearizing pFluc with *Nae* I, treating with T4 DNA polymerase and CIAP, and ligating with 0.2 which was excised from pT7Kv1.4 pA1,2 with *Xba* I and *Hinc* II and treated with T4 DNA polymerase. pFluc(1.2) and pFluc(1.0) containing the SV40 polyadenylation signal were generated by three fragment ligations. pFluc was digested with *Xba* I and *Nae* I and treated with CIAP. For pFluc

(1.2), the two insert fragments were generated by digesting pFluc(1.2) with *Xba* I and *Pac* I and digesting pT7Kv1.4 pA3 with *Pac* I and *Pvu* II. For pFluc(1.0), the two insert fragments were generated by digesting pFluc(1.2) with *Bgl* II, treating with T4 DNA polymerase, and digesting with *Pac* I and digesting pT7Kv1.4 pA3 with *Pac* I and *Pvu* II. The integrity of all plasmids was verified by DNA sequence analysis.

2.4. Kv1.4 mammalian expression constructs

pT7Kv1.4 Sma I was generated by digesting pT7Kv1.4 with *Eco* RI to remove sequences, treating with CIAP, and ligating annealed oligonucleotides to restore the 3' end of the cDNA. Oligonucleotides (2 nmol each) were phosphorylated with T4 polynucleotide kinase at 37°C for 45 minutes and annealed by incubating at 70°C for 10 minutes and cooling to room temperature. The following oligonucleotides pairs, 44asv3NCR/44as3NCR and 43s3NCR/45sv3NCR, were used:

44asv3NCR: 5' AATTCGAGCTCGGTACCCCGGGTGCATATTTAATAAAAAGATCT 3'

44as3NCR: 5' TAAGCCATAATTGCTGTAGCTGCTGGGTGCCTTATTTCTCCAAG 3'

43s3NCR: 5' AATTCCTTGGAGAAATAAGGCACCCAGCAGCTACAGCAATTATG 3'

45sv3NCR: 5' GCTTAAGATCTTTTTATTAAATATGCACCCGGGGTACCGAGCTCG 3'

To introduce a FLAG-epitope at the N-terminus of the Kv1.4 coding region, the *Nco* I restriction site within the Kv1.4 coding region of pT7Kv1.4 Sma I was mutated using KvdNcoI (+) and KvdNcoI (−) to generate pT7Kv1.4 Sma I dNco I (described below). Oligonucleotides, FLAG (−) (5' CATGCCCTTGTCATCGTCGTCCTTGTAGTCAAGAATTCC 3') and FLAGNcoI (+) (5' CATGGGAATTCTTGACTACAAGGACGACGATGACAAGGG 3'), were phosphorylated and annealed (as described above) and inserted into the remaining *Nco* I restriction site within pT7Kv1.4 Sma I dNco I.

Mammalian expression constructs encoding Kv1.4 followed by the ~ 0.2 kb or ~ 1.2 kb 3' NCR, pSV40PmFLAG-Kv1.4(0.2/1.2)pA and pSV40PmΔ5'NCR-FLAGKv1.4(0.2/1.2)pA, were constructed. The vector backbone containing the SV40 promoter and polyadenylation signal was derived from pRst1.0F by digestion with *Nhe* I and *Xba* I followed by treatment with T4 DNA polymerase. Kv1.4 sequences were derived by digesting pT7FLAGKv1.4 with *Hind* III and *Apa* I and pT7Kv1.4 Sma I with *Apa* I and *Bgl* II or *Sma* I for constructs containing the 5' NCR and digesting pT7Kv1.4 Sma I dNco I with *Nco* I and *Bgl* II or *Sma* I for constructs lacking the 5' NCR. For constructs lacking the 5' NCR, the *Nde* I fragment was excised and replaced with the equivalent fragment from pT7Kv1.4 Sma I to restore the wild type *Nco* I site within the coding region. All constructs were verified by restriction digest and sequence analysis.

2.5. PCR mutagenesis

pT7Kv1.4 (50 ng) was amplified with 125 ng of each oligonucleotide and *PfuTurbo* polymerase (Stratagene) using the following thermocycler parameters: (1) 95°C, 30 seconds, (2) 95°C, 30 seconds, (3) 55/60°C, 1 minute, (4) 68°C, 16 minutes (~ 2 min/kb). Steps 2–4 were repeated for 11 cycles. Following *Dpn* I digestion, a portion of the reaction was transformed into competent XL1-Blue bacteria.

Oligonucleotide primer pairs, pA1UC +/pA1AG −, pA2TGr +/pA2ACr −, and pA3TG +/pA3AC −, were used to mutate the three hexanucleotide sequences (designated A1, A1, and A3) at the third position uridine residue, while KvdNcoI (+) and KvdNcoI (−) were used to mutate an *Nco* I site within the Kv1.4 coding region. pA1UC +/pA1AG − and pA2TGr +/pA2ACr −

pA2ACr – while pA3TG +/pA3AC – were used to generate pT7Kv1.4 pA1,2 and pT7Kv1.4pA3, respectively. Mutations were verified by DNA sequence analysis.

pA1UC +: 5' TCAAACAAATGATACATCTTGGAGAAGAGGGAGGC 3'

pA1AG -: 5' TCATTTGTTTGATATGCAATACACGTTTAGTCACAATTATGCC 3'

pA2TGr +: 5' GAAGAGGGAGGCATGAAAAACAGCAGATC 3'

pA2ACr -: 5' GATCTGCTGTTTTTCATGCCTCCCTCTTC 3'

pA3TG +: 5' GATCTTTTTATGAAATATGCAACAAAATGCTAGATTTAAGTCCC 3'

pA3AC -: 5' TGTTGCATATTTTCATAAAAAGATCTTAAGCCATAATTGCTGTAG 3'

KvdNcoI (+): 5' TAGTGAAGAAGACCACGGGGATGGGTGTTTCG 3'

KvdNcoI (-): 5' CGAACACCCATCCCCGTGGTCTTCTTCACTA 3'

2.6. *In vitro* transcription

pRluc was linearized with *Bsr* BI, while pFluc was linearized with *Xba* I, *Pme* I, or *Ase* I to generate transcripts terminating immediately after the stop codon or containing vector sequences (~ 0.3 kb or ~ 1.0 kb), respectively. pFluc(1.2) and pFluc(1.0) were digested with *Pvu* II to generate transcripts containing the complete ~ 1.2 kb or the downstream ~ 1.0 kb, while pFluc(1.2)mA3 was linearized with *Bgl* II to generate transcript containing the upstream ~ 0.2 kb. pT7Kv1.4 *Sma* I was linearized with *Bgl* II or *Sma* I to generate Kv1.4 transcripts containing the ~ 0.2 kb or the full ~ 1.2 kb 3' NCR, respectively. Linearized plasmids were subjected to phenol/chloroform extraction and ethanol precipitation and resuspended in DEPC-treated H₂O.

In vitro transcribed RNAs for transient RNA transfections and *Xenopus* oocyte injections were generated using the mMessage mMachine T7 kit (Ambion). 1.0 µg of linearized template was incubated at 37°C for 2 hours, treated with DNase I at 37°C for 30 minutes, and precipitated with LiCl. Poly(A) tracts were added after DNase I digestion using the Poly(A) Tailing kit (Ambion).

2.7. Transient transfection

Cells were seeded 24 to 48 hours prior to transfection, generating monolayers at 50–80% or 80–90% confluency for DNA and RNA transfections, respectively. 35 mm and 60 mm dishes were used for luciferase assays and total RNA isolation, respectively. Equal molar quantities of plasmid DNA were transfected using FuGENE 6 (Roche Molecular Biochemicals) for 24 hours, while 1.0 µg firefly luciferase RNA and 0.5 µg *Renilla* luciferase RNA were transfected per 35 mm well for 15 hours using the Transmessenger transfection reagent (Qiagen) as previously described (Jang et al., 2004).

2.8. *Xenopus* oocytes experiments

Stage V oocytes were removed from *Xenopus laevis* adult females and prepared as previously described (Goldin, 1991). Oocytes were incubated in ND96 media (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5) supplemented with 0.1 mg/ml gentamicin, 0.55 mg/ml sodium pyruvate, and 0.5 mM theophylline. Potassium channel and firefly luciferase RNAs were injected at ~ 5.0 fmol/oocyte and incubated at 20°C in ND96. Potassium currents were recorded using a two-electrode voltage clamp at room temperature > 40 hours post-injection, while oocytes were Dounce homogenized in 1X passive lysis buffer

~ 24 hours post-injection for firefly luciferase assays. Potassium currents were recorded in ND96 without supplements using the oocyte clamp OC-725B (Warner Instruments, Hamden, CT), DigiData 1322A interface (Molecular Devices, Union City, CA), and pCLAMP software (version 9.1, Molecular Devices, Union City, CA). Oocytes were held at -100mV and depolarized to a range of potentials from -80mV to $+80\text{mV}$ in 20mV increments for 500ms with depolarizing pulses every 30s . Capacitive transients and leak currents were subtracted prior to analysis by P/4 subtraction.

2.9. Luciferase assays

Transfected cells were lysed with 1X passive lysis buffer 15 or 24 hours post-transfection and assays were performed using the Dual Luciferase Reporter Assay as previously described (Jang et al., 2004). Transfections were performed in triplicate and Fluc/Rluc ratios were determined for each sample. Average values are represented as fold relative to an arbitrary standard, usually pFluc.

2.10. Isolation of total RNA and oligo(dT) selection

Total RNA was isolated from cells and mouse brain tissue using TRI Reagent (Molecular Research Center, Inc.) and treated with DNase I (Worthington Biochemical Corporation) or subjected to oligo-dT selection using the Micro-FastTrack mRNA isolation kit (Invitrogen) followed by DNase I digestion. RNAs were incubated in 10 mM Tris-HCl, pH 7.7, 1 mM EDTA, 10 mM MgCl_2 , 1 mM DTT, 40 units RNasin, and $5.0\text{ }\mu\text{g}$ DNase I ($\sim 2.7\text{ units}/\mu\text{g}$) at 37°C for $30\text{--}60\text{ minutes}$. DNase I digestion was stopped by adding 10 mM EDTA, 0.3 M NaOAc, and 0.1% SDS and RNA was subjected to phenol/chloroform extraction and ethanol precipitation prior to resuspension with DEPC-treated H_2O or isolated using RNeasy columns (Qiagen).

2.11. Northern blot analysis

Total RNA from transiently transfected cells or oligo(dT) selected mRNAs were subjected to Northern blot analysis as previously described (Jang et al., 2004). mRNAs were detected using random hexamer primed ^{32}P -labeled probes against *Renilla* and firefly luciferase sequences, Kv1.4 coding region, 5' NCR, or 3' NCR sequences, and human β -actin cDNA sequences. The Kv1.4 coding region was excised from the genomic clone using *Sac* I, while Kv1.4 3' NCR sequences were excised from pGEM4Z-3' NCR [Kv1.4] (Jang and Semler, *unpublished*) with *Xba* I and *Eco* RI.

2.12. Western blot analysis

Cytoplasmic lysates were isolated from transiently transfected cells ~ 24 hours post transfection. Monolayers were rinsed twice with 1X PBS, resuspended in 1.0 ml cold 1X PBS, and centrifuged at $3,000\text{ rpm}$ for 5 minutes at 4°C . Cells were resuspended in 0.5 ml cold TSM Buffer (10 mM Tris, pH 7.6, 0.15 M NaCl, 5.0 mM MgCl_2), centrifuged at $3,000\text{ rpm}$ for 5 minutes at 4°C , resuspended in $200\text{ }\mu\text{l}$ of TSM containing 0.2% Nonidet P-40 and $100\text{ }\mu\text{M}$ phenylmethylsulfonyl fluoride, and incubated on ice for 20 minutes . Following centrifugation $16,000 \times g$ for 5 min at 4°C , the supernatant was recovered and stored at -20°C .

Protein was quantified using the BioRad Protein Assay. Equal micrograms of total protein were resolved on a 10% SDS-polyacrylamide gel at 30 mA/gel for $35\text{--}45\text{ minutes}$ and electroblotted to an Immobilon-P transfer membrane (Millipore) at 100 V for 80 minutes . Membranes were incubated in blocking buffer (5% milk powder, 0.1% Tween-20, 1X PBS) for 45 minutes at room temperature and washed with 0.1% Tween-20, 1X PBS. Membranes were incubated with primary antibodies [anti-Kv1.4 antibody ($1:3,000$ dilution; Upstate Technologies), and anti- β -actin antibody ($1:10,000$)], and anti-mouse antibody conjugated to alkaline phosphatase

(1:5,000) in 0.1% Tween-20, 1X PBS at room temperature for 45 minutes, repeatedly washed with 0.1% Tween-20, 1X PBS, and developed with NBT/NCIP (PIERCE).

3. Results

3.1. mKv1.4 mRNA 3' NCR sequences

mKv1.4 mRNA sequences were initially determined by aligning genomic sequences with cDNA sequences from other species (Wymore et al., 1994). Human, rat, and bovine 3' NCR cDNA sequences were reported to be ~79–84% identical to corresponding mouse genomic sequences (Wymore et al., 1994); however, subsequent re-sequencing of the genomic clone indicated some differences compared with the original reported sequence that are completely consistent with the existing GenBank genomic reference sequence (NT_039207 and Jang and Semler, *unpublished*). mKv1.4 mRNA 3' NCR sequences contain six AUUUA pentamers within the distal ~1.0 kb and two putative polyadenylation sites ~0.2 kb and ~1.2 kb downstream of the coding region (Figure 1). The putative upstream polyadenylation signal is composed of one of two hexanucleotide sequences (designated A1 and A2 in Figure 1) and U/GU-rich sequences. The putative downstream polyadenylation signal is composed of a hexanucleotide sequence (A3) and an extended GU-rich tract. A fourth hexanucleotide motif [nucleotides 3752–3757; denoted as (A4)] may indicate a third polyadenylation site; however, mRNA expression data in mouse tissues are not consistent with its use (Wymore et al., 1996). Utilization of these polyadenylation signals is supported by EST cDNA sequences which terminate nine (BU604649) and five to eighteen (BY648668, BB301808, BB316841, and BB537910) base pairs downstream of A1 and A3, respectively (Figure 1).

3.2. Effects of mKv1.4 3' NCR sequences on reporter expression in mammalian cells and cell-free extracts

To investigate possible effects of mKv1.4 3' NCR sequences on reporter expression in mammalian cells, firefly luciferase constructs containing the upstream ~0.2 kb, the downstream ~1.0 kb, or the complete ~1.2 kb were co-transfected into cells with pRLuc (Figure 2A). Hexanucleotide sequences were mutated (denoted mA) to inhibit 3' end processing at native mKv1.4 mRNA polyadenylation sites. The SV40 polyadenylation signal positioned downstream of inserted sequences mediated 3' end processing. For these constructs, nearly identical levels of firefly luciferase activity (normalized over *Renilla* luciferase values for transfection efficiency) were observed in the absence (–) and presence of the full-length mKv1.4 mRNA 3' NCR sequence (1.2) in SK-N-SH cells (Figure 2B) and HeLa cells (data not shown). For the construct containing the downstream 1.0 kb 3' NCR sequences (1.0), there was a moderate (~40%) increase in expression compared to the 1.2 kb construct. In addition, we found that a reporter construct harboring just the upstream 0.2 kb 3' NCR sequences (0.2) had somewhat lower levels (by ~30%) of luciferase activity compared with those found for the full length (1.2 kb) 3' NCR. In a separate transfection of SK-N-SH cells, mRNA expression levels from all constructs were within two-fold of each other (Figure 2C).

Somewhat different results were observed when *in vitro* transcribed reporter RNAs were examined in cells (Table 1) or *in vitro* (data not shown). In SK-N-SH cells, expression from capped and polyadenylated RNAs was highest (by ~3 fold) when the 0.2 kb sequences were present in the 3' NCR of the reporter RNA compared with levels detected for either the 1.0 or 1.2 kb sequences. Likewise, the presence of a shorter (0.3) non-specific vector sequence produced higher (~2.4 fold) normalized firefly luciferase values than those generated with a 1.0 kb vector sequence present in the 3' NCR (Table 1). Similar results were obtained in transfected HeLa cells (data not shown). Additionally, when *in vitro* transcribed, capped RNAs were translated *in vitro* using HeLa S10 cytoplasmic extract or rabbit reticulocyte lysate, similar

levels of firefly luciferase activity were observed in the absence or presence of mKv1.4 mRNA 3' NCR sequences (data not shown).

Collectively, these results are not consistent with previous findings observed in *Xenopus* oocytes and do not support an inhibitory role for the distal ~ 1.0 kb in mammalian cells. These data suggest that mKv1.4 mRNA 3' NCR sequences do not affect expression post-transcriptionally at the level of either RNA stability (Figure 2C; data not shown) or translation in the cell lines used in our experiments.

3.3. Exogenous Kv1.4 expression

Experiments performed by Wymore and colleagues used RNAs harboring Kv1.4 or Kv1.3 coding region sequences (Wymore et al., 1996). To determine if potassium channel coding region sequences are important for mKv1.4 mRNA 3' NCR activity, expression constructs encoding Kv1.4 were transiently transfected into cells and analyzed for RNA and protein expression (Figure 3). In HeLa cells, similar levels of mRNA were detected by Northern blot analysis using total RNA and oligo(dT) selected RNA (Figure 3A, left panel and right panel, respectively). In the absence of the ~ 1.2 kb 5' NCR, nearly equivalent levels of mRNA were expressed in HeLa cells regardless of the 3' NCR; 1.2 was 87% of 0.2 when normalized with β -actin mRNA (Figure 3A, lanes 4 and 5). Similar data were obtained in transfected SK-N-SH cells (data not shown). To resolve mRNAs containing the full 5' NCR and the ~ 1.2 kb 3' NCR, total RNA was subjected to oligo(dT) selection prior to Northern blot analysis (Figure 3A, right panel). When normalized with β -actin mRNA, the expression level of Kv1.4 mRNA containing the 1.2 3' NCR was 84% of that produced by the mRNA harboring the 0.2 sequences from the 3' NCR (Figure 3A, right panel, lanes 7 and 8).

When cytoplasmic fractions were analyzed by Western blot using a Kv1.4-specific antibody, two distinct bands (denoted by arrows) were detected in transiently transfected, but not mock transfected cells (Figure 3B, compare lanes 4 and 5–8). The faster migrating band co-migrated with a diffuse band detected in rat brain microsomes (rbm; Figure 3B, lane 1), a positive control for Kv1.4 expression. Bands of similar electrophoretic mobility were detected in lysates from cells inducibly expressing Kv1.4 (Figure 3B compare lane 3 to lanes 5 through 8). The amounts of protein mirror comparative RNA levels detected by Northern blot (Figure 3A, lanes 4, 5, 7, and 8); for both sets of RNA, the level of protein expressed from RNA containing the ~ 1.2 kb 3' NCR was 79–88% compared to expression from RNA containing the ~ 0.2 kb 3' NCR (Figure 3B, lanes 5–8). These results are consistent with experiments performed using firefly luciferase constructs (Figure 2B) and suggest that a putative role for the mKv1.4 mRNA 3' NCR sequences in mammalian cells is not dependent on Kv1.4 coding region sequences.

3.4. Kv1.4 expression in *Xenopus* oocytes

To determine whether the 3' NCR activities of mKv1.4 are *Xenopus* oocyte-specific, *in vitro* transcribed Kv1.4 mRNAs and firefly luciferase RNAs (utilized in Table 1) were examined for current and firefly luciferase activity in *Xenopus* oocytes, respectively (Figure 4). RNAs were capped *in vitro* and contain a post-transcriptionally added poly(A) tract. Representative current traces and average currents from eight independently injected oocytes depolarized at + 80 mV are shown (Figure 4A and 4B). When Kv1.4 mRNAs containing the ~ 1.2 kb mKv1.4 3' NCR were injected into oocytes, ~ 2-fold less current was measured compared to mRNAs containing the ~ 0.2 kb 3' NCR. (Figure 4B). Similar differences were observed at other depolarization potentials (– 80 to + 60 mV; data not shown). When oocytes were injected with reporter RNAs, similar levels of firefly luciferase activity were observed, independent of the presence of non-specific or Kv1.4-specific 3' NCR sequences (Figure 4C). Together these data suggest that Kv1.4 mRNA coding sequences may affect 3' NCR activity in oocytes, since ion

channel current, but not luciferase activity, differed between respective mRNAs containing the short (0.2 kb) compared to the long (1.2 kb) 3' NCR.

3.5. Expression of Kv1.4 mRNA species in mouse tissues and mammalian cell lines

Wymore and colleagues previously suggested that different polyadenylation signals in Kv1.4 pre-mRNAs may generate distinct mRNA species (Wymore et al., 1994). In a subsequent study, two distinct Kv1.4 mRNA species expressed in mouse brain tissue were demonstrated to differ only within their 3' NCR sequences (Wymore et al., 1996). We detected two mRNA species in RNA samples isolated from AKR/J adult mouse brain when probed for mKv1.4 mRNA 5' NCR sequences (Figure 5, lane 5). These species were not readily detected in RNA samples harvested from mouse skeletal muscle, heart, and liver tissue (data not shown). Low levels of Kv1.4 mRNA were previously detected in mouse cardiac and skeletal muscle (Wymore et al., 1996). Quantitation of the two mRNA species indicated that the larger mRNA species is 5–10 fold more abundant than the smaller RNA species in mouse brain tissue (Figure 5, lane 5). Differences in abundance are not due to decreased mRNA stability (Jang and Semler, unpublished observations). Kv1.4 mRNA expression was also examined in several mammalian cells/cell lines (Figure 5, lanes 1–4). Kv1.4 mRNA expression was not detected in HeLa cells or in a human neuroblastoma cell line (SK-N-SH). In contrast, ~ 2.2 kb and ~ 2.8 kb mRNA species were detected in a mouse muscle myoblast cell line (C2F3) and in primary rat cardiac myocytes (PrCM) prepared from neonatal rats, respectively, when hybridized with a probe against the mKv1.4 mRNA 3' NCR sequences (Figure 5, lanes 3 and 4). Expression of a single mRNA species in these cells suggests that polyadenylation of Kv1.4 pre-mRNAs may be cell-type-dependent. Differences in mRNA expression between adult cardiac tissue and neonatal cardiac myocytes are consistent with developmental regulation of Kv1.4 expression in rat ventricular myocytes (Figure 5 and data not shown) (Xu et al., 1996; Wymore et al., 1996).

3.6. mKv1.4 mRNA 3' NCR sequences determine RNA abundance

The significance of mKv1.4 mRNA 3' NCR sequences was further examined in mammalian cells using reporter constructs containing native Kv1.4 polyadenylation signals instead of the SV40 polyadenylation signal. The upstream ~ 0.2 kb (0.2), the distal ~ 1.0 kb (1.0), or the complete ~ 1.2 kb (1.2) 3' NCR along with flanking sequences were inserted downstream of the firefly luciferase coding region (Figure 6A). For the 1.2 construct, upstream hexanucleotide motifs (A1 and A2) were mutated at the third position uridine (mA1,2) to inhibit 3' end processing at the putative upstream polyadenylation site.

When reporter constructs (Figure 6A) were co-transfected into SK-N-SH cells with pRLuc (refer to Figure 2A), lower levels of firefly luciferase activity were observed with 0.2 sequences in the 3' NCR compared to constructs containing 1.2 or 1.0 sequences (Figure 6B). Similar patterns of expression were observed in HeLa cells, C2F3 cells, and primary rat cardiac myocytes (PrCM) (data not shown). Examination of RNA expression in transiently co-transfected SK-N-SH cells demonstrated that RNA abundance varied between constructs (Figure 6C). A ~ 2.5 kb mRNA species was expressed when cells were transfected with pFluc (Figure 6C, lane 2). Although the smallest mRNA expressed, this mRNA was also the most abundant and was arbitrarily set at 100%. The presence of mKv1.4 mRNA 3' NCR sequences (1.2 or 1.0) correlated with the expression of ~ 3.8 kb and ~ 3.5 kb mRNA species that were 49% and 62%, respectively, compared to RNA expressed from pFluc (Figure 6C, compare lanes 2–4). In contrast, the presence of the upstream 0.2 sequences generated an mRNA species of ~ 2.7 kb, expressed at only 11% of the levels of pFluc (Figure 6C, compare lanes 2 and 5). These results show that differences in expression observed between 0.2, 1.2, and 1.0 are due to altered RNA abundance and suggest that elements within the mKv1.4 mRNA 3' NCR contribute to such differences.

3.7. Distinct mRNA species and differences in RNA abundance are attributable to different polyadenylation signals

DNA transfection experiments using constructs harboring the SV40 polyadenylation signal demonstrated that the presence of mKv1.4 mRNA 3' NCR sequences alone does not significantly affect expression of reporter constructs (see Figure 2). Data from mRNA transfection/oocyte injection experiments and *in vitro* translation assays supported a similar conclusion (see Table 1 and Figure 4; data not shown). However, we found that the presence of mKv1.4 mRNA 3' NCR sequences containing native polyadenylation signals correlated with different levels of mRNA expression in cells (Figure 6). Mutation of polyadenylation determinants (Figure 7A) demonstrated that expression of specific mRNA species could be correlated with the presence of native mKv1.4 mRNA polyadenylation signals located ~0.2 kb and ~1.2 kb downstream of the coding region. For the 1.2 wt 3' NCR construct containing both wild type polyadenylation signals, two mRNA species were observed (Figure 7C, lane 7). When upstream hexanucleotide sequences (designated A1,2) were mutated in 1.2 (mA1,2), only the larger mRNA species was detected (Figure 6C, lane 3 and Figure 7C, lane 3). In contrast, for 0.2 and 1.2 mA3, only the smaller mRNA species was detected at a lower abundance (Figure 6C, lane 5 and Figure 7C, lanes 5 and 6). The 0.2 construct lacks sequences following the upstream polyadenylation signal, while the hexanucleotide sequence (A3) was mutated in 1.2 mA3. Expression of the smaller mRNA species alone at a lower overall abundance correlated with decreased levels of firefly luciferase activity (Figure 7B). When the upstream 0.2 sequences from the 3' NCR were deleted to generate the 1.0 construct, the presence of the remaining downstream polyadenylation signal (A3) correlated with the expression a single mRNA species migrating slightly faster than the one detected for the 1.2 construct (Figure 6C and Figure 7C, compare lane 3 and 4). This construct expressed luciferase levels nearly as high (82%) as those of the 1.2 wt construct (Figure 7B). Finally, when hexanucleotide sequences assumed to form the upstream polyadenylation signal were mutated to produce 0.2 mA1,2, a specific mRNA species could no longer be detected by Northern blot analysis (Figure 7C, lane 8), correlating with a further decrease in luciferase expression, 12% compared with 26% for the 0.2 construct (Figure 7B). Collectively, these data suggest that differences in RNA abundance observed in transiently transfected cells are attributable to distinct polyadenylation signals within mKv1.4 pre-mRNAs. The presence of the 1.2 kb 3' NCR results in higher levels of RNA expression compared to the 0.2 kb 3' NCR, reflecting more efficient utilization of the downstream polyadenylation signal relative to the upstream polyadenylation signal.

4. Discussion

The ~4.5 kb Kv1.4 mRNA species expressed in mouse brain and cardiac tissues contains a ~1.2 kb, AU-rich 3' NCR. Previous data from mRNA injection of *Xenopus* oocytes showed that inclusion of the distal ~1.0 kb correlated with decreased current and protein expression compared with RNA containing the proximal ~0.2 kb alone (Wymore et al., 1996). Removal and/or mutation of AUUUA pentamers within the distal ~1.0 kb relieved this inhibition of protein expression without affecting RNA stability, leading to the suggestion that mKv1.4 mRNA 3' NCR sequences inhibit translation (Wymore et al., 1996). In contrast, we found that in mammalian cells, the presence of mKv1.4 mRNA 3' NCR sequences did not significantly affect expression when inserted between the firefly luciferase coding region and the SV40 polyadenylation signal in transient DNA transfection experiments (Figure 2B). Similar results were observed when *in vitro* transcribed RNAs containing mKv1.4 mRNA 3' NCR sequences were examined in cell culture and *in vitro* (Table 1 and data not shown). The apparent discrepancies between our protein expression data in mammalian cells and those previously reported for *Xenopus* oocytes may be due, in part, to the use of a heterologous 5' IRES sequence from the genomic RNA of encephalomyocarditis virus in the expression constructs used by

Wymore and colleagues (Wymore et al., 1996). Such differences may also be due to the use of a tagged Kv1.3 coding sequence in the previous studies (rather than the Kv1.4 coding sequence or the firefly luciferase coding sequence present in our constructs) to monitor protein levels in injected oocytes or to subtle differences in the translation components in mammalian cells compared to those in oocytes.

When transiently co-transfected into mammalian cells/cell lines, reporter constructs lacking the SV40 polyadenylation signal mediated lower levels of expression in the presence of the upstream ~ 0.2 kb alone (0.2) compared with constructs containing the entire ~ 1.2 kb (1.2), the distal ~ 1.0 kb (1.0), or the SV40 polyadenylation signal alone (Figure 6). Deletion of sequences within the distal ~ 1.0 kb moderately decreased expression in the context of 1.2 wt, while the deletion of sequences within 0.2 further decreased expression, ~ 50% compared with 0.2 (data not shown). The presence of the upstream ~ 0.2 kb alone correlated with decreased mRNA abundance compared with constructs containing 1.2, 1.0, or the SV40 polyadenylation signal. Although reporter expression was shown to parallel RNA abundance, the presence of different regions of the 3' NCR did not specifically alter RNA stability in SK-N-SH cells (data not shown), indicating that differences in RNA abundance were not influenced at the level of RNA stability.

The expression of two mKv1.4 mRNA species differing by only ~ 1.0 kb within the 3' NCR was initially suggested to be the result of polyadenylation signals located ~ 0.2 kb and ~ 1.2 kb downstream of the coding region (Wymore et al., 1996). Our data showed that two mRNA species were detected when the complete 3' NCR was inserted downstream of firefly luciferase. Only the smaller mRNA species was detected at a lower abundance when the upstream ~ 0.2 kb alone was included or the downstream polyadenylation signal was mutated as in 1.2 mA3, while only the larger mRNA species was detected when the upstream polyadenylation signal was mutated in 1.2. EST and cDNA sequences terminating 178 (BU604649) and 1159-1176 (BY648668, BB301808, BB321534, BB316841, and BB537910) nucleotides downstream of the stop codon support the utilization of the identified polyadenylation signals. Other EST sequences terminating 958 (BE648550) and 1007 (AW123739 and BE952437) nucleotides downstream of the stop codon do not correlate with identifiable cleavage and polyadenylation determinants. EST sequences terminating downstream of A4, the only other potential polyadenylation determinant within the 3' NCR, have not been identified.

Although mKv1.4 mRNA 3' NCR sequences do not directly affect expression post-transcriptionally, the relative strengths of the upstream and downstream polyadenylation signals appear to determine RNA abundance, consistent with the observation that the ~ 4.5 kb mRNA species is ~ 5–10 fold more abundant than the ~ 3.5 kb mRNA species in mouse brain tissue. Perhaps changes in the efficiency of cleavage and polyadenylation contribute to Kv1.4 expression by altering RNA abundance. It will be of interest to determine if such a mechanism contributes to the control of ion channel activity in brain or cardiac tissues. It has been shown that 3' end processing can be regulated by cleavage and polyadenylation factors as well as non-constitutive factors. Increased expression of the 3' end processing factor, CstF-64, was shown to shift cleavage and polyadenylation from the membrane-specific polyadenylation site to the secretion-specific polyadenylation site for the immunoglobulin M heavy chain pre-mRNA (Takagaki et al., 1996). Additional studies demonstrated that increased binding between CstF-64 and the 100 kDa subunit of cleavage and polyadenylation specificity factor (CPSF-100) and polyadenylation sites correlated with B cell development and was not dependent on increased protein levels (Edwards-Gilbert et al., 1997). Non-constitutive factors may also affect 3' end processing by interfering with the association or activities of constitutive cleavage and polyadenylation factors. U1A protein, a component of U1 snRNP, auto-regulates expression of its own mRNA by binding specific elements within the 3' NCR and inhibiting polyadenylation through direct interactions with poly(A) polymerase (Boelens et al., 1993;

Gunderson et al., 1994). U1A binding at two distinct regions within the immunoglobulin M heavy chain pre-mRNA inhibits cleavage and polyadenylation at the secretion-specific polyadenylation site by two different mechanisms: preventing the association of CstF-64 and inhibiting poly(A) polymerase, respectively (Phillips et al., 2001; Phillips et al., 2004).

Some of the cells/cell lines or the conditions utilized in these studies may have limitations in their usefulness for analyzing Kv1.4 regulation. Among the cells/cell lines examined, only C2F3, the mouse skeletal myoblast cell line, and primary cardiac myocytes (PrCM) endogenously expressed a single mRNA species hybridizing to mKv1.4 mRNA 3' NCR-specific probe (refer to Figure 5). If these mRNA species actually encode functional Kv1.4 protein, this would suggest that a single polyadenylation signal is preferentially utilized in a cell-type-specific manner; however, transient transfection experiments indicated that both polyadenylation signals can be used in C2F3 and PrCM (data not shown). These mRNA species have yet to be characterized.

It should be noted that the studies described in this report have primarily examined mKv1.4 mRNA 3' NCR sequences in the absence and presence of the complete 5' NCR; however, within the nucleus, cleavage and polyadenylation are closely linked with transcription, splicing, and export. Therefore, native elements of the gene and/or RNA polymerase II transcription apparatus may be required for 3' NCR-specific activity. The presence of the native promoter may affect cleavage and polyadenylation by facilitating the recruitment of factors to the polymerase. Alternatively, native Kv1.4 coding region sequences may contribute to 3' NCR activities. Indeed, our potassium channel current data generated following injection of transcripts containing the complete Kv1.4 coding region showed differences when the 0.2 sequences versus the 1.2 sequences from the 3' NCR were included. Although slightly less dramatic in magnitude, our findings are in agreement with results of previous investigators who observed 3' NCR-mediated effects on Kv1.4 current recordings in *Xenopus* oocytes (Wymore et al., 1996). Future studies using intact Kv1.4 genomic sequences will be required to determine the individual contributions of the coding and non-coding sequences in the overall expression patterns of both Kv1.4 mRNAs and their encoded ion channel proteins.

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Abbreviations

mKv1.4	mouse voltage-gated potassium channel 1.4
NCR	noncoding region
SV40	simian virus 40

References

- Bakheet T, Frevel M, Williams BR, Greer W, Khabar KS. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res* 2001;29:246–254. [PubMed: 11125104]

- Bakheet T, Williams BR, Khabar KS. ARED 2.0: an update of AU-rich element mRNA database. *Nucleic Acids Res* 2003;31:421–423. [PubMed: 12520039]
- Berget SM. Exon recognition in vertebrate splicing. *J Biol Chem* 1995;270:2411–2414. [PubMed: 7852296]
- Boelens WC, Jansen EJ, van Venrooij WJ, Stripecke R, Mattaj IW, Gunderson SI. The human U1 snRNP-specific U1A protein inhibits polyadenylation of its own pre-mRNA. *Cell* 1993;72:881–892. [PubMed: 8458082]
- Calvo O, Manley JL. Strange bedfellows: polyadenylation factors at the promoter. *Genes Dev* 2003;17:1321–1327. [PubMed: 12782649]
- Chen CYA, Shyu AB. AU-rich elements - characterization and importance in messenger-RNA degradation. *Trends in Biochemical Sciences* 1995;20:465–470. [PubMed: 8578590]
- Edwards-Gilbert G, Veraldi KL, Milcarek C. Alternative poly(A) site selection in complex transcription units: means to an end? *Nucleic Acids Res* 1997;25:2547–2561. [PubMed: 9185563]
- Goldin AL. Expression of ion channels by injection of mRNA into *Xenopus* oocytes. *Methods Cell Biol* 1991;36:487–509. [PubMed: 1725803]
- Gunderson SI, Beyer K, Martin G, Keller W, Boelens WC, Mattaj LW. The human U1A snRNP protein regulates polyadenylation via a direct interaction with poly(A) polymerase. *Cell* 1994;76:531–541. [PubMed: 8313473]
- Jang GM, Leong LE, Hoang LT, Wang PH, Gutman GA, Semler BL. Structurally distinct elements mediate internal ribosome entry within the 5'-noncoding region of a voltage-gated potassium channel mRNA. *J Biol Chem* 2004;279:47419–47430. [PubMed: 15339906]
- Phillips C, Jung S, Gunderson SI. Regulation of nuclear poly(A) addition controls the expression of immunoglobulin M secretory mRNA. *EMBO J* 2001;20:6443–6452. [PubMed: 11707415]
- Phillips C, Pachikara N, Gunderson SI. U1A inhibits cleavage at the immunoglobulin M heavy-chain secretory poly(A) site by binding between the two downstream GU-rich regions. *Mol Cell Biol* 2004;24:6162–6171. [PubMed: 15226420]
- Proudfoot NJ. How RNA polymerase II terminates transcription in higher eukaryotes. *Trends Biochem Sci* 1989;14:105–110. [PubMed: 2658217]
- Takagaki Y, Seipelt RL, Peterson ML, Manley JL. The polyadenylation factor CstF-64 regulates alternative processing of IgM heavy chain pre-mRNA during B cell differentiation. *Cell* 1996;87:941–952. [PubMed: 8945520]
- Wymore RS, Korenberg JR, Kinoshita KD, Aiyar J, Coyne C, Chen XN, Hustad CM, Copeland NG, Gutman GA, Jenkins NA. Genomic organization, nucleotide sequence, biophysical properties, and localization of the voltage-gated K⁺ channel gene KCNA4/Kv1.4 to mouse chromosome 2/human 11p14 and mapping of KCNC1/Kv3.1 to mouse 7/human 11p14.3-p15.2 and KCNA1/Kv1.1 to human 12p13. *Genomics* 1994;20:191–202. [PubMed: 8020965]
- Wymore RS, Negulescu D, Kinoshita K, Kalman K, Aiyar J, Gutman GA, Chandy KG. Characterization of the transcription unit of mouse Kv1.4, a voltage-gated potassium channel gene. *J Biol Chem* 1996;271:15629–15634. [PubMed: 8663090]
- Xu H, Dixon JE, Barry DM, Trimmer JS, Merlie JP, McKinnon D, Nerbonne JM. Developmental analysis reveals mismatches in the expression of K⁺ channel alpha subunits and voltage-gated K⁺ channel currents in rat ventricular myocytes. *J Gen Physiol* 1996;108:405–419. [PubMed: 8923266]
- Zhao J, Hyman L, Moore C. Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol Mol Biol Rev* 1999;63:405–445. [PubMed: 10357856]

Figure 1.

Schematic representation of mKv1.4 mRNA 3' NCR. mKv1.4 mRNA 3' NCR sequences were determined from the genomic clone [(Wymore et al., 1996); Jang and Semler, *unpublished*]. Six AUUUA motifs (dark grey) are located within the distal ~ 1.0 kb between the two putative polyadenylation signals positioned ~ 0.2 kb and ~ 1.2 kb downstream of the coding region, while a seventh motif is present within the distal polyadenylation signal. Elements forming putative polyadenylation signals, the hexanucleotide sequence (black) and U/GU-rich sequences (white), are highlighted or boxed. Three hexanucleotide sequences, A(A/U)UAAA (denoted A1, A2, and A3), may contribute to polyadenylation. A fourth hexanucleotide (A4) located within the distal 1.0 kb does not correlate with known Kv1.4 mRNA species. A1 or A2 and downstream U/GU-rich sequences potentially comprise the upstream polyadenylation signal, while the downstream polyadenylation signal is likely to be composed of A3 and a long GU-rich tract. Nucleotide numbering is based on compiled mKv1.4 mRNA cDNA sequences. The 5' terminus defined by Wymore et al., 1996 begins eleven nucleotides upstream of the mRNA cDNA sequence and is set as the start of transcription. Asterisks denote sites of pre-mRNA cleavage according to published EST cDNA sequences.

Figure 2.

(A) Schematic representation of luciferase reporter constructs. Firefly luciferase constructs contain no 3' NCR sequences or different regions of the mKv1.4 mRNA 3' NCR between the firefly luciferase coding region and the SV40 polyadenylation signal. Reporter constructs contain the SV40 promoter (SV40pm; patterned box), a chimeric intron, the T7 promoter (T7pm; vertical line), and the SV40 polyadenylation signal (SV40pA; dark grey box). The 0.2 and 1.2 constructs include a small portion of the coding region (black box). mA1,2 and mA3 denote the mutation of hexanucleotide sequences and are indicated by asterisks (*). SK-N-SH cells were mock transfected (M) or transiently transfected with luciferase constructs for 24 hours. (B) Cell lysates were assayed for luciferase activity and (C) total RNA was isolated and

examined for firefly luciferase mRNA expression by Northern blot (as described in Materials and Methods).

Figure 3.

Exogenous Kv1.4 expression in HeLa cells. Kv1.4 expression constructs containing the ~ 0.2 kb or the ~ 1.2 kb mKv1.4 mRNA 3' NCR in the presence (+) or absence (Δ) of the complete mKv1.4 mRNA 5' NCR were transiently transfected into HeLa cells and examined for RNA and protein expression 24 hours post-transfection. (A) Total RNA (*left*) and oligo(dT) selected RNA (*right*) were subjected to Northern blot analysis using random hexamer primed probes to Kv1.4 coding region sequences and β-actin. HeLa cells were mock transfected (M, lanes 1 and 6) or transiently transfected with mammalian Kv1.4 expression constructs containing the ~ 0.2 kb 3' NCR (lanes 2, 4, and 7) or ~ 1.2 kb 3' NCR (lanes 3, 5, and 8) in the presence (lanes 2, 3, 7, and 8) or absence (lanes 4 and 5) of the authentic 1.2 kb 5' NCR of mKv1.4 mRNA. (B) Cytoplasmic lysates were examined for Kv1.4 protein expression by Western blot analysis (as described in Materials and Methods). As controls, rat brain microsomes (rbm; lane 1) and cell lysates from an inducible Kv1.4 expressing cell line were also used [Ind; lane 2 (uninduced) and lane 3 (induced)]. HeLa cells were mock transfected (M, lane 4) or transiently transfected with mammalian Kv1.4 expression constructs containing the ~ 0.2 kb 3' NCR (lanes 5 and 7) or ~ 1.2 kb 3' NCR (lanes 6 and 8) in the presence (lanes 5 and 6) or absence (lanes 7 and 8) of the 1.2 kb 5' NCR of mKv1.4 mRNA.

Figure 4.

Effects of the mKv1.4 mRNA 3' NCR in *Xenopus* oocytes. Kv1.4 and firefly luciferase RNAs (5.0 fmol) containing mKv1.4 mRNA 3' NCR sequences were injected into *Xenopus* oocytes and incubated at 20°C for ~ 40 and ~ 24 hours prior to current measurement and firefly luciferase assays, respectively. Kv1.4 RNAs contain the complete mKv1.4 mRNA 5' NCR, the coding region, and the ~ 0.2 kb (0.2) or the ~ 1.2 kb (1.2) 3' NCR. Current measurements were performed for 12 independent oocytes (0.2 kb 3' NCR) or 11 independent oocytes (1.2 kb 3' NCR), while firefly luciferase activity was assayed for four independent oocytes (refer to Materials and Methods). (A) Representative current traces and (B) average peak currents are shown for oocytes depolarized at a potential of + 80 mV. Error bars represent standard deviation from mean current traces ($p < 0.001$). (C) Firefly luciferase activities were measured for oocytes injected with RNAs containing no 3' NCR (–), vector sequences (0.3 and 1.0), and mKv1.4 mRNA 3' NCR sequences (1.2, 1.0, and 0.2) [refer to Materials and Methods]. Error bars represent standard deviation from mean luciferase values.

Figure 5.

Expression of Kv1.4 mRNA species in mouse brain tissue and mammalian cells/cell lines. Oligo(dT) selected mRNAs from AKR/J mouse brain tissue (6.0 μ g; lane 5) and mammalian cells/cell lines [HeLa, SK-N-SH, C2F3, and primary rat cardiac myocytes (PrCM)] (3.0 μ g; lanes 1–4) were analyzed by Northern blot analysis using probes complementary to mKv1.4 mRNA 3' NCR (lanes 1–4) or 5' NCR sequences (lane 5). AKR/J mouse tissues were obtained from Charles River Laboratories, and cells were maintained as described in Materials and Methods.

Figure 6.

(A) Schematic representation of firefly luciferase reporter constructs containing mKv1.4 mRNA 3' NCR sequences. pFluc (refer to Figure 2A) contains the SV40 promoter (SV40pm; patterned box), a chimeric intron, the T7 promoter (T7pm; vertical line), and the SV40 polyadenylation signal, but no additional 3' NCR sequences. The complete ~ 1.2 kb (1.2) 3' NCR, the downstream ~ 1.0 kb (1.0), and the upstream ~ 0.2 kb (0.2) were inserted into pFluc lacking the SV40 polyadenylation signal. In 1.2, hexanucleotide sequences, A1 and A2, were mutated at the third position uridine residue (indicated by mA1,2 and asterisks [*]). 0.2 and 1.2 include a small portion of the coding region (black box). Firefly luciferase constructs were transiently co-transfected into SK-N-SH cells with pRluc and examined for (B) firefly luciferase activity and (C) mRNA expression 24 hr post-transfection. pFluc was arbitrarily set

at 100% and transfections were performed in triplicate for luciferase assays. For RNA expression, SK-N-SH cells were mock transfected (M; lane 1) or transiently co-transfected with pRluc and pFluc (lane 2), pFluc(1.2) (lane 3), pFluc(1.0) (lane 4), or pFluc(0.2) (lane 5). Total RNA was subjected to Northern blot analysis using random hexamer-primed probe against Renilla and firefly luciferase coding region sequences. Blots were exposed to a phosphorimager screen and analyzed using Quantity One version 4.3.0 (BioRad). RNA size and relative abundance were determined for RNAs expressed in SK-N-SH cells. Renilla luciferase RNA was used to normalize firefly luciferase RNA levels.

Figure 7. Native Kv1.4 mRNA polyadenylation signals mediate expression of two mRNA species. (A) Schematic representation of mKv1.4 mRNA 3' NCR sequences containing wild type or mutant mKv1.4 mRNA polyadenylation determinants inserted into firefly luciferase constructs. Firefly luciferase constructs contain the SV40 promoter (SV40pm), a chimeric intron, and the T7 promoter (T7pm). The 0.2 and 1.2 constructs include a small portion of the coding region (black box). Firefly luciferase constructs were transiently co-transfected into SK-N-SH cells with pRluc. Cell lysates and total RNA were isolated 24 hours post-transfection in separate experiments and used to perform (B) luciferase assays and (C) Northern blot analysis. SK-N-SH cells were mock transfected (M; lane 1) or transiently co-transfected with pRluc and pFluc (lane 2; refer to Figure 2A) or pFluc containing mKv1.4 mRNA 3' NCR sequences with wild

type and/or mutated native mKv1.4 mRNA polyadenylation determinants: pFluc(1.2) (lane 3), pFluc(1.0) (lane 4), pFluc(0.2) (lane 5), pFluc(1.2 mA3) (lane 6), pFluc(1.2 wt) (lane 7), and pFluc(0.2 mA1,2) (lane 8). A1,2 and A3 denote hexanucleotide sequences comprising the upstream and downstream polyadenylation signals, respectively. 'm' denotes the mutation of hexanucleotide sequences, and '−' and '+' indicate the absence or presence of SV40 or wild type Kv1.4 polyadenylation signals. Mutated polyadenylation signals are indicated by asterisks (*) in (A).

Table 1

Effect of mKv1.4 mRNA 3' NCR sequences on RNA expression in SK-N-SH cells.

Effects of mKv1.4 mRNA 3' NCR sequences on protein expression in transfected SK-N-SH cells.

In vitro transcribed luciferase reporter RNAs were transfected into SK-N-SH cells, and cells were harvested for luciferase assays at 15 hr post-transfection. Firefly luciferase activities were normalized with Renilla luciferase activity and ratios were adjusted for differences in transcript size. Firefly luciferase RNAs contained no 3' NCR, additional vector sequences (0.3 and 1.0) or mKv1.4 mRNA 3' NCR sequences (0.2, 1.2, or 1.0).

3' NCR sequences ^a		Fluc/Rluc Ratio ^b	
		Average	S.D.
Vector	-	7.5	0.19
	0.3	5.8	0.10
	1.0	2.4	0.12
Kv1.4	0.2	5.8	0.19
	1.2	1.6	0.07
	1.0	1.8	0.03

^aFirefly luciferase RNAs contain no 3' NCR (-), vector sequences (0.3 and 1.0), or mKv1.4 mRNA sequences (0.2, 1.2, 1.0).

^bFirefly luciferase values were normalized with Renilla luciferase. Due to differences in transcript size, Fluc/Rluc ratios were also normalized for mole differences between equal micrograms of transfected RNAs. S.D. = standard deviation