Published in final edited form as:
Biochemistry. 2008 December 2; 47(48): 12664–12669. doi:10.1021/bi8010797.

Position and Stability Are Determining Factors for Translation Repression by an RNA G-Quadruplex-Forming Sequence within the 5′ UTR of the *NRAS* Proto-oncogene†

Sunita Kumari, **Anthony Bugaut**, and **Shankar Balasubramanian***
Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

Abstract

Nucleic acid secondary structures in the 5′ untranslated regions (UTRs) of mRNAs have been shown to play a critical role in translation regulation. We recently demonstrated that a naturally occurring, conserved, and stable RNA G-quadruplex element (5′-GGGAGGGGCGGGUCUGGG-3′), located close to the 5′ cap within the 5′ UTR of the *NRAS* proto-oncogene mRNA, modulates gene expression at the translational level. Herein, we show that the translational effect of this G-quadruplex motif in *NRAS* 5′ UTR is not uniform, but rather depends on the location of the G-quadruplex-forming sequence. The RNA G-quadruplex-forming sequence represses translation when situated relatively proximal to the 5′ end, within the first 50 nt, in the 5′ UTR of the *NRAS* proto-oncogene, whereas it has no significant effect on translation if located comparatively away from the 5′ end. We have also demonstrated that the thermodynamic stability of the RNA G-quadruplex at its natural position within the *NRAS* 5′ UTR is an important factor contributing toward its ability to repress translation.

Analysis of the human genome has revealed that only a small fraction of our genetic material, about 1.5%, codes for protein (1), while the vast majority (at least 60–70%) is transcribed on one or both strands of DNA (2). It appears that much of the noncoding genome may be involved in regulation of gene expression via multiple mechanisms (3, 4). In particular, mRNA untranslated regions (5′ and 3′ UTRs)[1] are involved in regulatory events

†We thank the BBSRC for project funding, Cancer Research UK for program funding, and the Cambridge Commonwealth Trust and Trinity College, Cambridge, for studentship funding.

*To whom correspondence should be addressed. Phone: +44 1223 336347. Fax: +44 1223 336913. sb10031@cam.ac.uk. .

NOTE ADDED IN PROOF A recently published computational study has revealed that G-quadruplex-forming sequences in 5′ UTRs exhibit a positional bias toward the 5′ end (40).

[1]Abbreviations:

UTR untranslated region

G guanine

NRQ *NRAS* RNA G-quadruplex-forming sequence

UV ultraviolet

CD circular dichroism

RRL rabbit reticulocyte lysate.

Europe PMC Funders Author Manuscripts

that ultimately affect protein biosynthesis. Such regulation can be mediated by cis-acting structural elements, for example stem-loops, in the UTRs (5). These RNA motifs can act either by serving as recognition sites for RNA-binding factors, such as proteins (6, 7), or by interacting directly with the translation initiation machinery (8, 9).

It is now established that certain G-rich nucleic acid sequences have a propensity to form stable four-stranded intramolecular structures called G-quadruplexes in the presence of physiological cations, notably K^+ and Na^+ (10). Putative G-quadruplex-forming sequences are prevalent throughout the human genome (11, 12), raising the possibility that such motifs may be associated with biological functions. Telomeric DNA G-quadruplex structures have been previously shown to inhibit the action of telomerase activity in vitro (13), which has inspired the development of G-quadruplex-based cancer therapeutic approaches (14). Recently, the formation of G-quadruplex DNA was shown to occur at the 3′ single-stranded overhang of ciliate telomere under the apparent control of telomere-end binding proteins and a protein phosphorylation event in vivo (15). It has also been proposed that DNA G-quadruplex motifs found within gene promoters (16) may act as regulatory elements for gene expression at the transcriptional level on the basis of chemical biology studies (17-19).

While the main focus in the field has been on DNA G-quadruplexes and their potential role in biology, it is notable that G-rich RNA is also known to fold into G-quadruplexes (20, 21). While the general importance of mRNA secondary structures as regulatory elements of gene expression is documented, little has been reported on RNA G-quadruplexes and their function(s). G-quadruplex formation in the 3′ UTR of insulin-like growth factor (IGF II) mRNA has been proposed to be involved in post-transcriptional processing (22). A recoding event (downstream frameshift) in a herpes simplex virus mutant is proposed to arise from the ability of its mRNA to form G-quadruplex structure (23). The fragile X mental retardation protein (FMRP) has been shown to bind to a G-quadruplex within the coding region of its mRNA and inhibit its translation (24, 25). An intramolecular RNA G-quadruplex motif found within the fibroblast growth factor (FGF-2) internal ribosome entry site (IRES) has been proposed to contribute to the IRES activity (26). Recently, Wieland and Hartig have showed that insertion of an artificial G-rich element that masks the ribosome binding site of an mRNA by folding into a G-quadruplex can be used to modulate gene expression in bacteria (27).

We recently demonstrated that a naturally occurring, conserved, and thermodynamically very stable RNA G-quadruplex element (NRQ, 5′-GGGAGGGGCGGGUCUGGG-3′) located close to the 5′ cap within the 5′ UTR of the *NRAS* proto-oncogene mRNA modulates gene expression at the translational level (28). A genome-wide computational analysis also revealed 3321 other 5′ UTR RNA G-quadruplex motifs in the human genome. These motifs are located at various positions throughout the 5′ UTRs of mRNAs. Furthermore, they can differ in the numbers of putative G-tetrads, the loop sizes, and the base compositions, all of which one would predict to influence the thermodynamic stability of the G-quadruplex structure on the basis of studies on DNA G-quadruplexes (29, 30).

The present study aimed to elucidate the effect of position and stability of a 5′ UTR RNA G-quadruplex-forming sequence on translation regulation. Translational control via secondary structure formations within the 5′ UTRs of mRNAs appears to be particularly predominant for growth factors, transcription factors, and oncoproteins, which generally possess long (>200 nt) and highly structured GC-rich 5′ UTRs, as opposed to the relatively short length, low GC content 5′ UTRs in other highly expressed cellular mRNAs (31, 32). We thus decided to address the question of the position and stability dependence of translation modulation by a 5′ UTR RNA G-quadruplex in the context of the full length (254 nt) 5′ UTR of the human *NRAS* proto-oncogene. We generated a series of mRNA

reporter constructs that contained either the wild-type *NRAS* RNA G-quadruplex-forming sequence (NRQ) located at different positions within the NRAS 5′ UTR or NRQ variants with altered thermodynamic stabilities at a fixed position, and we evaluated the corresponding translation efficiencies in a eukaryotic cell-free system.

MATERIALS AND METHODS

CD and UV Spectroscopy

For spectroscopic studies, RNA samples were prepared at 4 μM strand concentration in RNase-free water and degassed buffers containing 10 mM sodium cacodylate, pH 7.0, and varying KCl concentrations from 1 to 300 mM. The samples were annealed by heating at 90 °C for 10 min and then slow cooling to 5 °C at a controlled rate of 0.2 °C min^{-1}.

Circular dichroism (CD) experiments were performed using a Jasco J-810 spectropolarimeter equipped with a Peltier temperature controller. Typically, a 200 μL sample was placed in a 1 mm optical path length quartz cuvette, transferred in the spectropolarimeter, and allowed to equilibrate at 20 °C for 10 min. Five CD scans, over the wavelength range 220-320 nm, were performed at 50 nm min^{-1} with a 2 s response time, 1 nm pitch, and 1 nm bandwidth; their average was taken. For each experiment, a CD spectrum of the buffer was recorded and subtracted from the spectrum obtained for the RNA-containing solution. Data were zero-corrected at 320 nm.

Ultraviolet (UV)-melting studies were carried out on a Varian CARY 1E UV—visible spectrophotometer equipped with a Peltier temperature controller. Typically, a 100 μL sample was transferred to a 1 cm optical path length quartz cuvette and was covered with approximately 100 μL of mineral oil to prevent sample evaporation. This sample was transferred to the spectrophotometer and then heated to 90 °C and cooled to 5 °C, twice consecutively at a 0.25 °C min^{-1} temperature gradient; absorption data recorded at 295 nm were collected every 0.5 min on both annealing and melting steps. The thermal reversibility of the melting and annealing profiles allowed the use of the van't Hoff analysis to calculate the T_m values (33). The T_m values were taken as an average of three independent experiments and have an associated error of ± 1 °C.

Construction of Plasmids

To construct the plasmid pSKC14, which encoded the 236-nucleotide UTR(—)Q transcript, pSKC12 plasmid (28) was PCR amplified using *Pfu* DNA polymerase (Promega) and two sequence-specific primers: a forward primer (5′-TACTGTG**GG**ACGTCCCGTGTTGCGGCCTGCCGCATGACTCG-3′; bold GG indicate nucleotides that were mutated from AA in the natural 5′ UTR sequence to yield an efficient transcription template) and a restriction site *Eco*RI-tailed (underlined) reverse primer (5′TACTGTGAATTCTTACACGGCGATCTTTCCGCC-3′). This amplified product was purified using a PCR purification kit (Qiagen) and reamplified using a *Hin*dIII (underlined) and a minimal T7 promoter-tailed forward primer (5′TACTGTAAGCTTTAATACGACTCACTATAGGGACGTCCCGTGTTGCGGC-3′) and the above-mentioned *Eco*RI-tailed reverse primer. This PCR amplified product was digested with *Hin*dIII and *Eco*RI, purified by gel electrophoresis, and ligated with the *Hin*dIII and *Eco*RI digested linear pUC18 vector (Invitrogen). Positive clones were confirmed by sequencing both strands of the DNA.

Construction of the plasmid, pSKC11, containing the NRQ motif at its natural position (+14) in *NRAS* 5′ UTR had been described previously (28). Plasmids pSKC15, pSKC16, pSKC17, and pSKC18, which encoded UTR(+2)Q, UTR(+47)Q, UTR(+120)Q, and

UTR(+233)Q transcripts, respectively, were constructed by inserting the NRQ element in the 5′ UTR of the pSKC14 plasmid at positions +2, +47, +120, and +233, respectively. The insertions were performed using the QuickChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The insertion of the NRQ element at +2, +120, and +233 positions would have been followed by a single G nucleotide; therefore, a nucleotide T was inserted at the end of the NRQ element in the mutagenesis primers to avoid any alternative G-quadruplex structure formation at these positions. All the constructs were sequenced to confirm the presence of the intended changes.

Two plasmids, pSKC19 and pSKC20, were prepared containing a four G-tetrad and a two G-tetrad variant of the NRQ motif, respectively, at its natural position (+14) in the *NRAS* 5′ UTR. These plasmids were derived from the pSKC11 using the QuickChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Plasmid pSKC19 was constructed by inserting a G at the end of the first, third, and fourth G-stretch of the NRQ sequence in the 5′ UTR of the pSKC11 plasmid (the second G-stretch already contains a naturally occurring fourth G). Plasmid pSKC19 encodes the transcript UTR(+14G_4)Q. Plasmid pSKC20 was produced by mutating the last G of each G-stretch of the NRQ sequence to T. Plasmid pSKC20 encodes the transcript UTR(+14G_2)Q. All the intended changes were confirmed by sequencing both strands of the DNA.

In Vitro Transcription

The plasmids were linearized using the *Eco*RI restriction enzyme, which cuts at the 3′ end of the coding region of the luciferase reporter gene. 5′ capped transcripts were generated in vitro using the mMESSAGE mMACHINE T7 kit (Ambion), following the manufacturer's protocol. All the transcripts were purified on a 1% agarose gel, after DNase treatment. The RNA concentration was determined by UV spectroscopy. The integrity and the size of each transcript were confirmed by 1% agarose gel analysis.

In Vitro Translation and Luciferase Assay

In vitro translation of the mRNAs was carried out in a cell-free translation system consisting of extracts from nuclease-treated rabbit reticulocyte lysate (RRL) (Promega). Translation was carried out in a 10 μL reaction volume, containing 70% (v/v) RRL, 10 μM amino acid mixtures minus methionine, a 10 μM amino acid mixture minus leucine, and 200 ng of RNA, and incubated at 30 °C for 90 min. Firefly luciferase activity was measured using luciferase assay teagent (Promega) on an Orion II microplate luminometer (Berthold). A portion of the in vitro translated luciferase (4.5 μL) was added to the 50 μL of luciferase assay reagent. The luciferase light intensity was measured for 10 s after a delay time of 2.05 s. Luciferase intensity for each reaction was measured in duplicate.

RESULTS

Influence of the RNA G-Quadruplex Position

In order to allow manipulation of the position of the NRQ motif within the *NRAS* 5′ UTR, we generated pSKC14 luciferase reporter plasmid as a starting point. Plasmid pSKC14 was produced by deleting the unique, 18 nt, naturally occurring RNA G-quadruplex-forming-sequence from the wild-type *NRAS* 5′ UTR. This plasmid encodes for UTR(—)Q mRNA that comprises a 236-nucleotide 5′ UTR, which retains the full complexity of the *NRAS* 5′ UTR structure but does not contain the NRQ element, placed upstream of the firefly luciferase coding sequence. By using site-directed mutagenesis, plasmid pSKC14 was subsequently employed for producing constructs that contained a single copy of the NRQ element at various positions relative to the 5′ end of the *NRAS* 5′ UTR, starting from proximal to the 5′ cap to relatively close to the AUG start codon (Figure 1). In each case,

NRQ was inserted at least 10 nt from other G-stretches to prevent the formation of alternative G-quadruplex motifs. In addition to the natural position at +14 (with respect to the 5′ end), +2 and +47 positions were selected to investigate the modulation of translation by the RNA G-quadruplex when it is located relatively close to the 5′ cap. The other two selected positions, +120 and +233, were considered to examine the effect of the RNA G-quadruplex on translation when it is situated at an intermediate position and close to the 3′ end within the 5′ UTR, respectively.

For each construct, the corresponding 5′ capped RNA transcript (referred as UTR(+*X*)Q where *X* corresponds to the position of the first nucleotide of the inserted G-quadruplex relative to the 5′ end of the transcript) was generated by in vitro transcription. The transcripts were subjected to in vitro translation in rabbit reticulocyte lysate, and the translation efficiency was evaluated by the standard luminescence-based assay for luciferase activity (34). The translation efficiency of UTR(—)Q, which does not contain the NRQ element, was taken as the reference, and the translation activities of all the other constructs were normalized with respect to this system (Figure 2). The relative translation efficiency of the transcripts that comprised the NRQ element at positions +2, +14, and +47 was reduced to 47 ± 7%, 37 ± 4%, and 52 ± 5%, respectively. On the other hand, insertion of the G-quadruplex at positions +120 and +233 did not significantly affect the translation efficiency of the mRNAs, exhibiting 119 ± 17% and 107 ± 16% relative translation efficiency, respectively.

Influence of the RNA G-Quadruplex Stability

We further investigated how the thermal stability of the RNA G-quadruplex motif at its natural position (+14) in the *NRAS* 5′ UTR influences the level of translational repression induced. The stability of G-quadruplex structures has been shown to depend on the number of G-tetrads. The DNA and RNA G-quadruplex structures composed of relatively higher numbers of G-tetrads have been shown to have higher thermal stability compared to their counterparts with lower numbers of G-tetrads (27, 35). The NRQ structural motif is composed of three stacked G-tetrads. By using pSKC11 plasmid, which encodes for the UTR(+14)Q transcript, changes in the number of G-tetrads of the NRQ motif were performed using site-directed mutagenesis to alter its stability, while maintaining its natural position within the 5′ UTR (Figure 3). The stability of the G-quadruplex element was increased in transcript UTR(+14G_4)Q, as compared to the UTR(+14)Q, by inclusion of an extra (fourth) G-tetrad. This was done by inserting an additional G at the end of the first, third, and fourth G-stretches of the wild-type sequence. In contrast, the G-quadruplex stability was decreased by removal of a G-tetrad from the NRQ element after mutating the last G of each G-stretch to U.

First, using short synthetic ribooligonucleotides (Table 1), we performed a series of spectroscopic biophysical experiments (CD and UV-melting) to confirm and characterize intramolecular RNA G-quadruplex formation by the mutated sequences (SNRQG_4 and SNRQG_2) and to reveal the effects of mutations on the thermal stability of the folded G-quadruplexes.

At near physiological conditions of salt and pH (10 mM sodium cacodylate pH 7.0, 100 mM KCl), the CD spectra of both mutants exhibited a positive peak at around 263 nm and a negative peak at approximately 240 nm, comparable to those of the oligonucleotide corresponding to the wild-type G-quadruplex (SNRQG_3) (see Figure 1 of the Supporting Information). Furthermore, each sequence exhibited a clear hypochromic melting transition at 295 nm that is indicative of G-quadruplex formation (see Figure 2 of the Supporting Information). Melting and annealing were superimposable, and a study over a 20-fold oligonucleotide concentration range 1–20 μM showed no change in the melting temperature

(T_m) values of the transitions (see Table 1 of the Supporting Information): both are consistent with intramolecular G-quadruplex formation. It is also noteworthy that, for each sequence, the T_m of the transition depended on the nature of the monovalent cation added in the order $K^+ > Na^+ > Li^+$, which is a characteristic of G-quadruplex structures (see Table 2 of the Supporting Information). As expected, $SNRQG_4$ displayed a much higher thermal stability as compared to its two-tetrad counterparts $SNRQG_2$ in the same conditions (Table 2). At 100 mM KCl, the folded $SNRQG_4$ could not be melted, even at 95 °C, whereas $SNRQG_2$ unfolded with a T_m of 45 °C. At low potassium concentration (1 mM KCl), no structure-to-random coil melting transition was observed for $SNRQG_2$, while $SNRQG_4$ G-quadruplex exhibited a melting transition with a T_m of 67 °C. Under the same conditions, the G-quadruplex structure formed by $SNRQG_3$ was found to have a T_m of 63 °C. In the absence of any added stabilizing cations, the T_m values of $SNRQG_3$ and $SNRQG_4$ were found to be 43 and 58 °C, respectively. Taken together, these biophysical data indicate that all three sequences fold into intramolecular G-quadruplex with thermodynamic stabilities in the order $SNRQG_4 > SNRQG_3 >> SNRQG_2$ under near physiological pH and salt conditions.

The translation efficiencies of UTR(+14G_4)Q and UTR(+14G_2)Q transcripts were assessed in vitro in RRL and compared to that of the UTR(+14)Q transcript (Figure 4). The increased stability variant, UTR(+14G_4)Q, exhibited an equivalent level of luciferase expression as compared to the wild-type transcript, UTR(+14)Q. Conversely, a 2-fold enhancement in translation efficiency, as compared to the wild-type transcript, was observed for the decreased stability variant, UTR(+14G_2)Q.

DISCUSSION

Studies on translation regulation by 5′ UTR structural elements have hitherto been focused on double helix-based structures, for which stability and formation can be anticipated using predictive algorithms. However, RNA can adopt other structures involving noncanonical base-parings, which cannot be predicted using such tools. We recently showed that a four-stranded RNA G-quadruplex, naturally occurring within the 5′ UTR of *NRAS* proto-oncogene mRNA, inhibits translation (28). The present study aimed to investigate the position and stability dependence of translation regulation by this RNA G-quadruplex-forming sequence (NRQ). To address this, we assessed the in vitro translation efficiency of luciferase chimeric mRNAs that comprised (i) the NRQ element at different locations within the *NRAS* 5′ UTR or (ii) G-quadruplex mutants of decreased and increased stability at the natural position (+14) of the NRQ motif within the *NRAS* 5′ UTR.

Our study revealed that the NRQ element acts as a translational repressor when located within the first 50 nt at positions +2, +14, and +47 in the *NRAS* 5′ UTR, while it has no significant effect when located 120 or 233 nucleotides away from the 5′ end of the message. We considered the possibility that G-quadruplex formation might be influenced by position, but structure-prediction analysis of the various G-quadruplex regions confirmed that predicted hairpin structures were insufficiently stable to compete with G-quadruplex formation under the conditions used for translation (see the Supporting Information). Furthermore, translation efficiency did not show any correlation with the stability of predicted hairpin structures. This indicates that the translational effect of the NRQ element in *NRAS* 5′ UTR is not uniform but rather depends on the location of the structural motif. We also showed that the thermodynamic stability of the RNA G-quadruplex at its natural position (+14) in the *NRAS* 5′ UTR is an important parameter to mediate translation repression. Indeed, translation efficiency was significantly increased when the G-quadruplex structure contained just two G-tetrads, which showed considerably decreased stability as compared to the wild-type three G-tetrads NRQ. On the other hand, the addition of a

putative fourth G-tetrad to the RNA G-quadruplex had no significant effect on translation efficiency, which may be the consequence of a relatively modest additional stabilization of the G-quadruplex structure, as judged by UV-melting at low potassium concentration (Table 2). This may indicate that an intrinsically very stable structure, such as the NRQ element, requires a much larger degree of additional stabilization to induce a detectable further decrease in protein expression.

Our results suggest similarities with the effects observed from the insertion of artificial stem-loop structures in the 5′ UTRs of mRNAs, where it was found that translation was strongly inhibited when insertions were made proximal to the 5′ cap but the effect was reduced when hairpins were located further downstream (36-39). It was shown that translation inhibition by artificial RNA hairpins proximal to the 5′ cap is due to repression of the assembly of the translation preinitiation complex at the 5′ end of the message (38). Such studies also revealed that the translation inhibition induced by artificial hairpins inserted at a fixed position is generally related to their predicted thermodynamic stability, with the more stable hairpins contributing to greater translation inhibition (36, 37).

In conclusion, this study demonstrates that translation modulation by an RNA G-quadruplex-forming sequence is dependent on its position in the 5′ UTR of the message, with the inhibition of translation occurring only when the G-quadruplex-forming-sequence is proximal to the 5′ cap. Interestingly, this outcome suggests that the natural position of the RNA G-quadruplex-forming sequence in the 5′ UTR of the human *NRAS* proto-oncogene is quite optimal for translation inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

REFERENCES

1. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature. 2004; 431:931–945. [PubMed: 15496913]
2. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C. The transcriptional landscape of the mammalian genome. Science. 2005; 309:1559–1563. [PubMed: 16141072]
3. Mattick JS, Makunin IV. Non-coding RNA. Hum. Mol. Genet. 2006; 15:R17–29. [PubMed: 16651366]
4. Frith MC, Pheasant M, Mattick JS. The amazing complexity of the human transcriptome. Eur. J. Hum. Genet. 2005; 13:894–897. [PubMed: 15970949]
5. Pesole G, Mignone F, Gissi C, Grillo G, Licciulli F, Liuni S. Structural and functional features of eukaryotic mRNA untranslated regions. Gene. 2001; 276:73–81. [PubMed: 11591473]
6. Wilkie GS, Dickson KS, Gray NK. Regulation of mRNA translation by 5′- and 3′-UTR-binding factors. Trends Biochem. Sci. 2003; 28:182–188. [PubMed: 12713901]
7. Stripecke R, Oliveira CC, McCarthy JE, Hentze MW. Proteins binding to 5′ untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. Mol. Cell. Biol. 1994; 14:5898–5909. [PubMed: 8065323]
8. Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 1991; 266:19867–19870. [PubMed: 1939050]
9. Sonenberg N. mRNA translation: influence of the 5′ and 3′ untranslated regions. Curr. Opin. Genet. Dev. 1994; 4:310–315. [PubMed: 8032210]
10. Neidle, S.; Balasubramanian, S. Quadruplex Nucleic Acids. RSC Biomolecular Sciences; Cambridge, U.K.: 2006.
11. Huppert JL, Balasubramanian S. Prevalence of quadruplexes in the human genome. Nucleic Acids Res. 2005; 33:2908–2916. [PubMed: 15914667]

12. Todd AK, Johnston M, Neidle S. Highly prevalent putative quadruplex sequence motifs in human DNA. Nucleic Acids Res. 2005; 33:2901–2907. [PubMed: 15914666]
13. Zahler AM, Williamson JR, Cech TR, Prescott DM. Inhibition of telomerase by G-quartet DNA structures. Nature. 1991; 350:718–720. [PubMed: 2023635]
14. Neidle S, Parkinson G. Telomere maintenance as a target for anticancer drug discovery. Nat. Rev. Drug Discovery. 2002; 1:383–393.
15. Paeschke K, Simonsson T, Postberg J, Rhodes D, Lipps HJ. Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo. Nat. Struct. Mol. Biol. 2005; 12:847–854. [PubMed: 16142245]
16. Huppert JL, Balasubramanian S. G-quadruplexes in promoters throughout the human genome. Nucleic Acids Res. 2007; 35:406–413. [PubMed: 17169996]
17. Siddiqui-Jain A, Grand CL, Bearss DJ, Hurley LH. Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. Proc. Natl. Acad. Sci. U.S.A. 2002; 99:11593–11598. [PubMed: 12195017]
18. Bejugam M, Sewitz S, Shirude PS, Rodriguez R, Shahid R, Balasubramanian S. Trisubstituted isoalloxazines as a new class of G-quadruplex binding ligands: small molecule regulation of c-kit oncogene expression. J. Am. Chem. Soc. 2007; 129:12926–12927. [PubMed: 17918848]
19. Cogoi S, Xodo LE. G-quadruplex formation within the promoter of the KRAS proto-oncogene and its effect on transcription. Nucleic Acids Res. 2006; 34:2536–2549. [PubMed: 16687659]
20. Deng J, Xiong Y, Sundaralingam M. X-ray analysis of an RNA tetraplex (UGGGGU) (*4*) with divalent Sr(2+) ions at subatomic resolution (0.61 Å). Proc. Natl. Acad. Sci. U.S.A. 2001; 98:13665–13670. [PubMed: 11707581]
21. Pan B, Xiong Y, Shi K, Sundaralingam M. Crystal structure of a bulged RNA tetraplex at 1.1 a resolution: implications for a novel binding site in RNA tetraplex. Structure. 2003; 11:1423–1430. [PubMed: 14604532]
22. Christiansen J, Kofod M, Nielsen FC. A guanosine quadruplex and two stable hairpins flank a major cleavage site in insulin-like growth factor II mRNA. Nucleic Acids Res. 1994; 22:5709–5716. [PubMed: 7838726]
23. Horsburgh BC, Kollmus H, Hauser H, Coen DM. Translational recoding induced by G-rich mRNA sequences that form unusual structures. Cell. 1996; 86:949–959. [PubMed: 8808630]
24. Darnell JC, Jensen KB, Jin P, Brown V, Warren ST, Darnell RB. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell. 2001; 107:489–499. [PubMed: 11719189]
25. Schaeffer C, Bardoni B, Mandel JL, Ehresmann B, Ehresmann C, Moine H. The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. EMBO J. 2001; 20:4803–4813. [PubMed: 11532944]
26. Bonnal S, Schaeffer C, Creancier L, Clamens S, Moine H, Prats AC, Vagner S. A single internal ribosome entry site containing a G quartet RNA structure drives fibroblast growth factor 2 gene expression at four alternative translation initiation codons. J. Biol. Chem. 2003; 278:39330–39336. [PubMed: 12857733]
27. Wieland M, Hartig JS. RNA quadruplex-based modulation of gene expression. Chem. Biol. 2007; 14:757–763. [PubMed: 17656312]
28. Kumari S, Bugaut A, Huppert JL, Balasubramanian S. An RNA G quadruplex in the 5′ UTR of the NRAS protooncogene modulates translation. Nat. Chem. Biol. 2007; 3:218–221. [PubMed: 17322877]
29. Hazel P, Huppert J, Balasubramanian S, Neidle S. Loop-length-dependent folding of G-quadruplexes. J. Am. Chem. Soc. 2004; 126:16405–16415. [PubMed: 15600342]
30. Bugaut A, Balasubramanian S. A sequence-independent study of the influence of short loop lengths on the stability and topology of intramolecular DNA G-quadruplexes. Biochemistry. 2008; 47:689–697. [PubMed: 18092816]
31. Kozak M. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 1987; 15:8125–8148. [PubMed: 3313277]
32. Willis AE. Translational control of growth factor and proto-oncogene expression. Int. J. Biochem. Cell Biol. 1999; 31:73–86. [PubMed: 10216945]

33. Mergny JL, Lacroix L. Analysis of thermal melting curves. Oligonucleotides. 2003; 13:515–537. [PubMed: 15025917]
34. de Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 1987; 7:725–737. [PubMed: 3821727]
35. Smirnov I, Shafer RH. Effect of loop sequence and size on DNA aptamer stability. Biochemistry. 2000; 39:1462–1468. [PubMed: 10684628]
36. Kozak M. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. U.S.A. 1986; 83:2850–2854. [PubMed: 3458245]
37. Babendure JR, Babendure JL, Ding JH, Tsien RY. Control of mammalian translation by mRNA structure near caps. RNA. 2006; 12:851–861. [PubMed: 16540693]
38. Kozak M. Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. Mol. Cell. Biol. 1989; 9:5134–5142. [PubMed: 2601712]
39. Pelletier J, Sonenberg N. Photochemical cross-linking of cap binding proteins to eucaryotic mRNAs: effect of mRNA 5′ secondary structure. Mol. Cell. Biol. 1985; 5:3222–3230. [PubMed: 3837842]
40. Huppert JL, Bugaut A, Kumari S, Balasubramanian S. G-quadruplexes: the beginning and end of UTRs. Nucleic Acids Res. Oct 2.2008 doi: 10.1093/nar/gkn511.

Europe PMC Funders Author Manuscripts

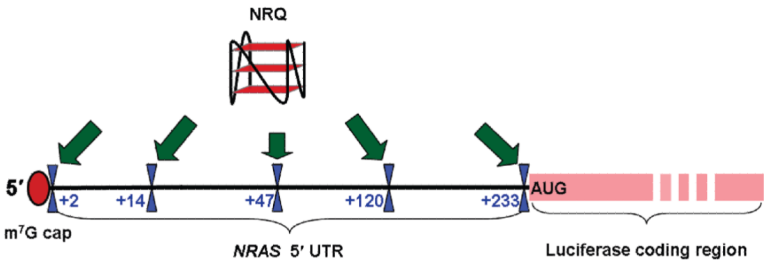

FIGURE 1.

Schematic showing the selected positions within the *NRAS* 5′ UTR at which the RNA G-quadruplex-forming-sequence (NRQ) was inserted. The position number refers to the location of the first 5′ nucleotide of the NRQ sequence relative to the 5′ end of the transcript. The +14 position is the natural position of the NRQ motif in the *NRAS* 5′ UTR.

Europe PMC Funders Author Manuscripts

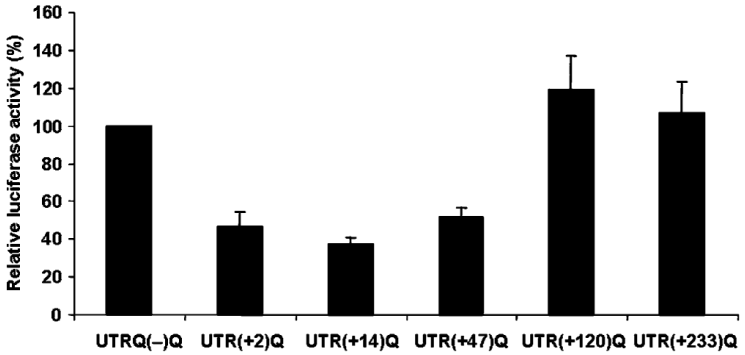

FIGURE 2.

Relative translation efficiencies of the constructs containing the NRQ element at different positions within the *NRAS* 5′ UTR, as judged by quantitation of the luciferase activity. Error bars represent the standard deviation from at least three independent experiments.

Europe PMC Funders Author Manuscripts

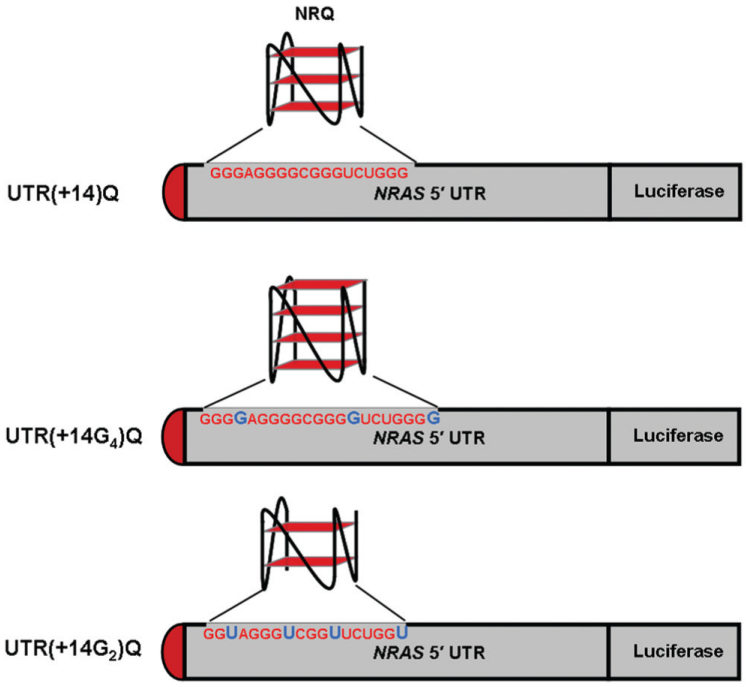

FIGURE 3.

Schematic representation of the UTR(+14)Q, UTR(+14G_4)Q, and UTR(+14G_2)Q transcripts showing the mutations (marked in blue) in the NRQ element to alter the number of G-tetrads at its natural position (+14) in *NRAS* 5′ UTR.

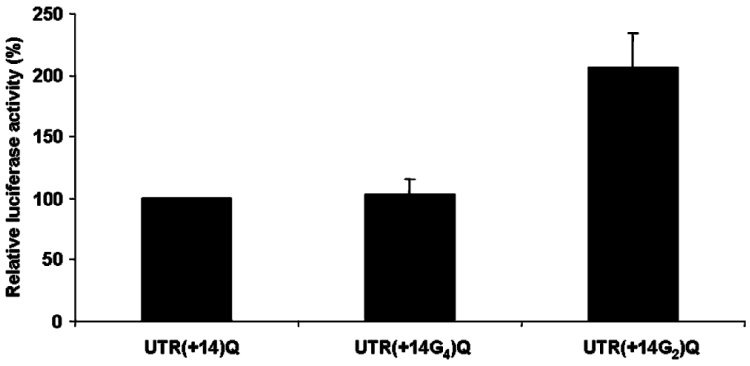

FIGURE 4.

Relative translation efficiencies of the constructs containing the NRQ G-quaduplex stability variants, as judged by quantitation of the luciferase activity. Error bars represent the standard deviation from at least three independent experiments.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Table 1

Sequences of the Synthetic RNA Oligonucleotides Used for the Biophysical Study[a]

name	sequence
$SNRQG_4$	5′-UGUGGGGAGGGGCGGGGUCUGGGG-3′
$SNRQG_3$	5′-UGUGGGAGGGGCGGGUCUGGG-3′
$SNRQG_2$	5′-UGUGGUAGGGUCGGUUCUGGU-3′

[a]Underlined nucleotides are runs of guanines capable of forming G-quartets.

Europe PMC Funders Author Manuscripts

Table 2

Melting Temperatures of $SNRQG_4$, $SNRQG_3$, and $SNRQG_2$ RNA G-Quadruplexes at Various KCl Concentrations[a]

K^+ concentration (mM)	$SNRQG_4$ T_m (°C)	$SNRQG_3$ T_m (°C)	$SNRQG_2$ T_m (°C)
0	58	43	N/A[c]
1	67	63	N/A[c]
10	80	74	N/A[c]
100	N/A[b]	N/A[b]	45
140	N/A[b]	N/A[b]	47
300	N/A[b]	N/A[b]	52

[a] T_m values are an average of three independent experiments and have an associated error of ±1 °C.

[b] N/A refers to cases where no melting was observed due to the high stability of the G-quadruplex.

[c] N/A refers to cases where no melting was observed owing to the absence of G-quadruplex formation.