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Translational control of SEPT9 isoforms is perturbed in disease

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A common feature of the mammalian septin gene family is complex genomic architecture with multiple alternate splice variants. Septin 9 has 18 distinct transcripts encoding 15 polypeptides, with two transcripts (SEPT9_v4 and v4*) encoding the same polypeptide. We have previously reported that the ratio of these distinct transcripts is altered in neoplasia, with the v4 transcript being the usual form in normal cells but v4* becoming predominant in tumours. This led us to ask what the functional differences between these two transcripts might be. The 5' UTR of v4 and v4* have distinct 5' ends encoded by exons 1β (v4) and 1ζ and 2 (v4*) and a common 3' region and initiating ATG encoded within exon 3. Here we show that the two mRNAs are translated with different efficiencies and that cellular stress can alter this. A putative internal ribosome entry site can be identified in the common region of the v4 and v4* 5' UTRs and translation is modulated by an upstream open-reading frame in the unique region of the v4 5' UTR. Germline mutations in hereditary neuralgic amyotrophy (HNA) map to the region which is common to the two UTRs. These mutations dramatically enhance the translational efficiency of the v4 5' UTR, leading to elevated SEPT9_v4 protein under hypoxic conditions. Our data provide a mechanistic insight into how the HNA mutations can alter the fine control of SEPT9_v4 protein and its regulation under physiologically relevant conditions and are consistent with the episodic and stress-induced nature of the clinical features of HNA.

INTRODUCTION

The genetic screens of Hartwell for cell division cycle mutants led to the discovery of a series of genes with functions in cytokinesis (1). Among these were a series of genes that were shown to encode P loop GTP-binding regions whose products were localized to the bud neck during cytokinesis in *Saccharomyces cerevisiae*. Such genes have been called septins and are now known to be evolutionarily conserved with multiple members in species in all kingdoms except plants (2,3). In yeast, septin proteins can form filaments and localize to the cytokinetic ring during cytokinesis, acting as membrane diffusion barriers. In animals cells, the range of septin functions appears to be much greater, and they have roles in cytokinesis, vesicle trafficking, cell polarity, cytoskeletal dynamics, apoptosis, neurodegeneration and neoplasia (2–4). At least 13 septin genes are known in mammals such as man and extensive alternate splicing is a common feature. The subcellular localization of human septin proteins is diverse (5), and a wide range of hetero-oligomeric septin complexes are believed to participate in cellular processes (6). The properties and functions of these septin-containing complexes remain poorly understood but interactions with cytoskeletal elements including actin and tubulin as well as interactions with a range of other cellular proteins such as MAP4, HIF1α and signalling molecules such as rhoetokin have recently been described (7–10).

Some clues to septin functions have come from the study of human disease, and there is compelling evidence for septins having a role in neoplasia (11). For example, several septins have been identified as in-frame fusions with MLL in leukemia (12), and SEPT9 was originally identified at a region of allelic imbalance in ovarian and breast cancer (13,14). Moreover, the murine SEPT9 locus is a common site for integration by lymphomagenic retroviruses (15). Altered expression of several septins has been observed in diverse malignancies (2,16,17). Our own laboratory has focussed on SEPT9, and we have shown this to have 18 distinct transcripts (based on multiple transcription start sites) that encode 15 polypeptides (18). A range of studies have shown that in neoplasia there are altered patterns of SEPT9 transcripts expressed with over-
expression of SEPT9_v4 and v4+ (17,19). Of note is that there are two distinct transcripts, SEPT9_v4 and v4+, both with very long 5'-UTRs that encode the same polypeptide and that in neoplasia we see a dramatic alteration of the normal ratio of these species (17,19). The SEPT9_v4 protein has a number of properties that suggest a role in neoplasia. For example, over-expression of this protein induces altered cell polarity and increased motility (21). We have also observed that expression of this isoform can alter microtubule dynamics (our unpublished results) and displace high molecular SEPT9 isoforms from filamentous structures (21).

Septins have also been implicated in other diseases and, in particular, in neurological conditions (2). Recently, Kuhlenbaum et al. (22) reported that germline mutations could be found in SEPT9 in six of nine kindreds with the autosomal dominant condition hereditary neuralgic amyotrophy (HNA). Haplotype analysis excluded a founder effect in these kindreds (22). In this condition, patients experience episodic pain and wasting of muscles in the limbs and especially the arms, often triggered by inter-current infection, prolonged exercise and other stress (23,24). Five of the mutations map to exon 3 of SEPT9. Although these mutations contribute to the open-reading frame of the long SEPT9 isoforms (v1, v2 and v3), we have not found that these nonsense mutations have a discernible phenotype when engineered in expression constructs (unpublished data). Since exon 3 is a common element of the 5'-UTRs of both SEPT9_v4 and v4+, we speculated that the mutations might alter the function of these long untranslated regions. A clue to the role of translational control being important in the production of the SEPT9_v4 protein isoform came from its expression from two distinct SEPT9 transcripts that both have long >700 bp 5'-UTRs. The majority of mRNAs has short 5'-UTRs <200 bp; however, in the subset with long 5'-UTRs, the majority represents genes involved in growth control or other regulatory mechanisms (25,26). We therefore investigated the properties of 5'-UTRs in SEPT9_v4 and v4+ and discovered that they are translated with different efficiencies and respond differently to cellular stresses. Furthermore, we found that the disease-associated HNA mutations alter these responses. The data presented here provide new insight into septin gene regulation and the perturbations that occur in both neoplasia and HNA.

RESULTS

The SEPT9_v4 and SEPT9_v4+ 5'-UTRs have potential for differential translational control

The 5'-UTRs of SEPT9_v4 and _v4+ transcripts differ only in their extreme 5'ends, i.e. exon 1B and exons 1c and 2, respectively (Fig. 1A). Then both splice into the same acceptor site of exon 3 (18), with coding beginning with the AUG at position 400 in this exon. Examination of the sequences indicates that both UTRs are G/C-rich (>60%). Modelling using mFold suggests both have the potential for extensive secondary structure (data not shown) with high predicted free energy 6G < -300 Kcal/mol. Both UTRs contain a number of uAUGs within their unique regions (v4 has five uAUGs and v4+ has two uAUGs) and one in their common region of exon 3 (Fig. 1B). Closer examination of the context of the sequence surrounding these uAUGs (Fig. 1B) indicates that only two uAUGs at positions 7 and 240 of exon 1B are in strong context relative to the Kozak consensus sequence (A/G)CC(A/G)CCAGG (27) Crucially, these contain a G at position +4 and a purine at position -3. These therefore represent potential upstream open-reading frames (uORFs) of eight (uORF 1) and 31 amino acids (uORF 2), respectively (Fig. 1B). It is noteworthy that these are in frame with exon 3 AUG from which translation of the SEPT9_v4 protein begins.

The SEPT9_v4 and SEPT9_v4+ transcripts are translated with different efficiencies

To investigate potential translational control, both 5'-UTRs were cloned upstream of the SEPT9_v4 ORF in pcDNA3.1MycHis(+)B and used to transfect HeLa cells. Translation from the SEPT9_v4+ construct was consistently higher than that observed with the SEPT9_v4 construct when controlled for transfection efficiency (v5-tagged protein in Fig. 2A) and loading (tubulin in Fig. 2A). This difference is not due to transcriptional effects, since relative mRNA copy numbers are not significantly different when compared with real-time RT-PCR (Fig. 2B).
A further comparison of the two full-length mRNAs was carried out by investigating their response to etoposide-induced stress. Etoposide inhibits DNA topoisomerase II, thereby inhibiting DNA synthesis. Cells transfected with full-length transcripts in pCDNA3.1MycHis(+/−)B were treated with increasing concentrations of etoposide. To determine copy number, real-time RT–PCR shows that both constructs exhibit a comparable reduction in transcription when etoposide is added to the medium (Fig. 2D), but thereafter there is no significant difference in transcript levels. Translation from the SEPT9_v4 construct decreases with increasing concentration of etoposide. This is particularly evident in longer exposures of western blots of the SEPT9_v4-derived Myc-tagged protein (as in Fig. 2C). This was confirmed by densitometry (Supplementary Material, Fig. S1).

For the SEPT9_v4* construct, there was an initial decrease in translation at the lowest drug concentration (presumably as a result of reduced global cellular transcription), but thereafter translation was refractory to increasing concentration of drug (Fig. 2C). These observations are again consistent with different translatability of the two SEPT9_v4 splice variants. Comparable observations were made with other stresses such as hydrogen peroxide (data not shown).

We next used a luciferase reporter construct to compare the two 5′-UTR sequences. Both full-length 5′-UTRs and the common and the unique regions were inserted upstream of the firefly luciferase reporter gene in the PGL-2 promoter vector (Fig. 3A) in both orientations. These constructs were co-transfected into Cos-7 cells along with a Renilla luciferase plasmid (phRL-TK) for 24 h. Firefly luciferase activity was measured and normalized to Renilla luciferase activity as transfection efficiency control and expressed relative to the activity of parental PGL-2 vector (Fig. 3B). The results indicate that the SEPT9_v4, SEPT9_v4*/C3 full-length 5′-UTRs and the common region of the two transcripts all enhance reporter gene activity significantly (*P < 0.05) when compared with parental vector. Constructs containing the regions of 5′-UTR in reverse orientation had a minor inhibitory effect due to the insertion of ~700 bp upstream of a reporter (data not shown). Greatest reporter activity was observed with the common region. This was followed by the SEPT9_v4*/C3 full-length 5′-UTRs and the common region of the two transcripts all enhance reporter gene activity significantly (**P < 0.001). Northern blotting for firefly luciferase (Fig. 3C) shows that this is not due to a transcriptional effect nor is it due to altered transfection efficiencies (Renilla blot in Fig. 3C).

To investigate potential promoter activity in any of these regions, we cloned the same 5′-UTR fragments into the promoterless PGL-2 Basic vector (PGL-2B). These were co-transfected into Cos-7 cells and normalized and expressed relative to the common region.
PGL-2P promoter (+ve control containing SV40 promoter). No promoter activity was observed when compared with the parental PGL-2B (Fig. 3D), which suggests that the unique regions of \textit{SEPT9}_v4 and \textit{SEPT9}_v4/C3 may modulate activity of a positive translational effect mediated by the common region.

**Translation of \textit{SEPT9}_v4 splice variants is regulated by an IRES**

The data presented thus far are consistent with a role for sequence(s) in the common region exerting an effect on translation of the \textit{SEPT9}_v4 ORF. To determine whether a discrete part of the common region was responsible, fragments of the common region were amplified, cloned into PGL2-P and transfected into Cos-7 cells (Fig. 4A). All fragments in the reverse orientation reduced translation to a level below that of vector (data not shown). Fragments 123–174, 123–278 and 243–278 generated a comparable reduction in translation even in the forward orientation. In contrast, fragments 0–174, 0–278, 123–416 and 243–416 caused no such inhibition of translation and indeed may have had a minor positive effect. However, none of these reached statistical significance. The fact that the effect is only seen with the full-length fragment suggests that the translational effect may be multi-partite and probably structural or conformational in nature.

We therefore speculated that such a structure which could influence translation might be an internal ribosome entry site (IRES) which should also facilitate cap-independent translation. To test this, the \textit{SEPT9}_v4, \textit{v4}/C3, \textit{common} and \textit{unique} region of 5'-UTRs when cloned upstream of the luciferase reporter in the promoterless PGL2-Basic luciferase construct (PGL-2B). These were co-transfected into Cos-7 cells, normalized to \textit{Renilla} luciferase transfection control and expressed relative to the SV40 promoter containing PGL-2 promoter vector. There were no significant differences. v4 or v4F \textit{SEPT9}_v4/v4 ORF in forward orientation; v4Un or v4UnF \textit{SEPT9}_v4/v4 unique region in forward orientation; ComF \textit{SEPT9}_v4/v4 common region in forward orientation; v4 or v4R \textit{SEPT9}_v4/v4 unique region in reverse orientation; v4Un or v4UnR \textit{SEPT9}_v4/v4 unique region in reverse orientation; ComR \textit{SEPT9}_v4/v4 common region in reverse orientation.
Cos-7 cells were transfected with these constructs indicate that all three constructs facilitate the second cistron expression 1.5–2.5-fold the c-Myc IRES activity (Fig. 5B). Interestingly, in contrast to monocistronic constructs (Fig. 3B), the SEPT9_v4 5′-UTR in this model exhibits greater activity than the SEPT9_v4/C3 and common region (Fig. 5B), possibly indicating that a cap-dependent control mechanism is negated in the context of the bicistronic vector. Northern blot analysis of RNA from Cos-7 transfections proved difficult; however, bands of the appropriate size, up-shifted when compared with parental PRF vector were observed (Fig. 5C, upper panel). Furthermore, no lower monocistronic or alternatively spliced products were visualized when probed for firefly luciferase, indicating that transcripts are full length. The differences in transcript levels appear to be a result of transfection efficiency, as when the blot was re-probed for Renilla luciferase (Fig. 5C, lower panel), similar intensity of bands was observed. In addition, we demonstrate that neither the SEPT9_v4 or _v4/C3 sequences contain a cryptic promoter, since deletion of the SV40 promoter leads to a dramatic reduction in activity (Fig. 4D).

We also demonstrated that these observations cannot be accounted for by ribosome shunting from the first to second cistron. We cloned the 5′-UTRs and the common region into the PhpRF vector which contains a stable hairpin upstream of cistron one and which dramatically inhibits translation and therefore prevents shunting. Transfection of these constructs into the Cos-7 cell line showed that the presence of a hairpin had no effect on the second cistron expression in PhpRMF, PhpRv4F and PhpRv4_F (Fig. 5F), whereas the first cistron activity is dramatically decreased in all four vectors (Fig. 5E).

Taken together, these observations are consistent with the presence of an IRES in the common region of the 5′-UTRs of the SEPT9_v4 and v4* transcripts. However, our initial data had shown different translational efficiencies of the two full-length transcripts, and we therefore speculated that this might be due to sequence(s) in their respective unique regions which regulate the IRES. We have previously noted two in-frame uAUGs (A7T and A240T) in a strong context in the SEPT9_v4 unique region. We used site-directed mutagenesis to mutate these AUGs (Fig. 6A) and observed that mutation of A7T caused a dramatic reduction in protein expression. Mutation of A240T had no effect on translation (Fig. 6B). These observations cannot be explained by variable transfection efficiencies or loading effects (V5-tagged control and tubulin blots, respectively, in Fig. 6B). Real-time RT–PCR also indicates that these differences are not due to transcriptional effects (Fig. 6C). We therefore provide compelling evidence for translational control of the SEPT9_v4 protein by two distinct mRNAs.

HNA-associated mutations alter translational control of SEPT9_v4

The translational control of the SEPT9_v4 protein by two distinct mRNAs casts new light on the functional significance of recently described germline mutations in HNA. Although these mutations are described as mapping to coding sequence in SEPT9 isoforms v1–v3 (22), we noted that they are also within the region of exon 3, which we now define as important for regulation of translation of SEPT9_v4 (i.e. the common region). We therefore engineered into full-length SEPT9_v4 and v4* constructs a disease-associated mutation (C262T) reported in several distinct kindreds affected by HNA (Fig. 7A). Although these mutations may cause amino acid substitutions in SEPT9_v1, v2 and v3 protein, it is notable that they lie in predicted stem-loop structures of the
**SEPT9_v4** 5'-UTR common region. The v4* wild-type and C262T mutant function similarly under normoxic as well as hypoxic conditions (Supplementary Material, Fig. S2) or other stresses (data not shown). In stark contrast, although under normoxic conditions the wild-type and C262T **SEPT9_v4** constructs behave similarly, the C262T is much more efficiently translated under hypoxic conditions (Fig. 7B). The real-time RT–PCR analysis of RNA indicates that the relative transgene transcript levels show a comparable increase in the hypoxic cells (Fig. 7C) and that under normoxia and hypoxia, the transcript levels are not significantly different.
DISCUSSION

A wide range of mechanisms exist to ensure that cells have the correct levels of particular polypeptides for normal cellular function. Although many genes are regulated by mechanisms that control the production of mRNA species, post-transcriptional mechanisms are also of crucial importance, and considerable evidence now points to the importance of control of translation. The translation of mRNAs in eukaryotic cells is principally controlled by 5-methyl-guanine cap dependent mechanisms, but there is increasing evidence for the role of internal ribosome entry as a second physiologically relevant mechanism (29). Although originally described in picornaviruses, a range of cellular circumstances have been discovered, in which cap-dependent translation is perturbed and where IRES can allow effective translation (30). For example, this phenomenon is well described in development, differentiation (31,32) and also during apoptosis and the cell cycle (29,33). It also occurs when cells are stressed by genotoxins, hypoxia, nutrient restriction and physical insults such as heat and cold shock (30). Many of these processes are characterized by partial or global shutdown of normal transcriptional and other biosynthetic processes (including cap-dependent translation) or where there is a need for highly regulated (spatially and/or temporally) production of specific polypeptides.

Cellular IRESs have recently been uncovered in transcripts that encode various growth-related proteins. It is proposed that translation occurs utilizing the IRES in situations where cap-dependent translation is inhibited (30,34,35). However, unlike viral IRES, which can usually be mapped to discrete sequences, cellular IRESs frequently comprise multiple non-overlapping sequences and their major determinant may be their three-dimensional structure (36). The presence of upstream start codons (uAUGs) and uORFs also influence translational efficiency from IRES or canonical AUG by processes such as stalling, re-initiation and shunting. uORFs can also encode polypeptides that can act on the translational machinery itself (37). For example, genes as diverse as CD36, MDM2, ERBB2, SOC1 and RARB have experimentally characterized uORFs that regulate translation (37).

The range of genes whose transcripts are regulated by IRES-based mechanisms is rapidly growing (30; and see IRES data base at http://www.iresite.org/) and includes transcription factors (c-myc, N-myc, c-jun, RUNX1 and so on), stress response factors (Apaf-1, Bag-1, APC and so on), growth factors and their receptors (ERα, VEGF, PDGF, FGF-2, IGF-1 receptor and so on), cytoskeletal and cell–cell contact proteins (ARC, MAP2, connexins), signalling molecules and kinases, components of the translational machinery itself (eIF4G1, NAT1) as well as an array of channel proteins, transporters and other proteins (betaF1-ATPase, neurogranin, ...
property of those isoforms (SEPT9_v1, v2 and v3) that utilize exon 3 as part of an open-reading frame (unpublished data). In contrast, we do see a clear effect of mutations in exon 3 used as part of the 5’-UTR of the SEPT9_v4 and v4* transcripts. These results indicate that the germline mutation in patients with HNA may have a significant effect on SEPT9_v4 translation during stress such as hypoxia and therefore is physiologically relevant to the episodic nature of induction of HNA.

The data presented here provide new insights into these phenomena by demonstrating the potential for translational control of the SEPT9_v4 transcripts with the existence of a regulatable (v4) and a non-regulatable (v4*) transcript. Thus, we have discovered that the SEPT9_v4 protein is regulated by translational control. We demonstrate the presence of an IRES in the common region of the 5’-UTR of these two transcripts and show that the unique regions confer on this differential translational efficiencies. We show that an in-frame upstream AUG in a strong context facilitates translation from the IRES and that stress responses modulate the translational efficiency of the IRES. Finally, we demonstrate that the disease-associated HNA mutations alter these responses. It is of note that other septin genes produce multiple transcripts by alternate splicing and SEPT8 (for example) also produces two distinct transcripts that encode the same polypeptide (47). It may therefore be that translational control is a general mechanism for the regulation of septin polypeptide expression, and we would posit that the proteins made by this mechanism have crucial regulatory roles in septin biology. An important future step will be to determine the physiological circumstances under which the SEPT9 transcripts are regulated by IRES mechanisms. For example, do these mechanisms have a role during the cell cycle? This is an attractive model since septins were first identified as alleles of genes with cytokinesis phenotypes. In addition, there is abundant evidence for spatially regulated translational control of genes in neuronal physiology and this may be relevant to SEPT9 and possibly to other septins.

On the basis of the data presented here and our previous data (21), a model might then be proposed where SEPT9_v4* is a non-regulated transcript and where the v4 transcript is highly regulatable. Introduction of the disease-associated C262T mutation leads to deregulation of SEPT9_v4 translation and, under hypoxic stress, enhanced SEPT9_v4 protein production. Given that the SEPT9_v4 polypeptide can have dramatic effects of cellular behaviour and that SEPT9 is known to influence microtubule behaviour, perturbed SEPT9_v4 protein expression could alter microtubule function in critical cells such as long axons. HNA is associated with episodic bouts of axonal dysfunction that can be precipitated by inter-current illness, infection and extreme exercise. Such stresses may then normally regulate SEPT9 isoform expression. Loss of this crucial isoform regulation in the presence of C262T HNA-associated mutations may lead to altered stoichiometry of SEPT9 isoforms and hence altered function. Similarly, in neoplasia, the ratio of these transcripts changes (17,19,20) by altered methylation and perhaps by other mechanisms, resulting in a predominance of the non-regulatable SEPT9_v4* transcript and leading to increased levels of SEPT9_v4 protein again with altered stoichiometry.
and altered function. The result of these disease-associated events is to deregulate the normal control of the SEPT9_v4 protein with its accumulation in cells. This protein can alter other SEPT9 isoforms, for example, displacing SEPT9_v1 from filaments (21), and induce a number of cellular alterations including perturbation of polarity, motility and microtubule dynamics. SEPT9_v1 can also stabilize HIF1α via SEPT9_v1. It would appear that cells use many methods to regulate septin gene expression.

**MATERIALS AND METHODS**

**DNA manipulations and construct generation**

The SEPT9_v4 and SEPT9_v4* 5′-UTRs were amplified from cDNA of the ovarian carcinoma cell line PEO4, as it was previously shown to contain both transcripts (20). The 5′-UTRs were amplified using primers incorporating HindIII restriction sites (v4HindIIIF-5′-ACTAGAAGCTTTGATGAGG AGGCCGA-3′, v4HindIIIF-5′-CTCTAAGCTTTGCCGCC CGGCGCTTCA-3′, v4HindIIIF-5′-AGATAAGCTTCC TGGCTGCTGCTGACT-3′) and inserted upstream of the firefly luciferase reporter in the PGL-2 promoter (Promega) vector generating the plasmids v4F/R and v4′F/R. Similarly, deletion constructs were produced by PCR amplifying and subcloning fragments of exon 3 into PGL-2 promoter, generating ComF/R, v4UnF/R, v4-v4′IIR, B-v4F/R, B-v4F/R, v4/v4′IIR, 0-174, 0-278, 123-174, 123-278, 123-416, 243-278, 243-416 (ComHindIIF-5′-CGGCGCA_5′-CTCGAAAGCTTCTTGCAGGAACCAAGACA-3′, v4UnHindIIIR-5′-CTCGAGAAGCTTTCTGCGAGAAGCCAAGACA-3′, v4′UnHindIIIR-5′-ACGATAAGCTTCTGGGCACTCGAGTCACCA-3′, 174R-5′-AGACTAAAGCTTTGCGAGGCAGGAGTTGGAG-3′, 278R-5′-AGACTAAAGCTTTGCGAGGAGTTGGAG-3′). Promoterless constructs were achieved by excision and sub-cloning into the HindIII site in the MCS of pGL-Basic vector (Promega) upstream of the firefly luciferase reporter to generate Bv4F/R, B-v4′F/R and B-CF/R.

Full-length constructs were generated by overlapping PCR utilizing products generated by amplification of 5′-UTR using forward primers v4HindIIIF/v4′HindIIIF and reverse primer in exon 3 (A0B16F-5′-GCATCTGGATCCCTCCCCTTGG-3′); with product amplified from SEPT9_v1 construct utilizing forward primer in exon 3 (A0B15F-5′-CGGCCGAT CGAGCTGCTCCAT-3′) and reverse primer to the extreme 3′ end of coding region incorporating an XhoI restriction site (FLXhoIR-5′-AGGTTGCTGCAGACCTCCGGGT-3′). These products were combined and amplified with extreme 5′ (v4HindIIIF/v4′HindIIIF) and 3′ primer (FLXhoI) and subjected to further PCR and the resulting full-length product digested and inserted into the HindIII and XhoI restriction sites in the MCS of pCDNA3.1-Myc/His B(+) vector (Invitrogen). In all full-length constructs, the 3′ splice variant ‘a’ was used (18). Point mutants, A7 → T, A240 → T; C262 → T, were introduced by site-directed mutagenesis using the QuikChange II SDM kit (Stratagen), using oligonucleotide primers (A7TF-5′-AGCTAAGCTTGGACAGTCACTGAGGAGGAG CGGGACC-3′, A7TF-5′-GGTCCGCCCTCCTCAAACTGAC AAGCTTAACT-3′, A240TF-5′-GAAGTGCTGGGATTTGG CGCAGGAGC-3′, A240TR-5′-GCTCCTGCAGCAATCCCC AGCCACCTC-3′, C262TF-5′-CGAGCCGATCGCTCCTGGC GACTGAGCTG-3′, C262TR-5′-CACTGCACTGGGAGCC CAACCCGCTCGGG-3′) according to manufacturers’ instructions.

Bicistronic constructs PRF, PRMF and PRRMF have been described previously (48) and were a kind gift of Prof. Anne Willis. Further constructs were generated by PCR amplification of 5′-UTRs and common region from PGL-2 constructs utilizing forward primers containing SpeI restriction site (v4SpeIF-5′-ACTAGAAGCTTTGATGAGG AGGCCGA-3′, v4SpeIF-5′-CTCTAAGCTTTGCCGCC CGGCGCTTCA-3′, v4SpeIF-5′-AGATAAGCTTCC TGGCTGCTGCTGACT-3′, ComSpeIF-5′-ACTGAACTAGTCCTGGGAA GAACCTTTGAAGGTGCCGAAG-3′, v4′SpeIF-5′-CTCTAAGCTTGGGACTTGGGAGG GCCGAAG-3′, v4′SpeIF-5′-CTCTAAGCTTGGGACTTGGG AGGCCGA-3′) and inserted into those sites in the intercistronic region of PRF or hairpin containing construct PhpRF to generate PRv4F, PRv4′F and PRComF. Excision of the SV40 promoter from the PRF, PRMF, PRv4F and PRv4′F was achieved by sequential digestion with NheI and EcoRV, blunt ending using the Klenow fragment of DNA PolI and re-ligation. All PCRs were carried out with Proofreading Platinum Taq Hi-Fi (Invitrogen) according to manufacturers’ instructions, with annealing temperatures of 55–63°C. All plasmids were isolated using Plasmid Maxi Midi/Mini-prep kits (Qiagen) according to manufacturers’ instructions and fully sequenced to confirm identity and orientation.

**RNA analysis**

RNA was extracted with RNA Stat-60 (Ambion) according to manufacturers’ instructions, resuspended in DEPC-treated sterile distilled water and subjected to RQ1 DNase digestion (Promega) to remove any contaminating genomic and plasmid DNA. RNA for northern blotting was further purified using RNAeasy kit (Qiagen), eluted and added to MMF loading buffer to final concentration: 50% formamide, 6% formaldehyde, 1× Blue juice (Invitrogen), denatured for 5 min at 65°C and separated in 1.2% denaturing agarose gel containing 20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, 6.3% formaldehyde and 1 mg/ml ethidium bromide. Transfer of RNA to Hybond N nylon membrane was carried out by vacuum transfer, membranes washed in 2× SSC (0.3 M NaCl, 0.03 M C6H12O7·2H2O), dried and cross-linked by exposure to UV. Northern probes were labelled using ready-to-go DNA labelling beads (Amersham), according to manufacturers’ instructions, to which α-32PdCTP (2500 Ci/mmol) was added. Membranes were hybridized with radio-labelled probe overnight in hybridization buffer (0.332 M NaH2PO4·2H2O, 0.158 M Na2HPO4 anhydrous) at 65°C and washed with decreasing concentration of SSC containing 0.1% SDS as required and autoradiography carried out at ~80°C.

For quantitative PCR, 1 μg of DNase-treated RNA was reverse transcribed using the M-MLV RT kit (Invitrogen) and random primers to produce cDNA according to manufacturers’ instructions. Quantitative RT–PCR was carried out under standard conditions using DyNaMo SYBR Green qPCR kit (Finnzymes). Denaturation was carried out for
10 min at 94°C, followed by 35 cycles of 94°C for 10 s, 55°C for 10 s and 72°C for 10 min and a final extension of 5 min at 94°C. Myc-tagged transgene transcript levels were determined utilizing forward primer in exon 12 (s9ex12F-5'-GTGGAGAAGAGCCGA-3') and reverse primer to vector-encoded Myc-tag (MycR-5'-CAGATCCTCTCGTAGATGAG-3'). β-Actin was quantified as loading control (BA7-5'-CCCAAGATCATTTGGACCTTC-3', BA8-5'-GCCAGGCGCTACAGGGGATA-3' primers). Standard curves were generated from 10-fold serial dilutions of plasmid containing appropriate sequence and copy number calculated by the comparative Ct method and expressed as a percentage of the appropriate control/parental message and normalized to β-actin level.

RNA models were produced using the mFold (49,50) RNA structure prediction programme available online (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/).

Cell culture

Cos-7 cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal calf serum, 2 mM l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 1 mM sodium pyruvate; HeLa cells were cultured in RPMI 1640 (Life technologies) with l-glutamine, supplemented with 10% FCS, 2.8 mM l-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator containing 5% CO2. Cells were grown to 60–80% confluence and transfected with 2 μg/well plasmid DNA in six-well plates for western blotting and RNA extractions for RT–PCR, 500 ng per well in 24 well-plates for luciferase assays or 10 μg per 90 mM dish for RNA extraction for northern blotting. Transfections were carried out using genejuice (Novagen) according to manufacturers’ instructions and harvested 48 h post-transfection. For hypoxia experiments, cells were transferred to a hypoxic chamber (1% O2, 5% CO2) (Novagen) according to manufacturers’ instructions and harvested 40 min at 94°C for 10 s, 55°C for 10 s and 72°C for 10 min and a final extension of 5 min at 94°C. Myc-tagged transgene transcript levels were determined utilizing forward primer in exon 12 (s9ex12F-5'-GTGGAGAAGAGCCGA-3') and reverse primer to vector-encoded Myc-tag (MycR-5'-CAGATCCTCTCGTAGATGAG-3'). β-Actin was quantified as loading control (BA7-5'-CCCAAGATCATTTGGACCTTC-3', BA8-5'-GCCAGGCGCTACAGGGGATA-3' primers). Standard curves were generated from 10-fold serial dilutions of plasmid containing appropriate sequence and copy number calculated by the comparative Ct method and expressed as a percentage of the appropriate control/parental message and normalized to β-actin level.

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Protein analysis and reporter gene assays

Proteins were subjected to SDS–PAGE using the Laemmli method. Following separation, proteins were transferred to PVDF and blocked for 1 h in 5% milk containing 10 mM tris incubated overnight at 4°C with appropriate primary antibody. Membranes were washed three times with TBS-T (10 mM tris pH 8.0, 0.15 M NaCl, 0.1% triton X-100) following primary and HRP-conjugated secondary (DAKO) incubations and bands revealed by chemiluminescence (Pierce SuperSignal) as described previously (51). Myc-tag (Clone 9E-10) and β-actin monoclonal antibodies were purchased from Sigma and V5-tag monoclonal from Invitrogen. Cells transfected with reporter constructs were lysed in Passive Lysis Buffer (Promega) and luciferase expression was determined using the firefly LAR(20 mM tricine, 2.67 mM MgSO4·7H2O, 0.1 mM EDTA, 33.3 mM DTT, 530 μM ATP, 270 μM acetyl coenzyme A (lithium salt), 0.5 mM luciferin, 0.25 mM (MgCO3)4Mg(OH)2·5H2O) and Renilla coelenterazine (Nanoprobes) substrate (0.02 mg/ml). Briefly, 20 μl of each lysate was dyplated-plate and fired (10s) and Renilla (2s) luciferase activity was assayed in an EG&G Berthold microplate luminometer LB96V after addition of 50 μl of substrate to each well.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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