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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'-UTRs of v4 and v4* have distinct 5' ends encoded by exons 1b (v4) and 1c 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 rhotekin 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 leukaemia (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_v1 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 (17,19,20). 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, Kuhlenbaumer 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 missense 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 different efficiencies and respond differently to cellular stresses.

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 1β and exons 1ζ 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 

\[
\Delta G < -300 \text{ Kcal/mol.}
\]

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 1β are in strong context relative to the Kozak consensus sequence (A/G)CC(A/G)CCUGG (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 (C3/C3 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/C3 5'-UTR construct, which showed significantly more activity than the SEPT9_v4 5'-UTR (**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
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 SEPT9_v4 and SEPT9_v4/C3 may modulate activity of a positive translational effect mediated by the common region.

**Translation of 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 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 SEPT9_v4, v4+/C3_5′-UTRs and also the common region were inserted into the intercistronic region of the PRF bicistronic vector (28) (Fig. 5A). Bicistronic vectors are considered the ‘gold standard’ when defining IRES activity, and this vector encodes Renilla luciferase as the first cistron and firefly luciferase as the second cistron. The ratio of second/first cistron (firefly/Renilla luciferase) expression can be used as a measure of cap-independent translation and IRES activity; with the important caveats that alternative splicing, promoter activity and ribosome shunting can generate second cistron activity independently of internal ribosome entry and bias the assay. The data generated when
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
The effect on translation of \textit{SEPT9}_v4 and \textit{SEPT9}_v4\_C3-UTR common region. The v4\_C3-UTR 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 \textit{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.

\textbf{Figure 5.} The effect on translation of \textit{SEPT9}_v4 and \textit{SEPT9}_v4\_C3-UTRs in a bicistronic luciferase reporter gene analysis. (A) Representation of a series of bicistronic constructs which were generated to include an intercistronic insertion of 5\_UTRs, location of hairpin upstream of \textit{Renilla} luciferase and excision of SV40 promoter. (B) Bicistronic luciferase assay in Cos-7 cell line, expressed as ratio of second cistron activity (firefly luciferase) over first cistron activity (\textit{Renilla} luciferase) expressed relative to the activity of c-myc IRES. (C) Northern blot analysis of RNA from bicistronic luciferase reporter construct transfection in Cos-7 cell line, probed for firefly luciferase open-reading frame stripped and re-probed for \textit{Renilla} luciferase. (D) Effect of promoter excision on luciferase activity from transfection of bicistronic construct in Cos-7 cell line. Data represent luciferase activity of promoterless construct expressed relative to that of parental construct containing an SV40 promoter. (E and F) Luciferase activity following insertion of hairpin upstream of first cistron (\textit{Renilla} luciferase) and transfection into Cos-7 cell line. Figures represent raw \textit{Renilla} (E) and firefly (F) luciferase activity with/without hairpin. All luciferase figures represent the results of at least three independent experiments carried out in triplicate.
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.

Figure 7. Effect of HNA mutation on expression of SEPT9_v4 in response to hypoxia. (A) Site-directed mutagenesis of full-length constructs in pCDNA3.1 Myc-tagged vector was used to generate the C → T transversion in SEPT9_v4 commonly observed in the germline of patients with HNA. (B) Western blot analysis of transient transfection in HeLa cell line of SEPT9_v4 wild-type and the C262T point mutant under normoxic (N) and hypoxic (H) conditions, re-probed for V5-tagged MEKK as transfection control and β-tubulin loading control. (C) Results of real-time PCR control for Myc-tagged wild-type and mutant constructs normalized to β-actin levels and expressed relative to SEPT9_v4 wild-type levels under normoxia and hypoxia. The figure represents the results of at least three independent experiments carried out in triplicate.

FMR1). Moreover, it is increasingly recognized that disease states can be associated with mutations leading to alterations in the translational control of particular mRNA species. For example, considerable data now exist, which suggests that neoplasia can be associated with perturbed translational control of key cellular regulators including FGF-2, c-myc, p53, HIF1α and VEGF (38–40). Neurological diseases are also associated with alterations in translational control (41–43). For example, IRES activity has been shown to be disrupted in the 5'-UTR of connexin-32 mRNA, leading to perturbed connexin levels and a subtype of Marie–Charcot–Tooth disease (44), and the gene encoding fragile X syndrome protein has an IRES (45). A characteristic of these physiological and pathological situations is the need for careful regulation (spatial and temporal) of particular protein isoforms.

A feature of the septins is the formation of heterooligomeric protein complexes (2,3,6) involving multiple septin species. Evidence also exists for homotypic association of septin proteins and the potential formation of complexes made up of different isoforms of a particular septin (46; Pentland, Russell and Hall, unpublished data). In this regard, SEPT9 is of note, since 15 polypeptides are produced from 18 transcripts generated by alternate splicing (18). Alterations in SEPT9 expression have been described in neoplasia and, in particular, alterations in the ratio of SEPT9_v4 and v4* transcripts (19,20). Furthermore, germline mutations have been found in exon 3 of SEPT9 in HNA (22). We can find no evidence of these mutations altering any so far detectable
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, and altered SEPT9_v4 levels can thus perturb 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'-AGTAGAAGCTTCTAGATGGAGG AGGGCCCTCA-3', v4HindIIIF-5'-CTCTAAAGCTTGTGGGCC CGGGOCTCCA-3', v4HindIIIF-5'-AGTTAACGCTTCTCACGGAG CCTCTCA-3'), and inserted upstream of the firefly luciferase reporter in the PGL-2 promoter (Promega) vector generating the plasmids p4F/R and p4F/R. Similarly, deletion constructs were produced by PCR amplifying and subcloning fragments of exon 3 into PGL-2 promoter, generating ComF/R, v4UnF/R, v4F/R, 0–174, 0–278, 123–1274, 123–278, 123–416, 243–278, 243–416 (ComHindIIIF-5'-AC TGAAGCTTCTTGAAAGAAGACTTGAACGAGAGGAGTC TACCA-3'), v4UnHindIIIF-5'-ATCGAAGCTTCTTGCAAGGACCCAAGACA-3', v4HindIIIF-5'-ACGATAAGCTTCTGGGAGCCAGTGCTACCA-3', 174R-5'-AGAGTACGCTTGGGAGCTTGAAGGAGTTCACA-3', 278R-5'-AGAGTACGCTTGGGAGCTTGAAGGAGTTCACA-3', 243F-5'-AGAGTACGCTTGGGAGCTTGAAGGAGTTCACA-3', 243R-5'-AGAGTACGCTTGGGAGCTTGAAGGAGTTCACA-3', using RNAeasy kit (Qiagen), eluted and added to MMF and reverse transcribed using the M-MLV RT kit (Invitrogen) according to manufacturers' instructions. Quantitative RT–PCR was carried out with 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 C6H12Na2O7·2H2O), 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 (25 μCi) 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 (5′-GTTGAGGAGAGGAGCCAGA-3′) and reverse primer to vector-encoded Myc-tag (MycR-5′-CAGATCTCTTTCTGAGATGAG-3′). β-Actin was quantified as loading control (BA7-5′-CCCAGATCATGTGAGACCTTC-3′, BA8-5′-GCCAGAGGCTACAGGGATA-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, 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% confluency 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 well 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) 24 h post-transfection and harvested following 24 h hypoxia. For etoposide (Sigma) treatments, cells were switched to media containing the drug 24 h post-transfection and incubated for a further 24 h before harvest. For monocistronic luciferase reporter experiments, the phRL-TK (Promega) plasmid was co-transfected at a ratio of 1:4 with firefly luciferase construct to normalize for transfection efficiency, whereas full-length constructs were co-transfected with V5-tagged MEKK expression construct.

**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 luciferrin, 0.25 mM (MgCO₃)₆Mg(OH)₂·5H₂O and Renilla coelenterazine (Nanoprobe) substrate (0.02 mg/ml). Briefly, 20 μl of each lysate was duplicate-plated and firefly (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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