We report that hnRNP K, an RNA-binding protein implicated in multiple aspects of post-transcriptional gene control, is essential for axon outgrowth in Xenopus. Its intracellular localization was found to be consistent with one of its known roles as an mRNA shuttling protein. In early embryos, it was primarily nuclear, whereas later it occupied both the nucleus and cytoplasm to varying degrees in different neuronal subtypes. Antisense hnRNP K morpholino oligonucleotides (MOs) microinjected into blastomeres suppressed hnRNP K expression from neural plate stages through to at least stage 40. Differentiating neural cells in these embryos expressed several markers for terminally differentiated neurons but failed to make axons. Rescue experiments and the use of two separate hnRNP K MOs were carried out to confirm that these effects were specifically caused by knockdown of hnRNP K expression. For insights into the involvement of hnRNP K in neuronal post-transcriptional gene control at the molecular level, we compared effects on expression of the medium neurofilament protein (NF-M), the RNA for which binds hnRNP K, with that of peripherin, another intermediate filament protein, the RNA for which does not bind hnRNP K. hnRNP K knockdown compromised NF-M mRNA nucleocytoplasmic export and translation, but had no effect on peripherin. Because eliminating NF-M from Xenopus axons attenuates, but does not abolish, their outgrowth, hnRNP K must target additional RNAs needed for axon development. Our study supports the idea that translation of at least a subset of RNAs involved in axon development is controlled by post-transcriptional regulatory modules that have hnRNP K as an essential element.

KEY WORDS: Cytoskeleton, Neurofilament, Peripherin, Post-transcriptional regulation, Ribonucleoprotein

INTRODUCTION

Axonal outgrowth depends on coordinating the expression of functionally interrelated genes. For example, synthesis of cytoskeletal proteins and the associated factors that regulate and organize their assembly must be matched with bouts of axonal extension to ensure that the axon is neither under- nor oversupplied. The rapidly shifting dynamics of this outgrowth makes it difficult to envision how this balance is achieved through transcriptional control alone. Placing the synthesis of cytoskeletal proteins under direct translational control would both complement transcriptional regulatory mechanisms and offer advantages to the growing axon for matching expression with demand. The dramatic shift from untranslated to translated pools undergone by the middle neurofilament (NF-M) mRNA in retinal ganglion cells during optic nerve regeneration indicates that proteins involved in building the axon are indeed under strong translational control (Ananthakrishnan et al., 2008). However, the identities of specific elements involved in the translational control of such messages and the degree to which such elements are necessary for axon outgrowth remain unresolved issues.

The life histories of mRNAs as they pass through the cell are largely governed by continually evolving sets of ribonucleoproteins (RNPs) (Moore, 2005). One idea is that RNPs may act as components of regulatory modules targeting subsets of specific functionally related messages, providing an additional level of control beyond transcription (Keene and Tenenbaum, 2002). In the nervous system, RNPs are already known to play important roles in neural plasticity and development (Perrone-Bizzozero and Tenenbaum, 2002; Si et al., 2003; Yao et al., 2006). Heterogeneous nuclear RNP K (hnRNP K) is the founding member of the K-homology domain family of RNPs. Although abundantly expressed in nervous system, its role there is not understood. It binds directly to the NF-M 3'-untranslated region (3'-UTR), along with additional RNPs, including HuB and hnRNPs E1 and E2 (Antic et al., 1999; Thyagarajan and Szaro, 2004). hnRNP K is thought to function as a central scaffolding component of evolving mRNP complexes whose compositions are modulated by multiple kinases, thereby providing a mechanism whereby mRNA fate can be coupled with cell-signaling events (Bomsztyk et al., 2004).

Two observations highlight the potential importance of the interactions of hnRNP K with neuronal mRNAs during development. In neuroblastoma cells, hnRNP K directly antagonizes Hu binding to p21 mRNA to promote proliferation over differentiation (Yano et al., 2005). In mammalian brain, it associates with the mRNAs of three NF subunits [light (NF-L), medium (NF-M) and heavy (NF-H)] more strongly in postnatal than in adult neocortex (Thyagarajan and Szaro, 2008). To explore its role in the intact developing nervous system, we first examined the cellular distribution of hnRNP K in developing Xenopus and then suppressed its expression with antisense morpholino oligonucleotides (MOs) injected into blastomeres. Suppressing hnRNP K expression both blocked axonal outgrowth and inhibited the translation of NF-M. These observations are consistent with the hypothesis that hnRNP K plays an essential role in the translation of a subset of proteins involved in building the axon.

MATERIALS AND METHODS

Microinjection, rearing and culturing of embryos

Two-cell stage periodic albino Xenopus laevis embryos (Hoperskaya, 1975) were microinjected into one or both blastomeres (10 nl each; 1 ng/ml) with MO (Gervasi and Szaro, 2004). The following MOs (Gene Tools) were used: antisense hnRNP K MO1, 5'-GCT GCT ACC TTT CTC CTA CGC CGA C3'- starting from nucleotide –54; a second, non-overlapping antisense hnRNP K MO (MO2), 5'-TTC TCT CTG CTC TGT CTC CAT CTT CT3' (the initiation codon is underlined); antisense hnRNP E MO, 5'-GGC TAT
TGT CAG AAG CTG TAC AAA G3' starting from nucleotide –50; and a
standard control MO. 5′/CCT CTT ACC TCA GTT ACA ATT TAT A3′. Fluorescent
dextranx (FDx) were co-injected to label cells descended from the
injected blastomere (0.75% w/v lysinated fluorescein-isothiocyanate
dextran; 0.1-0.25% Cascade Blue Dextran, Molecular Probes).

For rescue experiments, cDNA spanning the complete coding sequence of X. laevis hnRNK K (GenBank BC044711) was obtained by RT-PCR
(Superscript II RT; Platinum Pfx DNA polymerase, Invitrogen) from total
RNA of stage 29/30 embryos using the following primers, which had NorI
sites added to their 5′-end to facilitate cloning: 5′/ATA GGC GCC GCT
AAA AGA AGA TGG AGA CAG AGC AGG3′ (forward); and 5′/ATA
GGC GCC GAG AAC TCA AAA CCC ATA AGA ATA ATC3′ (reverse).
PCR products were ligated into the NorI site of a modiﬁed pGem3Z
expression vector (pSP6-XhnRPNK), downstream of the SP6 promoter and
upstream of the 3′ of rabbit β-globin (Lin and Szaro, 1996). The
insert sequence was conﬁrmed to match the GenBank sequence. The plasmid
was subsequently linearized with XhoI for use as template for in vitro
transcription of 5′-capped RNA (mMESSAGE mMACHINE SP6, Ambion).
Rescue was performed by coinjecting two-cell embryos with
hnRNK K MO1 (10 ng) plus hnRNK K RNA (50-100 pg).

Dissociated embryonic spinal cord-myotomal cultures were prepared
from spinal cord, including extra caudal hindbrain together with
surrounding myotomes, of stage 22 embryos. Each culture was prepared from
a single embryo and grown at 22.5°C on 35 mm polystyrene dishes
(Δ

Table 1. Numbers of embryos processed for immunohistochemistry and in situ hybridization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>hnRNK K</th>
<th>NF-M protein</th>
<th>NF-M mRNA</th>
<th>Peripherin protein</th>
<th>Peripherin mRNA</th>
<th>Neuronal β-tubulin</th>
<th>HNK-1</th>
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<tr>
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<td>215</td>
<td>183</td>
<td>67</td>
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<tr>
<td>hnRNK K MO2</td>
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<td>n/a</td>
<td>52</td>
<td>n/a</td>
<td>46</td>
<td>n/a</td>
</tr>
<tr>
<td>Control MO</td>
<td>196</td>
<td>164</td>
<td>25</td>
<td>143</td>
<td>19</td>
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<td>28</td>
</tr>
<tr>
<td>Dye alone</td>
<td>115</td>
<td>86</td>
<td>11</td>
<td>22</td>
<td>21</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Uninjected control</td>
<td>186</td>
<td>108</td>
<td>24</td>
<td>102</td>
<td>21</td>
<td>93</td>
<td>21</td>
</tr>
<tr>
<td>hnRNPK MO1+mRNA</td>
<td>60</td>
<td>55</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>31</td>
<td>n/a</td>
</tr>
<tr>
<td>Dye alone</td>
<td></td>
<td></td>
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</tbody>
</table>
hnRNPK and axon development

RESULTS

Developmental expression pattern of hnRNPK

Previous biochemical studies had shown that hnRNPK in Xenopus is initially expressed as a maternal protein in oocytes and is present throughout early development, with expression increasing as cells differentiate (Marcu et al., 2001; Iwasaki et al., 2008). Results from whole-mount immunocytochemistry were consistent with these earlier biochemical studies and further found that hnRNPK was present in all the germ layers from blastulae stages onwards (illustrated at stages 10, 15, 22, 37 and 42 in Fig. 1). Throughout development, hnRNPK was prominently expressed in nuclei. From gastrula stages onwards, hnRNPK became progressively more highly expressed in ectodermal and mesodermal derivatives, such as neural plate and somites, than in endodermal ones. This trend continued through early tadpole stages, where hnRNPK staining in differentiating tissues, especially in nervous system, became increasingly more cytoplasmic (Fig. 1F).

To improve resolution of intracellular structures, we next examined hnRNPK expression in dissociated embryonic spinal cord-myotomal co-cultures. At 24 hours after plating, hnRNPK immunostaining colocalized with DAPI-stained nuclei in both neurons and muscle cells (Fig. 2A-C); it was detectable neither in neurites nor in growth cones (Fig. 2C). In histological sections of differentiating tissues, especially in nervous system, became increasingly more cytoplasmic (Fig. 1F).

Suppression of hnRNPK expression with antisense morpholino oligonucleotides

To study its function during development, we suppressed hnRNPK expression with antisense MOs. The bilateral symmetry of the embryo enabled the application of anatomical criteria to identify affected cellular subtypes in unilaterally injected embryos; FDx was co-injected to identify the injected side. To control for inadvertently targeting genes other than hnRNPK, we injected two separate hnRNPK antisense MOs targeting non-overlapping regions of the 5′-UTR (Heasman, 2002). As an additional control, we injected a third MO targeting the 5′-UTR of Xenopus hnRNPK (Gravina et al., 2002), the closest relative of hnRNPK (Ostareck-Lederer et al., 1998). A standard control MO was added to control for non-specific MO effects.

Up to gastrula stages, neither hnRNPK MO had any discernible effect on hnRNPK expression (Fig. 3A1,A2). This was most likely because of a large store of maternal protein (Iwasaki et al., 2008). By neural plate stages, however, expression was strongly suppressed throughout the injected half of the embryo (Fig. 3B1,B2). The efficacies of the two hnRNPK MOs at suppressing hnRNPK expression were similar and the resulting phenotypes (described later) were indistinguishable. Neither hnRNPK MO had any effect on hnRNPE expression (not shown), nor did the hnRNPE and standard control MOs have any effect on hnRNPK (see Fig. S2 in the supplementary material). Suppression of hnRNPK expression persisted through at least stage 40 (Fig. 3C-E). The suppression of hnRNPK was also assessed by western blots of stage 29/30 embryos (see Fig. S3 in the supplementary material). Neither unilateral nor bilateral injections of hnRNPK MO1 had any effect on GAPDH expression. Embryos receiving control MO expressed 97±5% (s.d.) (three blots) as much hnRNPK as un.injected ones, and embryos receiving unilateral or bilateral injections of hnRNPK MO1 expressed 57±6% and 8±3%, respectively, as much as uninjected ones.

hnRNPK shuttles between the nucleus and cytoplasm, escorting its RNA targets to assist their recruitment into mRNP granules and ribosomes (Piñol-Roma and Dreyfuss, 1992; Moore, 2005). hnRNPK immunostaining was not found in axons (e.g. optic nerve and ventral spinal cord white matter; see Fig. S1 in the supplementary material), indicating that hnRNPK is unlikely to play a major role in axonal transport of mRNAs.
Effects of hnRNP K knockdown on development of intact embryos

By external criteria, hnRNP K MO-injected embryos developed normally through stage 20. At stage 22, the injected sides of hnRNP K MO-injected animals began to constrict, leading to a ‘bent’ phenotype. Despite this bending and additional mild defects in anterior cranial and optic vesicle formation, rates of survival through stage 40 (assayed in 19 spawnings) were indistinguishable between hnRNP K MO-injected embryos and controls: hnRNP K MO1, 91±4% of 2042 embryos; hnRNP K MO2, 92±3% of 1039; control MO, 94±5% of 1739; FDx alone, 93±6% of 1106; and uninjected embryos, 94±4% of 2598. None of these survival rates differed significantly (P>0.1, one-way ANOVA).

We next examined effects of hnRNP K knockdown in neurons by whole-mount immunostaining of tadpoles (stage 37/38) for several neuronal antigens typically found in both perikarya and axons. In all cases, phenotypes obtained with either of the two hnRNP K MOs were indistinguishable. We first looked at a phosphorylation-independent epitope of NF-M (RMO270). In contrast to uninjected and control MO-injected animals (Fig. 4A), NF-M immunostaining of optic (not shown), motor and sensory nerves was severely compromised on the hnRNP K MO-injected side (MO1, Fig. 4B; MO2, Fig. 4C). Rostrocaudal spinal tracts on the injected side were also stained less intensely than on the uninjected side (separated by the broken line, Fig. 4B,C). Because these tracts contain decussated axons, we believe that such staining on the injected side may have originated from the uninjected side. The absence of any NF-M immunostained perikarya on the injected side supported this possibility and further indicated that NF-M protein expression was also likely to have been affected. Western blots of stage 29/30 embryos were used to confirm this suppression (see Fig. S3 in the supplementary material). Whereas control MO-injected animals expressed 88±7% (±s.d.) (three blots) as much NF-M as uninjected ones, animals unilaterally and bilaterally injected with hnRNP K MO1 expressed only 56±4% and 9±4% as much, respectively.

Fig. 2. Transition of hnRNP K from predominantly nuclear to mixed nuclear and cytoplasmic localization during neuronal development. (A-C) hnRNP K immunofluorescence (A), DAPI-fluorescence (B) and phase-contrast (C) images of embryonic muscle cell and neurons in dissociated cell culture. hnRNP K immunostaining colocalized with DAPI-stained nuclei (arrowheads, A,B); neurites and growth cones (gc) had no immunostaining (arrows in A,C). (D,E) hnRNP K immunoperoxidase staining of sections of juvenile frog retina (D) and spinal cord ventral horn (E). Retinal ganglion cells (RGC) exhibited more intense cytoplasmic than nuclear staining; motoneuron staining was both in the nucleus and cytoplasm (arrowhead, E). Arrow in E indicates interneuron with predominantly nuclear staining.

Fig. 3. Suppression of hnRNP K expression by antisense MO. Embryos were unilaterally injected with two separate non-overlapping hnRNP K MOs (MO1 in A-D; MO2 in E). (A1,A2) Stage 10 gastrula; persistent maternal hnRNP K expression. (B1,B2) Stage 15 neural plate stage; suppression of hnRNP K expression on the injected side (left of broken line). Upper arrowhead, neural fold; lower arrowhead, somitic mesoderm, on the uninjected side. (C1,C2) Stage 22 tailbud stage; hnRNP K expression in myotome, spinal cord and brain (arrowheads, left to right, respectively) was suppressed on the injected side (C2) when compared with the uninjected side (C1). Because the animals are bent, the optical section in C2 also contained some cells from the uninjected side of the embryo (rectangle). (D,E) Three-day tadpole (stages 39 and 40); the uninjected side exhibited normal staining and morphology, but the injected side exhibited only background immunostaining and some defects in somites. (A1-C2,E) Confocal optical sections; (D) fluorescence dissecting microscopic view of whole animal. hnRNP K immunostaining, red; co-injected FITC-dextran, green.
As the absence of NF-M stained axons might therefore have been due to the suppression of NF-M expression, we next stained animals for three additional neuronally expressed antigens normally present in neuronal perikarya and axons (Fig. 4D1-H). Two of these were cytoskeletal proteins, neuronal β-tubulin (visualized with antibody JDR.38B) and peripherin (visualized with a rabbit antiserum). These proteins are abundant in all developing *Xenopus* axons from the time of neurite initiation onwards (Moody et al., 1996; Gervasi et al., 2000; Undamatla and Szaro, 2001). The HNK-1 antibody targets an extracellular carbohydrate moiety, and in *Xenopus* developing spinal cord, it stains terminally differentiated neurons and their axons (Nordlander, 1989). On the uninjected side, all three exhibited patterns of staining that are typical for these antibodies: i.e. clusters of cell bodies in spinal cord and peripheral ganglia, as well as robustly stained CNS axon tracts, motor axons and peripheral sensory axons. On the injected side, clusters of neuronal perikarya were well stained, indicating that the hnRNP K MO had little effect on their overall expression. This conclusion was confirmed by comparing the relative intensities of peripherin immunofluorescence quantitatively from confocal microscopic images. On the injected side, the mean pixel intensity per cell was 95% that of the uninjected side [142±33 (s.d.) in 120 cells versus 150±26 in 114 cells, respectively]. Thus, the absence of axons cannot reasonably be attributed to faint immunostaining. On the *hnRNP K* MO-injected side, spinal cord CNS tracts were only very lightly stained, and only a very few short wispy peripheral axons were seen emanating from spinal cord and cranial ganglia. In all control MO-injected cases, staining with these antibodies was bilaterally symmetric and indistinguishable from that of uninjected animals. The incidence of these effects is summarized in Table 2. Because staining with three independent markers failed to reveal axons but was unaffected in perikarya, we concluded that hnRNP K knockdown compromised axonal outgrowth but not neuronal determination.

**Rescue of effects on axon outgrowth and NF-M expression by co-injection of hnRNP K RNA**

To confirm that effects on axon outgrowth and NF-M expression were specific to *hnRNP K*, we co-injected in vitro transcribed *Xenopus* *hnRNP K* RNA lacking the MO targeting site (50-1000 pg) plus *hnRNP K* MO1 (10 ng). Injection of 1000 pg of RNA proved lethal (33 embryos), but at 100 and 250 pg per embryo, *hnRNP K* expression was largely restored (30 embryos each). Co-injected animals continued to bend towards the injected side to varying degrees among animals, but less so than in animals receiving *hnRNP K* MO alone, providing external confirmation of partial rescue. *hnRNP K* immunostaining intensity on the injected side also varied among animals but was generally distributed as in normals (Fig. 5A). For example, *hnRNP K* immunostaining on the injected side appeared in somitic myocyte nuclei, although myotomes were generally smaller and nuclei were less well aligned than normal.
Next, embryos co-injected with hnRNP K MO and 100-250 pg of RNA were immunostained in whole mount for neuronal β-tubulin and NF-M. Both peripheral axon outgrowth and NF-M expression were effectively restored (Table 2; Fig. 5B,C), with the degree of restoration inversely correlating with the severity of the ‘bent’ phenotype. Thus, MO effects on axon outgrowth and NF-M protein expression can be attributed to the suppression of hnRNP K expression.

Table 2. Incidence of axonal defects in unilaterally injected intact embryos*

<table>
<thead>
<tr>
<th>Markers for axon staining</th>
<th>Morpholino</th>
<th>Severe axon outgrowth defects</th>
<th>Slight to moderate axon outgrowth defects</th>
<th>Normal axon outgrowth</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-M</td>
<td>MO1</td>
<td>175</td>
<td>8</td>
<td>0</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>MO2</td>
<td>116</td>
<td>10</td>
<td>0</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>CM</td>
<td>0</td>
<td>3</td>
<td>161</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>MO1+RNA</td>
<td>5</td>
<td>45</td>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>Peripherin</td>
<td>MO1</td>
<td>176</td>
<td>16</td>
<td>0</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>MO2</td>
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<td>143</td>
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<tr>
<td></td>
<td>MO1+RNA</td>
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<td>25</td>
<td>3</td>
<td>31</td>
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<tr>
<td>Neuronal β-tubulin</td>
<td>MO1</td>
<td>149</td>
<td>8</td>
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<td>CM</td>
<td>0</td>
<td>0</td>
<td>28</td>
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</table>

*Embryos were unilaterally injected at the two-cell stage and assayed at stage 37/38 by whole-mount immunostaining. Severe defects include virtually no peripheral axonal outgrowth on the injected side, except for a few wispy fibers. Slight to moderate defects include axons formed, but with visibly less robust outgrowth or staining intensity.

Endogenous association of hnRNP K with NF-M but not peripherin mRNA

hnRNP K has been implicated in multiple aspects of post-transcriptional regulation (Bomsztyk et al., 2004). To gain insights into its specific role in post-transcriptional gene expression, we examined effects of hnRNP K knockdown on NF-M expression, a known target of hnRNP K. We compared these effects with those on peripherin, a functionally similar protein whose RNA is not a target. NF-M is a Type IV and peripherin is a Type III neuronal intermediate filament protein. Although both are found together in developing axons, peripherin is generally expressed earlier than NF-M, and thus their expressions are separately controlled (Gervasi et al., 2000).

hnRNP K has been shown in vitro in both Xenopus and rat to bind directly to the NF-M 3′-UTR, but, as of yet, this interaction has only been demonstrated to occur endogenously in rat (Thyagarajan and Szaro, 2004; 2008). To confirm an endogenous association in Xenopus, hnRNP K-containing RNP-RNA complexes extracted from juvenile frog brain were co-immunoprecipitated with anti-hnRNP K, then assayed by RT-PCR and agarose gel electrophoresis for NF-M (Fig. 6A). Co-IP NF-M PCR product was readily visible and enriched (lane 3) over pre-IP product (TIC, lane 2). Product was missing from IPs performed with purified anti-β-galactosidase monoclonal antibody (lane 4), confirming the specificity of the hnRNP K antibody. Xenopus EFTα, which is not an hnRNP K target (lane 5), was also missing from the anti-hnRNP K co-IP, confirming the specificity of the interaction with NF-M mRNA. No peripherin RT-PCR product was detected in the hnRNP K-IP, even after two rounds of amplification with nested sets of primers (Fig. 6B; lanes 4 and 5). Thus, in Xenopus brain, NF-M mRNA associates endogenously with hnRNP K but peripherin mRNA does not.

Effects of hnRNP K suppression on mRNA expression of NF-M and peripherin

Unilaterally injected hnRNP K MO tadpoles (stage 39/40) expressed NF-M mRNA in neuronal perikarya on both sides (Fig. 6C), indicating that the loss of NF-M protein expression was not due primarily to transcriptional effects. Although in some instances NF-M mRNA staining was less intense on the injected side, this was not always the case (Fig. 6D, seen with FISH). Because, in dissociated cell culture, NF-M expression increases...
hnRNP K and axon development

Fig. 6. Binding to hnRNP K and expression of NF-M mRNA in vivo. (A,B) Co-immunoprecipitation and RT-PCR of NF-M (A) and peripherin (B) mRNAs with hnRNP K from juvenile brain. (1) NF-M PCR from plasmid template with Xenopus NF-M cDNA insert, which served as a positive control for NF-M PCR amplification. (2) NF-M RT-PCR of sample prior to co-IP, demonstrating NF-M mRNA presence in the lysate. (3) NF-M RT-PCR of anti-hnRNP K co-IP. (4) NF-M RT-PCR of anti-β-galactosidase co-IP, a control for non-specific IP. (5) EF1-α RT-PCR of anti-hnRNP K co-IP, demonstrating absence of mRNAs not associating with hnRNP K. (6) EF1-α RT-PCR of TIC, demonstrating its presence in lysate. (B) (1,2) peripherin PCR from plasmid template, which served as positive controls for peripherin PCR with each primer set. (3) peripherin RT-PCR of lysate prior to co-IP using the first pair of primers (30 cycles). (4,5) peripherin RT-PCR of anti-hnRNP K co-IP with the first (30 cycles) and second (15 additional cycles) pair of nested primers, respectively. Std, 1 kb DNA ladder (NE Biolabs). (C-E) Expression of NF-M and peripherin mRNAs in unilaterally injected hnRNP K knockdown animals. Dorsal views in whole mount of the entire animal (C), spinal cord (C1) and head (C2) of a stage 39/40 tadpole, processed for NF-M in situ hybridization (digoxigenin-alkaline phosphatase). Stained neurons are on both sides of the spinal cord (C; arrowheads in C1) as well as in brain, trigeminal ganglion (Vth) and retinal ganglion cells (Rgc, C2). (D) Horizontal confocal optical section of a unilaterally injected stage 39/40 hnRNP K MO tadpole processed for NF-M FISH. Rostral is towards the upper left. (E) Dorsal view of the head of a stage 39/40 hnRNP K MO tadpole stained for peripherin mRNA. R, rhombomeres.

EFFECTS OF hnRNP K KNOCKDOWN ON NEURONAL DEVELOPMENT IN DISSOCIATED CELL CULTURE

Dissociated embryonic spinal cord-myotome cell cultures were used to determine whether the effects on axon outgrowth seen in intact embryos were cell-autonomous and to provide independent confirmation of the antibody staining in whole mount that axon outgrowth is compromised; in culture, it is readily seen under phase-contrast illumination without immunostaining. Suppression of hnRNP K expression was as effective in culture as in whole mount-contrast illumination without immunostaining. Inhibition of outgrowth began as early as axon initiation, we concluded that inhibition of outgrowth began as early as axon initiation.

Effects of hnRNP K knockdown on neuronal development in dissociated cell culture

Dissociated embryonic spinal cord-myotome cell cultures were used to determine whether the effects on axon outgrowth seen in intact embryos were cell-autonomous and to provide independent confirmation of the antibody staining in whole mount that axon outgrowth is compromised; in culture, it is readily seen under phase-contrast illumination without immunostaining. Suppression of hnRNP K expression was as effective in culture as in whole mount-contrast illumination without immunostaining. Inhibition of outgrowth began as early as axon initiation.

Effects of hnRNP K knockdown on intracellular localization of NF-M mRNA

Because hnRNP K has been implicated in shuttling RNAs from the nucleus (Bomszyk et al., 1997; Bomszyk et al., 2004; Mikula et al., 2006), we used FISH to localize NF-M mRNA intracellularly during hnRNP K knockdown. In whole mount, on the uninjected side of hnRNP K MO tadpoles, spinal cord neuronal nuclei were better defined by circumferential cytoplasmic FISH-staining than those on the injected side (Fig. 8A,A’). For better resolution of this phenomenon, we performed NF-M double FISH-immunohistochemistry in culture. In control cultures, NF-M protein extended into neurites and the RNA surrounded the nucleus (labeled with DAPI), as is normal (Fig. 8B1-3). In unilaterally injected hnRNP K MO-cultures, such ‘normal’ neurones could be seen adjacent to cells that were unstained for NF-M protein and had
NF-M were positive for Arrowheads in A and B indicate cells identified as neurons because they mRNA-positive cells that had neurites. In parentheses NF-M are the total number of cultures that was expressed in that was retained in the nucleus, when compared with ~0.03% for other groups, an ~80-fold RNA translation was directly affected, we performed polysomal profiling by sucrose gradient ultracentrifugation. In this assay, more actively translated mRNAs fractionate with polysomes, which lie to the left of the monosomal fraction. Earlier work in Xenopus showing that loss of NF-M attenuates axon elongation without completely abolishing it (Szarow, 1995; Lin and Szaro, 1996; Walker et al., 2001), along with results from knockout mice (Elder et al., 1998), argue that more than a loss of NF-M protein must be at work to account for the loss of axons in hnRNP K knockdown animals. To gain some further insights into this issue, we thus looked at effects in ‘neurons’ (as identified by peripherin and neuronal tubulin staining) on the three axonal cytoskeletal elements whose synthesis does not require hnRNP K. In bilaterally injected hnRNP K MO cultures, peripherin formed twisted filaments and aggregates, and neuronal β-tubulin formed densely packed, ring-like structures, ~2-6 μm in diameter in perikarya (Fig. 10D,F). By contrast, their distributions appeared normal in control MO cultures (Fig. 10C,E). F-actin staining with fluorescent phalloidin was also abnormal. Normally in Xenopus cultures, even newly initiated neurites and filopodia stand out with fluorescent phalloidin-staining (Smith et al., 2006), but in bilaterally injected hnRNP K MO-cultures, neither filopodia nor neuritic processes were detectable in any cells (Fig. 10B). Instead, F-actin staining was generally circumscribed around the perikaryal periphery, usually with more on one side of the cell than the other, suggesting cells may be polarized despite their failure to initiate neurites. By contrast, neuritic and growth cone lamellipodia and filopodia on the three axonal cytoskeletal components (to the right) are considered translationally silent. In control embryos, NF-M mRNA underwent a marked, leftward shift into polysomal fractions between stages 25 (early axonal outgrowth) and 29/30 (the peak of spinal axonal outgrowth), indicating increased translational efficiency. A comparable shift occurs during optic nerve regeneration (Ananthakrishnan et al., 2008), suggesting that this shift in embryos is associated with increased axonal outgrowth. At stage 25, peripherin mRNA was already present in polysomal fractions and exhibited a less dramatic shift at stage 29/30, consistent with its being expressed earlier than NF-M (Gervasi et al., 2000). In hnRNP K MO animals, the leftward shift was virtually eliminated for NF-M mRNA, leaving the mRNA in monosomal and lighter fractions. By stark contrast, the peripherin mRNA profile was unaffected, confirming that hnRNP K is not essential for its translation. Such a selective effect indicates that hnRNP K is essential for the translation of only a subset of mRNAs.

Effects of hnRNP K knockdown on other cytoskeletal components

RNA-staining that appeared to overlap nuclei (Fig. 8C1,2). When counterstained with DAPI, this staining was clearly seen to overlap with nuclei (Fig. 8D1-4) in both unilaterally and bilaterally injected cultures. Moreover, in bilaterally injected cultures, NF-M protein was absent from virtually all (>98%) NF-M mRNA positive cells, and these cells had no neurites.

To validate our conclusion that hnRNP K plays an important role in shuttling NF-M message effectively from the nucleus, we quantified NF-M and peripherin RNA from nuclear and cytoplasmic subcellular fractions of bilaterally injected hnRNP K MO- and control (stage 29/30) tadpoles. The cytoplasmic/nuclear ratio (determined as ACmRNA from qRT-PCR) was significantly less for NF-M mRNA in hnRNP K MO embryos than for either RNA in the other groups (P<0.01, one-way ANOVA). Approximately 2.4% (~2 ACmRNA that was expressed in hnRNP K MO embryos was retained in the nucleus, when compared with ~0.03% for other groups, an ~80-fold reduction in the efficiency of nucleocytoplasmic export.

Biochemical assessment of NF-M mRNA translation in hnRNP K knockdown animals

To test whether NF-M RNA translation was directly affected, we performed polysomal profiling by sucrose gradient ultracentrifugation. In this assay, more actively translated mRNAs fractionate with polysomes, which lie to the left of the monosomal peak seen in A260 plots (Fig. 9). Messages in monosomal or lighter fractions (to the right) are considered translationally silent. In control embryos, NF-M mRNA underwent a marked, leftward shift into polysomal fractions between stages 25 (early axonal outgrowth) and 29/30 (the peak of spinal axonal outgrowth), indicating increased translational efficiency. A comparable shift occurs during optic nerve regeneration (Ananthakrishnan et al., 2008), suggesting that this shift in embryos is associated with increased axonal outgrowth. At stage 25, peripherin mRNA was already present in polysomal fractions and exhibited a less dramatic shift at stage 29/30, consistent with its being expressed earlier than NF-M (Gervasi et al., 2000). In hnRNP K MO animals, the leftward shift was virtually eliminated for NF-M mRNA, leaving the mRNA in monosomal and lighter fractions. By stark contrast, the peripherin mRNA profile was unaffected, confirming that hnRNP K is not essential for its translation. Such a selective effect indicates that hnRNP K is essential for the translation of only a subset of mRNAs.

DISCUSSION

hnRNP K knockdown by antisense MOs inhibited axon outgrowth, dramatically reducing otherwise robust nerves to the occasional wispy fiber in intact animals and virtually eliminating neuritic outgrowth altogether in culture. This deficiency arose not from inhibiting neuronal differentiation per se, as the expressions of several neuronal markers were unaffected. Instead, it most probably arose from the loss of specific key axonal cytoskeletal proteins whose mRNAs are influenced by hnRNP K, leading to the disrupted cytoarchitectures of essential axonal polymers. Comparing the differential effects on peripherin and NF-M further indicated at the molecular level that this RNA binding protein in developing Xenopus is needed for efficient nucleocytoplasmic shuttling and loading of selective target mRNAs onto polysomes for translation.
hnRNP K and axon development

**Fig. 8. FISH and immunohistochemistry for NF-M.** (A, A') NF-M FISH of single confocal optical sections from opposite sides of spinal cord or unilaterally injected hnRNP K MO1 tadpole (stage 39/40). Broken outlines surround individual neurons. (B1-D4) Cells from dissociated cell culture. (B1-B3) Control MO neuron stained for NF-M protein (B1), RNA (B2) and RNA/DAPI merged (B3). (C1, C2) Adjacent cells from a unilaterally injected hnRNP K MO culture, viewed for NF-M protein (C1) and RNA (C2). Arrow indicates normal staining for both protein and RNA; arrowhead indicates a cell with no protein and FISH pattern typical of hnRNP K MO-injected spinal cord neurons from whole mount. (D1-D4) Neurons from a bilaterally injected hnRNP K MO culture stained for protein (D1), RNA (D2), DAPI (D3) and RNA/DAPI merged (D4). Scale bars in B1,C1 and D2 apply to B1-3,C1-2 and D1-4, respectively. Bar graph shows qRT-PCR of nuclear and cytoplasmic fractions for NF-M and peripherin RNAs from bilaterally injected hnRNP K MO and control animals assayed at stage 29/30. ΔCT, mean (±s.d.) difference in the number of PCR cycles to reach threshold (see text). *ΔCT was significantly less for NF-M RNA in hnRNP K MO embryos than for other groups (P<0.01, one-way ANOVA).

**Fig. 9. Polysome profiling of NF-M and peripherin mRNAs.** Black bars indicate the distribution of NF-M or peripherin mRNA across fractions, represented as a percentage of the total of each respective mRNA in the gradient (right ordinate). The black line depicts A260 values (left ordinate) across fractions. 'M' indicates the position of the monosomal peak in the A260 trace. (Top four panels) During normal development, NF-M (left) and peripherin (right) profiles shift from right to left, indicating mRNA moving from poorly translated to efficiently translated fractions. (Bottom two panels) Bilaterally injected hnRNP K MO1 embryos processed at stage 29/30. hnRNP K knockdown strongly inhibited the shift into heavier polysomal fractions for NF-M but not peripherin mRNA.
The ubiquitous and early expression of hnRNP K originally led us to anticipate that its knockdown would probably produce an early embryonic lethal. In retrospect, the survival of embryos through early stages of development may have been a fortuitous consequence of the persistence of maternal hnRNP K through gastrula stages, possibly allowing embryos to bypass earlier defects on such processes as cellular proliferation. hnRNP K and its Drosophila homolog bancal, for example, have both been implicated in promoting cell proliferation in neuroblastoma cells and developing appendages (Charroux et al., 1999; Yano et al., 2005). Even so, we saw no evidence of any overall inhibition of cellular proliferation in Xenopus, even after hnRNP K expression was thoroughly suppressed: (1) tail formation, which requires cell proliferation, was overtly normal; (2) proliferating-cell-nuclear-antigen (PCNA) was expressed in hnRNP K MO-injected descendants (data not shown); and (3) neuronal cell counts (i.e. cells expressing neuronal β-tubulin and NF-M mRNA) in dissociated cell culture did not differ significantly between hnRNP K MO- and control cultures. Experiments in culture also ruled out any significant cell-autonomous effects on neuronal cell survival, although we can rule out neither secondary trophic effects in the intact animal owing to the loss of axons nor the possibility that hnRNP K plays additional roles at later times.

At the molecular level, hnRNP K knockdown significantly reduced the efficiency of NF-M mRNA export from the nucleus and inhibited its loading onto polysomes for translation, leading to suppression of NF-M protein expression. These data, along with its cellular localization, support a role for hnRNP K as a nuclear-cytoplasmic RNA shuttling protein in Xenopus, despite the apparent absence of the KNS nuclear localization signal of human hnRNP K (Sioni et al., 1993; Michael et al., 1997). Shuttling could nonetheless be mediated by alternative sequences in Xenopus hnRNP K. For example, it shares an ERK1/2 phosphorylation site with human hnRNP K, which has been implicated in cytosolic localization in HeLa cells (Habelhah et al., 2001). Drosophila bancal also exhibits shuttling behavior, despite lacking the KNS motif. Instead, it has an M9 motif, which is known to mediate shuttling of human hnrNPs A1 and A2/B1 (Charroux et al., 1999).

Because hnRNP K has been implicated in multiple, sometimes seemingly contradictory facets of RNA regulation, drawing an explicit connection with any one aspect of mRNA regulation has historically proved difficult (Bomsztyk et al., 1997; Bomsztyk et al., 2004; Ostareck-Lederer et al., 1998). The contemporary view is that hnRNP K serves as the central element of a docking platform that accompanies RNAs from nucleus to cytoplasm, selectively recruiting various elements to the RNA to dictate its fate at any moment (Bomsztyk et al., 2004; Mikula et al., 2006). Our results support such an idea. This raises future questions about how hnRNP K function is regulated. In addition to changes in hnRNP K expression, its actions can be modulated by competition with other RNP s, such as hnRNP E1 (Ostareck et al., 1997; Ostareck-Lederer and Ostareck, 2004) and Hu proteins (Yano et al., 2005), both of which also target NF-M mRNA (Antic et al., 1999; Thyagarajan and Szaro, 2004). Such proteins could therefore participate jointly in NF-M post-transcriptional regulatory modules. In other instances, hnRNP K actions are regulated by phosphorylation, potentially linking its actions to cell signaling events (Ostareck-Lederer et al., 2002). Our results indicate that the developmental changes in hnRNP K-NF-M RNA interactions seen during postnatal cortical development (Thyagarajan and Szaro, 2008) may underlie regulated changes in NF-M translation.

The most surprising result was the loss of axon outgrowth. The near ubiquitous penetrance of this effect in both intact animals and culture makes it exceedingly unlikely that only a few neuronal subtypes were targeted. Equally unlikely is that axonal loss was caused solely by the loss of NF-M. Instead, the failure of neurites to be initiated, together with the disrupted cytoarchitectures of essential axonal polymers, argue that the axonal defects result from the failure of one or more proteins needed for organizing these polymers to be expressed. These findings raise the intriguing possibility that the translational control of multiple proteins involved in building the axon may be linked through shared elements such as hnRNP K. Our earlier findings that hnRNP K binding to NF-M RNA is developmentally regulated and that NF-M itself is under strong translational control during optic axon regeneration are consistent with hnRNP K being part of such a regulatory module rather than a constitutive facet of NF-M RNA metabolism (Thyagarajan and Szaro, 2004; Thyagarajan and Szaro, 2008; Ananthakrishnan et al., 2008). Future studies to identify the cofactors of hnRNP K, its additional targets during axonogenesis and its mode of regulation should provide the necessary information to distinguish between whether hnRNP K acts as part of a regulatory module influencing several targets via the same mechanism or as one shared element of multiple modules differentially affecting each target separately. Regardless of the outcome of such studies, the current work affirms the importance of post-transcriptional control of selective mRNAs for axon development and identifies hnRNP K as one of the crucial elements.

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