Internal initiation of translation of the TrkB mRNA is mediated by multiple regions within the 5′ leader

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ABSTRACT

Translational regulation of the dendritically localized mRNA encoding for the neurotrophin receptor TrkB has important ramifications for synaptic function. We examined whether the TrkB mRNA is translated through an internal initiation entry site (IRES). The human TrkB 5′ leaders are derived from the use of alternative promoters and alternative splicing, but all 5′ leaders share a common exon. Insertion of a full-length 5′ leader, as well as the common exon into the intercistronic region of a dicistronic luciferase construct, yielded luciferase activity generated from the second cistron that was either equivalent or higher than that observed from the encephalomyocarditis virus IRES. Moreover, inhibiting cap-dependent translation *ex vivo* and in *in vitro* lysates had only a minimal effect on the translation of mRNA containing the TrkB 5′ leader. Dissecting the 5′ leader showed that the IRES is located in the exon common to all TrkB 5′ leaders. Moreover, six regions ranging from 2 to 25 nt were identified that either promoted or inhibited IRES activity. Taken together, these results suggest that the 5′ leader of the human TrkB mRNA contains multiple *cis*-elements that regulate internal initiation of translation and that this mechanism may contribute significantly to the translation of the TrkB mRNA in neuronal dendrites.

INTRODUCTION

Neurotrophin receptors constitute a small family of tropomyosin-related tyrosine kinases (Trk) and include TrkA, TrkB and TrkC [for review see (1)]. The receptors display a relative specificity for their neurotrophin ligands nerve growth factor, brain-derived neurotrophic factor (BDNF) and NT4, and NT3, respectively. In addition, an unrelated receptor p75 belonging to the tumor-necrosis family also binds the neurotrophin ligands with equal affinity.

Multiple cellular functions are influenced by neurotrophin receptor activation (2). Trk receptors are enriched in the nervous system and play a large role both in its development and maintenance. During neural development, Trk receptor activity leads to neurite outgrowth, cellular proliferation and neuronal differentiation, whereas in the adult, Trk receptors alter synaptic efficacy and are essential for hippocampus-mediated learning (3). For example, BDNF can be released both pre- and post-synaptically and bind to the TrkB receptor located on the post-synaptic density. TrkB activation in turn increases local protein synthesis and glutamate receptor phosphorylation leading to changes in dendritic morphology and synaptic efficacy (4–6).

The TrkB receptor also plays a role in cell survival. In response to stressful stimuli including ischemia (7), TrkB activation is often anti-apoptotic (8). Tumor cells utilize this feature by upregulating the level of TrkB receptors (and BDNF) and consequently are resistant to many chemotherapeutic reagents (9).

Owing to its role in multiple cellular activities, Trk receptor expression is regulated at the level of transcription (10), mRNA stability (11) and protein half-life (12). However, regulation of Trk receptor protein synthesis is not well understood. This mechanism is likely very pertinent in neurons. In response to neural activity, the TrkB gene is transcribed and the mRNA is one of the small group of RNAs that are transported to dendrites and translated (13,14). Local protein synthesis in dendrites provides a mechanism to temporally and spatially regulate protein synthesis (15). Indeed, translation of the dendritically localized mRNA encoding for the alpha subunit of CAMKII contributes to learning and memory (16). Since TrkB can alter many synaptic processes, translational regulation of the dendritically localized mRNA would be important.

The major mechanism for regulating protein synthesis is at the initiation step. Historically, the main mechanism of

translation initiation has been called cap-dependent translation (17). Processing of mRNAs from Pol II transcripts results in the 5′ end of the RNA containing a 7-methyl guanosine or cap structure. The cap structure recruits eukaryotic initiation factor (eIF)-4E (cap-binding protein) and in turn through its binding to eIF-4G recruits the translational machinery, including the 40S ribosome (18).

Another mechanism to initiate translation occurs independent of the cap structure whereby the translational machinery contacts the mRNA directly at sites called internal ribosomal entry sites (IRESs) either in the 5′ leader or in the coding sequence (generating a truncated protein) (19–21). IRESs have been best described in viruses. The major characteristics of viral IRESs include a long 5′ leader containing multiple upstream open reading frames (uORFs) as well as a high G/C nucleotide content generating a secondary structure vital for translation initiation.

To date, only a subset of eukaryotic mRNAs has been shown to contain IRESs (22). Many of these mRNAs encode for proteins that contribute to cell growth and proliferation, including FGF2, c-myc and IGF-2. IRES-dependent translation is thought to occur at periods in which cap-dependent translation is impaired or inhibited, including during mitosis and in response to stressful stimuli, such as ischemia, amino acid deficiency and DNA damage. For example, the mRNA encoding for vascular endothelial growth factor (VEGF), a growth factor that stimulates angiogenesis contains an IRES (23–25). In response to decreased blood flow and consequently decreased oxygen, VEGF is transcribed and the mRNA is translated through an IRES (26).

The mechanism and regulation of IRES-dependent translation of eukaryotic mRNAs is not well understood (20,21). Studies have supported both structural and sequence-specific motifs as being integral for internal initiation (27,28). However, a methodical study identifying the *cis*-elements critical for internal initiation of a eukaryotic IRES has not been completed.

Cap-dependent translation initiation has been observed in neuronal dendrites (29,30), but it may be insufficient to ensure efficient translation of dendritically localized mRNAs. eIF-4E, which is the rate-limiting protein in the cell body for cap but not IRES-dependent translation (31), may be present in a lower concentration in dendrites. Ribosomes may also be present in limiting amounts in dendrites and an IRES located in the 5′ leader of a dendritically localized mRNAs could recruit ribosomes and ensure efficient translation (32). Furthermore, synaptic activity leads to a large increase in intracellular calcium resulting in the activation of the calcium-dependent enzyme calpain (33). Both eIF-4E and eIF-4G are calpain substrates. Interestingly, calpain-mediated cleavage of eIF-4E inhibits cap-dependent translation (34), while proteolytic cleavage products of eIF-4G can upregulate IRES-dependent translation (35).

IRES-dependent translation may be a major mechanism for initiating translation in neuronal dendrites. A group of dendritically localized mRNAs, including the alpha subunit of CaMKII, activity-related cytoskeleton protein (ARC) and microtubule associated protein 2 (MAP2), have all been shown to contain IRESs (29). Since many growth factor receptors as well as dendritically localized mRNAs contain IRESs, it was hypothesized that the TrkB mRNA would also

contain an IRES. In the present study, evidence is provided demonstrating that the 5′ leader of the human TrkB 5′ leader contains an IRES. In an attempt to ascertain the mechanism by which the TrkB IRES operates, the 5′ leader was dissected. Multiple regions ranging from 2 to 10 contiguous nucleotides were found to promote IRES activity, while a 25 nt *cis*-element inhibited IRES activity. These results imply that not only can the TrkB mRNA be translated through an IRES, but that IRES-dependent translation of the TrkB mRNA may be regulated.

MATERIALS AND METHODS

Constructs

The TrkB 5′ leaders were PCR amplified from a human brain cDNA library (Clontech) and inserted into the dual luciferase vector-RP (36,37) (a generous gift from Dr Anne Willis, University of Leicester) with EcoRI and NcoI endonuclease restriction sites. For *in vitro* translation, the dicistronic construct was digested with EcoRV and BamHI releasing the *Renilla* and *Photinus* gene and the SV40 3′-untranslated region (3′-UTR). The two luciferase genes were inserted into the multiple cloning site of the SK+ vector (Stratagene) downstream of the T7 promoter. The monocistronic vector for the *ex vivo* experiments was created by digesting the RP vector with EcoRI and BamHI. The digest released the 5′ leader, the *Photinus* luciferase gene and the SV40 3′-UTR, which were cloned into the pGL3 vector (Promega). The monocistronic vector for the *in vitro* experiments was created by inserting the above *Photinus* gene into the SK+ Bluescript vector (Stratagene) downstream of a T7 promoter. The 5′ leaders as well as the *Photinus* gene and SV40 3′ UTR were isolated with EcoRI and BamHI and inserted into the backbone of the PGL3 vector (Promega).

Serial truncations were produced by PCR amplification with 5′ and 3′ primers containing EcoRI and NcoI endonuclease restriction sites, respectively. Internal deletions were created using either the Quick Change Multi Site-Directed Mutagenesis kit (Stratagene) or Gene Tailor Site-Directed Mutagenesis System (Invitrogen). The 5′ leader was subsequently sequenced and re-cloned into the dual luciferase or the monocistronic luciferase vectors.

The promoterless construct was created by digesting the dual luciferase vector with SmaI and EcoRV and religating the construct. This procedure deletes the SV40 promoter and intron located upstream of the *Renilla* luciferase ORF.

In experiments using a hypophosphorylated form of 4E-BP1 (containing Thr-37-Ala/Thr-46-Ala mutations), plasmids expressing this protein or the parent vector (both based on pACTAG-2) were co-transfected with the monocistronic constructs described above, using an 8-fold molar excess of the 4E-BP1 or control expression constructs (38). The 4E-BP1 double mutant and control expression plasmids were generously provided by Dr Nahum Sonenberg (McGill University, Montreal).

Cell culture/luciferase assays

N2a and C6 cells were obtained from ATCC and cultured in DMEM, 10% fetal bovine serum and 200 mM L-glutamine. Cells were transfected with 2 µg of DNA using Fugene

transfection reagent (Roche). After 24 h, the cells were lysed with 500 µl of lysis buffer (Promega). Forty microliters of the supernatant were used for the luciferase assays using the Dual-Luciferase Reporter Assay System and analyzed in a Luminoskan luminometer.

In vitro translation

The Bluescript SK+ vector containing the 5′ leaders upstream of the *Photinus* luciferase gene was linearized with BamHI and *in vitro* transcribed using mMessage Machine (Ambion) producing capped mRNA. The mRNA was extracted with phenol/chloroform and a sample was run on an agarose gel to ensure RNA integrity. An aliquot of 0.5 µg of the mRNA, cap analog (Ambion) and 1.6 nM methionine was added to rabbit reticulocyte lysate (Speed Read, Novagen) and incubated for 1 h at 30°C. The sample was subsequently assayed for *Photinus* and *Renilla* luciferase activity.

RESULTS

The 5′ leader of the TrkB mRNA initiates translation within a dicistronic construct *ex vivo*

The characteristics of 5′ leaders that correlate with their ability to internal initiate translation include a long leader containing a high G/C nucleotide content and multiple uORFs (19,23,39). The human TrkB 5′ leader exhibits both of these traits; it is composed of five exons that are created through alternative promoters and alternative splicing to yield leaders ranging from 392 to 824 nt (40) with a G/C nucleotide content of 60–70% and two to four uORFs. All of the TrkB 5′ leaders contain exon 5.

To determine whether the TrkB 5′ leader exhibits IRES activity, 353 nt of exon 5 that is common to all 5′ leader variants was inserted into the intercistronic region of a dicistronic luciferase construct (36). In addition, the β-globin 5′ leader and the intercistronic region (a multiple cloning site) in the dual luciferase (RP) construct were chosen as negative controls, while the leader from EMCV was used as a positive control for IRES activity. The dicistronic constructs were individually transfected into the C6 and N2a neural cells lines. After 24 h, the cells were harvested and assayed for luciferase activity (Figure 1A). The *Photinus*:*Renilla* luciferase (P:R) ratio obtained from the control RP dicistronic construct was normalized to one. The P:R ratio derived from the construct containing the β-globin 5′ leader was equivalent to the control RP construct. In C6 cells, both the EMCV and TrkB leaders exhibited a similar P:R ratio that was higher than that observed for the dicistronic constructs containing the leader from the control construct or the β-globin 5′ leader (Figure 1A). In N2a cells, the P:R ratio from the TrkB construct increased, while that from the EMCV construct decreased in comparison with their respective P:R ratios observed in C6 cells. This initial result suggests that the TrkB 5′ leader exhibits IRES activity and that the regulation of IRES activity may be cell-type dependent.

To determine whether translation of the second cistron (*Photinus* luciferase) was due to alternative splicing of the dicistronic mRNA or the creation of a cryptic promoter in the intercistronic region, a northern blot analysis was
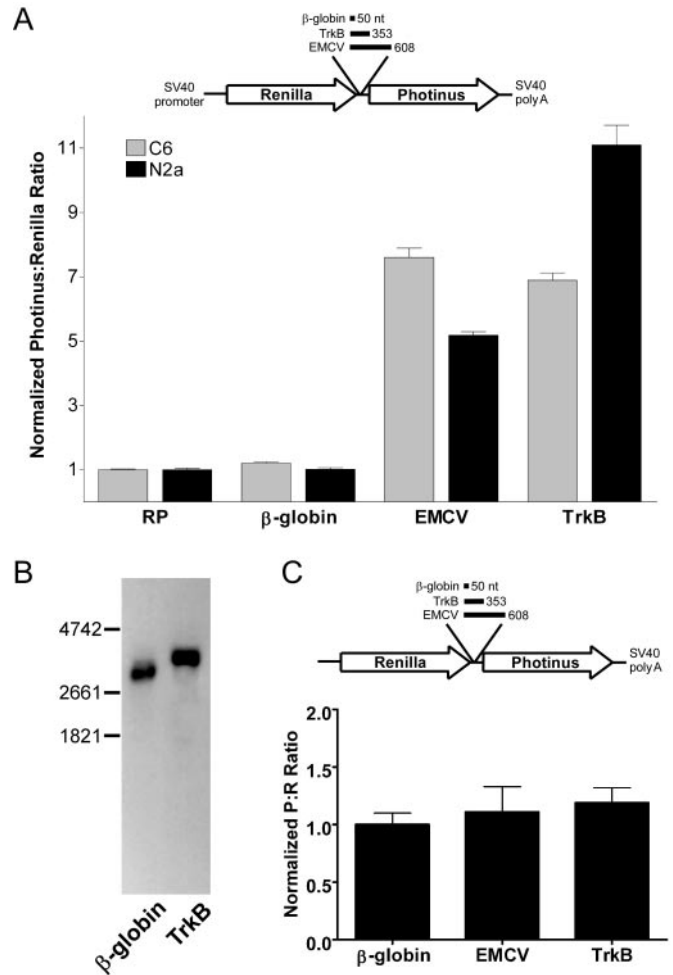

Figure 1. The TrkB 5′ leader exhibits IRES activity. (**A**) Dicistronic luciferase constructs containing the multiple cloning site (RP) of the 5′ leader from the β-globin, TrkB or mRNA or EMCV RNA inserted into the intercistronic region was transfected individually into C6 or N2a cells. Luciferase activity is shown as the ratio of *Photinus* luciferase to *Renilla* luciferase (P:R) and is normalized to the activity of the control construct RP. A P:R ratio that is above that obtained from RP indicates the presence of an IRES. (**B**) Northern blot analysis of transfected dicistronic luciferase constructs. A cRNA probe directed against the *Photinus* luciferase gene identified a single mRNA species from RNA isolated from C6 cells transfected with the dicistronic luciferase construct containing the β-globin or TrkB 5′ leader. (**C**) Promoterless dicistronic luciferase constructs containing the β-globin, TrkB or EMCV 5′ leader inserted into the intercistronic region were transfected individually into C6 cells. The P:R ratio is normalized to the activity obtained from the β-globin construct.

performed. RNA was obtained from C6 cells transfected with the dicistronic constructs containing the β-globin or the TrkB 5′ leader inserted into the intercistronic region. A 1 kb probe directed against *Photinus* luciferase, the second cistron, yielded single mRNA species (even after a lengthy exposure) of the predicted length of the dicistronic construct (Figure 1B). This result suggests that cryptic splicing or cryptic promoter activity is not a major contributor to the enhanced *Photinus* luciferase activity seen with the dicistronic construct containing the TrkB 5′ leader.

Northern blot analysis may be insensitive to detect minor mRNA species that represent monocistronic mRNA

containing *Photinus* luciferase (41). To overcome this limitation and further determine whether the TrkB 5′ leader contains a cryptic promoter, the promoter and intron were deleted from the dicistronic constructs containing the β-globin, EMCV, or TrkB 5′ leader and transfected into C6 cells (Figure 1C). If the TrkB 5′ leader exhibits cryptic promoter activity, it would generate a significantly higher P:R ratio than that obtained from the other dicistronic constructs. However, the P:R ratio obtained from all three dicistronic constructs was similar. Taken together, these results demonstrate that the human TrkB 5′ leader contains an IRES.

The TrkB 5′ leader exhibits IRES activity in a dicistronic and monocistronic mRNA *in vitro*

To determine whether the TrkB 5′ leader is able to internally initiate translation *in vitro*, capped dicistronic mRNA was translated in rabbit reticulocyte lysate. In the presence of increasing concentrations of cap analog, *Renilla* luciferase activity generated from translation of the first cistron decreased, demonstrating that the translation of the first cistron was cap dependent (Figure 2A). However, luciferase activity from the translation of the second cistron (*Photinus* luciferase) increased to 125% of control values in the presence of 5 μM cap analog and gradually decreased to ∼105% of control value after the addition of 50 μM cap analog. This result suggests that the translation of the second cistron is not due to cap-dependent mechanisms including reinitiation or leaky scanning, but instead internal initiation of translation mediated by the TrkB 5′ leader is responsible for the translation.

To determine whether the TrkB 5′ leader can internally initiate translation of a monocistronic mRNA, capped monocistronic mRNAs were translated containing the β-globin or TrkB 5′ leader upstream of the *Photinus* luciferase gene. This experiment also determines whether the upstream sequence encoding for the *Renilla* luciferase gene contributed to the IRES activity found in the *in vitro* translation of the dicistronic mRNA (Figure 2A).

In the presence of cap analog, translation of the *Photinus* luciferase mRNA was differentially regulated depending upon the 5′ leader that was present (Figure 2B). Increasing the concentration of the cap analog led to a linear decrease in the translation of the mRNA containing the β-globin 5′ leader, whereas translation of the mRNA containing the TrkB 5′ leader increased in the presence of 10 and 25 μM cap analog. Addition of the highest amount of cap analog (50 μM) resulted in *Photinus* luciferase activity that was slightly above the control value. This result suggests that the TrkB 5′ leader can initiate cap-independent translation. Moreover, the increase in the translation of the mRNA containing the TrkB 5′ leader in the presence of cap analog implies there may be competition between the cap structure and the IRES for the translational machinery.

Sequential truncations of the TrkB 5′ leader result in a gradual decrease in IRES activity

As an initial attempt to identify regions that promote internal initiation of translation, serial truncations of the TrkB 5′ leader were created. Sequential 5′ deletions ranging from 47 to 75 nt of the TrkB 5′ leader were inserted into the intercistronic
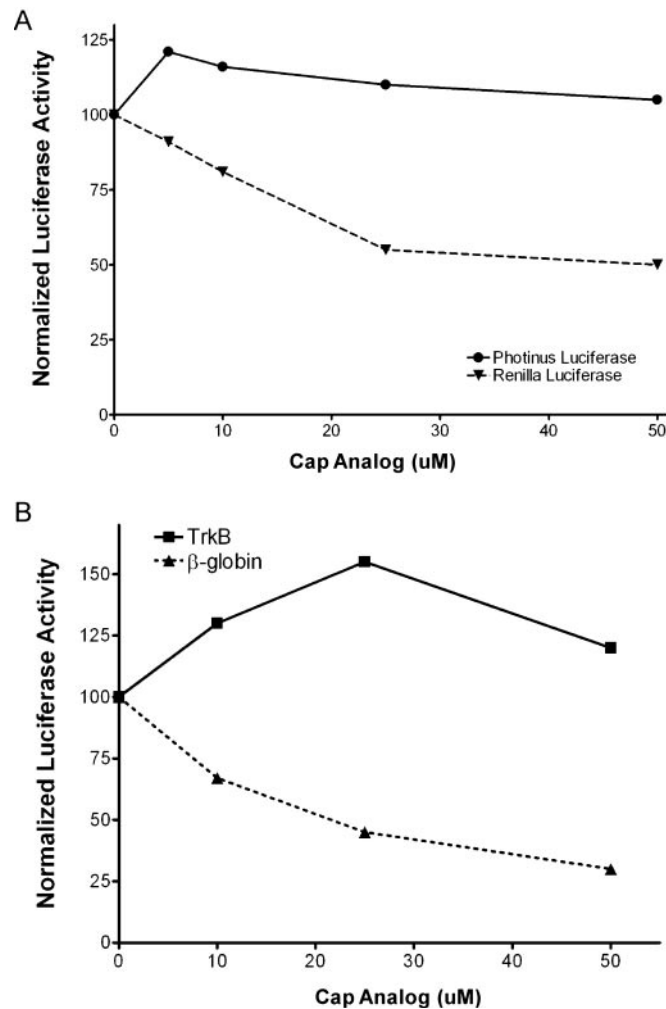

Figure 2. *In vitro* translation of dicistronic and monocistronic luciferase mRNA. (**A**) Dicistronic mRNA containing the β-globin or TrkB 5′ leader was translated in rabbit reticulocyte lysate in the presence of increasing concentrations of cap analog. The initial P:R ratio from the dicistronic mRNA containing the two 5′ leaders was normalized to 100. (**B**) Monocistronic mRNA containing the β-globin or TrkB 5′ leader was translated in rabbit reticulocyte lysate in the presence of increasing concentrations of cap analog. The initial level of *Photinus* luciferase activity from each monocistronic mRNA was normalized to 100.

region of the RP construct and transfected into C6 cells (Figure 3). The initial truncation of 75 nt led to an increase in the P:R ratio, while subsequent truncations resulted in an almost step-wise decrease in the P:R ratio. The 3′ 64 nt did not exhibit any IRES activity. However, deleting the 3′ 64 nt alone decreased the P:R ratio by ∼41% compared with the intact 353 nt 5′ leader (data not shown). This result suggests that the 3′ region of the TrkB 5′ leader is important for positioning the IRES in context with the translational start site.

A PCR amplification of the 353 nt 5′ leader resulted in an internal deletion of 25 nt (−307−−283). Inserting this leader into the intercistronic region of the dicistronic construct yielded the highest P:R ratio suggesting that the deleted nucleotides may inhibit IRES activity (Figure 3). Taken together, the dicistronic construct transfection results suggest
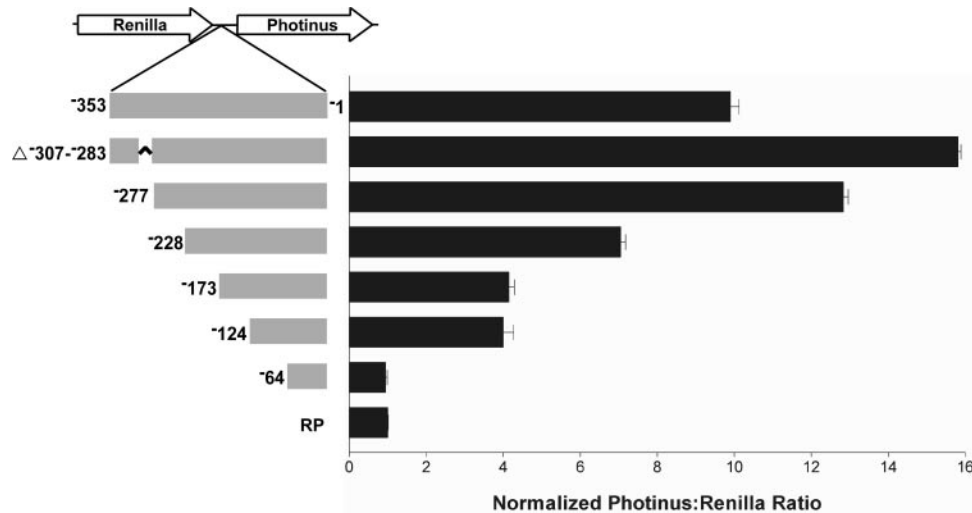
Figure 3. Truncated TrkB 5′ leaders exhibit variable levels of IRES activity. Dicistronic luciferase constructs containing 5′ truncations of the TrkB 5′ leader or an internal deletion in the TrkB 5′ leader inserted into the intercistronic region were transfected into C6 cells. Luciferase activity is shown as the P:R ratio and is normalized to the activity from the control RP construct.

that multiple regions in the TrkB 5′ leader affect internal initiation of translation.

Truncated TrkB 5′ leaders mediate varying levels of cap-independent translation of monocistronic mRNAs *ex vivo*

Most mammalian mRNAs are monocistronic and placing an IRES in the intercistronic region of a dicistronic mRNA may compromise its ability to initiate translation. Consequently, the 353 nt and truncated TrkB 5′ leaders as well as the β-globin 5′ leaders were inserted upstream of the *Photinus* luciferase gene. The plasmids were co-transfected with a plasmid encoding for a hypophosphorylated form of 4E-BP1 (38) or a control plasmid into C6 cells. In its hypophosphorylated state, 4E-BP1 will bind eIF-4E and inhibit cap-dependent translation (38). A western blot using antibodies directed against 4E-BP1 showed that equivalent amounts of 4E-BP1 were co-transfected (Figure 4B). The results are shown as the percentage of luciferase activity for each plasmid co-transfected with 4E-BP1 compared with the control co-transfection (Figure 4A). In the presence of 4E-BP1, the level of *Photinus* luciferase activity derived from the mRNA containing the β-globin 5′ leader decreased by 70%. However, translation of the mRNA containing the 353 nt TrkB 5′ leader only decreased by 30%. This result suggests that the presence of the TrkB 5′ leader can initiate translation when cap-dependent translation is inhibited.

Truncating the 5′ 78 nt of the 5′ leader did not affect the level of translation in the presence of 4E-BP1 (Figure 4A). Larger truncations led to a processive decrease in luciferase activity, with the 3′ 64 nt generating luciferase activity equivalent to that observed from the β-globin 5′ leader. Conversely, the internal deletion of 25 nt (Δ−307−−283) resulted in only a 10% reduction of luciferase activity in the presence of 4E-BP1. These results parallel those obtained from the dicistronic constructs (Figure 3), suggesting that both assays are measuring similar IRES activity and imply that multiple regions throughout the TrkB 5′ leader promote and inhibit IRES activity.

Deletion analysis confirms the presence of an IRES inhibitory element

The initial truncation of the 353 nt TrkB 5′ leader led to an increase in IRES activity (Figure 3). Internally deleting 25 of the initial 78 nt also produced an increase in IRES activity in both the dicistronic and monocistronic luciferase constructs (Figures 3 and 4). These results suggest that the 25 nt region is inhibiting internal initiation mediated by IRES elements located 5′ and/or 3′ to this region. To determine whether the region upstream of the putative inhibitory 25 nt promotes internal initiation of translation, serial truncations of 10 nt were created from the 5′ end of the TrkB 5′ leader containing the Δ−307−−283 deletion. The 5′ leaders were inserted into the intercistronic region of the dicistronic luciferase construct and transfected into C6 cells. Surprisingly, the initial 10 nt deletion led to a decrease in IRES activity that was equivalent to deleting the entire 50 nt (Figure 5A). Additional truncations did not affect the IRES activity.

Smaller truncations of 2 nt were created to identify further the region at the 5′ end of the TrkB 5′ leader that exhibited IRES activity in the absence of the putative inhibitory 25 nt (Figure 5B). The initial 2 nt truncation (−353−−352) had no effect on the level of IRES activity. But truncating the adjacent 2 nt (−351−−350) led to a loss of IRES activity that was equivalent to that observed after the entire 50 nt is deleted (Figure 5A). Four additional 2 nt serial truncations did not further alter the IRES activity, implying that the 2 nt segment (−351−−350) is a critical element for promoting internal initiation of the TrkB 5′ leader.

To confirm that the presence of the −307−−283 nt region was inhibiting IRES activity at the 5′ end of the leader, truncations were created in the intact 353 nt TrkB 5′ leader. In this context, the initial 10 nt truncation did not have any effect on IRES activity (Figure 5C). This result suggests that the presence of the nucleotides −307−−283 masks the IRES activity mediated at the 5′ end of the leader. Moreover, these 25 nt also inhibit the IRES activity promoted 3′ to this region as the P:R ratio increased after the 5′ truncation encompassed the inhibitory 25 nt (−328−−283).
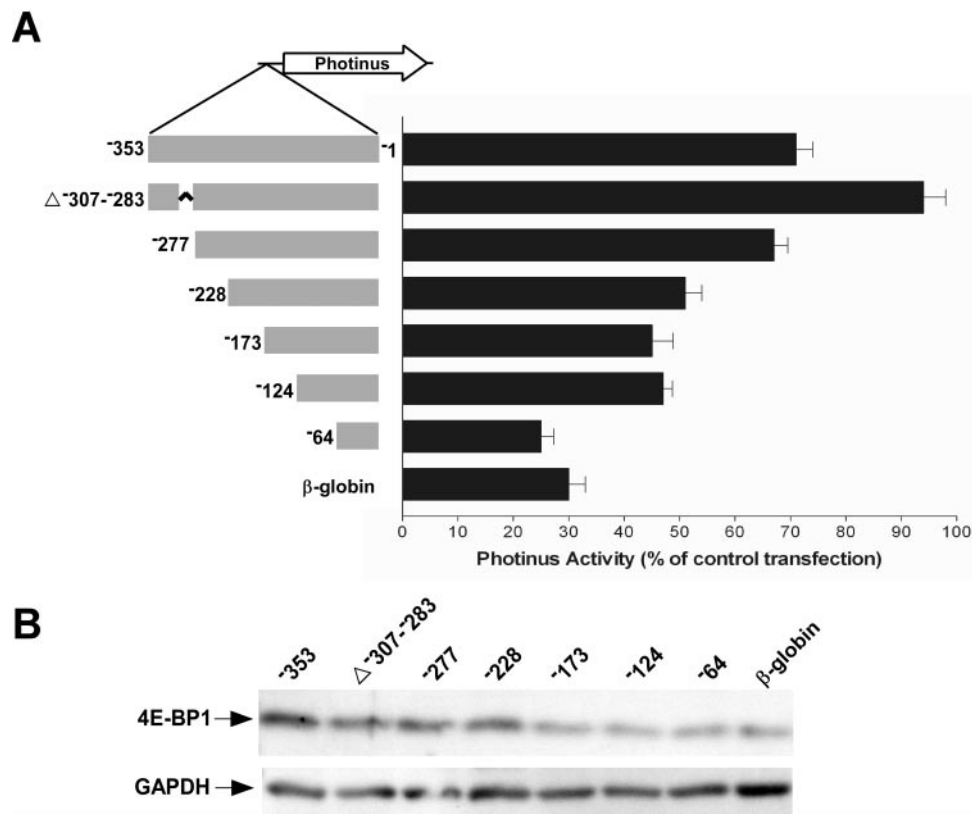
Figure 4. Inhibiting cap-dependent translation of monocistronic mRNA by overexpressing 4E-BP1. (**A**) Monocistronic *Photinus* luciferase constructs containing the β-globin, TrkB 5′ leader, 5′truncations of the TrkB 5′ leader or an internal deletion in the TrkB 5′ leader were co-transfected with either a plasmid expressing hypophosphorylated 4E-BP1 or a control plasmid and assayed for luciferase activity. The activity obtained in cells co-transfected with 4E-BP1 is represented as a percentage of the activity obtained in cells co-transfected with the control plasmid. (**B**) Western blot analysis using antibodies directed against 4E-BP1 or GAPDH (as a loading control) of lysates from the co-transfections in (A).

10 nt regions contribute to TrkB IRES activity

To identify regions within the TrkB 5′ leader that contribute to the IRES activity, more defined deletions were produced. Ten nucleotide truncations were created in the three larger truncations that exhibited a loss of IRES activity, −278−−228, −228−−173 and −124−−64 (Figure 6). The truncated 5′ leaders were inserted into the intercistronic region of the dicistronic luciferase constructs and transfected into C6 cells.

The initial three serial 10 nt truncations that encompassed −278−−249 each led to a minor, but reproducible decrease in IRES activity (Figure 6A). The fourth 10 nt truncation (−248−−239) resulted in the largest decrease in IRES activity while the fifth truncation did not result in any change in IRES activity. Truncating −248−−238 resulted in <50% of the total decrease in IRES activity seen by truncating the entire 51 nt. This result suggests that the nucleotides located further upstream (−278−−249) also contribute to the IRES activity.

In contrast to the serial truncations of the first segment, the initial four serial 10 nt truncations of the −228−−173 segment did not affect IRES activity (Figure 6B). The final 10 nt truncation (−187−−178) led to a loss of IRES activity that was equivalent to truncating the entire 54 nt.

Serial truncations of the final segment (−124−−65) yielded two truncations (−124−−115 and −94−−85) that were responsible for the loss of IRES activity result from truncating the entire 60 nt (Figure 6C). Overall, the multiple serial

truncations led to the identification of four regions consisting of 10 contiguous nucleotide that when deleted result in a significant decrease in IRES activity mediated by the 353 nt TrkB 5′ leader.

Internal deletions of putative IRES elements alters IRES activity

Serial truncations of the intact 353 nt TrkB 5′ leader led to the identification of four 10 nt segments that contribute to internal initiation of translation. The four 10 nt segments are denoted on a model of the secondary structure [Figure 7, Mfold (42,43)] of the 353 nt TrkB 5′ leader [(A) −248−−239, (B) −187−−178, (C) −124−−115 and (D) −94−−85]. The red parenthesis identifies the IRES inhibitory region and the blue parenthesis demarcates 2 nt, which when serially truncated in the absence of the inhibitory region results in a reduction of IRES activity (Figure 5B). The model is limited in that it does not display tertiary interactions (e.g. pseudoknots), the presence of which would greatly affect the secondary structure.

The serial truncations may create novel IRES promoting regions possibly through changing the secondary structure of the remaining RNA. To confirm whether these 10 nt segments promote IRES activity, the segments were deleted individually and in combination within the context of the intact 353 nt TrkB 5′ leader. The internally deleted constructs
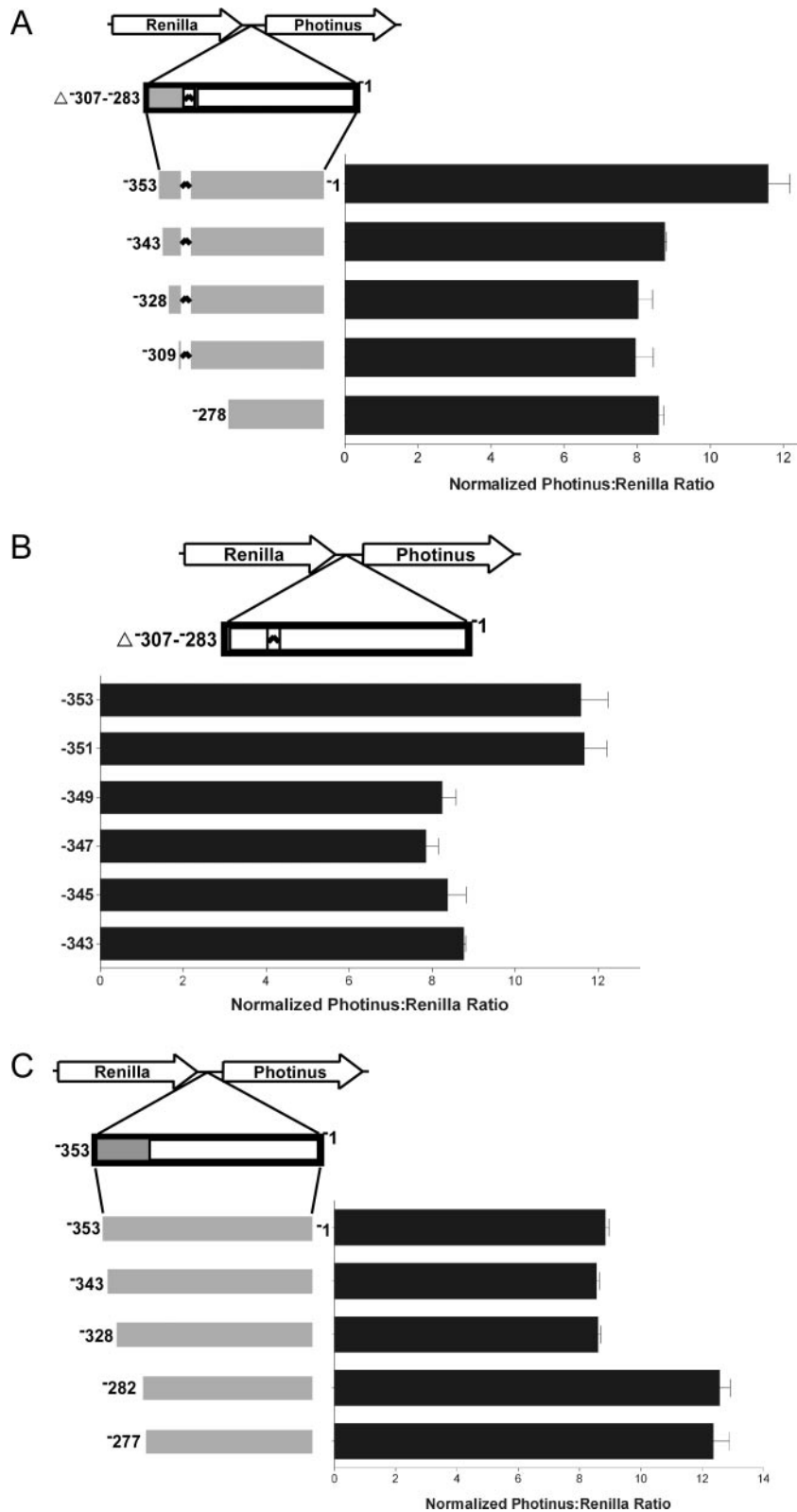
Figure 5. IRES activity is observed in the 5′ end of the TrkB 5′ leader in the absence of a putative inhibitory region. (**A**) The 5′ serial truncations were produced in a TrkB 5′ leader containing an internal deletion of 25 nt. (**B**) Serial truncations of 2 nt were produced in a TrkB 5′ leader containing an internal deletion of 25 nt. (**C**) 5′ serial truncations similar to that created in (A) were produced in the 353 nt TrkB 5′ leader (containing the putative inhibitory 25 nt). All 5′ leaders were inserted into the intercistronic region of a dicistronic luciferase construct and transfected individually into C6 cells. The P:R ratios were normalized to that obtained from the control RP construct.

Figure 6. Serial truncations identify IRES promoting regions in the TrkB 5′ leader. Serial truncations of 4–11 nt were produced in the TrkB 5′ leader containing the 3′ 278 nt (**A**), 3′ 227 nt (**B**) or the 3′ 124 nt (**C**). The leaders were inserted into the intercistronic region of a dicistronic luciferase construct and transfected individually into C6 cells. The P:R ratios were normalized to that obtained from the control RP construct.
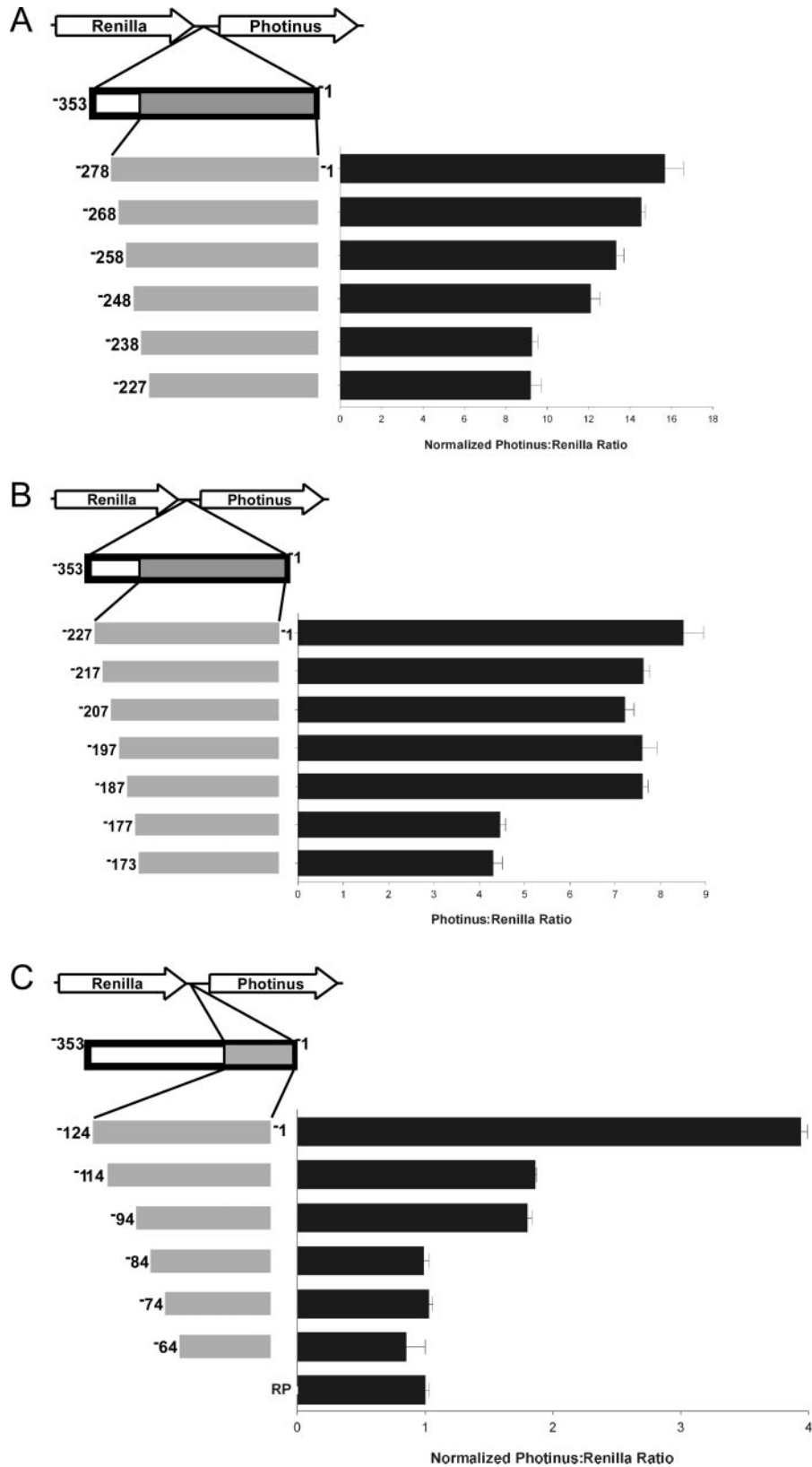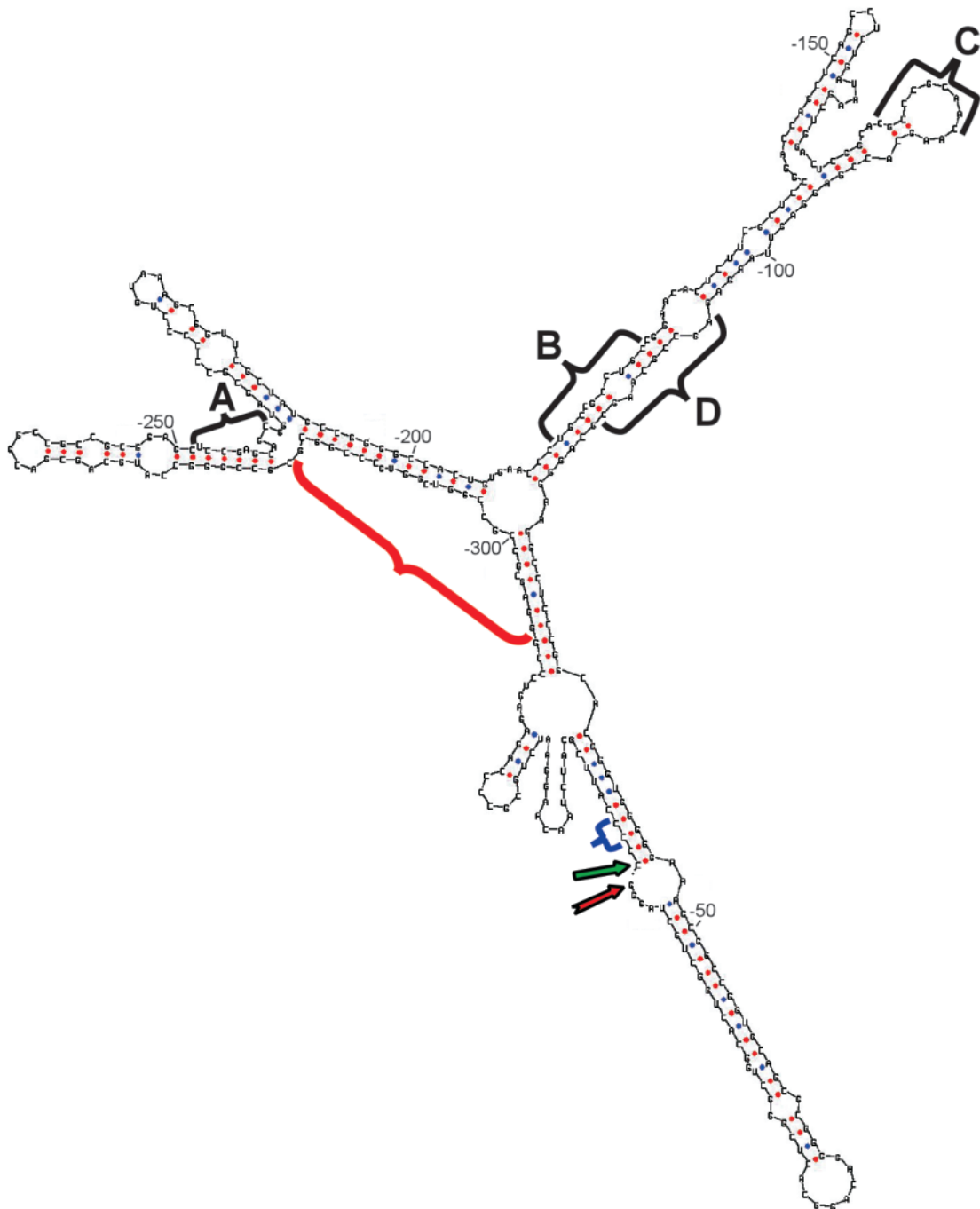
Figure 7. A model [Mfold (42,43)] of the secondary structure of the 353 nt TrkB 5′ leader identifying regions that promote and inhibit IRES activity. The green and red arrows define the 5′ and 3′ end of the 5′ leader. Parentheses labeled (**A–D**) demarcate 10 nt, which when serially truncated result in a loss of IRES activity. The area delineated by the red parenthesis identifies 25 nt that when internally deleted result in an increase in IRES activity. The blue parenthesis demarcates 2 nt, which when serially truncated in the absence of the inhibitory region results in a loss of IRES activity.

were inserted into the intercistronic region of the dicistronic luciferase construct and transfected into C6 cells. Individually deleting three of the four 10 nt segments (B, C and D) led to a marked decrease in IRES activity of ∼50% (Figure 8). However, deleting segment A had no affect on IRES activity. The 10 nt serial truncations in this region revealed that deletion of nucleotides upstream of segment A also resulted in a loss of IRES activity (Figure 6A), leaving

the possibility that these regions may compensate for the loss of segment A. However deletions of the 10 nt regions upstream (−278−−269, −268−−259 and −258−−249) individually or in combination with the deletion of segment A had no effect on IRES activity (data not shown). Moreover, deleting the entire 50 nt (−278−−228) in the context of the intact 5′ leader did not affect internal initiation (data not shown). This result implies that the IRES activity mediated
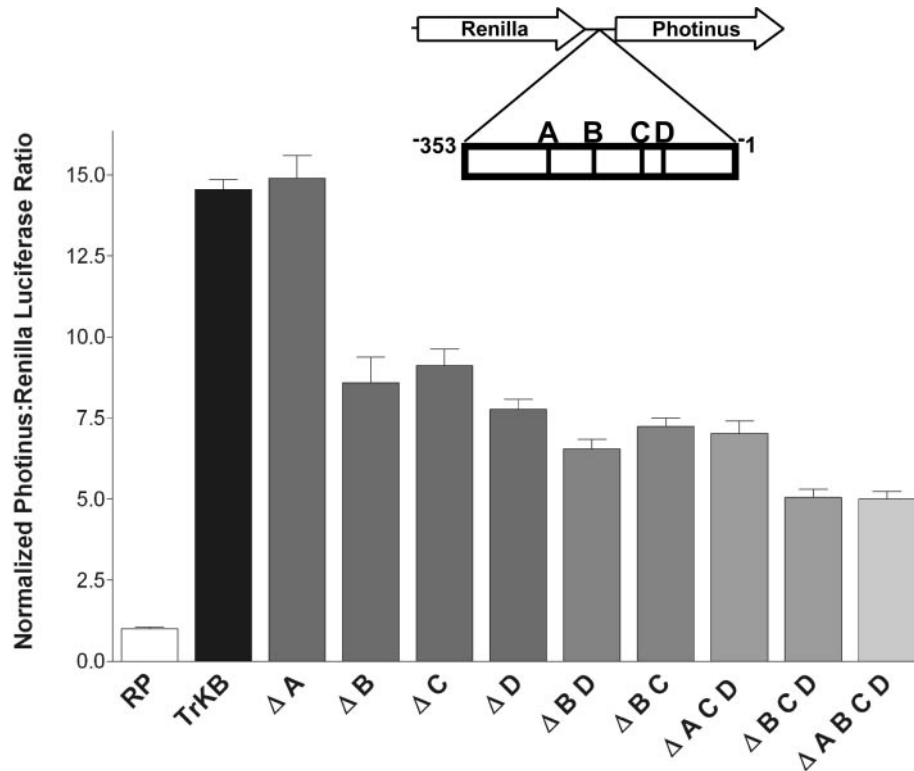
Figure 8. Internal deletions of the TrkB 5′ leader reveal regions that promote IRES activity. The 10 nt regions that were identified from the serial truncation analysis (Figure 6) were deleted individually or in different combinations within the TrkB 5′ leader. The 5′ leaders were inserted into the intercistronic region of a dicistronic luciferase construct and transfected individually into C6 cells. The P:R ratios were normalized to that obtained from the control RP construct.

by segment A is an artifact of the serial truncations. However, it also demonstrates that the random deletion of 10–50 nt region within the TrkB 5′ leader does not affect IRES activity.

Deleting multiple segments produced a further, albeit proportionately smaller, decrease in IRES activity (Figure 8). Removal of segments B, C and D in combination yielded the largest decrease in IRES activity, equivalent to approximately one-third of the value obtained from the intact 5′ leader. Deleting segment A in context with the other segments did not further decrease IRES activity. These results confirm that at least three 10 nt regions contribute to internal initiation of translation within the 353 nt TrkB.

IRES elements affect translation of a full-length TrkB 5′ leader

Exon 5, the exon common to all human TrkB 5′ leaders, has been dissected to uncover multiple regions that affect internal initiation. To determine whether these elements also affect IRES activity of a full-length TrkB 5′ leader, the TrkB 5′ leader containing exons 1, 2 and 5 was PCR amplified from a human brain cDNA library. The 689 nt full-length leader was inserted into the dicistronic luciferase construct and yielded a P:R ratio similar to that observed from the 353 nt leader (Figure 9A). Deleting the three 10 nt IRES elements (segments B, C and D) resulted in a dramatic decrease in the P:R ratio of ∼70%.

In vitro translation of a monocistronic mRNA containing a leader consisting of TrkB exons 1, 2 and 5 was performed to confirm the *ex vivo* results. Addition of cap analog did not affect the translation of the mRNA (Figure 9B). Indeed, there was an increase in the translation after the addition of 10 and 25 μM cap analog, similar to that observed from the 353 nt leader (see Figure 2B). Deleting the segments B, C and D within the context of the full-length TrkB leader resulted in a loss of cap-independent translation. This result was similar to that observed from the loss of translation in the presence of cap analog from the mRNA containing the β-globin 5′ leader. Taken together, these results suggest that the IRES in the TrkB 5′ leader resides in exon 5, and segments B, C and D within that exon are the major *cis*-elements promoting IRES activity.

DISCUSSION

In this paper, we demonstrate that the 5′ leader of the mRNA encoding for the human TrkB receptor internally initiates translation. In addition, the IRES was localized to the exon common to all of the TrkB 5′ leader variants. A dissection of the common exon identified multiple regions that either promoted or inhibited IRES activity, suggesting that IRES-dependent translation is regulated.

Demonstrating internal initiation of translation

The general method for identifying IRESs has been through the use of dicistronic DNA constructs (36,37). Recent reports suggest that cryptic promoter or cryptic splicing of a dicistronic luciferase construct can generate monocistronic *Photinus* luciferase mRNA and artificially inflate the P:R ratio (41).
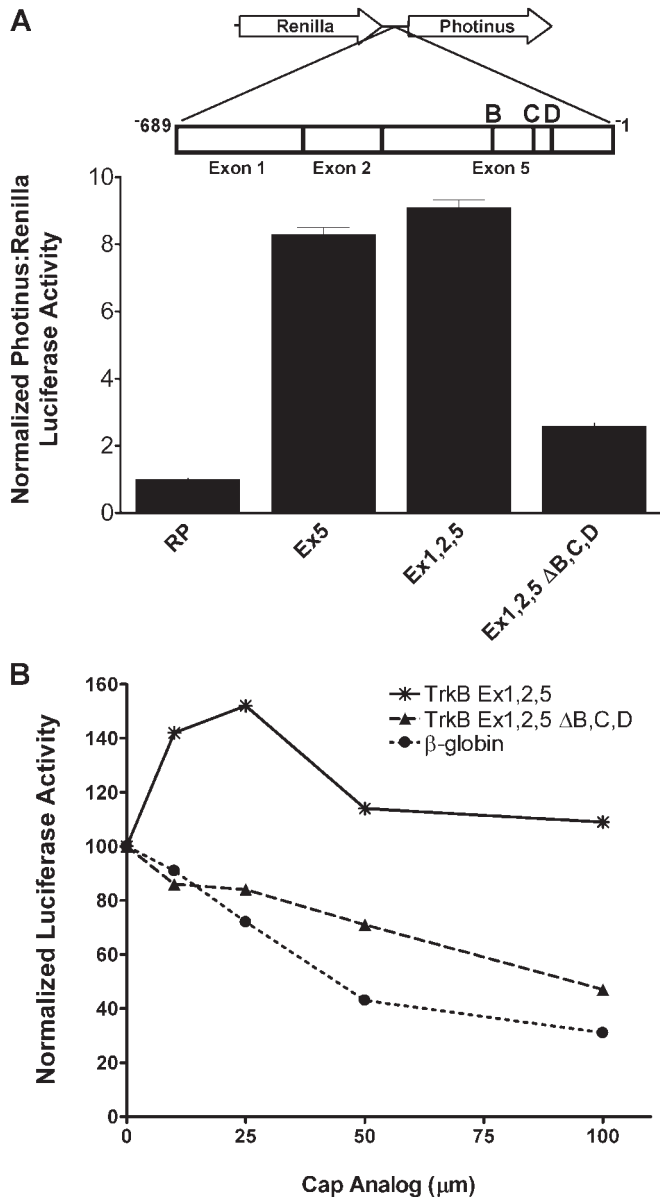
A

B

Figure 9. IRES activity within a full-length TrkB 5′ leader resides in exon 5. (**A**) The TrkB 5′ leader containing exon 5, a full-length TrkB 5′ leader consisting of exons 1, 2 and 5, and the full-length TrkB 5′ leader with IRES promoting regions B, C and D deleted were inserted into the intercistronic region of a dicistronic luciferase construct and transfected individually into C6 cells. The P:R ratios were normalized to that obtained from the control RP construct. (**B**) Monocistronic mRNA containing the β-globin, a full-length TrkB 5′ leader consisting of exons 1, 2 and 5, and the full-length TrkB 5′ leader with IRES promoting regions B, C and D deleted were translated in rabbit reticulocyte lysate in the presence of increasing concentrations of cap analog. The initial level of *Photinus* luciferase activity from each monocistronic mRNA was normalized to 100.

Neither a long exposure of a northern of the dicistronic RNA did produce any lower mRNA species (Figure 1B) nor did deleting the promoter in the dicistronic luciferase construct provide evidence of a cryptic promoter in the TrkB 5′ leader (Figure 1C). Moreover, additional experiments conclusively demonstrated that the TrkB 5′ leader exhibits IRES activity: *ex vivo* inhibition of cap-dependent translation by overexpressing 4E-BP1 only modestly decreased the translation of an

mRNA containing the TrkB 5′ leader and addition of cap analog to *in vitro* lysates only had a marginal effect on the translation of a dicistronic or monocistronic mRNA containing the TrkB 5′ leaders (Figures 2 and 4).

Characterizing the TrkB IRES

Serial truncation analyses of eukaryotic 5′ leaders, including the present one, have shown a gradual or step-wise decrease in IRES activity, suggesting that multiple regions contribute to full IRES activity (44,45). The truncations could affect IRES activity by altering the structure of the residual RNA. Indeed, truncation of one 10 nt region (segment A) led to a substantial decrease in IRES activity, but no change in IRES activity was observed when this region was internally deleted in the context of the intact leader. However, this result implies that all internal deletions are not deleterious for IRES activity. Internal deletions of three other 10 nt regions all resulted in a substantial loss of IRES activity when they were internally deleted, demonstrating that serial truncation analysis of IRESs may be a useful approach.

The internal deletion studies demonstrate that the main IRES elements are located in the exon 5, which is common to all TrkB 5′ leaders. The function of the other upstream exons is unknown. They could contribute to RNA stability. Owing to their high G/C content (52–70%), exons 1–4 may also affect cap-dependent translation by generating a structured RNA impeding ribosomal scanning from the cap structure.

Elucidating the mechanism for eukaryotic IRES-dependent translation

Both secondary structure and sequence-specific motifs are proposed to be integral to IRES-dependent translation (27,28). It is not known whether deletions of the segments affecting IRES activity in the TrkB 5′ leader are altering critical structural domains and/or inhibiting binding by IRES transactivating factors (ITAFs). Individual deletions of the two major structural domains in the c-myc IRES resulted in the loss of IRES activity similar to that observed with the 10 nt deletions in the TrkB 5′ leader (46). However, the segments may be binding ITAFs. Sequences to which ITAFs bind have not been well identified. The best known is PTB, but the IRES promoting regions do not contain any long contiguous stretches of pyrimidines.

An alternative hypothesis for recruiting the ribosome to the IRES is through complementary base pairing of the 5′ leader with the 40S ribosomal RNA (28,47). Segments B, C and D all show some degree of complementarity with 5, 6 and 7 contiguous nucleotides, respectively, potentially base pairing to 18S RNA. Other reports have demonstrated that extensive ribosomal base pairing can inhibit translation (32) and the inhibitory region in the TrkB 5′ leader contains a stretch of 13 out of 16 nt that are complementary to 18S RNA. Whether these regions do indeed base pair with the 40S ribosome and affect translation is unknown. There are additional regions in the TrkB 5′ leader that are complementary to 18S RNA, including seven contiguous nt within the 3′ 64 nt, a region that does not exhibit any IRES activity. This observation implies that all regions that can potentially base pair with the 18S RNA are likely not involved in translation.

Identifying a region in the TrkB 5′ leader that inhibits IRES-dependent translation

Most analyses to date have focused on identifying *cis*-elements and *trans*-acting factors that promote IRES activity. However, elements and factors may exist to inhibit IRES activity in order to regulate the level of protein synthesis. Indeed, a region of 25 nt near the 5′ end of the exon 5 in the TrkB leader structure when deleted results in an increase in IRES activity. This inhibitory region appears to mask IRES promoting elements both upstream and downstream in the 5′ leader. Although serial truncations in other studies have identified putative inhibitory regions in other IRESs (44,48,49), this is one of the first studies to demonstrate it by internally deleting the region.

The secondary structure of the TrkB 5′ leader containing the inhibitory region may be less favorable for ribosomal binding. An example of this phenomenon is observed with the IRES located in the 5′ leader of the mRNA encoding for the cationic amino acid transporter (cat-1) (50). The IRES is constitutively inactive. However, translation of an uORF alters the downstream secondary structure that results in an RNA structure capable of promoting internal initiation. A similar result is also observed by truncating the cat-1 5′ leader. Serial truncation of the TrkB 5′ leader that included the inhibitory region also results in an increase in IRES activity, suggesting that a similar mechanism may occur.

Which mechanism of translation initiation is used for the TrkB mRNA *in vivo*?

Inhibiting cap-dependent translation both *ex vivo* and *in vitro* only modestly affected the translation of an mRNA containing the TrkB 5′ leader. Thus, it is tempting to speculate that the major mechanism for initiating translation of the TrkB mRNA *in vivo* is through an IRES. However, inhibiting cap-dependent translation *ex vivo* may lead to increased transcription of ITAFs and consequently higher levels of IRES activity. In many instances, in which cap-dependent translation is inhibited *in vivo*, including mitosis (48), IRES-dependent translation is increased.

In neurons, the TrkB mRNA is localized to both neuronal perikarya and dendrites (13,14). It is not known whether cap- and IRES-dependent translation of the mRNA occurs simultaneously or whether it is spatially and temporally regulated. For example, many dendritically localized mRNAs have been shown to contain IRESs (29), and IRES-dependent translation of these mRNAs may occur only in dendrites. However, cap-dependent translation of exogenous mRNA does occur in dendrites (29,30), and IRES-dependent translation is observed in the perikarya of non-neuronal cells. Alternatively, translation of mRNAs containing IRESs may occur during periods when cap-dependent translation is inhibited, such as in response to hypoxia and other apoptotic stimuli (26,51). Indeed, TrkB mRNA is synthesized during periods of low oxygen concentration and activation of the receptor may promote cell survival (52).

Internal initiation of neurotrophin receptor mRNAs

Many mRNAs encoding for growth factors contain IRESs, including IGF-2, FGF-2 and VEGF (24,26,53–55). However, TrkB is the first growth factor receptor mRNA to contain an IRES. It would be of interest to determine whether other neurotrophin receptor mRNAs utilize IRES-dependent translation. The 5′ leader of the human TrkC mRNA exhibits similar characteristics to TrkB in that it is long (629 nt), G/C-rich (62%) and contains upstream AUGs. The human TrkA receptor 5′ leader is significantly shorter at only 83 nt, but it contains 79% G/C content. 5′ leaders as short as 140 nt can contain an IRES (29). The 5′ leader of the human p75 receptor is incomplete but contains 79% G/C content in the 114 nt published to date. Thus, all neurotrophin receptors appear to contain a highly structured 5′ leader, which may contribute to lower levels of protein synthesis by inhibiting cap-dependent ribosomal scanning. However, in response to unique stimuli, including apoptosis or neural activity, IRES-dependent translation of these mRNAs may occur to respond to the cellular requirement for enhanced Trk protein expression.

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