A 5' Leader of Rbm3, a Cold Stress-induced mRNA, Mediates Internal Initiation of Translation with Increased Efficiency under Conditions of Mild Hypothermia*

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Although mild hypothermia generally reduces protein synthesis in mammalian cells, the expression of a small number of proteins, including Rbm3, is induced under these conditions. In this study, we identify an Rbm3 mRNA with a complex 5' leader sequence containing multiple upstream open reading frames. Although these are potentially inhibitory to translation, monocistronic reporter mRNAs containing this leader were translated relatively efficiently. In addition, when tested in the intercistronic region of dicistronic mRNAs, this leader dramatically enhanced second cistron translation, both in transfected cells and in cell-free lysates, suggesting that the Rbm3 leader mediates cap-independent translation via an internal ribosome entry site (IRES). Inasmuch as Rbm3 mRNA and protein levels are both increased in cells exposed to mild hypothermia, the activity of this IRES was evaluated at a cooler temperature. Compared to 37 °C, IRES activity at 33 °C was enhanced up to 5-fold depending on the cell line. Moderate enhancements also occurred with constructs containing other viral and cellular IRESes. These effects of mild hypothermia on translation were not caused by decreased cell growth, as similar effects were not observed when cells were serum starved. The results suggest that cap-independent mechanisms may facilitate the translation of particular mRNAs during mild hypothermia.

Mammalian cells exposed to conditions of mild hypothermia continue to be metabolically active; however, there is a general inhibition of protein synthesis (1) and a concomitant increase in expression of a small number of cold-shock mRNAs and proteins (3, 4). Some of these changes appear to protect cells, enabling them to more effectively tolerate hypothermic or other stressful conditions (reviewed in Refs. 2 and 3). In addition, some of these molecular changes may be physiologically relevant, for example, in testis and skin, where cell temperatures are typically 30–34 °C and ~33 °C, respectively (3). Two of the mRNAs induced in cells exposed to mild cold stress encode Rbm3 and the cold-inducible RNA-binding protein (Cirp), also known as A18 (4–7). These are two highly homologous members of the glycine-rich RNA-binding protein family, and both proteins are thought to affect the posttranscriptional regulation of gene expression, perhaps functioning as RNA chaperones that facilitate translation at colder temperatures (4, 6, 8).

The focus of this study is the 5' leader of the mRNA encoding mouse Rbm3, which affects its translation at 37 °C and under conditions of mild cold stress. The Rbm3 cDNA identified in this study contains a 720-nucleotide 5' leader that appears to be a longer variant of that described by Danno et al. (7). This longer variant contains 12 upstream open reading frames (uORFs) that should, in the absence of other mechanisms, block translation of the main open reading frame (9, 10). However, when tested in the 5'-UTR of a reporter gene, the Rbm3 leader sequence mediated a higher level of translation than expected both in transfected cells and in cell-free lysates. This suggested that Rbm3 mRNA might contain sequences that enable the translation machinery to bypass these potentially inhibitory uORFs. This notion was supported by showing that the Rbm3 5' leader could internally initiate translation in a cap-independent manner. The sequence or sequences that mediate this activity will be referred to as an internal ribosome entry site (IRES).

The induction of Rbm3 mRNA in cells exposed to mild cold stress prompted an investigation of the relative efficiency of its IRES at a lower temperature. The results showed that the activity of the Rbm3 IRES was enhanced in some cell lines when cells were cultured at 33 °C compared with cells cultured at 37 °C. Moderate enhancements of internal initiation were also observed with the c-myc and poliovirus (PV) IRESes, but not with the encephalomyocarditis (EMCV) IRES. These effects of mild hypothermia on IRES activity did not appear to be caused by decreased cell growth, as similar effects were not observed under conditions of serum starvation. This study suggests that internal initiation of translation may be an important component of the cold shock response in mammalian cells, ensuring the translation of particular proteins even though overall protein synthesis is inhibited.

**Experimental Procedures**

Isolation of Rbm3 cDNA Clones—Rbm3 cDNA clones were selected in the course of another study2 that used a PCR-based screen to identify novel RNA-binding proteins that might affect post-transcriptional events in the mammalian nervous system as they do in Drosophila and Caenorhabditis (11–14). Degenerate PCR primers modeled on the ribo-

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1 The abbreviations used are: uORF, upstream open reading frame; CAT, chloramphenicol acetyltransferase; UTR, untranslated region; IRES, internal ribosome entry site; PV, poliovirus; EMCV, encephalomyocarditis; PCR, polymerase chain reaction; RNP, ribonucleoprotein.

2 G. C. Owens, unpublished results.
nucleoprotein (RNP) 1 and RNP2 sequences in the first RNA-binding domain of the Drosophila musashi gene product were used to amplify cDNA fragments (~140 base pairs) containing a RNP consensus sequence type RNA-binding domain (15) or an RNA recognition motif (16). PCR products amplified from postnatal day 14 mouse C57BL/6 brain poly(A) + cRNA were cloned and used to screen a mouse brain cDNA library in ZAP II (Stratagene) to identify a larger Rbm3 cDNA clone. As the 5' leader of this cDNA clone appeared to be a longer variant of those described previously, its authenticity was confirmed by nested PCR of cDNA from different sources: poly(A)+ mRNAs derived from adult mouse brain and total RNA extracted from mouse fibroblast NIH-3T3 cells. Primer sets used for amplification were based on sequences contained within the Rbm3 cDNA identified in this study. PCR reactions using mouse brain cDNA as a template were carried out using primer set 1 (sense primer, 5'-CGAGGCGATCCACCTAGCTCTTAC-3'; antisense primer 5'-CGTTTTAGCTTATTCTCTTGGTATT-3'). After a first round of PCR, an aliquot of the reaction was reamplified with the same primer set. PCR reactions were also carried out with cDNA from both mouse brain and fibroblast NIH-3T3 cells as templates using nested primers. The initial amplification was carried out using primer set 2 (sense primer, 5'-AACAGCAAGAAATATATGACTA-3'; antisense primer, 5'-CTCTTCCATTCTAGTCTCTAC-3'). Nested amplification was carried out using primer set 3 (sense primer, 5'-GACTGAAAGCCAGTCAACAACCG-3'; antisense primer, 5'-GCTAAGCATGAGCGAC-3'). PCR products obtained after two rounds of PCR were inserted into pCR2.1 TOPO (Invitrogen) and sequenced. All Rbm3 constructs described in this study utilized this 5' leader.

Sequence comparisons to GenBank were performed using the BLAST sequence comparison algorithm (Genetics Computer Group). The BESTFIT program (Genetics Computer Group) was used to compare the Rbm3 sequence to segments of the mouse 18 S rRNA (accession no. X00686). The significance of the sequence matches was evaluated by using BESTFIT quality scores with those obtained when the sequence matches were randomly shuffled 100 times and compared with 18 S rRNA.

Analysis of Rbm3 5' Leader in Reporter Gene Constructs—To determine its effect on translation, the Rbm3 5' leader was tested in the 5'-UTR of the firefly (Photinus) luciferase coding region. This construct (Rbm3/PhotinusCAT) derived from the pGL3-control plasmid (Promega), contained the chloramphenical acetyltransferase (CAT) gene (Promega) as a second cistron, expressed via the EMCV IRES (nucleotides 286–860, accession no. X74312). Expression of the CAT enzyme should reflect dicistronic mRNA levels and allows estimation of the translation efficiency of the Photinus cistron. To test whether the Rbm3 5' leader, contained an IRES, this sequence was tested in the intercistronic region of the RP and RPh (hairpin) dicistronic reporter vectors, which were kindly provided by Dr. Anne E. Willis (University of Leicester, Leicester, United Kingdom). These constructs encode dicistronic mRNAs with the sea panay (Renilla) luciferase coding region as the first cistron and Photinus luciferase coding region as the second cistron. The initiation codon of the Photinus luciferase gene is contained within an Ncol site. This restriction site was used to clone the Rbm3 5' leader immediately upstream of the Photinus luciferase coding region. Other inserts cloned into the RP and RPh vectors were: the EMCV IRES; the human PV (Mahoney strain) IRES, nucleotides 1–743 (kindly provided by Dr. Peter Sarnow, Stanford University, Stanford, CA); the human c-myc IRES (nucleotides 2501–4519 contained within the c-myc gene, accession no. J00012); and the mouse g-globin 5'-untranslated region (UTR), nucleotides 1–52 (nucleotides 2666–2717 in g-globin gene, accession no. J00413).

The reporter constructs (0.5 µg) were transfected into 1 × 10⁶ cells using PuGENE 6 (Roche Molecular Biochemicals). Where indicated, transfection efficiencies were normalized by co-transfection with the pCMVβ vector (CLONTECH). Cells were harvested 24 h after transfection and luciferase, CAT, and β-galactosidase activities determined (17). IRES activities in this study refer to the Photinus:Renilla luciferase expression ratio. Northern blot analysis was performed as described previously (18) using total RNA prepared from transiently transfected cells.

For cell-free translation studies, constructs were linearized using BamHI and cRNA was transcribed using the MESSAGE mMachine T7 transcription kit (Ambion). C6 cell-free lysate was prepared as described previously (19). Translation reactions were carried out in the presence or absence of GTP or the cap analogue (m7G(5'ppp5'G)) as indicated.

For cold stress studies, cells were transfected and assayed as described above, except that, following transfection, cells were recovered for 24 h at 37 °C; medium was changed, and cells were incubated at 33 °C for an additional 48 h. Parallel controls were similarly treated but maintained at 37 °C throughout. For serum deprivation studies, cells were also recovered for 24 h following transfection. After 24 h, medium was removed, cells were washed with phosphate-buffered saline three times, and incubated in Dulbecco's modified Eagle's medium, 0.5% fetal bovine serum for 16 h. Parallel controls were treated similarly but incubated in Dulbecco's modified Eagle's medium, 10% fetal bovine serum throughout.

RESULTS

Isolation of Rbm3 cDNA Clones and Validation of the Rbm3 5' Leader—A PCR-based screen to identify novel RNA-binding proteins that might mediate mRNA processing in the brain identified a cDNA fragment designated U9, the predicted amino acid sequence of which corresponds exactly to that of the mouse Rbm3 protein (7), a glycine-rich RNA-binding protein. This protein is closely related to Cirp and to the predicted translation product of a human sequence found on chromosome 1q24–1q25 (accession no. Z98751). All three of these protein sequences are distantly related to that of heteronuclear RNP G (4, 20).

The Rbm3 cDNA fragment U9 was used as a probe to isolate an approximately 1.4-kilobase pair Rbm3 cDNA clone from a mouse neonatal brain cDNA library (GenBank™ accession no. Y052560). The 5' leader contained within this cDNA is 720 nucleotides long, and is considerably longer than the mouse and human sequences that have been described previously (6, 7). Comparison of the leader sequence identified in this study to the published leader sequences revealed a match of 59 nucleotides immediately upstream of the initiation codon when compared with the other mouse sequence (7), and of 107 nucleotides when compared with the human sequence (6). The authenticity of the 720-nucleotide 5' leader identified in this study was confirmed by nested PCR and sequencing of the PCR products. In independent experiments, sequences containing the 5' leader and coding sequences were amplified using cDNA templates prepared from two different sources: adult mouse brain and mouse fibroblast NIH-3T3 cells. The sequences of the PCR products obtained with both cDNA templates matched those of the isolated Rbm3 cDNA clone.

Sequence Analysis of the Rbm3 5' Leader—The Rbm3 5' leader sequence is 720 nucleotides long and contains 13 uORFs, none of which overlap the Rbm3 coding sequence. Seven of the upstream AUGs contain a purine at position –3 and might therefore be in good context to function as initiation codons (21). In addition, sequence comparisons of the Rbm3 5' leader sequence to those contained within GenBank identified several matches. These included a reverse complementary match to a segment of the p84 gene, as well as to numerous different ESTs, which may indicate that there are several alternatively spliced variations of the Rbm3 5' leader.

A striking feature of the Rbm3 5' leader sequence is that it contains numerous complementary matches to 18 S rRNA. Fifteen of these sequence matches, ranging from ~80 to 100% over ~8–20 nucleotides, are documented in Table I.

Reporter mRNAs Containing the Rbm3 5' Leader Are Translated Relatively Efficiently despite Numerous uORFs—To determine the effect on translation of the Rbm3 leader sequence, it was tested in the 5'-UTR of a dicistronic mRNA with the Photinus luciferase reporter gene as the first cistron and the CAT gene, driven by the EMCV IRES, as the second cistron (Rbm3/PhotinusCAT, Fig. 1). Although the presence of numerous uORFs within the 5' leader of Rbm3 mRNA might be expected to completely block translation of the Photinus reporter gene (10), this was not the case. When tested in the rat glioma C6 cell line, the translation efficiency of the Rbm3/PhotinusCAT mRNA was 28% of the efficiency of the P/EMCV/CAT mRNA (Fig. 1). When tested in three other cell lines,
human neuroblastoma SK-N-SH (SK), mouse fibroblast NIH-3T3, and mouse neuroblastoma N2a, the translation efficiency of the Rbm3/P/EMCV/CAT mRNA was 64, 16, and 72% of the efficiency of the P/EMCV/CAT mRNA, respectively. A similar monocistronic mRNA containing the Rbm3 5’ leader in the 5’-UTR of the Photinus luciferase gene was transcribed in vitro and translated in a cell-free lysate prepared from the C6 cell line and compared with the parent construct. The translation efficiency of this full-length transcript was ~30%, which was similar to the 28% efficiency obtained when the Rbm3/P/EMCV/CAT construct was transfected into C6 cells, indicating that the Rbm3 5’ leader affected the translation efficiency of the monocistronic mRNAs. These results are consistent with the notion that other mechanisms such as internal initiation, leaky scanning, or reinitiation (21) might be involved in the translation mediated by the Rbm3 5’ leader. A consistent observation in all four cell lines was that the expression of the second cistron (CAT driven by the EMCV IRES) was lower from the construct containing the Rbm3 5’ leader compared with the control construct. This suggested that the presence of this 5’ leader decreased mRNA levels, perhaps by affecting mRNA stability.

The Rbm3 5’ Leader Functions as an IRES in Transfected Cells—To determine whether the Rbm3 5’ leader could internally initiate translation, it was cloned in the intercistronic region of a dicistronic mRNA and tested in transfected cells (Fig. 2). The dicistronic mRNAs used in these studies contain Renilla and Photinus luciferases as the first and second cistrons, respectively (RP; Ref. 22). The presence of the Rbm3 5’ leader in the intercistronic region of this dual luciferase dicistronic mRNA (Rbm3/RP) enhanced expression of the downstream Photinus luciferase gene ~22-fold over background when expressed in the N2a cell line (Fig. 2A). The presence of the Rbm3 5’ leader in the dicistronic mRNA resulted in decreased translation of the first cistron (Fig. 2A, compare Renilla luciferase activities for RP versus Rbm3/RP). This may indicate that the stability of this mRNA was decreased compared with the control, which is consistent with the results obtained with monocistronic mRNAs containing the Rbm3 5’ leader.

To begin to evaluate whether the enhanced translation of the second cistron mediated by the Rbm3 5’ leader was independent of the translation of the first cistron, a stable hairpin-forming structure (b) was used to block its translation. Hairpin structures can inhibit translation by physically blocking scanning ribosomes (see Ref. 23), and in the present studies, this sequence appeared to inhibit expression of the upstream Renilla luciferase cistron by >80% in N2a cells. This inhibition ranged from 67–93% in other cell lines. In contrast, the expression of the downstream Photinus luciferase cistron remained high in the construct containing the Rbm3 5’ leader (Rbm3/ RP). Indeed, inhibiting the translation of the Renilla cistron resulted in an apparent increase in the expression of the Photinus luciferase cistron (Fig. 2A).

To evaluate whether the enhanced translation of the downstream cistron resulted from shorter monocistronic mRNAs generated by weak promoter activity, RNA fragmentation, or unusual splicing events, the dicistronic mRNA was analyzed using Northern blots. The results indicated that the construct was expressed as a full-length dicistronic mRNA (Fig. 2B). Although these results are consistent with the Rbm3 5’ leader functioning as an IRES, Northern analyses have limited sensitivity, so the Rbm3 5’ leader was further evaluated for its ability to mediate internal initiation in an intact full-length mRNA in cell-free lysates.

The Rbm3 5’ Leader Functions as an IRES in Cell-free Lysates—Capped dicistronic mRNAs that lack (RP) or contain the Rbm3 5’ leader (Rbm3/RP) were transcribed in vitro and translated in lysates prepared from C6 cells (Fig. 3). Translation of the parent RP mRNA yielded Photinus luciferase activities that were indistinguishable from the background obtained from control reaction mixtures that lacked Photinus luciferase mRNAs. In contrast, an equimolar concentration of the Rbm3/RP mRNA consistently yielded Photinus luciferase activities that were ~15-fold higher than background. To evaluate if the translation mediated by the Rbm3 leader was cap-independent, in vitro transcribed and capped Rbm3/RP mRNAs were translated in the presence of an increasing concentration of m⁷GpppG, a cap analogue that blocks cap-dependent translation by binding to initiation factor eIF4E (24). With the RP mRNA, translation of the Renilla luciferase cistron was blocked by ~90%, but was not affected by similar concentrations of non-methylated GTP, which was tested as a control (data not shown). Translation of the Photinus luciferase cistron remained unchanged at a background level in the absence or presence of the cap analogue. With the Rbm3/RP mRNA, the presence of the cap analogue also blocked translation of the first cistron by ~90%; however, translation of the Photinus luciferase cistron increased ~2-fold. The enhanced translation of the second cistron in this experiment was consistent with the results obtained in transfected cells when expression of the first cistron was blocked by a stable stem-loop structure. Taken together, the results of the transfection and cell-free translation studies support the notion that the Rbm3 5’ leader contains an IRES.

The Rbm3 IRES Is Relatively Active in Several Cell Lines—The activity of the Rbm3 IRES was compared with that of another cellular IRES from the c-myc mRNA, and to two picornaviral IRESes: PV, an enterovirus IRES, and EMCV, a cardiovirus IRES. The 5’-UTR of the β-globin mRNA served as a negative control for these experiments, as the translation of this mRNA is cap-dependent (e.g. Refs. 25–27). These constructs were transiently transfected into four cell lines, N2a, NIH-3T3, C6, and SK, and luciferase activities were measured after 24 h (Fig. 4). The results showed that the activity of the Rbm3 IRES ranged from ~10- to 40-fold over background depending on the cell line. These activities were generally higher than those of the c-myc, PV, and EMCV IRESes in these cell lines (28, 29).

Rbm3 IRES Activity Is Enhanced under Conditions of Mild Cold Stress—Expression of Rbm3 mRNA and protein are induced by mild hypothermia (7); the activity of this IRES was therefore assessed in cells exposed to this condition. Dicistronic constructs containing the Rbm3 5’ leader were transfected into

| Rbm3 5’ leader | Similarity to 18 S rRNA | p by chance |
|----------------|------------------------|------------|
| −19 to −31     | 92.3% in 13 nucleotides | <0.01      |
| −70 to −85     | 81.3% in 16 nucleotides | <0.05      |
| −163 to −173   | 100% in 10 nucleotides  | <0.01      |
| −217 to −228   | 90.9% in 11 nucleotides | <0.01      |
| −285 to −298   | 92.9% in 14 nucleotides | <0.01      |
| −301 to −310   | 100% in 10 nucleotides  | <0.05      |
| −371 to −380   | 90% in 10 nucleotides   | <0.05      |
| −429 to −437   | 100% in 9 nucleotides   | <0.01      |
| −483 to −506   | 85.7% in 14 nucleotides | <0.01      |
| −532 to −539   | 100% in 8 nucleotides   | <0.01      |
| −543 to −562   | 85% in 20 nucleotides   | <0.01      |
| −603 to −614   | 91.7% in 12 nucleotides | <0.01      |
| −648 to −655   | 100% in 8 nucleotides   | <0.01      |
| −659 to −672   | 92.9% in 14 nucleotides | <0.01      |
| −677 to −693   | 88.2% in 17 nucleotides | <0.01      |

The data in Table I further support the notion that the Rbm3 5’ leader contains an IRES.
A Cold Stress-responsive IRES in 5′ Leader of Rbm3 mRNA

**FIG. 1.** Transfection analysis of the Rbm3 5′ leader in the 5′-UTR of a reporter mRNA. A schematic representation of the dicistronic constructs used in this analysis is indicated with the sequence components represented to scale. Constructs include the parent P(EMCV)/CAT vector and the Rbm3P/EMCV/CAT construct, which contains the Rbm3 5′ leader (gray bar). The EMCV IRES is indicated as a diagonal hatched bar. Constructs were transfected into C6 cells. Photinus luciferase and CAT activities were normalized for transfection efficiency (see "Experimental Procedures") and normalized to 1.0 for the activities of the control construct P(EMCV)/CAT. P/CAT is the ratio of the normalized Photinus luciferase and CAT activities. Numbers in parentheses represent S.E.

| Normalized Enzyme Activities |
|-------------------------------|
| P(EMCV)/CAT                        |
| Photinus       | CAT             | P/CAT        |
| Rbm3P/EMCV/CAT                  | 1.0 (0.05) | 1.0 (0.04)  | 1.0 |

**FIG. 2.** A, transfection analysis of the Rbm3 5′ leader sequence in dicistronic mRNAs. A schematic representation of the dicistronic constructs used in this analysis is indicated with the Rbm3 5′ leader sequence indicated as a gray bar. Constructs are based on the RP (Renilla-Photinus) vector. Luciferase activities from dicistronic constructs that lack (RP and Rbm3/RP) or contain (RP and Rbm3/RPh) the inverted repeat are shown after transfection into N2a cells. Renilla and Photinus luciferase activities and P/R ratios have been normalized to 1.0 for the activity of the RP construct. Numbers in parentheses represent S.E. B, Northern blot of total RNA purified from N2a cells transfected with the Rbm3/RPh and RP dicistronic constructs and probed with a riboprobe complementary to the Photinus luciferase coding sequence. The positions of the 28 and 18 S rRNAs are indicated.

**DISCUSSION**

In this study, we have demonstrated that a 5′ leader of the Rbm3 mRNA contains a potent IRES that was more active in some cell types when exposed to mild cold stress. Even at 37 °C, however, the Rbm3 IRES was relatively more active than the

c-myc, PV, and EMCV IRESes when tested in the same dicistronic system in these cells. Sequence analysis of the Rbm3 5′ leader revealed a number of uORFs, a feature that has also been noted in other 5′ leaders that contain IRESes (e.g., Refs. 31–33). At present, however, it is unknown if any of the uORFs in the Rbm3 leader are translated or if other mechanisms, in addition to internal initiation, such as leaky scanning, reinitiation, or shunting, affect the translation of the Rbm3 cistron. In addition to uORFs, the Rbm3 5′ leader contains numerous segments with complementarity to 18 S rRNA. In previous studies we showed that complementary sequence matches in other mRNAs could bind to 40 S ribosomal subunits by inter-
acting with the 18 S rRNA, and affect translation (19, 34, 35).

Complementary sequence matches to 18 S rRNA have also been identified in other cellular IRESes and in short nucleotide sequences that function as IRES modules (e.g. Refs. 17, 18, and 36–38). Indeed, complementarity to 18 S rRNA appears to be a defining feature of some IRESes (Refs. 17 and 18; data not shown). Thus far, several short nucleotide sequences have been identified within the Rbm3 IRES that facilitate or affect internal initiation.3 Whether any of these cis-acting sequences directly bind to 40 S ribosomal subunits by interacting with 18 S rRNA is the focus of ongoing investigations.

Internal initiation during cold stress resembles several other cellular situations in which IRESes seem to facilitate the translation of some mRNAs even though overall translation is reduced, as occurs during picornaviral infection, hypoxia, apoptosis, and the G2/M phase of the cell cycle (33, 39–42). During poliovirus infection, for example, cap-dependent translation is reduced; however, the poliovirus IRES and some cellular IRESes enable the production of particular proteins (33). Under conditions of mild cold stress, the activities of the Rbm3, c-myc, and PV IRESes were enhanced in some of the cell lines tested. In contrast, the relative efficiency of the EMCV IRES was not enhanced in any of these cell lines. This variation in the ability of individual IRESes to initiate translation under conditions of mild cold stress may reflect differences in the stability of higher order RNA structures at colder temperatures, which might affect ribosome recruitment or subsequent movement of the translation machinery to the initiation codon. Alternatively, variations in the efficiency of these IRESes during mild hypothermia in different cell types may reflect differential expression of trans-acting factors that affect these IRES activities.

There is evidence that the molecular changes that accompany mild hypothermia can protect cells from subsequent hypoxia-induced damage (1), an observation that has been exploited in the treatment of patients with certain types of brain injury (e.g. Ref. 43). The expression properties of Rbm3 and the closely related CIRP genes suggests that the Rbm3 and CIRP proteins may therefore contribute to the physiological changes that accompany mild hypothermia. This has been demonstrated for murine CIRP in studies showing that expression of this protein prolongs the G1 phase of the cell cycle and is responsible for slow cell growth during mild cold stress (8). It has also been suggested that CIRP and Rbm3 function as RNA chaperones and might facilitate translation at colder temperatures by binding to single-stranded RNA and disrupting secondary structures that become more stable at these temperatures. This raises the possibility that the efficiency of some IRESes during mild hypothermia may be affected by Rbm3, CIRP, or by other related protein(s).

The expression properties and possible protective role of the Rbm3 protein during exposure to cold stress may be similar to that of another stress-induced gene, that for the X-linked inhibitor of apoptosis, which after γ-irradiation or serum withdrawal is translated via an IRES, and which protects cells from apoptosis (40). Further studies are needed to investigate the

FIG. 4. Dicistronic analysis of the Rbm3 5′ leader in four cell lines. A schematic representation of the dicistronic constructs used in this analysis is indicated. Inserts include the Rbm3 5′ leader (gray bar), the EMCV and PV IRESes (diagonal hatched lines), and the β-globin 5′-UTR (black bar). Histograms represent the ratio of Photinus to Renilla luciferase activities (P:R) after transfection into N2a, NIH-3T3, C6, or SK cells. These ratios have been normalized to 1.0 for that of the RP construct. Horizontal bars represent S.E.

FIG. 5. The effects of mild cold stress on internal initiation of translation mediated by the Rbm3 and other IRESes. As in Fig. 4, constructs are schematically represented and luciferase activities were similarly determined after transfection into NIH-3T3 and C6 cells when cultured either at 33 °C or at 37 °C (see “Experimental Procedures”). P:R ratios were determined at 33 °C and at 37 °C and normalized to 1.0 for the activity of RP at each temperature. The relative changes in P:R ratios (33 °C: 37 °C) are represented in the histogram. Horizontal bars represent S.E.

FIG. 6. The effects of serum starvation on internal initiation of translation mediated by the Rbm3 and other IRESes. Constructs are schematically represented and luciferase activities determined as in Fig. 4. Transfected NIH-3T3 cells were cultured either in medium containing low (0.5%) or high serum (10%; see “Experimental Procedures”). P:R ratios were determined and normalized to 1.0 for the activity of RP from transfected cells grown under the same serum conditions and are represented as the relative changes in P:R ratios (low serum:high serum). Horizontal bars represent S.E.

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possible role of the Rbm3 protein during mild cold stress, to
determine if other mRNAs induced by mild hypothermia, such as
Cirp, also contain IRESes, and to understand how internal
initiation is differentially regulated under these conditions.

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