

# The leader region of Laminin B1 mRNA confers cap-independent translation

Michaela Petz<sup>1</sup>, Daniela Kozina<sup>2</sup>, Heidemarie Huber<sup>1</sup>, Tanja Siwiec<sup>1</sup>,  
Joachim Seipelt<sup>3</sup>, Wolfgang Sommergruber<sup>2</sup> and Wolfgang Mikulits<sup>1,\*</sup>

<sup>1</sup>Department of Medicine I, Division: Institute of Cancer Research, Medical University of Vienna, Borschke-Gasse 8a, A-1090 Vienna, <sup>2</sup>Boehringer Ingelheim Austria, Dr Boehringer Gasse 5-10, A-1120 Vienna and <sup>3</sup>Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Medical Biochemistry, Medical University of Vienna, Dr Bohr-Gasse 9, A-1030 Vienna, Austria

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## ABSTRACT

Translation initiation of eukaryotic mRNAs generally occurs by cap-dependent ribosome scanning. However, certain mRNAs contain internal ribosome entry sites (IRES) allowing cap-independent translation. Several of these IRES-competent transcripts and their corresponding proteins are involved in tumorigenesis. This study focused on IRES-driven translation control during the epithelial to mesenchymal transition (EMT) of hepatocytes that reflects crucial aspects of carcinoma progression. Expression profiling of EMT revealed Laminin B1 (Lamb1) to be translationally upregulated. The 5'-untranslated region (UTR) of Lamb1 was potent to direct IRES-dependent mRNA utilization of a bicistronic reporter construct. Stringent assays for cryptic promoter and splice sites showed no aberrantly expressed transcripts, suggesting that the reporter activity provided by the leader region of Lamb1 mRNA exclusively depends on IRES. In accordance, Lamb1 expression increased upon negative interference with cap-dependent translation by expression of human rhinovirus 2A protease or heat shock of cells. Finally, the enhanced expression of Lamb1 during EMT correlated with an elevated IRES activity. Together, these data provide first evidence that the 5'-UTR of Lamb1 contains a *bona fide* IRES that directs translational upregulation of Lamb1 during stress conditions and neoplastic progression of hepatocytes.

## INTRODUCTION

Ribosome scanning is the widely used mechanism of translation initiation in eukaryotes. The scanning

mechanism requires recognition of the cap structure at the 5' end of the mRNA by the cap-binding protein eIF4E, followed by recruitment of the activated 40S ribosomal subunit and subsequent downstream scanning along the 5'-untranslated region (UTR) to a suitable start codon (1). Nucleotide composition, length and secondary structures of the 5'-UTR determine the efficiency of translation initiation. Long, GC-rich and highly structured 5'-UTRs reduce migration of the 43S pre-initiation complex along the RNA (2). Alternatively, a structural motif of the 5'-UTR, referred to as internal ribosome entry site (IRES), allows ribosomes to directly bind upstream of the start codon and to initiate translation independently of the cap structure (3).

Initially described in picornavirus, the IRES-mediated translation initiation has been identified in cellular mRNAs (4,5). Escaping multiple mechanisms to regulate cap-dependent translation, internal initiation selectively allows sustained or enhanced translation of transcripts during situations with high need of corresponding proteins. Cellular IRES have been demonstrated to be active during apoptosis (6), angiogenesis (7), the G2/M phase of the cell cycle (8) or stress conditions such as hypoxia (9), amino acid starvation and heat shock (10). The observation that IRES elements are recognized in gene products regulating these processes suggests an important function of internal translational initiation in the posttranscriptional control of gene expression. IRES-driven translation further might have severe implications in tumorigenesis, since it affects the expression of platelet-derived growth factor (PDGF-B) or c-myc, which are involved in proliferation, and of apoptotic protease activating factor 1 (Apaf1) and vascular endothelial growth factor (VEGF), which govern programmed cell death and angiogenesis, respectively (11–14). Evidence for the relevance of IRES-mediated translation in cancer is particularly provided by the proto-oncogene c-myc. Although deregulated transcription of c-myc is frequently observed in many cancer types,

\*To whom correspondence should be addressed. Tel: +43 1 4277 65250; Fax: +43 1 4277 65239; Email: wolfgang.mikulits@meduniwien.ac.at

oncogenic gain-of-function can arise from changes in translation such as the C–T mutation in the IRES of c-myc that results in increased expression in cells derived from patients with multiple myeloma (15).

Ras and PI3K/AKT signalling pathways, which are frequently activated in tumours, play an important role in translational regulation and malignant transformation (16). For example, PI3K/AKT/mTOR regulates protein synthesis by activating the ribosomal protein S6 and translation initiation factors important for the ribosome recruitment to the mRNA (17). Overexpression of the eukaryotic initiation factors (eIF)4G and eIF4E has been demonstrated in various types of tumours which leads to the increased efficiency of cap-dependent translation (16,17). Elevated levels of rate-limiting eIF4E selectively enhance the translation of highly structured mRNAs which even contain IRES such as VEGF, ornithine decarboxylase (ODC) or fibroblast growth factor 2 (18).

The acquisition of invasive and metastatic properties of carcinoma cells is a frequent event in the late stage tumourigenesis. Loss of epithelial characteristics and the gain of a fibroblastoid phenotype during the progression in malignancy represent a phenomenon referred to as epithelial to mesenchymal transition (EMT) (19). A predominantly occurring molecular alteration in hepatocellular carcinoma is the overexpression of transforming growth factor (TGF)- $\beta$  that induces EMT in cooperation with active Ras (20,21). In this study, we performed expression profiling of hepatocellular EMT by employing total versus polysome-bound transcripts on DNA microarrays, and found increased translation of several components of the extracellular matrix (ECM) among them laminin B1 (LamB1). LamB1 is one of the three different  $\beta$ -subunits that form together with  $\alpha$ - and  $\gamma$ -chains over 14 heterotrimeric laminin isoforms with diverse functions (22). Integrin- and non-integrin-mediated laminin signalling activate several regulatory pathways that are involved in metastasis of cancer cells (23,24). Here, we demonstrate that the 5'-UTR of LamB1 directs translation of a bicistronic mRNA that is devoid of cryptic promoter or splice sites, indicating the presence of a functional IRES. This finding was corroborated by the persistent expression of LamB1 after cleavage of eIF4G by 2A protease or during heat shock. In addition, upregulated LamB1 was found to be associated with increased IRES activity upon hepatocellular EMT, demonstrating the involvement of cap-independent translation of LamB1 transcripts.

## MATERIALS AND METHODS

### Construction of the plasmids

The LamB1 5'-UTR was amplified by employing a cDNA library of human HeLa cells. Primers were used according to the GenBank sequence NM\_002291. Amplification products were cloned into pGem-Easy and transformed into *E. coli* JM109. The human LamB1 5'-UTR contained a short intron that was present after the cloning procedure, probably due to incomplete splicing. To obtain the correct intronless 5'-UTR, the sequence was

stepwise amplified. For construction of bicistronic plasmids, Firefly luciferase was inserted into pIRES (Promega, Madison, USA), resulting in pEMCV-F, followed by insertion of Renilla luciferase resulting in pR-EMCV-F. Bicistronic pR-Lam-F was constructed by replacing the EMCV sequence of pR-EMCV-F with the amplified LamB1-5'UTR. A bicistronic control plasmid pR-F was obtained by deletion of the LamB1 5'-UTR sequence from pR-Lam-F. Monocistronic plasmids harbouring either the EMCV or the LamB1-5'-UTR upstream of Firefly luciferase were constructed by removal of the Renilla luciferase sequence from pR-Lam-F or pR-EMCV-F, resulting in pLam-F or pEMCV-F, respectively. The monocistronic control plasmid pF exclusively containing Firefly luciferase was constructed by excision of EMCV from pEMCV-F. Plasmids for the cryptic promoter assay were constructed by inserting Firefly luciferase into pGEM-3Zf(-) (Promega, Madison, USA), resulting in pGEM-F. Cloning of the LamB1 5'-UTR into pGEM-F led to pGEM-Lam-F. Vectors expressing 2A protease of human rhinovirus serotype 2 were generated by an in-frame fusion of N-terminal-enhanced green fluorescent protein (GFP) with either wild-type 2A protease cDNA (p2Awt) or the inactive mutant C106A (p2Amut) in pCIneo (Promega).

### Cell culture

Immortalized murine MIM-1-4 hepatocytes were grown on collagen-coated culture dishes in RPMI 1640 plus 10% foetal calf serum (FCS), 40 ng/ml human TGF- $\alpha$  (Sigma, St. Louis, USA), 30 ng/ml human insulin-like growth factor II (IGF-II, Sigma, St. Louis, USA), 1.4 nM insulin (Sigma, St. Louis, USA) and antibiotics, as described previously (25). Malignant epithelial MIM-R hepatocytes were generated by stable retroviral transmission of MIM-1-4 cells with oncogenic v-Ha-Ras and GFP as outlined recently (26). Human SW480 colon carcinoma cells were cultured in DMEM and 10% FCS. All cells were kept at 37°C and 5% CO<sub>2</sub> and routinely screened for the absence of mycoplasma. Human TGF- $\beta$  1 (R&D Systems, Minneapolis, USA) was used at a concentration of 2.5 ng/ml for the first 72 h of EMT induction. For long-term treatment of MIM-R hepatocytes, TGF- $\beta$  1 was supplemented to the medium at a concentration of 1 ng/ml, resulting in fibroblastoid MIM-RT cells after cytokine administration for >2 weeks.

### Heat shock

Medium supplemented with 10 mM HEPES was used for heat shock experiments. Cells were incubated for 8 min at 44°C in a water bath and subsequently for 2 min at room temperature (27).

### RNA isolation and DNA microarray analysis

Epithelial MIM-R hepatocytes and metastatic fibroblastoid MIM-RT cells were used for expression profiling. Total RNA of each cell population was isolated using RNeasy as recommended by the manufacturer (Quiagen, Hilden, Germany). Polysome-associated mRNA populations were isolated through fractionation of cytoplasmic

extracts in sucrose gradients as described (28). Briefly, cellular extracts were prepared and supplemented with 20 mM dithiothreitol, 150 µg/ml cycloheximide, 665 µg/ml heparin and 1 mM phenylmethylsulphonyl fluoride after removal of nuclei and mitochondria. The resulting supernatants were layered onto 10 ml linear sucrose gradients [15–40% sucrose (w/v)] and centrifuged in a SW41Ti rotor (Beckman, Palo Alto, USA) at 38 000 r.p.m. for 120 min at 4°C. The RNA extracted from 19 harvested fractions were subsequently analyzed for integrity and association with (poly)ribosomes. Poly(A)<sup>+</sup> mRNA from total RNA preparations and those pooled from fractions 12–19 corresponding to polysome-associated RNA was isolated using oligo-dT beads (Quiagen, Hilden, Germany). Each cRNA preparation for hybridization was performed with 5 µg RNA according to the protocol provided by manufacturer (Affymetrix, Santa Clara, USA). RNA labelled by incorporation of biotinylated CTP and UTP was purified with RNeasy columns (Qiagen, Hilden, Germany) and quantified by a spectrophotometer. Affymetrix Mu11K GeneChips consisting of two individual subarrays (subA and subB), in total containing roughly 11 000 probe sets of oligonucleotides, were subsequently hybridized at 45°C for 16 h. Following washes, probe arrays were scanned three times at 6-µm resolution using the GeneChip confocal scanner (Hewlett-Packard, Waldbronn, Germany). GeneChip 3.0 (Affymetrix, Santa Clara, USA) was used to quantify the scanned image. To correct for minor differences in overall chip fluorescence, intensity values were scaled to a level that the overall fluorescence intensity of each chip of the same type was equivalent. Differentially translated mRNAs were considered as significantly induced or repressed by ≥2-fold or ≤0.5-fold regulation of polysome-bound RNA, respectively, and a ≤2-fold or ≥0.5-fold variation of total RNA.

#### Reverse transcriptase polymerase chain reaction (RT-PCR)

Poly(A)<sup>+</sup> mRNA was extracted and reverse transcribed using an mRNA isolation and first-strand cDNA synthesis kit (Roche, Mannheim, Germany). Aliquots of the resulting products were employed as templates for specific PCR amplifications using Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The conditions for PCR reaction were optimized for each primer pair. The following forward and reverse primers were used for specific amplifications: mouse LamB1 5'-ATGAAGCGGAGGAAGCCAAC-3' and 5'-TCACAC TGCCGAGCATAAC-3'; mouse RhoA 5'-GTGGAAT TCGCCTTGCATCTGAGAAGT-3' and 5'-CACGAA TTCAATTAACGCATGAGGCT-3'; pR-Lam-F and pR-F plasmid 5'-GCTAACGCAGTCAGTGCTTC-3' and 5'-CTCACGCAGGCAGTTCTATG-3'. The amplification products were subsequently analyzed by electrophoresis on 1% agarose gels and staining with ethidium bromide. Signals were quantified with ImageQuant 5.0 (Amersham Biosciences, Little Chalfont, UK).

#### Real-time PCR

PCR reactions were performed with Taq Man according to the recommendations of the manufacturer (Invitrogen Corporation, CA, USA), and quantified with the ABI prism 7700 sequence detection system and the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The following probes, forward and reverse primer sequences were used: mouse RhoA: 5'-FAM-ACCTGAAGAAGGCAGAGATATGGCAA-TAMRA-3', 5'-AATGAAGCAGGAGCCGGTAA-3' and 5'-CCCAAAGCGCCAATCC-3'; mouse LamB1: 5'-FAM-ATATCCAAGGAACCCAAAACCTGCTAAC-TAMRA-3', 5'-AGGCGATTAACAAGCTGATGAG-3' and 5'-AAGCTGCCGTTTCAGATTCAA-3'; Renilla luciferase: 5'-FAM-TCAAGATAAGATCAAAGCAAT AGTTCACGCTGAAAGT-TAMRA-3', 5'-GCTTGTTT GGCATTTTCATTATAGCT-3' and 5'-TTCACGAGG CCATGATAATGTT-3'; Firefly luciferase: 5'-FAM-AAACGGATTACCAGGGATTTTCAGTCGATGTAC-TAMRA-3', 5'-GCAAAAAAAGCTCCCAATCATC-3' and 5'-AAGGACTCTGGCACAAAATCGT-3'.

#### Western blot analysis

The preparation of cellular extracts, separation of proteins by SDS-polyacrylamide gel electrophoresis and immunoblotting were carried out as described recently (20). The protein extract from  $1 \times 10^5$  cells per sample was loaded onto gels and immunological detection of proteins was performed with the SuperSignal detection system (Pierce Chemical Company, Rockford, USA). The following primary antibodies were used: anti-LamB1 (Santa Cruz Biotechnology, California, USA), 1:1000; anti-LamB1 (Neo Markers, Fremont, USA), 1:1000; anti-actin (Sigma, St. Louis, USA), 1:1000; anti-hsp70 (Neo Markers, Fremont; USA), 1:1000; anti-eIF4G (29), 1:5000. Secondary antibodies (Calbiochem, LaJolla, USA) were used at dilutions of 1:10 000. Signals on the autoradiographs were scanned and quantified with ImageQuant 5.0 (Amersham Biosciences, Little Chalfont, UK).

#### Transient transfections and luciferase reporter assays

Cells were plated at a density of  $2.5 \times 10^4$  cells per 24-well plate or  $1 \times 10^5$  cells per 6-well plate 1 day before transfection. Lipofectamine Plus was used for transient transfections as recommended by the manufacturer (Invitrogen, Carlsbad, USA). Relative Luciferase activity of mono- or bicistronic plasmids was determined by co-transfection of 0.4 µg plasmid and 0.1 µg β-galactosidase reporter (30) per 24-well or 1 µg plasmid and 0.25 µg β-galactosidase reporter per 6-well. For assays to interfere with cap-dependent translation, vectors of wild type (p2Awt) or mutated 2A protease (p2Amut) were co-transfected with bicistronic plasmid with amounts as described above. Cells were lysed 48 h post-transfection and the luciferase activity was determined using a Luminoskan (Labsystems, Farnborough, UK) as previously described (31). Light emission was measured for 3 s after addition of each luciferase substrates and

integrated over an interval of 10 s. Assays were performed in duplicate and results represent the averages of two independent experiments after normalization to  $\beta$ -galactosidase activities. In case of heat shock and ectopic 2A protease expression, Firefly luciferase activity was normalized to the RNA level after reverse transcription and quantitation of cDNA. Renilla and Firefly luciferase activities of bicistronic plasmids were determined using a Dual-Luciferase<sup>®</sup> Reporter Assay System according to the protocol of the manufacturer (Promega, Madison, USA). The expression ratio of the two reporter genes was normalized to the empty control reporter (pRF).

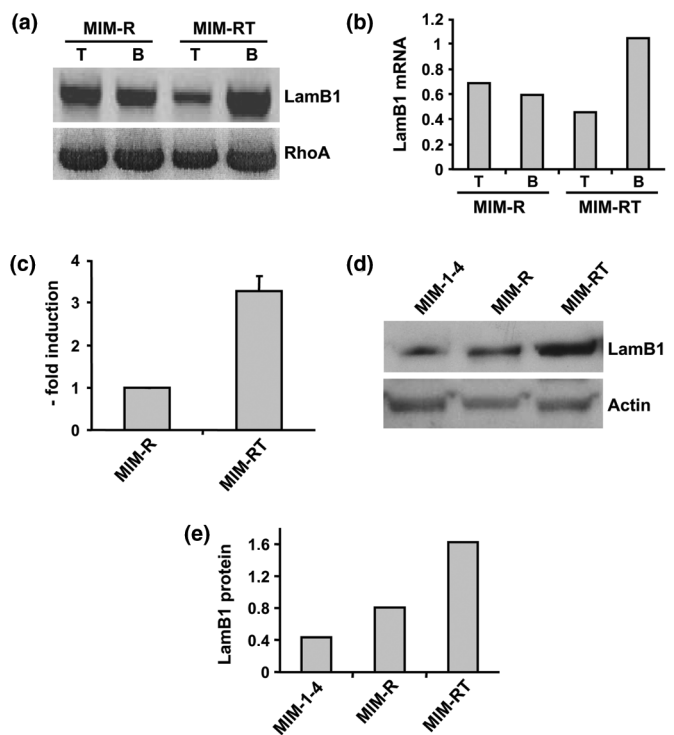
## RESULTS

### Identification of candidate mRNAs for differential translation upon hepatocellular EMT

We previously described a model of EMT that depends on the synergism of active Ras and TGF- $\beta$  in hepatocytes, and faithfully reflects several aspects of tumour progression (25). TGF- $\beta$  exerts its tumour progressive role by endowing neoplastic Ras-expressing hepatocytes (MIM-R) with metastatic fibroblastoid features (MIM-RT) (20). We performed expression profiling of epithelial MIM-R and MIM-RT derivatives by employing total versus polysome-bound mRNAs in order to gain insight into changes of translation control upon hepatocellular EMT (28). Differential hybridization revealed 128 up- and 84 translationally downregulated mRNAs (data not shown). To identify putative candidates of IRES-mediated translation, transcripts with 5'-UTRs longer than 200 nt were selected. Notably, the list of candidate mRNAs corresponds to genes involved in remodulation of the ECM during EMT including LamB1. The translational upregulation of LamB1 upon EMT was confirmed by semiquantitative RT-PCR of total versus polysome-bound RNA isolated from MIM-R and MIM-RT cells (Figure 1a). Total LamB1 mRNA showed a variation of 1.5-fold in MIM-R versus MIM-RT cells whereas polysome-bound LamB1 mRNA was 2-fold upregulated (Figure 1b). Quantitative real-time PCR (QRT-PCR) revealed a 3.5-fold increase by calculating expression levels of total and bound RNA normalized to the constitutive expression of RhoA (Figure 1c). The upregulation of LamB1 at the polypeptide level was additionally confirmed by western blot analysis (Figure 1d and e). Together, this experimental approach allowed to identify an overall 3–4-fold translational upregulation of Lam B1 expression during hepatocellular EMT.

### The 5'-UTR of human LamB1 enhances the activity of a monocistronic reporter gene and directs translation from a bicistronic mRNA

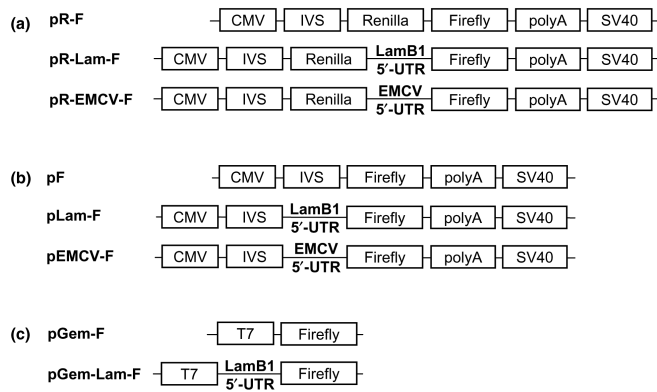
The LamB1 5'-UTR consists of 335 nt with a GC content of 68%. Analysis of the LamB1 5'-UTR based on Zukers algorithm predicted a highly stable secondary structure with a minimal free energy of  $-154$  kcal/mol (data not shown) (32,33). The secondary structure of the 5'-UTR would negatively affect translation dependent on ribosome



**Figure 1.** Translational regulation of LamB1 upon hepatocellular EMT. (a) Semiquantitative RT-PCR of LamB1 using total (T) and polysome-bound mRNA populations (B) of MIM-R and MIM-RT cells. The constitutive expression of RhoA is shown as control. (b) Quantitation of LamB1 mRNA shown in Figure 1a after normalization to the level of RhoA expression. (c) Quantitative real-time PCR of total and polysome-associated RNA fractions as used for conventional RT-PCR. Comparative expression levels of bound to total RNA were normalized to RhoA levels. LamB1 upregulation in MIM-RT cells was calculated relative to MIM-R hepatocytes. The mRNA expression of MIM-R was set to the level of 1. (d) Western blot analysis of LamB1 in parental MIM-1-4, neoplastic MIM-R and fibroblastoid MIM-RT cells. (e) Quantitation of LamB1 protein expression after normalization to the level of constitutive actin.

scanning of a heterologous reporter gene. In order to examine the effect of the LamB1 5'-UTR on translation, we generated a construct with the 5'-UTR arranged upstream of the Firefly luciferase reporter gene that is driven by a CMV promoter flanked by a short intron sequence (IVS; Figure 2b). Plasmids with (pLam-F) and without (pF) the LamB1 5'-UTR were transfected into MIM-R hepatocytes and assayed for the relative Firefly luciferase activity. As shown in Figure 3a, a strongly enhanced reporter activity mediated by the LamB1-5'-UTR was monitored, suggesting the presence of an initiation mechanism alternative to ribosomal scanning. This high reporter activity of LamB1 5'-UTR compared to EMCV might be affected by the Ras hyperactivation in MIM-R hepatocytes.

The bicistronic assay has been widely used to detect the activity of sequence elements involved in IRES-mediated translation. While the upstream Renilla luciferase reporter is translated via cap-dependent scanning, the downstream Firefly luciferase reporter is translated dependent on the upstream 5'-UTR containing an IRES (pR-LamF, Figure 2a). A similar bicistronic vector carrying

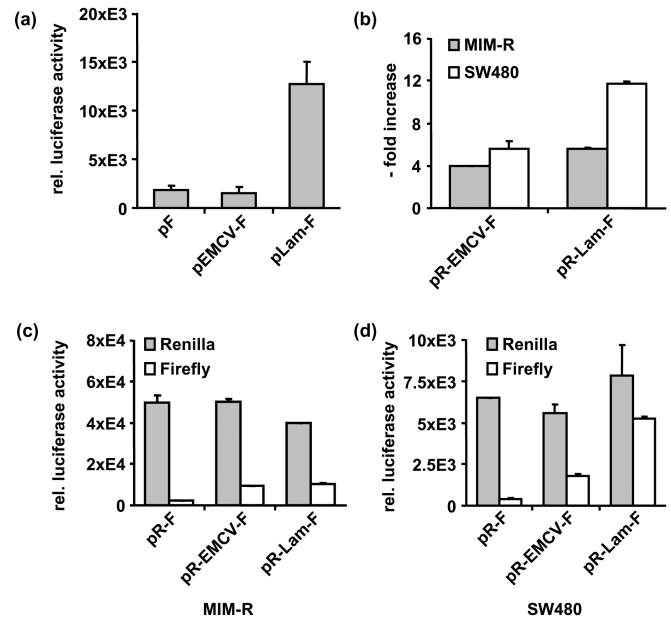


**Figure 2.** Schematic diagram of vectors employed in this study. (a) The bicistronic vectors contain an EMCV or LamB1 5'-UTR in the linker region between the Renilla and Firefly luciferase reporter. (b) Similarly constructed monocistronic vectors are devoid of the upstream Renilla luciferase. (c) The Firefly luciferase reporter in the presence or absence of the upstream LamB1 5'-UTR and downstream of a bacteriophage-derived T7 promoter was employed for the cryptic promoter assay.

the encephalomyocarditis virus (EMCV) 5'-UTR provided a positive control for IRES-mediated translation (pR-EMCV-F), and an empty bicistronic plasmid was used as a negative control (pR-F) (Figure 2a). These vectors were each transfected into MIM-R hepatocytes and into human SW480 colon carcinoma cells, and the ratios of Firefly:Renilla luciferase activities were evaluated. Figure 3b shows a 6-fold upregulation of the pR-Lam-F plasmid compared to the empty control pR-F in MIM-R cells, and a 12-fold induction in SW480 cells (Figure 3b). These data were further analyzed by individual evaluation of Renilla and Firefly luciferase activity. In both MIM-R and SW480 cells, cap-dependent luciferase activities of Renilla cistrons of pR-F, pR-EMCV-F or pR-LamF revealed no significant variations (Figure 3c and d). Yet, comparison of cap-independent luciferase activities of Firefly cistrons of pR-F versus pR-LamF showed an increase in MIM-R hepatocytes (Figure 3c) which was even stronger in SW480 carcinoma cells (Figure 3d). In conclusion, these data suggest IRES-driven translation of LamB1 in both mouse and human carcinoma cells.

### The human LamB1 5'UTR contains no cryptic promoter or splice sites

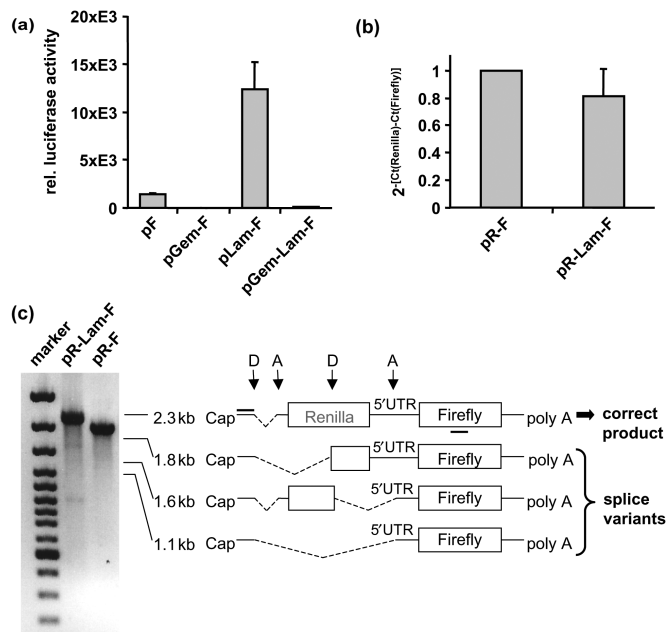
The bicistronic assay for IRES activity has been subjected to the criticism that the downstream cistron activity could arise from the presence of cryptic promoter or splice sites rather than from a *bona fide* IRES (34). In order to reliably analyze cryptic promoters, we constructed a plasmid containing a bacteriophage-derived T7 promoter in the presence or absence of the LamB1 5'-UTR upstream of Firefly luciferase, termed pGem-Lam-F and pGem-F, respectively (Figure 2c). These vectors were each transfected into MIM-R hepatocytes and analyzed for Firefly luciferase activity. Since the T7 promoter is not recognized by eukaryotic transcriptional machinery, the cDNA insert



**Figure 3.** Mono- and bicistronic reporter assays to detect IRES activity. (a) Firefly luciferase assay of MIM-R hepatocytes, transfected either with monocistronic pF, pEMCV-F or pLam-F. Cells were lysed 48 h post-transfection and the Firefly luciferase activity was normalized to  $\beta$ -galactosidase levels. (b) Dual luciferase assay of MIM-R hepatocytes and human SW480 cells transfected with bicistronic plasmids. Each cell type either expressed pR-EMCV-F or pR-Lam-F. Cells were lysed 48 h after transfection and Firefly:Renilla ratios were calculated relative to the value obtained with empty plasmid (pR-F) after normalization to  $\beta$ -galactosidase. (c) Renilla and Firefly luciferase activity each on its own of pR-F, pR-EMCV-F and pR-Lam-F in MIM-R hepatocytes and (d) in SW480 cells.

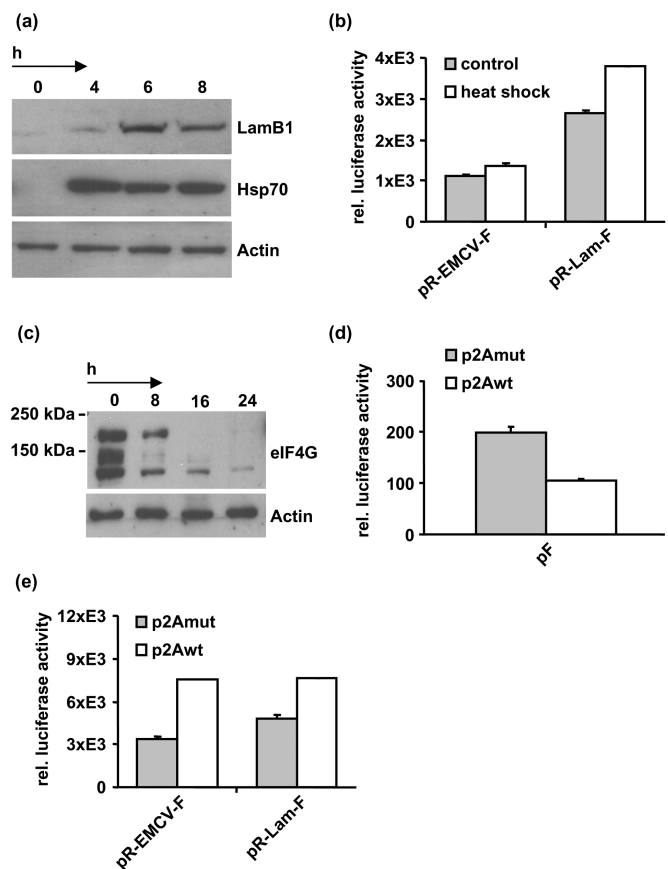
would not be transcribed unless a cryptic promoter is present. As shown in Figure 4a, luciferase failed to display activity, indicating that no cryptic promoter sites are located in the LamB1 5'-UTR that direct aberrant transcription and subsequent cap-dependent translation.

In addition, we found a putative acceptor splice site in the LamB1 5'-UTR and two possible donor splice sites in Renilla luciferase upon screening with NetGene2, as shown in Figure 4c (35,36). A similar combination of a Renilla donor splice site and 5'-UTR acceptor splice site was recently shown to be capable of removing the majority of the Renilla open reading frame (ORF) leading to reduced Renilla activity that was misinterpreted as IRES activity of X-linked inhibitor of apoptosis (XIAP) (37). To test the presence of splice sites within the LamB1 5'-UTR, total RNA from MIM-R cells transfected with the bicistronic pR-Lam-F plasmid or the empty bicistronic pR-F vector was analyzed by RT-PCR. QRT-PCR was employed to determine the ratio of Renilla and Firefly luciferase cistrons, as reported recently (38). QRT-PCR analysis revealed that the ratios of Renilla and Firefly luciferase cistrons were comparable between pR-F and pR-Lam-F by showing a minor decrease, respectively (Figure 4b). These results suggest that splicing products derived from the Renilla donor and the LamB1 5'-UTR acceptor splice are hardly detected. In order to confirm these data, primers were designed for the amplification of



**Figure 4.** Detection of putative cryptic promoter and splice sites located in the 5'-UTR of LamB1. **(a)** The cryptic promoter assay was performed with a vector harbouring a T7 promoter that is inactive in eukaryotic cells. Firefly luciferase activity was measured in MIM-R cells either transfected with pGem-Lam-F, pGem-F or the monocistronic vectors pLam-F and pF. Luciferase activities determined 48 h post-transfection were normalized to  $\beta$ -galactosidase activities. **(b)** Quantitative RT-PCR to detect cryptic splice sites in the Lam B1 5'-UTR. The bicistronic pR-Lam-F vector as well as the empty bicistronic pR-F plasmid were each transfected into MIM-R cells. Total RNA was prepared 48 h post-transfection and quantitative RT-PCR was performed to analyze ratios of Renilla and Firefly luciferase cistrons. The Renilla luciferase:Firefly luciferase ratio was calculated as  $2^{-[Ct(Renilla)-Ct(Firefly)]}$ . The Renilla:Firefly luciferase ratio of pR-F was set to the level of 1. **(c)** Semiquantitative RT-PCR to detect cryptic splice sites in the Lam B1 5'-UTR. Bicistronic pR-Lam-F and pR-F plasmids were each transfected and total RNA was prepared as in Figure 4b. Schematic drawings of pR-Lam-F are included that represent possible aberrant splice products. The arrowheads indicate putative donor (D) and acceptor (A) splice sites that are based on *in silico* analysis of the LamB1 5'-UTR, a short intervening sequence of the vector and a recently found splice site in the Renilla luciferase. The thick bars at the 5' end close to the cap and in the Firefly luciferase indicate the position of the forward and reverse primers.

the bicistronic construct starting directly at the transcriptional start site and terminating within the Firefly luciferase ORF (position +633). As outlined in Figure 4c, the resulting PCR product includes (i) the short intervening sequence located in the vector downstream of the CMV promoter, (ii) the ORF of Renilla luciferase, (iii) the LamB1 5'-UTR and (iv) the 5' part of the Firefly luciferase ORF. Thus, this PCR is convenient to detect all possible splice variants. RT-PCR resulted in one amplification product corresponding to the correctly spliced transcript of pR-Lam-F with the intervening sequence being the only intron, and importantly, no cryptic splice variants could be detected. From these data, we concluded that the activity of the LamB1 5'-UTR of the bicistronic vector was indeed mediated by the IRES activity of LamB1 rather than generated by aberrantly expressed transcripts.



**Figure 5.** LamB1 translation after heat shock or intervention with ribosome scanning through 2A protease-dependent cleavage of eIF4G. **(a)** LamB1 expression in MIM-R cells 4, 6 and 8 h after heat shock as detected by western blotting. **(b)** Firefly luciferase assay of MIM-R hepatocytes transfected either with pR-EMCV-F or pR-Lam-F bicistronic plasmids. Cells were exposed to heat shock 12 h post-transfection. 48 hours after transfection, Firefly luciferase activity was determined and normalized to the RNA level after reverse transcription and quantitation of cDNA. **(c)** Western blot analysis of MIM-R cells showing the cleavage of eIF4G1/II. MIM-R cells were transfected with wild-type 2A protease expressing plasmid (p2Awt) and lysed at the indicated times. **(d)** Firefly luciferase assay of MIM-R hepatocytes co-transfected with pR-F and either p2Awt or p2Amut. **(e)** Firefly luciferase assay of MIM-R cells co-transfected either with pR-EMCV-F or pR-Lam-F and p2Awt or p2Amut, respectively. Cells were lysed 48 h post-transfection and the Firefly luciferase activity was normalized to the RNA level after reverse transcription and quantitation of cDNA.

### IRES-mediated translation of LamB1 mRNA increases during heat shock

Heat shock induces cellular stress and concomitantly impairs cap-dependent translation (39). Internal ribosomal initiation allows to maintain or enhance translation of cellular mRNAs during situations of transient stress such as heat shock. Thus, we analyzed the impact of heat shock on IRES-mediated translation of LamB1 by introducing bicistronic pR-F, pR-EMCV-F or pR-Lam-F plasmid into MIM-R cells (Figure 2a). As expected, we detected increased IRES-mediated translation of LamB1 (Figure 5b). Heat shock was verified by the induced expression of heat shock protein 70 (hsp70). In line with

these data, western blot analysis of heat shock kinetics indicated an increased level of endogenous murine LamB1 (Figure 5a).

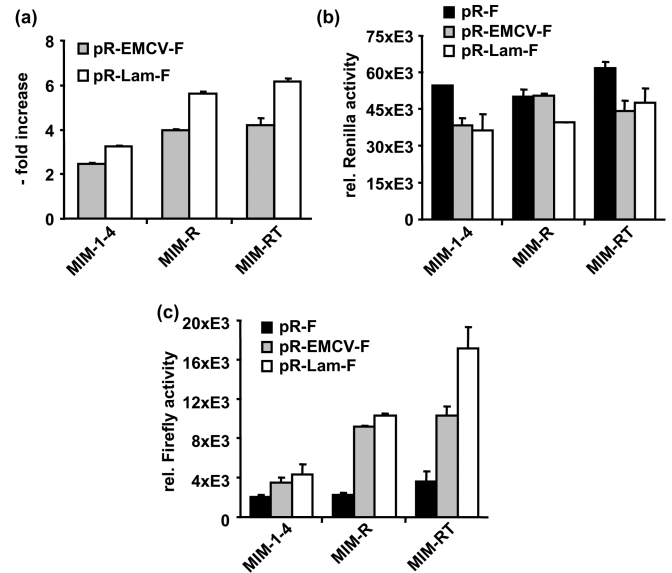
### Human LamB1 5'-UTR-mediated translation sustains after negative interference with ribosome scanning by 2A protease expression

Picornavirus is known to shut off cap-dependent translation of infected host cells through 2A protease-mediated cleavage of eIF4G, a scaffolding protein that bridges the cap-binding protein eIF4E with the ribosomal subunit (40). Thus, the first cellular IRES element in immunoglobulin heavy-chain-binding protein (BiP) was identified due to its continuous activity in poliovirus-infected cells (41). In order to study 2A protease-mediated inhibition of cap-dependent translation on the expression of LamB1, we co-transfected vectors expressing wild-type 2A protease (p2Awt) or an inactive mutant C106Ala (p2Amut) with bicistronic ECV (pR-EMCV-F) or LamB1 (pR-Lam-F) into MIM-R cells for determination of the relative Firefly luciferase activity. The activity of 2A protease was verified by western blotting of p2Awt transfected MIM-R cells showing that eIF4G was effectively cleaved after 16 h (Figure 5c) (42–44). As expected, cap-dependent translation of p-F was reduced (Figure 5d), while pEMCV-F and pLam-F showed elevated levels of luciferase activity (Figure 5e). Thus, these data indicate that the human LamB1 5'-UTR confers translation independently of intact eIF4G.

### IRES-mediated translation of LamB1 is upregulated in hepatocellular EMT

As analyzed by western blotting of parental MIM1-4, malignant MIM-R and metastatic MIM-RT cells, LamB1 expression increased ~2-fold in malignant MIM-R cells compared to normal MIM1-4 cells which was further 2-fold elevated in metastatic MIM-RT cells (Figure 1d and e). We subsequently addressed the question on the impact of IRES-mediated translation of LamB1 expression during EMT. The bicistronic vectors pR-F, pR-EMCV-F, pR-Lam-F were transfected into MIM1-4, MIM-R and MIM-RT cells, and luciferase activities were analyzed. Similar to the increase of protein expression (Figure 1d and e), the luciferase activities were elevated in Ras-transformed MIM-R cells as compared to MIM1-4 cells, and the highest rates of luciferase activity could be detected in MIM-RT cells by the synergy of Ras and TGF- $\beta$  (Figure 6a). Individual assessment of cap-dependent luciferase activity of Renilla cistrons revealed no significant variations (Figure 6b) whereas cap-independent luciferase activity of Firefly cistrons showed a strong increase in MIM-1-4 versus MIM-RT cells (Figure 6c). These results indicate that internal ribosome initiation significantly contributes to the activation of LamB1 protein expression upon EMT.

Next, we examined the involvement of IRES-mediated regulation on LamB1 translation in the induction phase of EMT. Bicistronic pR-F, pR-EMCV-F or pR-Lam-F plasmids were transfected into MIM-R hepatocytes, and EMT was induced by administration of TGF- $\beta$  1 for 24 h.

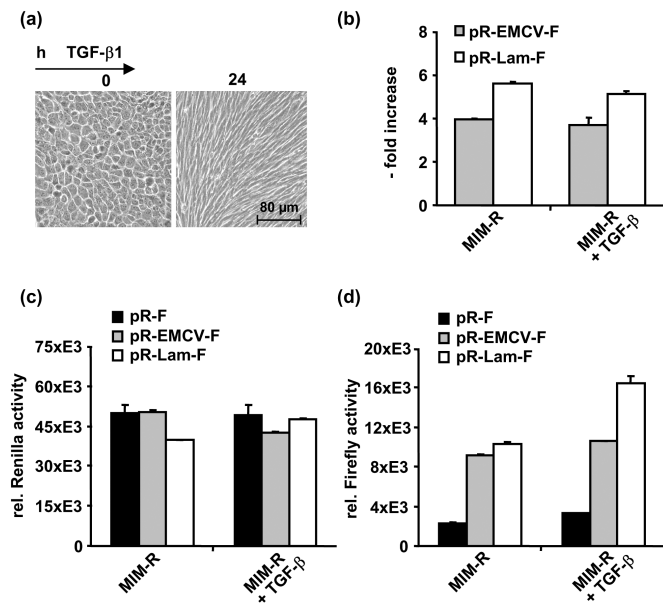


**Figure 6.** Cap-independent expression of LamB1 during hepatocellular EMT. (a) Dual luciferase assay of MIM-1-4, MIM-R and MIM-RT cells transfected with bicistronic plasmids pR-F, pR-EMCV-F or pR-Lam-F. Cells were lysed 48 h after transfection and Firefly:Renilla ratios were calculated relative to empty plasmid (pR-F) after normalization to  $\beta$ -galactosidase. (b) Renilla and (c) Firefly luciferase activity each on its own of pR-F, pR-EMCV-F and pR-Lam-F.

Noteworthy, MIM-R cells synchronously undergo EMT and complete morphological alterations 24 h post-TGF- $\beta$  treatment (Figure 7a) (45). This approach revealed an increased ratio of luciferase activity in TGF- $\beta$ -treated cells, suggesting an IRES activity in LamB1 transcripts during the early phase of EMT (Figure 7b). Individual assessment of cap-dependent luciferase activity of Renilla cistrons revealed no significant variations (Figure 7c), whereas cap-independent luciferase activity of Firefly cistrons showed a strong increase in MIM-R hepatocytes versus those induced to EMT (Figure 7d).

## DISCUSSION

As a subunit of the heterotrimeric Laminin, LamB1 is involved in ECM–cell interactions which influences multiple cellular processes such as cell adhesion, migration, proliferation and differentiation (23). Interaction of malignant cancer cells with Laminins is considered as a key event in tumour progression (24). Neoplastic epithelial cells frequently display aberrantly expressed Laminin receptors which directly correlate with enhanced invasiveness. Laminin signalling is mediated by integrin and non-integrin receptors which activate central pathways such as MAPK or PI3K. In human hepatocellular carcinoma, the differential expression of the  $\alpha$ 6  $\beta$ 1 and 67-kDa receptor results in FAK tyrosine phosphorylation, leading to FAK-GRB2 association and ERK cascade activation which promotes cell migration (46,47). The expression of LamB1 has been described to be tightly regulated at the transcriptional level by retinoic acid, but little is known about the translational regulation (48). In this study,



**Figure 7.** Cap-independent expression of LamB1 during induction of EMT. (a) Phase contrast microscopy of polarized MIM-R hepatocytes, which synchronously establish the fibroblastoid morphology within 24 h of TGF- $\beta$ 1 treatment (2.5 ng/ml). (b) Dual luciferase assay of MIM-R cells transfected with the bicistronic plasmids pR-F, pR-EMCV-F or pR-Lam-F. Cells were untreated or treated with 2.5 ng/ml TGF- $\beta$  1 for 24 h prior to lysis (48 h post-transfection). Firefly:Renilla ratios were calculated relative to empty control (pR-F) after normalization to  $\beta$ -galactosidase. (c) Renilla and (d) Firefly luciferase activity each on its own of pR-F, pR-EMCV-F and pR-Lam-F. Cells were untreated or treated with 2.5 ng/ml TGF- $\beta$  1 for 24 h prior to lysis (48 h post-transfection).

we present first evidence for a cap-independent translation initiation of LamB1 that allows an immediate response to changes under (patho)physiological conditions.

Experimental evidence for an initiation mechanism of LamB1 that is alternative to cap-dependent translation has been provided by the finding that the 5'-UTR located upstream of a heterologous reporter gene led to increased expression despite the presence of the strong secondary structure with a free energy of  $-154$  kcal/mol. This stem-loop motif is likely to decrease the efficiency of cap-dependent translation as it has been shown for secondary structures with a free energy value up to  $-50$  kcal/mol (49,50). Therefore, we employed a bicistronic assay revealing that the LamB1 5'-UTR is capable to direct translation in murine and human cells. Recently, this frequently used assay has been challenged by the possibility that cells transfected with bicistronic constructs may give rise to transcripts that are aberrantly processed either by the presence of a cryptic promoter or by RNA splicing which could be misinterpreted as IRES activity (34). Since commonly used methods for the detection of aberrant transcripts such as northern blotting have been criticized for being too insensitive, we relied on RT-PCR for the detection of cryptic splice variants. Our results indicated neither the presence of a cryptic promoter nor splice sites, suggesting an activity of the bicistronic assay mediated by IRES rather than by aberrantly expressed transcripts (Figure 4).

The biological significance of internal initiation implicates selective translation of cellular mRNAs in situation of transient cellular stress when cap-dependent translation is impaired. In accordance with these findings, we observed an increased IRES activity of LamB1 in heat shocked cells. Moreover proteolysis of eIF4G by 2A protease also resulted in elevated IRES activity. These observations underline the independence of LamB1 protein synthesis on cap-mediated translation (Figure 5).

Several IRES elements have been identified in mRNAs corresponding to key regulatory proteins which contribute to tumourigenesis such as survival factors (Bag-1) (10), growth factors (PDGF-B, c-myc) (11,12), cell cycle control (ODC) or angiogenesis (VEGF) (8,14). A crucial and frequent event in late stage carcinogenesis is the breakdown of epithelial polarity and homeostasis. This phenomenon, referred to as EMT is characterized by loss of epithelial organization and gain of a mesenchymal, invasive phenotype (19). We observed the translational upregulation of LamB1 in EMT that depends on the collaboration of active Ras and TGF- $\beta$  in hepatocytes, a model closely mimicking frequently occurring molecular alternations in hepatocellular carcinoma (20). TGF- $\beta$  signalling cooperates with Ras through activation of downstream effectors such as the ERK/MAPK and PI3K/AKT pathway (51,52). It is noteworthy, both ERK/MAPK and PI3K/AKT pathways regulate cap-dependent translation by activation of eIF4E (53). Overexpression of eIF4E has been described in a wide range of tumours and predominantly increases cap-dependent translation of IRES-competent transcripts which is usually reduced due to the long structured leader regions of mRNAs (18). In our experimental approach, the expression of LamB1 was elevated in Ras-transformed hepatocytes and further increased upon TGF- $\beta$ -induced EMT (Figure 1). Enhanced cap-dependent translation induced by Ras signalling provides a possible mechanism for the translational upregulation of LamB1 that still remains to be investigated. Most notably, however, the upregulation of LamB1 correlates with elevated IRES activity as observed in bicistronic assays. The IRES activation points to cellular stress which arises from TGF- $\beta$  signaling upon the induction EMT. Most likely, cellular stress is provided by TGF- $\beta$ -mediated induction of cell cycle arrest and apoptosis that is prevented by Ras-induced ERK/MAPK signalling (26). Our recent data revealed that hepatocellular EMT consists of an induction phase in which MAPK/ERK signaling establishes the resistance against TGF- $\beta$ -mediated effects, and a maintenance phase in which a concomitant PI3K/AKT signalling is activated by TGF- $\beta$  autocrine production (45). Accordingly, high IRES activation of LamB1 could be observed during the early phase of EMT upon TGF- $\beta$  treatment of Ras-transformed hepatocytes (Figure 7).

In conclusion, our data indicate that the internal initiation of LamB1 translation provides a novel cap-independent control mechanism for the regulation of LamB1 expression which is involved in the tissue invasion of carcinoma cells. IRES-controlled translation during EMT might therefore present an important event, since



additional mRNA species predicted to internal ribosome entry have an impact in tumour progression.

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