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CATHEPSIN L EXPRESSION IS UP-REGULATED BY HYPOXIA IN HUMAN MELANOMA CELLS: ROLE OF ITS 5'-UNTRANSLATED REGION

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Running Title

Hypoxia up-regulates cathepsin L expression

Abbreviations

EMCV; encephalomyocarditis virus; IRES, internal ribosome entry site; moAb, monoclonal antibody; RT-PCR, reverse transcription–polymerase chain reaction; ScFv, single chain variable fragment; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UTR, untranslated region.

Summary

Overexpression of cathepsin L, a cysteine protease and consequently procathepsin L secretion switch the phenotype of human melanoma cells to highly tumorigenic and strongly metastatic. This led us to identify the DNA regulatory sequences involved in the regulation of cathepsin L expression in highly metastatic human melanoma cells. Data demonstrated presence of inhibitory sequences in the 3' region downstream of cathepsin L gene and in the 3'- and 5'-flanking region of GC/CCAAT sites of its promoter. In addition, we established that the 5'-untranslated region (5'-UTR) was the most important region for cathepsin L expression. This 5'-UTR integrated an alternative promoter and sequences involved in post-transcriptional regulation. Transfection experiments of bicistronic reporter vectors and RNAs demonstrated that cathepsin L 5'-UTR contained a functional internal ribosome entry site (IRES). This complete IRES was present only in one of the three splice variants, which differed in their 5'-UTR. Then, we analyzed cathepsin L expression in this human melanoma cell line grown under hypoxia. We demonstrated that under moderate hypoxic conditions (1% O₂) intracellular expression of cathepsin L was up-regulated. Hypoxia increased significantly only the expression of the transcript which contains the complete IRES but inhibited promoter activity. These data suggested that presence of an IRES allowed cathepsin L mRNA translation to be efficient under hypoxic conditions. Altogether, our results emphasized that *in vivo* tumor hypoxic environment up-regulates cathepsin L expression which promotes tumor progression.

Key words

Hypoxia, Tumor Progression, IRES, Cathepsin L.

INTRODUCTION

Cathepsin L, a lysosomal cysteine protease, plays an important role in the catabolism of proteins and consequently in the behaviour of human cells. Evidences support that increase of cathepsin L expression is involved in tumor progression. Indeed, we previously demonstrated that cathepsin L overexpression in human melanoma cells transfected with cathepsin L cDNA switches their phenotype to highly tumorigenic and strongly metastatic [1, 2]. In addition, malignant melanoma cells of patient tumors expressed higher concentrations of cathepsin L than normal cells [3]. Specific inhibitors of cathepsin L activity inhibited distant bone metastasis [4] and invasive capacity of melanoma cells [5]. Down-regulation of cathepsin L by antisense cDNA in melanoma cells significantly reduced invasiveness [6].

Consequently to cathepsin L overexpression, procathepsin L secretion increases and contributes to the switch to highly tumorigenic and metastatic phenotype of human melanoma cells. Indeed, pretreatment of tumorigenic and highly metastatic human melanoma cells with polyclonal anti-cathepsin L antibody, which acts at the extracellular level, strongly inhibited tumor formation and significantly decreased lung metastasis in *nude* mice [7]. Moreover, focusing on procathepsin L secretion, we generated a single chain variable fragment (ScFv) from a monoclonal anti-cathepsin L antibody prepared in our laboratory [8]. We demonstrated that intracellular expression of anti-cathepsin L ScFv in human melanoma cells strongly inhibited: 1) procathepsin L secretion, without modifying the intracellular amount or processing pattern of cathepsin L; 2) human melanoma tumor growth and metastasis development in *nude* mice [9].

Secreted procathepsin L, by acting on a wide range of extracellular components, contributes to human melanoma progression, facilitates invasion, angiogenesis and inhibits immune system. For instance, we demonstrated that secreted procathepsin L cleaves human C3, the third component of complement, consequently inhibiting complement-mediated cell lysis and thus contributing to human melanoma resistance to the immune system [7, 10, 11]. Furthermore, procathepsin L generated C3 fragments which could trigger regulatory functions on normal and tumor cells by interacting with specific surface receptors. C3 fragments are involved in a large spectrum of biological functions and regulations [2].

Thus, these data emphasize the need to identify the molecular events involved in the regulation of human cathepsin L gene. We previously characterized human cathepsin L promoter and identified a region, essential for basal cathepsin L transcription, which contains one CCAAT motif and two GC boxes (GC/CCAAT sites), localized 60 bp upstream of the major transcription initiation site. Transcription factors as the nuclear factor Y (NF-Y) and members of the Sp-family

(Sp1, Sp2 and Sp3) bind on these sites [12]. More recently, we demonstrated that distinct molecular mechanisms regulate expression of cathepsin L in tumor cells. Indeed, cathepsin L gene silencing in lymphoma cells was due to genomic DNA methylation. Cathepsin L overexpression in melanoma cells involved either gene amplification or unidentified transcriptional mechanism at the promoter level [13]. Expression of several cathepsin L mRNA splice variants, which differed in their 5'-untranslated ends, could also constitute another level of regulation in melanoma cells [12].

Altogether these data led us to analyze the regulation of human cathepsin L gene, under normoxic and hypoxic conditions, in A375SM, a highly tumorigenic and metastatic melanoma cell line. We herein demonstrate that: **1)** 5'-untranslated region (5'-UTR) of cathepsin L mRNA is essential for its expression; **2)** cathepsin L 5'-UTR contains an alternative promoter and an internal ribosome entry site (IRES); **3)** hypoxia up-regulates expression of cathepsin L; **4)** this increase correlates with higher expression of the splice variant which contains the IRES.

MATERIALS and METHODS

Cell lines and culture conditions

The A375SM and DM-4 human melanoma cell lines were kindly provided by Dr M. Bar-Eli (M. D. Anderson Cancer Center, Houston, TX, U.S.A.) and the H1299 lung carcinoma cell line was obtained from the ATCC. The highly tumorigenic and metastatic human melanoma A375SM cells were established from *nude* mice lung metastases produced by the A375 human cell line isolated from a lymph node [14]. Cells were maintained in culture as adherent monolayers in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal calf serum, Glutamax-1 (2 mM), non-essential amino acids (0.1 mM), and penicillin/streptomycin (Invitrogen) at 37°C in a CO₂ incubator.

Hypoxia treatment

Cells were maintained under hypoxic conditions at 37°C within a modular incubator chamber (Billups-Rothenberg) filled with 1% O₂ and 5% CO₂ (balanced with N₂) for 26 h. A standard tissue culture incubator was used for a normoxic environment (21% O₂, 5% CO₂). For transient transfected cells, hypoxic conditions were applied 24 h after transfection.

Plasmid constructs

The mammalian expression vectors encoding a fusion protein of procathepsin L with a C-terminal V5 epitope (pcDNA3.1-V5-His-ProCTSL and pcDNA3.1-V5-His-5'-UTR-ProCTSL) were generated from preprocathepsin L cDNA, amplified by PCR using a 5' primer complementary either to the coding region or the 5'-UTR of cathepsin L cDNA (**Table I**) and cloned into pcDNA3.1-V5-HisA (Invitrogen), using *Bam*HI and *Xho*I. The cathepsin L promoter-firefly luciferase constructs pGL3-P4/U/I-(-1/-3263), pGL3-P1/U/I-(-1/-1646), pGL3-U1/I-(-1/-1388) and pGL3-P1-(-1465/-1646) and glyceraldehyde-3-phosphate dehydrogenase promoter-firefly luciferase construct pGL3-GAPDH were previously described [12]. Other monocistronic reporter constructs (pGL3-U/I-(-1/-1489), pGL3-P1/U-(-1211/-1646), pGL3-P2-(-1465/-1929), pGL3-P3-(-1465/-2085) and pGL3-P4-(-1465/-3263) were generated by PCR using specific primers (**Table I**) and by subcloning in the promoterless pGL3-Basic vector (Promega) digested with *Bgl*II and *Mlu*I. The 1500 bp genomic fragment in 3' of cathepsin L gene was generated by PCR from the genomic DNA of melanoma cells using a primer downstream of stop codon and a primer complementary to the 3' region (**Table I**). PCR products were subcloned in the promoterless pGL3-Basic, pGL3-P1/U/I-(-1/-1646) and pGL3-P4/U/I-(-1/-3263) digested with *Bam*HI and *Sal*I, to generate pGL3-Basic+1500, pGL3-P1/U/I-(-1/-1646)+1500 and pGL3-

P4/U/1-(-1/-3263)+1500. Mutagenesis of the CCAAT motif and of the two GC boxes to generate pGL3-P1/U/I-(-1/-1646)Mut and pGL3-P1-(-1465/-1646)Mut was described previously. To create pRL-FL and pSV-RL-FL bicistronic vector constructs, the coding region of *Renilla* luciferase (RL) without or with the SV40 early promoter was amplified by PCR from the pRL-SV40 vector (Promega) and subcloned in the promoterless pGL3-Basic vector digested with *SacI* and *MluI*. DNA fragments of cathepsin L 5'-UTR and the encephalomyocarditis virus (EMCV) IRES were generated by PCR using specific primers (**Table I**) from pcDNA3.1-V5-His-5'-UTR-ProCTSL or pIRES2-AcGFP1 vector (Clontech), respectively. PCR products were then inserted into pRL-FL and pSV-RL-FL vectors at *MluI* and *BglII* sites to generate bicistronic vector constructs used in this study. Amplification reactions were performed using the GeneAmp high-fidelity PCR system (Applied Biosystems) and constructs were confirmed by sequence analysis (Genome Express). Nomenclature used for functional elements in reporter constructs are: P1 to P5 for cathepsin L promoter fragments; I for intron 1 of cathepsin L gene; U and U1 to U5 for 5'-UTR of cathepsin L cDNA.

DNA transfection and reporter assays

Expression and monocistronic reporter vectors were transfected into melanoma cells using Lipofectin reagent (Invitrogen) as previously described [12]. Transfections were performed with 600 ng of reporter constructs and 200 ng of pRL-null (Promega) or with 600 ng of cathepsin L expression vectors and 80 ng of pRL-CMV (Promega). Reporter vectors which encode *Renilla* luciferase are used for normalization of transfection efficiency. Bicistronic reporter vectors (800 ng) were transfected using the Gene Porter 2 reagent (Gene Therapy System) in accordance with the manufacturer's protocol. Cell extracts were prepared 48 h after transfection. Quantification of firefly and *Renilla* luciferase activities was performed with the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was calculated by normalizing transfection efficiency with the *Renilla* luciferase activity. The transfections were performed in triplicate and similar results were obtained from at least three independent experiments.

Reverse Transcription-PCR Analysis

To estimate mRNA expression, semi-quantitative reverse transcription-PCRs (RT-PCRs) were performed as previously described [9]. Briefly, total RNA was isolated from melanoma cells 48 h after transfection using TRIzol reagent (Invitrogen) and then reverse transcribed. The PCR reaction was carried out with 24 cycles of amplification for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 25 cycles for firefly luciferase and 30 cycles for RB18A. Sequences of specific primers were previously described for GAPDH [9] and were: 5'-

TTGCTTTTACAGATGCAC-3' and 5'-AGGATCTCTGGCATGCGA-3' for firefly luciferase; 5'-ATGGAGCATCACAGTGGTAGT-3' and 5'-ACTGCTTTTCATCTTCCCTG-3' for RB18A. To analyze cathepsin L mRNA splicing, PCR was performed with 30 cycles of amplification using primers complementary to sequences downstream and upstream the first intron: 5'-GACAGGGACTGGAAGAGAGGAC-3' and 5'-AAAGGCAGCAAGGATGAGTGTAGGATTCAT-3'. To analyze splicing of bicistronic mRNA, cDNA were prepared from cells transfected with bicistronic constructs and PCR was carried out with 30 to 50 cycles of amplification using a primer 5'-GAGCTATTCCAGAAGTAGTGAGGAG-3' complementary of the *Renilla* luciferase 5'-UTR and a primer 5'-GCCTTATGCAGTTGCTCTCCAGCGGTTCC-3' specific of the firefly luciferase coding region. To quantify mRNA expression, real-time PCR was performed by Cogenics (Meylan, France) with an Applied Biosystems 7500 Fast Real-Time PCR System using SYBR Green. Luciferase amplification primers were 5'-GCCTGAAGTCTCTGATTAAGT-3' and 5'-ACACCTGCGTCGAAGATGT-3'. The amount of luciferase transcript in each RNA sample was quantified and normalized to GAPDH content. Relative amounts of luciferase cDNA were calculated by the relative comparative $2^{-\Delta\Delta C_t}$ method and are expressed as a percentage of luciferase cDNA measured in cells transfected with pGL3-P1/U1(-1/-1646).

Western blot Analysis

Protein expression and secretion were analyzed in conditioned media and cell extracts by western blot as previously described [8], using anti-cathepsin L moAb (clone 3D8) prepared in our laboratory, anti-GAPDH moAb (moAb374; Chemicon International), anti-RB18A Ab (anti-TRAP220; C19; Santa Cruz Biotechnology), anti-lamin A/C moAb (MCA1429; Serotec) or anti-V5-Tag moAb (MCA1360; Serotec). Bound antibodies were detected with peroxidase-linked secondary antibody (Dako) and the enhanced chemiluminescence system (Amersham Biosciences).

In vitro RNA synthesis and RNA transfection

In vitro synthesis of capped RNAs was carried out using the mMACHINE kit (Ambion). Briefly, pSV-RL-FL and pSV-RL-U1-FL containing bicistronic transcription units under the control of the T7 promoter were linearized downstream from the 3' end of the firefly luciferase coding sequence at the *Xba*I restriction site. Transcript was synthesized from 1 μ g of the linearized plasmid DNA with T7 RNA polymerase in the presence of cap analog. Poly A tails were added to the newly synthesized mRNAs using the poly(A) tailing kit, as described by Ambion. Capped and polyadenylated mRNAs were examined for integrity by electrophoresis in a

denaturing agarose gel and concentration was estimated by measuring absorbance at 260 nm. RNA transfections were carried out using Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol. A375SM melanoma cells were seeded in 24-wells tissue culture plates, cultured for 24 h and transfected with 0.8 μg of *in vitro* synthesized mRNA and 2 μl of the Lipofectamine 2000 reagent. After incubation for 6 h, the cells were washed and transfection medium was replaced by culture medium. Cells extracts were prepared 16 h later and luciferase activities were measured.

Stage 2(a) POST-PRINT

RESULTS

DNA regulatory sequences of human cathepsin L gene

Our previous demonstration that in A375SM human melanoma cells, cathepsin L overexpression correlated with high promoter activity and that CCAAT motif and GC boxes were not directly involved in this regulation [13], led us to identify others DNA regulatory sequences involved in cathepsin L gene expression.

Thus, we subcloned 3'- and 5'-flanking region of cathepsin L gene in the luciferase reporter vector pGL3-Basic. These constructs were cotransfected into A375SM cells with the promoterless phRLnull plasmid, used as an internal control and luciferase activities were quantified (**Figure 1**). As shown **Figure 1(A)**, deletion mutants in 3' of GC/CCAAT sites were compared to pGL3-P1/U/I-(-1/-1646) reporter vector, which contained the 1646 bp sequence upstream of the translation start site of human cathepsin L gene [12]. Deletion of the first intron (-1210/-1) of cathepsin L gene induced a 1.7 fold decrease of luciferase activity (**Figure 1A**). Deletion of the -1211/-1464 sequence corresponding to the 5'-untranslated region (5'-UTR) of cathepsin L mRNA induced an additional 5.2 fold decrease of luciferase activity (**Figure 1A**). Then, reporter vectors containing fragments of 5'-flanking region of GC/CCAAT sites were compared to pGL3-P1-(-1465/-1646) construct which contained the 181 bp sequence upstream of the major transcription start site (-1489) of cathepsin L gene [12]. The 1.4 fold decrease in luciferase activity measured with the construct pGL3-P4-(-1465/-3263) supported the presence of negative regulator sequences between -2086 and -3263 (**Figure 1B**).

Then, presence of transcriptional regulatory region in 3' of cathepsin L gene was investigated. The 1500 bp sequence in 3' of the stop codon was subcloned downstream the luciferase reporter gene into the promoterless pGL3-Basic vector and into pGL3-P1/U/I-(-1/-1646) and pGL3-P4/U/I-(-1/-3263) vectors to generate pGL3-Basic+1500, pGL3-P1/U/I-(-1/-1646)+1500 and pGL3-P4/U/I-(-1/-3263)+1500. As shown **Figure 1(C)**, luciferase activity was not detected with the pGL3-Basic+1500 construct supporting that the 3' region of cathepsin L gene was not able to initiate transcription. A 1.4 fold decrease in luciferase activity was observed in cells transfected with the pGL3-P1/U/I-(-1/-1646)+1500 and pGL3-P4/U/I-(-1/-3263)+1500 vectors, in comparison with the pGL3-P1/U/I-(-1/-1646) and pGL3-P4/U/I-(-1/-3263) vectors, respectively (**Figure 1C**). Altogether, these data supported presence of negative regulatory sequences in 3'- and 5'-flanking region of GC/CCAAT sites and in the 3' region of cathepsin L gene.

5'-UTR is involved in regulation of cathepsin L gene expression

As the deletion of the -1211/-1464 sequence of the 5'-UTR induced the highest inhibition of luciferase activity, we focused on the analysis of its contribution in cathepsin L gene expression. Thus, A375SM cells were transfected with reporter vector constructs in which cathepsin L promoter was deleted, *i.e.* pGL3-U/I(-1/-1489) and pGL3-U1/I(-1/-1388) constructs, containing only sequences downstream of the major transcription initiation site (**Figure 2A**). No significant luciferase activity was detected with pGL3-U1/I(-1/-1388) construct, as compared to the promoterless pGL3-Basic vector. However, a 58% luciferase activity was detected with the pGL3-U/I(-1/-1489) vector, as compared to the activity of pGL3-P1/U/I(-1/-1646) construct. These results supported that the region between -1389 and -1489 contained a promoter. Then, A375SM cells were transfected either with wild-type pGL3-P1(-1465/-1646) and pGL3-P1/U/I(-1/-1646) vectors or with mutated pGL3-P1(-1465/-1646)Mut and pGL3-P1/U/I(-1/-1646)Mut vectors in which CCAAT motif and GC boxes were disrupted [12]. Luciferase activities showed (**Figure 2A**) that these mutations totally inhibited promoter activity of the P1 region (-1465/-1646) and induced a 76% decrease of the activity of the P1/U/I region (-1/-1646). Differences in relative luciferase activities of pGL3-U/I(-1/-1489) and pGL3-P1/U/I(-1/-1646)Mut, *i.e.* 58% and 24% respectively, suggested that the promoter in 5' of the major transcription initiation site inhibits the activity of the promoter localized between -1389 and -1489.

Then, we analyzed expression of firefly luciferase mRNA by real-time quantitative RT-PCR in cells transfected with reporter vector constructs (**Figure 2B**). Transfection efficiencies, determined by measuring *Renilla* luciferase activity, were similar. The amount of firefly luciferase transcript in each RNA sample was normalized to GAPDH content and expressed as a percentage of luciferase cDNA measured in cells transfected with pGL3-P1/U/I(-1/-1646). Data demonstrated that luciferase mRNA expression levels correlated with luciferase activities in cells transfected with pGL3-P1/U/I(-1/-1646) and pGL3-U/I(-1/-1489) in agreement with a transcriptional regulation (**Figure 2B, columns 1 and 2**). In contrast, despite a strong 87% difference (shown in **Figure 2A**) between luciferase activities of cells transfected with pGL3-P1/U/I(-1/-1646) or pGL3-P1(-1465/-1646), luciferase mRNA expression levels were similar (**Figure 2B, columns 1 and 3**). Identical results were observed when firefly luciferase mRNA levels were analyzed by semi-quantitative PCR analysis (**Figure 2C**). Indeed, intensities of amplified luciferase PCR products are very similar between cells transfected with pGL3-P1/U/I(-1/-1646) or pGL3-P1(-1465/-1646) (**Figure 2B, lanes 1 and 2**). Thus, both quantitative and semi-quantitative PCR analyses demonstrated that inhibition of luciferase activity induced by the deletion of 5'-UTR was not supported by a transcriptional mechanism.

In addition, cathepsin L cDNA was subcloned with or without the 5'-UTR in an expression vector under the control of the cytomegalovirus promoter (CMV). The pcDNA3.1-V5-His-ProCTSL and pcDNA3.1-V5-His-5'-UTR-ProCTSL vectors encode a fusion preprocathepsin L with a C-terminal V5 epitope. Expression of ectopic procathepsin L, characterized by a 44 kDa molecular weight, was analyzed by western blot using anti-V5 moAb, in cell extracts and in conditioned media prepared from transfected A375SM cells (**Figure 2D**). To take into account transfection efficiency variation, *Renilla* luciferase reporter vector was cotransfected with cathepsin L expression vectors and samples were loaded according to their *Renilla* luciferase activities. Data demonstrated a strong increase of the intracellular expression and secretion of procathepsin L when cathepsin L coding region was associated to its 5'-UTR. Altogether, these data supported that 5'-UTR was involved in the regulation of cathepsin L expression independently of transcriptional mechanism.

Presence of an internal ribosome entry site (IRES) in the 5'-UTR of cathepsin L

The 5'-UTR of cathepsin L mRNA is 293 bp long and 68% GC-rich. Folding of the 5'-UTR RNA using the Mfold program [15] allowed to predict a very stable structure ($\Delta G = -126$ kcal/mol), suggesting that cap-dependent translation will be inefficient. This analysis and the ability of cathepsin L 5'-UTR to stimulate expression suggested that translation might be proceeded by internal ribosome recruitment. IRES, first identified in the 5'-UTR of poliovirus and encephalomyocarditis virus (EMCV), was also described in several cellular mRNAs [16]. To investigate the putative presence of an IRES in cathepsin L 5'-UTR, we used pSV-RL-FL, a bicistronic vector containing two reporter genes: the *Renilla* luciferase (upstream cistron driven by the SV40 promoter) and firefly luciferase (downstream cistron). DNA fragments of cathepsin L 5'-UTR were subcloned within the intercistronic spacer between these two coding regions (**Figure 3A**). As control, we used the pRL-FL, a SV40 promoter-less bicistronic vector which allowed to detect presence of cryptic promoter in the subcloned region.

The bicistronic vectors pSV-RL-U1-FL and pRL-U1-FL (U1 corresponds to -1/-1388 region, **Figure 3A**) were transfected into A375SM cells. As shown **Figure 3(B)**, *Renilla* luciferase activity was detected only with vectors which contained the SV40 promoter (pSV-RL-FL and pSV-RL-U1-FL) supporting transcription of the bicistronic mRNA. Firefly luciferase activity was 5.3-fold higher in cells transfected with pSV-RL-U1-FL than with control pSV-RL-FL (**Figure 3C**). *Renilla* (**Figure 3D**) and firefly (**Figure 3E**) luciferase activities were also determined in A375SM cells transfected with pSV-RL-U2-FL and pRL-U2-FL (U2 corresponds to -1371/-1489 region, **Figure 3A**). High firefly luciferase activity detected with both constructs (**Figure 3E**)

also supports presence of a promoter in cathepsin L 5'-UTR between -1371 and -1489, as described in **Figure 2**. These data suggested presence of an IRES in fragment -1/-1388 of cathepsin L 5'-UTR.

However, the presence of cryptic splice sites in the cathepsin L 5'-UTR could also lead to firefly luciferase activity, without the presence of an IRES. To clarify this point, RT-PCRs were performed on total RNA isolated from A375SM cells transfected with pSV-RL-U1-FL vector or pRL-U1-FL control vector, using primers specific to the 3' end of the SV40 promoter sequence (downstream the transcription start site) and to the firefly luciferase coding region. A single amplified product was obtained from transfected cells with pSV-RL-U1-FL, which size corresponded to the full length bicistronic RNA around 1600 bp (**Figure 4A, lane 4**). Absence of smaller PCR products using 30, 40 and 50 cycles of amplification supported absence of splicing. Then, as direct transfection of dicistronic RNAs were previously used to confirm IRES activity in cells [17, 18], we generated *in vitro* capped and polyadenylated mRNAs from control pSV-RL-FL and pSV-RL-U1-FL (**Figure 4B**) for transfection experiments into A375SM cells. As shown **Figure 4(C)**, relative luciferase activity obtained from A375SM cells transfected with RL-U1-FL mRNA was 2.6-fold greater than the one obtained from cells transfected with control RL-FL mRNA. RNA transfections indicated that observed firefly luciferase activity was RNA-dependent and not due to the presence of a cryptic promoter or a splicing. Thus, altogether these data strongly supported the presence of an IRES in cathepsin L 5'-UTR.

To localize more precisely the region necessary for IRES activity, deletions were introduced in the U1 fragment (-1/-1388) and were subcloned into the pSV-RL-FL bicistronic vector (**Figure 5A**). These constructs were then transfected into A375SM cells and their relative luciferase activities were measured (**Figure 5B**). A decrease of 34% IRES activity was observed when -1302/-1388 DNA fragment was deleted from the 5' end of the U1 fragment (-1/-1388), to generate the U4 fragment (-1/-1301). U3 fragment (-1301/-1388) showed a very slight activity. In addition, deletion of -1/-11 fragment corresponding to exon 2 at the 3' end of U1 fragment, to generate the U5 fragment (-1211/-1388), did not modify IRES activity. These data supported that IRES was mainly localized in the first exon between -1211 and -1388 and suggested that progressive rather than abrupt loss of activity occur when 5'-UTR fragments are deleted, consistent with modular property of cellular IRES elements, described by others [19].

We next compared activities of cathepsin L IRES and encephalomyocarditis virus (EMCV) IRES, one of the most powerful elements known for internal initiation of translation in a broad range of host cells [20]. Thus, the cathepsin L IRES and the EMCV IRES, subcloned into pSV-RL-FL bicistronic vector, were transfected into A375SM melanoma cells and H1299 lung

carcinoma cells (**Figure 5C**). Data demonstrated only a 2-fold difference between cathepsin L IRES activity and EMCV IRES activity in both cell lines, despite these IRES activities were lower in H1299 cells. Thus, cathepsin L IRES activity was not restricted to melanoma cells and was efficient to initiate translation.

Regulation of human cathepsin L gene expression in hypoxic conditions

IRES were identified in genes induced under hypoxic conditions as the vascular endothelial growth factor (VEGF) [21, 22] and the hypoxia-inducible factor (HIF-1 α) [23]. Our herein demonstration of the presence of an IRES in cathepsin L mRNA and the involvement of cathepsin L in angiogenesis [9] led us to analyze its regulation by hypoxia.

Thus, cathepsin L expression was analyzed by western blot in A375SM cells grown under hypoxic conditions, during 26 h (**Figure 6A**). Data demonstrated that both cathepsin L forms (*i.e.* the 29 kDa cathepsin L mature and the 41 kDa procathepsin L forms) were clearly increased. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, usually used as control, was slightly increased under hypoxic conditions (**Figure 6A**), as described in other cell types [24]. Thus, we used two other controls, *i.e.* RB18A (TRAP220/DRIP205) and lamin A/C, which expressions were not modified between normoxic and hypoxic conditions (**Figure 6A**). Similar results were obtained with DM-4, another human melanoma cell line. Indeed, hypoxic conditions also induced in DM-4 cells a significant increase of cathepsin L forms and especially for the procathepsin L form (**Figure 6B**). Patterns of intracellular 41 kDa procathepsin L, 34 and 29 kDa cathepsin L mature forms were different between these two melanoma cell lines, as we already described [13].

Furthermore, human melanoma cells express three splice variants differing in their 5'-UTR [12]. These transcripts, named, hCATL-A, hCATL-A II and hCATL-A III, resulted of the splicing of intron 1 using the same 3' splice site located 11 nucleotides upstream of the ATG and different 5' splice sites located 1210, 1300 and 1355 nucleotides upstream of the ATG, respectively. Analysis by semi-quantitative RT-PCR, showed that hypoxia induced a significant increase of only hCATL-A expression (**Figure 6C**). Interestingly, the complete IRES (-1211/-1388) is enclosed only within the hCATL-A transcript variant. In control, expression of RB18A mRNA was not modified under hypoxia (**Figure 6C**). These results demonstrated that hypoxia induced modification in the ratio of splice variants by increasing expression of the transcript which contains the IRES.

In addition, we compared cathepsin L promoter activity under both normoxic and hypoxic conditions. The pGL3-P1/U/I(-1/-1646) construct, which contained the firefly luciferase gene

driven by the cathepsin L promoter, was transfected into A375SM cells, with the reporter vector pRL-SV40, used as an internal control. We also verified that SV40 promoter activity was not modified in A375SM cells grown under hypoxia conditions (data not shown). Hypoxia induced a 37% decrease of relative luciferase activity in transient transfected cells (**Figure 6D, Part 1**). In comparison, GAPDH promoter activity was increased under hypoxic conditions (**Figure 6D, Part 2**). In contrast of cathepsin L, up-regulation of GAPDH mRNA level (**Figure 6C**) is in correlation with the increase of its promoter activity, which supported that GAPDH was regulated by transcriptional mechanism in melanoma cells, as described by others in endothelial cells [24, 25]. Data demonstrated that up-regulation of cathepsin L expression under hypoxic conditions was not due to transcriptional mechanisms. Using the pSV-RL-U1-FL bicistronic construct (-1/-1388 fragment of cathepsin L 5'-UTR), cathepsin L IRES activity was analyzed in A375SM cells grown under normoxic or hypoxic conditions. As shown in **Figure 6(D), Part 3**, relative luciferase activities were identical, supporting that the cathepsin L IRES is functional under both conditions.

DISCUSSION

First, we focused on the identification of DNA regulatory sequences involved in cathepsin L gene overexpression in A375SM, a highly tumorigenic and metastatic human melanoma cell line.

We established that cathepsin L gene regulation is certainly complex, as 5'-flanking sequence, 3'-flanking sequence and untranslated exons/intron region were involved. Indeed, our data demonstrated presence of: **1)** inhibitory sequences of transcription in the 5' region of cathepsin L promoter, upstream the GC/CCAAT sites; **2)** silencers in the 3' region that regulated cathepsin L promoter activity. This was supported by cloning the 1500 bp sequence from the end of the stop codon of cathepsin L in 3' of luciferase gene downstream of the SV40 late poly(A) signal. In these conditions, luciferase mRNA sequence was not modified, which allowed to measure only transcriptional regulation and to exclude effect on mRNA stability or translation by the 3'-UTR of cathepsin L gene; **3)** negative regulatory sequences localized in the intron and the two exons upstream of the translation initiation site. Inside this region, the 5'-UTR of cathepsin L mRNA is the most important for cathepsin L expression. It was described, that long 5'-UTRs often confer an inhibitory effect on gene expression due to the inhibition of cap-dependent translation by stable secondary structure, upstream open reading frames or presence of specific sequence for inhibitory proteins as the iron-responsive element [26]. In contrast, cathepsin L 5'-UTR increased expression, as shown using luciferase reporter constructs and expression vectors encoding an ectopic cathepsin L cDNA with or without the 5'-UTR.

In addition, we demonstrated that the 5'-UTR contained an alternative promoter localized between -1389 and -1489 from the translational start site. Transfection of reporter constructs containing mutations in GC boxes and CCAAT motif demonstrated that the main promoter in 5' of the major transcription initiation site inhibited the activity of the alternative promoter. However, we previously identified minor transcription initiation sites between -1371 and -1382 using RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) in melanoma cells [12]. Indeed, this alternative promoter seems to be active in melanoma cells and allows synthesis of shorter cathepsin L mRNAs. This -1389/-1489 promoter could mediate around 20% of cathepsin L transcription in melanoma cells, based on the percentage of the sequenced RLM-RACE products (22%) [12], the 24% luciferase activity of the pGL3-P1/U/I-(-1/-1646)Mut construct and the absence of activity of pGL3-P1-(-1465/-1646)Mut construct. The -1389/-1489 promoter is also functional in other cell types, as shorter cathepsin L mRNAs lacking nucleotides at the 5'-end were identified in a human malignant kidney tumor [27].

We also herein demonstrated, for the first time, the presence of an IRES in the 5'-UTR of cathepsin L or a member of the lysosomal cysteine protease family, using the bicistronic reporter strategy. Indeed, transfection experiments of bicistronic reporter vectors or RNAs confirmed that cathepsin L 5'-UTR contains a functional IRES, which is involved in translational regulation. Several human cathepsin L mRNA species differing in the length of the 5'-UTR have been reported earlier [27, 28]. However we demonstrated that human melanoma cells synthesize three different splice variants, hCATL-A, hCATL-A II and hCATL-A III [12]. The complete IRES (-1210/-1301) is enclosed only in the hCATL-A transcript variant. Interestingly, cellular distribution analysis in some normal and tumor tissues and cell lines showed that hCATL-A is widely expressed [27].

Second, we analyzed cathepsin L regulation under hypoxic conditions, as IRES involvement was described in the regulation of gene induced under hypoxic conditions [29]. We herein demonstrated, for the first time, that under moderate hypoxic conditions (1% O₂) cathepsin L intracellular expression was up-regulated in human melanoma cells. Interestingly, in rodent cells grown under severe hypoxic conditions (< 0.1% O₂) an increase of cathepsin L expression was mentioned [30, 31].

In addition, we demonstrated that hypoxia increased cathepsin L mRNA expression and especially of the hCATL-A transcript variant which contains the complete IRES. However, promoter activity was inhibited under hypoxic conditions supporting that cathepsin L was not up-regulated at the transcriptional level. Specific up-regulation of the hCATL-A transcript suggested that hypoxia induced either selective modification of mRNA stability of individual splice variants or modification in cathepsin L splicing. Indeed, both mechanisms were involved in gene regulation under hypoxia: **1)** increase in the stability of VEGF mRNA contribute to its induction [32] and this stabilization depended on the cooperation of elements in each of the 5'-UTR, the coding region, and the 3'-UTR [33]; **2)** differential splicing was also described for others genes, as telomerase [34] and X-box binding protein 1 [35]. It should be noted that in both cases, splicing was linked to up-regulation of transcription. Indeed, from our data, down-regulation of cathepsin L transcription suggested that hCATL-A variant contains elements required for efficient mRNA stabilization by hypoxia.

Our results supported that hCATL-A, the only cathepsin L transcript in which the complete IRES is present, is involved in up-regulation of cathepsin L expression by hypoxia. However, cathepsin L IRES activity was not modified by hypoxia. Similar discrepancy was also observed under hypoxic conditions with other genes which contained an IRES. Indeed, IRES activity of VEGF [21, 22] and HIF [23] were not increased under hypoxic conditions and IRES activity of

Tie2 was only slightly stimulated [18] in bicistronic reporter assays. However, these authors using polysome profile analysis showed that presence of IRES allowed translation of these genes during hypoxic conditions [18, 22, 23]. It is well known that hypoxia downregulates protein synthesis by inhibiting cap-dependent translation, which accounts for the translation of most mRNAs. Then, translation of factors that are critical for the hypoxic response is maintained by initiation via an internal ribosomal entry mechanism [29]. Altogether, these results suggest that the IRES in the hCATL-A transcript mediates cathepsin L mRNA translation under hypoxic conditions and contributes to the up-regulation of cathepsin L expression in melanoma cells. Further analyses are needed to explore whether other molecular mechanisms are involved in cathepsin L regulation by hypoxia.

In conclusion, our data clearly demonstrate that 5'-UTR region of cathepsin L mRNA plays a major role in its regulation. For the first time, the presence of an IRES was identified within this 5'-UTR. In addition, hypoxia up-regulates intracellular expression of cathepsin L with a selective increase of the variant which contains this IRES. The increase of cathepsin L expression induced under hypoxic conditions, should be related to the properties of cathepsin L to regulate angiogenic factors [36-38] and to contribute to neovascularization in ischemia [39] and in tumor progression [9]. Altogether, these data emphasize that hypoxic environment of tumors up-regulates cathepsin L expression which promotes *in vivo* tumor angiogenesis, tumor growth and metastasis.

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LEGENDS

FIGURE 1. Regulatory sequences within human cathepsin L gene.

Reporter assays were performed into A375SM cells. Schematic representation of transfected firefly luciferase (FLuc) reporter constructs are shown. Localizations of the major (-1489) and minor (-1377) transcription initiation sites, of the 50 bp region containing the two GC boxes and the CCAAT motif (GC/CCAAT) and of the first intron are indicated. **(A) Regulation by DNA regions in 3' of GC/CCAAT sites.** Cells were transfected with reporter vectors containing cathepsin L promoter in which the first intron and the 5'-UTR of cathepsin L were deleted. **(B) Regulation by DNA regions in 5' of GC/CCAAT sites.** 5'-deletion mutants of cathepsin L promoter subcloned into reporter vector were used. **(C) Regulation by the 3' region of the cathepsin L gene.** Reporter vectors, in which the 1500 bp sequence in 3' of the stop codon of cathepsin L gene was subcloned downstream the luciferase gene, were transfected. Relative luciferase activities are presented as percentages. Results are means \pm S.D.

FIGURE 2. Involvement of the 5'-UTR in cathepsin L expression.

(A) Reporter vectors containing DNA regions of human cathepsin L gene with 5' or 3' deletions and/or mutations in GC/CCAAT sites were transfected into A375SM cells. Relative luciferase activities are presented as percentages of activity obtained in cells transfected with pGL3-P1/U/I(-1/-1646) vector. Results are means \pm S.D. **(B)** Expression of firefly luciferase cDNA was analyzed by real-time quantitative RT-PCR into A375SM cells transfected with reporter vectors (**columns 1-3**) or non-transfected (n.t.) (**column 4**). Luciferase transcript levels were normalized to GAPDH mRNA and are expressed as a percentage of the transcript level measured in cells transfected with pGL3-P1/U/I(-1/-1646) vector. **(C)** Transfected cells cDNA was also assayed for firefly luciferase expression using semi-quantitative PCR. GAPDH cDNA, also amplified from the same cDNA, was used as control for DNA integrity and equal loading. **(D)** Western blot using anti-V5 moAb of extracts and conditioned media of A375SM cells transfected with empty pcDNA3.1-V5-His vector (**lane 1**), pcDNA3.1-V5-His-ProCTSL (**lane 2**) or pcDNA3.1-V5-His-5'-UTR-ProCTSL (**lane 3**) expression vectors, which encode tagged procathepsin L cDNA without or with 5'-UTR. PRL-CMV reporter vector, which contained the reporter gene *Renilla* luciferase driven by the CMV promoter, was introduced in each transfection. Gel loading was normalized to the luciferase activity to correct for variations of transfection efficiency. Results are means \pm S.D.

FIGURE 3. Presence of a potential IRES in the human cathepsin L 5'-UTR.

(A) Schematic representation of bicistronic constructs containing regions of cathepsin L 5'-UTR in the intercistronic region between the *Renilla* (RLuc) and firefly (FLuc) luciferase open reading frames. The major (-1489) and minor (-1377) transcription initiation sites are identified by dots and arrows indicate the splice sites of the first intron. Expression is driven by the SV40 promoter (pSV40) in pSV-RL-FL vectors while pRL-FL vectors, used as a control, did not contain promoter element. (B-E) A375SM cells were transfected with bicistronic vectors containing different regions of cathepsin L 5'-UTR. *Renilla* luciferase activities are shown in (B) and (D) and firefly luciferase activities in (C) and (E). Results are means \pm S.D.

FIGURE 4. Absence of splicing or cryptic promoter activity in the fragment -1/-1388 of the human cathepsin L 5'-UTR.

(A) **RT-PCR analysis.** RT-PCRs were performed on total RNA isolated from A375SM cells non-transfected (n.t.) or transfected with different bicistronic vectors containing fragment -1/-1388 of cathepsin L 5'-UTR, using 30, 40 and 50 amplification cycles. Molecular size markers (M) are indicated. The location of the PCR primers used in this amplification are schematically shown. (B-C) **mRNA transfection into A375SM cells.** Capped and polyadenylated mRNAs were generated from bicistronic vectors pRL-FL and pRL-U1-FL *in vitro* and analyzed by denaturing agarose gel electrophoresis (B). Then, mRNAs were transfected in A375SM cells and the relative luciferase activities were calculated. Results are means \pm S.D (C).

FIGURE 5. Mapping of cathepsin L IRES and comparison to EMCV IRES activity in A375SM melanoma and H1299 lung carcinoma cells.

(A) Schematic representation of bicistronic constructs containing regions of cathepsin L 5'-UTR in the intercistronic region between the *Renilla* (RLuc) and firefly (FLuc) luciferase open reading frames in pSV-RL-FL vectors. The major (-1489) and minor (-1377) transcription initiation sites are identified by dots and arrows indicate the splice sites of the first intron. (B-C) Bicistronic vectors containing cathepsin L IRES or EMCV IRES were transfected in A375SM melanoma cells or in H1299 lung carcinoma cells and relative luciferase activities were calculated. Results are means \pm S.D.

FIGURE 6. Regulation of cathepsin L gene expression in hypoxia.

A375SM and DM-4 human melanoma cells were grown under normoxia (N) or hypoxia (H) for 26 h. Cell extracts (A, B) and cDNA (C) were prepared. (A-B) Cell extracts were separated by SDS-PAGE and analyzed by western blot with anti-cathepsin L, anti-glyceraldehyde-3-phosphate

dehydrogenase (GAPDH), anti-RB18A and anti-lamin A/C antibodies. (C) Cathepsin L mRNA splicing or GAPDH and RB18A mRNAs expression were analyzed by semi-quantitative RT-PCR. (D) A375SM cells were transfected with monocistronic reporter vectors containing human cathepsin L promoter (pGL3-P1/U/I(-1/-1646)), human GAPDH promoter (pGL3-GAPDH) or bicistronic reporter vector containing DNA fragment -1/-1388 of cathepsin L 5'-UTR (pSV-RL-U1-FL). Hypoxic treatment was initiated twenty-four hours after transfection. Relative luciferase activities are presented as percentages of activity measured in normoxic conditions. Results are means \pm S.D.

Table 1 Sequence of primers used for PCR amplification

Vector constructs	Sense Primer	Antisense Primer
Expression vectors : Amplification of human cathepsin L cDNA with or without its 5'-UTR		
proCTSL	5'-GCGGATCCATGAATCCTACACTCATCCTT-3'	5'-CCGCTCCGAGCACAGTGGGGTAGCTGGCT-3'
5'-UTR-proCTSL	5'-CGGGATCCGCCAGAACCGCGACCTCCGCAACC-3'	5'-CCGCTCCGAGCACAGTGGGGTAGCTGGCT-3'
Monocistronic reporter vectors : Amplification of 5' region of cathepsin L gene		
-1/-1489	5'-CCGACGCGTAGAACCGCGACCTCCGCAAC-3'	5'-GAAGATCTGTGTTTTAAAACCTAGGAAGGGAAC-3'
-1211/-1646	5'-CCGACGCGTAGTCAGTAAACAAGCCACGAAC-3'	5'-GAAGATCTGTGTCCACCCGGCTCGGAGGCTGGC-3'
-1465/-1929	5'-CCGACGCGTGTGTTGTCTGTGGACTAAACAGGAGC-3'	5'-GAAGATCTTCAAGGTTGCGGAGGTCGCGGTTCT-3'
-1465/-2085	5'-CCGACGCGTAGTCTCCTCCCTCCTAGGGTCT-3'	5'-GAAGATCTTCAAGGTTGCGGAGGTCGCGGTTCT-3'
-1465/-3263	5'-CCGACGCGTTTCTACACATTGTGACTGGTTCT-3'	5'-GAAGATCTTCAAGGTTGCGGAGGTCGCGGTTCT-3'
Monocistronic reporter vectors : Amplification of 3' region of cathepsin L gene		
	5'-CGGGATCCGCTGGTGGACGGTGATGAGGAAG-3'	5'-ACGCGTCCGACCCCTTATTGACTAGCTGCTCTAG-3'
Dicistronic reporter vectors : Amplification of Renilla luciferase cDNA with or without SV40 promoter		
RL	5'-CCGGAGCTCATGACTTCGAAAGTTTATGATCC-3'	5'-CCGACCGGTTTATTGTTCAATTTTTGAGAACTC-3'
SV-RL	5'-CCGGAGCTCGCGCAGCACCATGGCCTGAA-3'	5'-CCGACCGGTTTATTGTTCAATTTTTGAGAACTC-3'
Dicistronic reporter vectors : Amplification of cathepsin L 5'-UTR		
U1 : -1/-1388	5'-CCGACGCGTGACAGGGACTGGAAGAGAGGAC-3'	5'-GAAGATCTGTTTTAAAACCTGTGTCCACCC-3'
U2 : -1371/-1489	5'-CCGACGCGTAGAACCGCGACCTCCGCAAC-3'	5'-GAAGATCTTCAAGGCCAGCAGAGTTCGGGGTAGA-3'
U3 : -1301/-1388	5'-CCGACGCGTGACAGGGACTGGAAGAGAGGAC-3'	5'-GAAGATCTTCAAGGCCAGCAGAGTTCGGGGTAGA-3'
U4 : -1/-1301	5'-CCGACGCGTAGGTGGGGAAGCCGGGGAGGGC-3'	5'-GAAGATCTGTTTTAAAACCTGTGTCCACCC-3'
U5 : -1211/-1388	5'-CCGACGCGTGACAGGGACTGGAAGAGAGGAC-3'	5'-GAAGATCTGTGTCCACCCGGCTCGGAGGCTGGC-3'
Dicistronic reporter vectors : Amplification of ECMV IRES		
	5'-CCGACGCGTATCCGCCCTCTCCCTCC-3'	5'-GAAGATCTTGTGGCCATATTATCATCGTG-3'

The underlined restriction sites were added to the 5'-end of primers to facilitate subcloning.

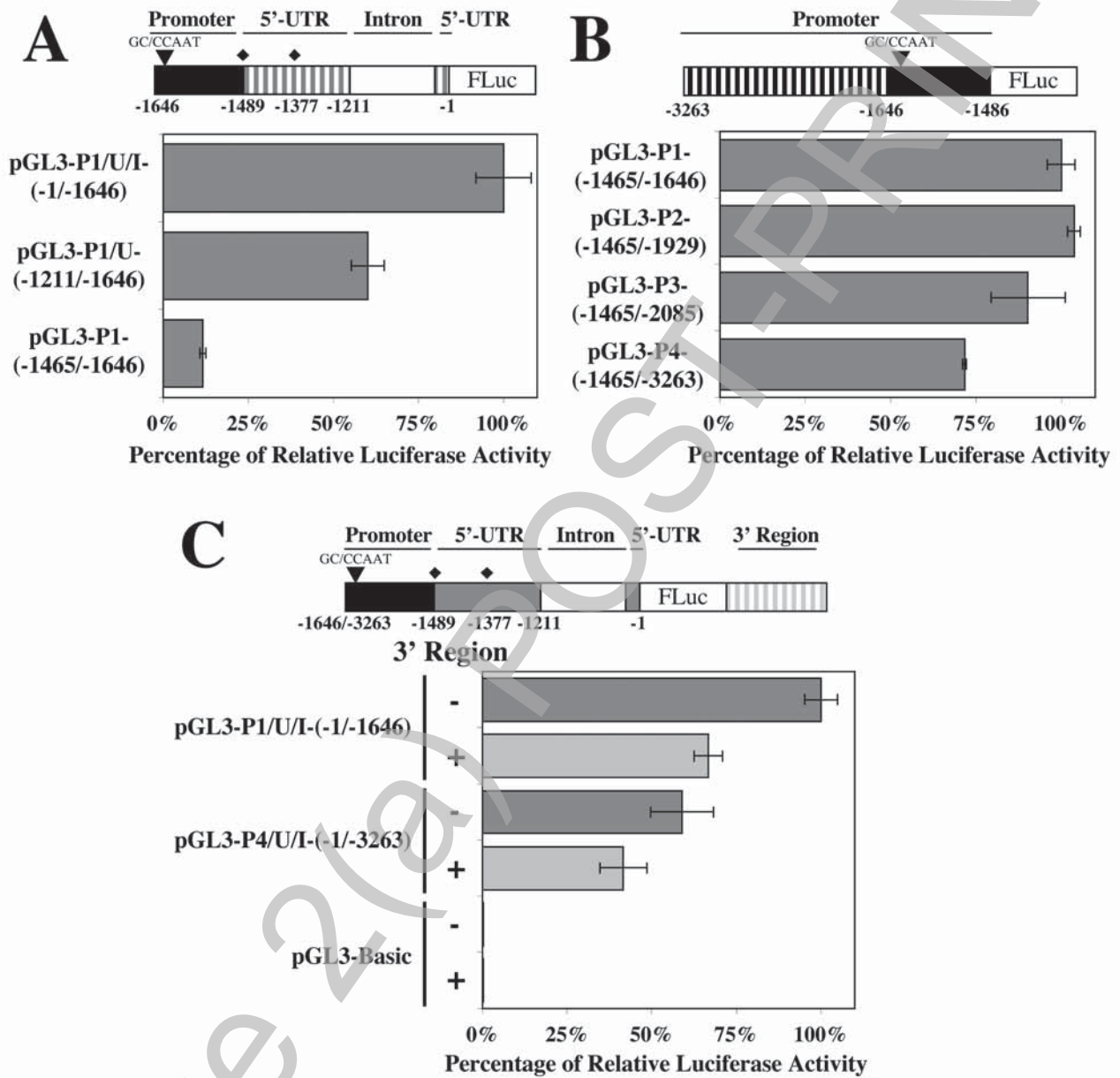


Figure 1

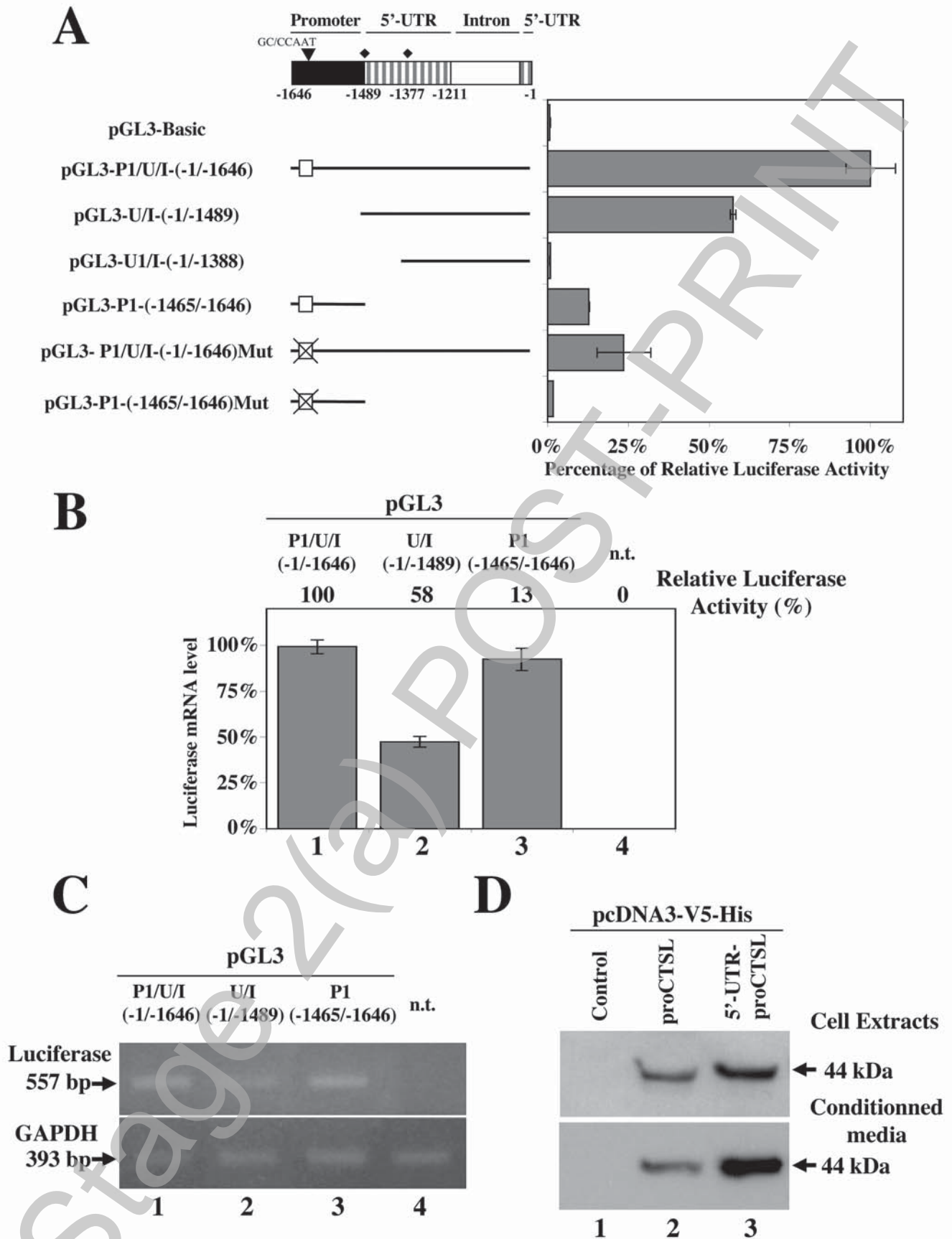


Figure 2

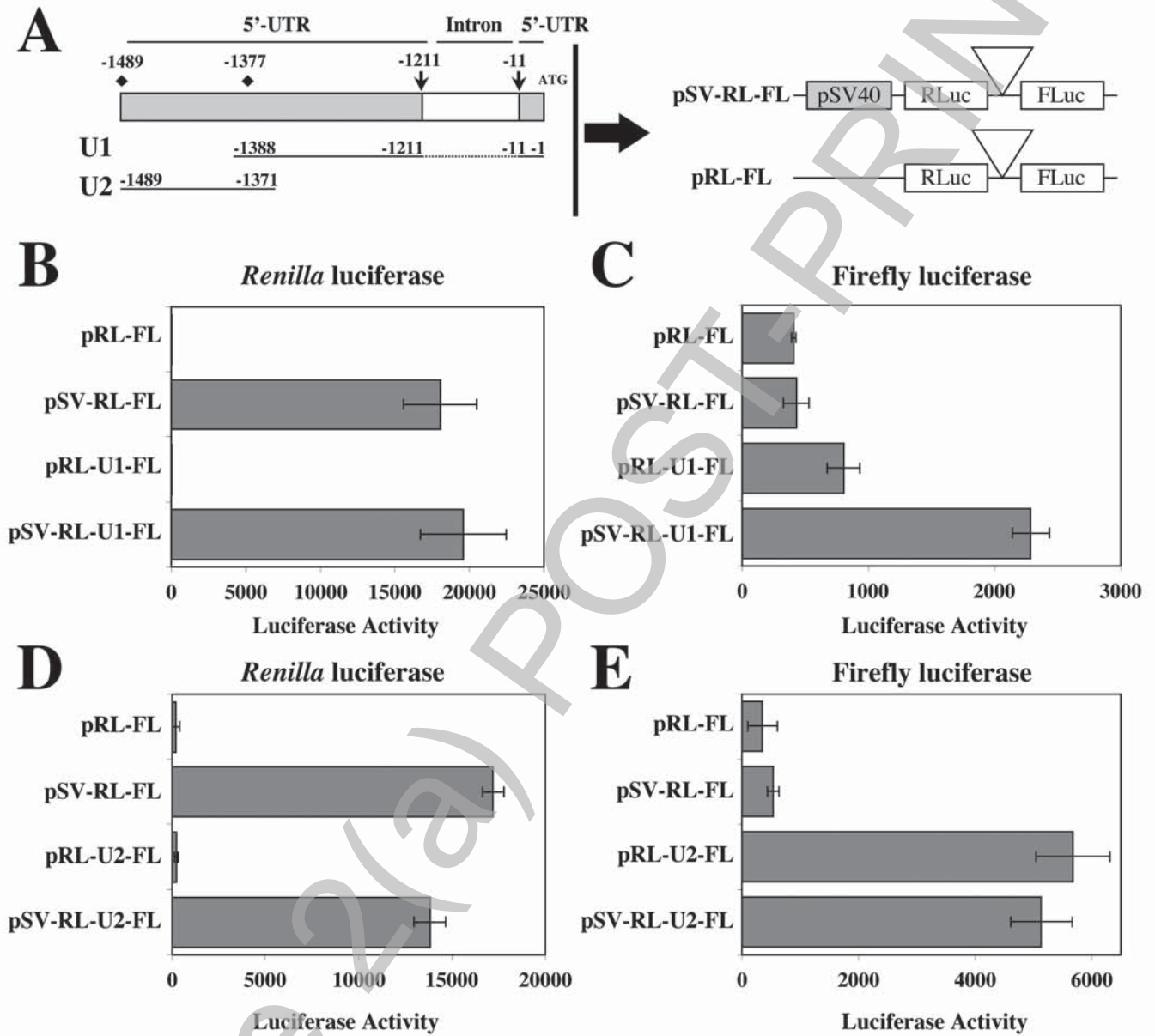


Figure 3

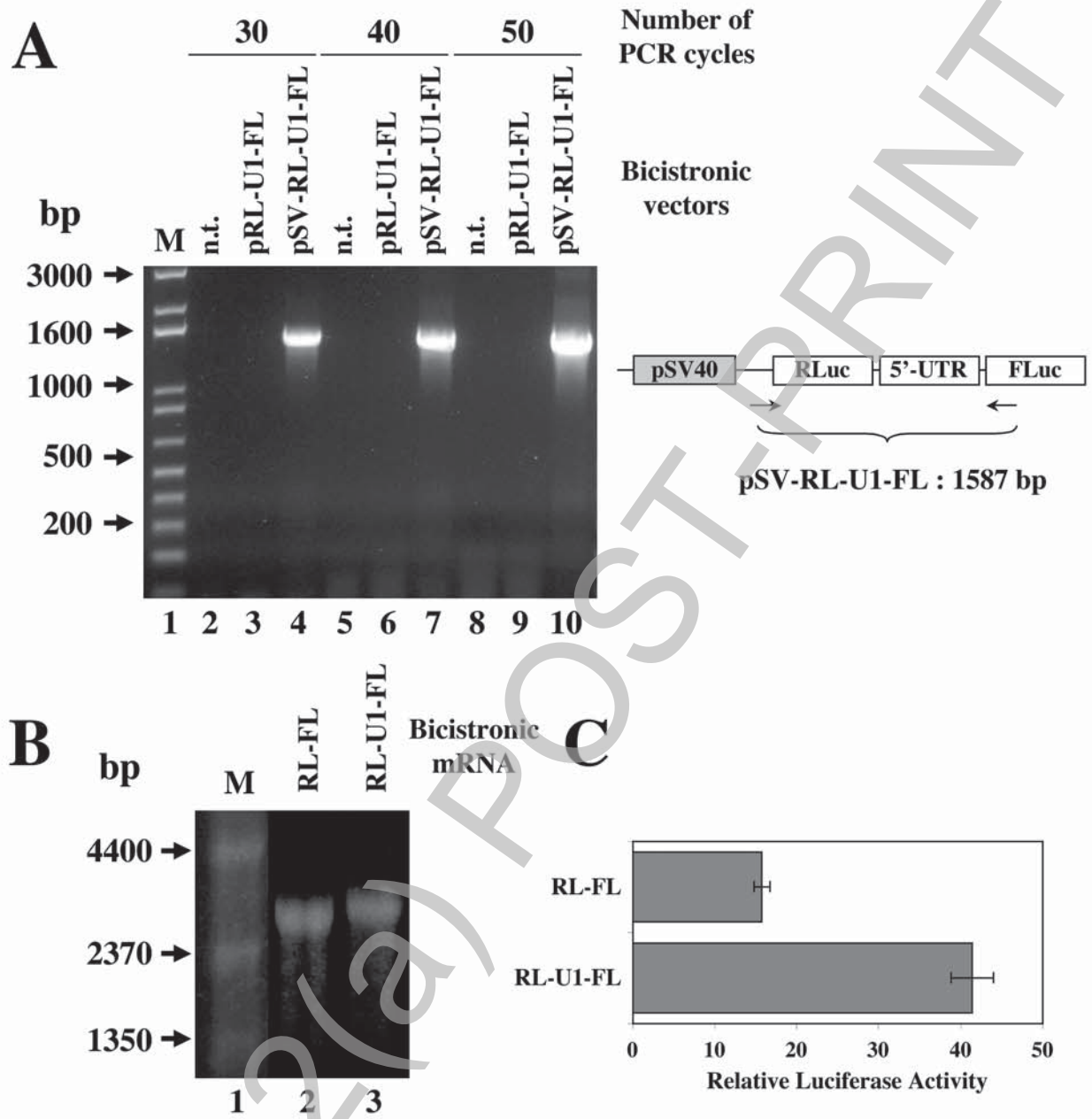


Figure 4

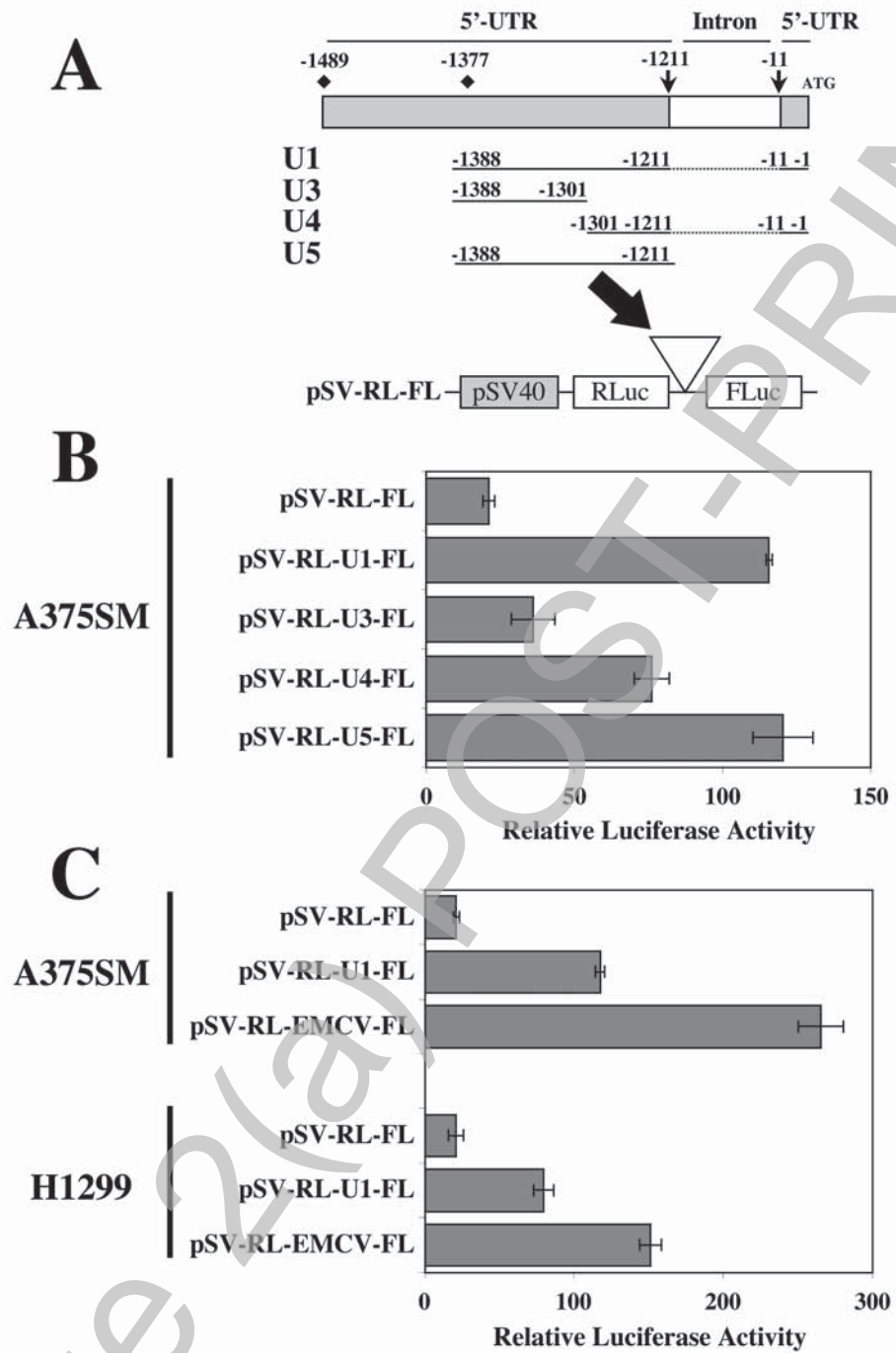


Figure 5

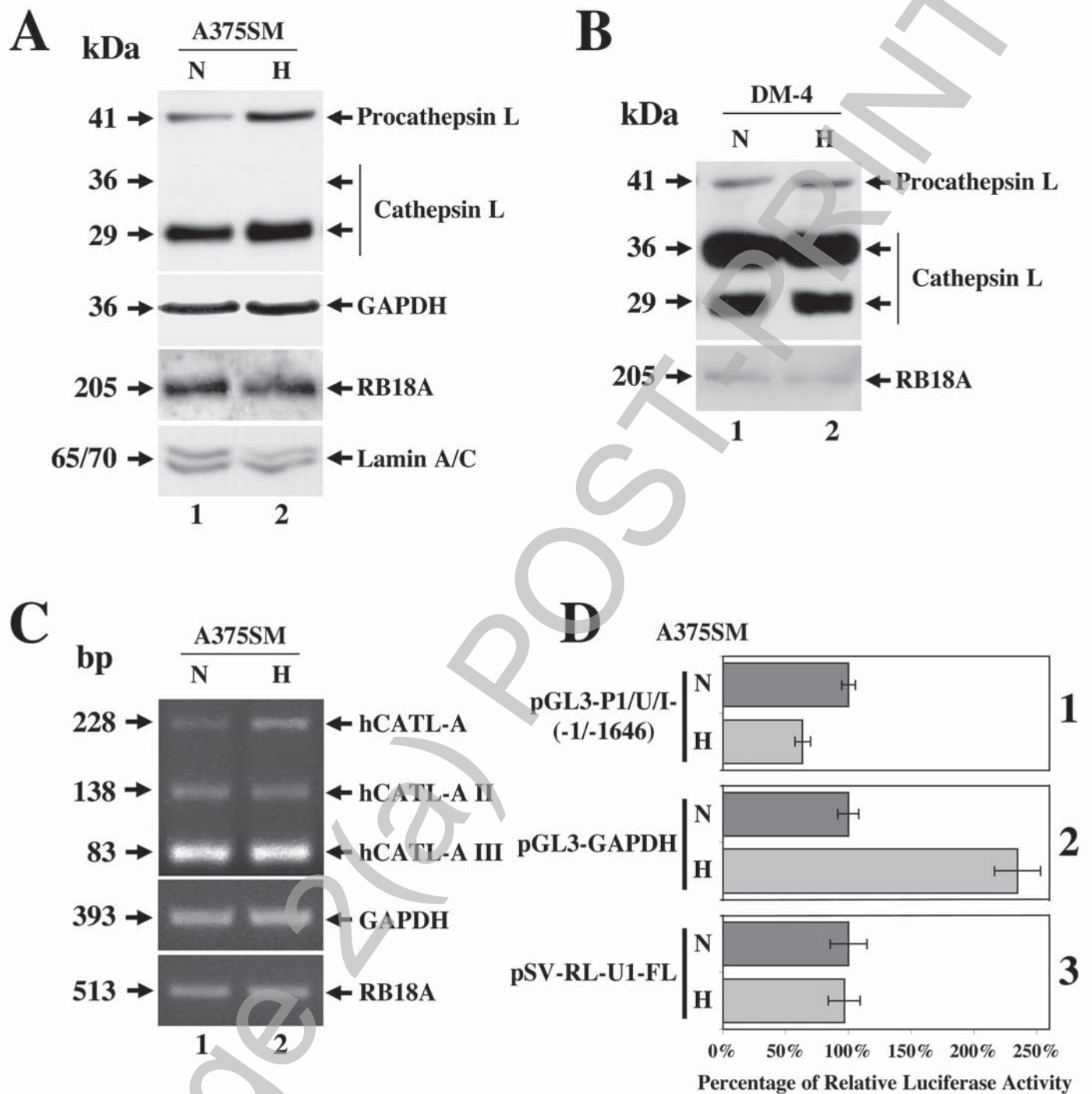


Figure 6