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Fabienne Brenet[1,#], Nadège Dussault[1,#], Christine Delfino[1], Françoise Boudouresque[1], Olivier Chinot[1,2], Pierre-Marie Martin[1,2] and L'Houcine Ouafik[1,2,*]

Affiliation: Université de la Méditerranée, Aix-Marseille II
[1]Laboratoire de Cancérologie Expérimentale, Inserm EMI 0359
[2]Laboratoire de Transfert d'Oncologie Biologique
Faculté de Médecine Secteur Nord, IFR Jean Roche
Bd. Pierre Dramard, 13916 Marseille Cedex 20, France

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# Both authors contributed equally to this work.

*Corresponding author : Dr. L'Houcine Ouafik
Voice : (33) 491698882
Fax : (33) 491090171
E-mail : ouafik.h@jean-roche.univ-mrs.fr

Abstract

Adrenomedullin (AM) is a multifunctional regulatory peptide with important angiogenic and mitogenic properties. Here we identify a region of stable secondary structure in the 5'-untranslated region (5' UTR) of human AM mRNA. Reverse transcriptase polymerase chain reaction of the 5' UTR consistently resulted, in addition to the product with the expected size of 155 base-pair (bp), in a second product with a ~65-bp deletion from the central region of the 5' UTR, suggesting the presence of a secondary structure. The presence of a stem-loop structure was confirmed by probing the 5' UTR with RNases with selectivity for single- or double-stranded RNA. We investigated the role of this stem-loop structure in expression of luciferase reporter gene in cultured cell lines. Reporter assays using a chimeric mRNA that combined luciferase and the 5' UTR of AM mRNA demonstrated a dramatic decrease of the reporter activity due to a decreased translation, while the deletion of the stem-loop structure localized between nt +31 to +95 from the cap site led to the recovery of activity. Gel migration shift assays using cytosolic extracts from mammalian cell lines demonstrate a specific binding of a cytosolic protein to riboprobes containing the 5' UTR of AM but not to riboprobes either corresponding to other areas of the message or containing the 5' UTR but lacking the region of secondary structure. Although we conclude that the 5' UTR of the human AM mRNA can modulate the translation of AM mRNA *in vivo*, and that the predicted stem-loop structure is necessary for this inhibition, the functional consequences of the *cis* element-binding activity remain to be determined.

Introduction

The translational control of specific mRNAs is a prominent mechanism of gene regulation contributing to diverse physiological processes in many cell types. Although many features of a transcript can contribute to its translation, most control elements are located within the untranslated regions (UTRs) of the mRNA. The 5' UTR has a large influence on translation and plays a key role in post-transcriptional gene regulation (Kozak *et al*., 1987, 1991 and 1996; Sonenberg *et al*., 1994; van der Valden *et al*., 1999; Willis *et al*., 1999). The efficiency of translation initiation is largely governed by the composition and structure of the 5' UTR of the mRNA, which is determined by both its length and its sequence. Stable secondary structure and sequence-specific signals and one or two small upstream open reading frames (uORFs) within a 5' UTR can profoundly inhibit protein translation. Most highly expressed mRNAs have relatively short (20-100 nucleotides) 5' UTRs that lack uORFs and extensive secondary structures (Kozak *et al*., 1987). In contrast, mRNAs encoding growth factors, transcription factors, oncoproteins, and other regulatory proteins are poorly translated and are often long, highly structured 5' UTRs with multiple upstream ATGs (Kozak *et al*., 1991, van der Valden *et al*., 1999; Willis *et al*., 1999). Regulatory proteins interacting with specific sequences of the 5' UTR have also been shown to control the rate of translation (Leibold *et al*., 1988; Rouault *et al*., 1988). One or more of these mechanisms could operate in the case of Adrenomedullin (AM) mRNA.

Adrenomedullin (AM) is a widely distributed multifunctional peptide with critical roles in vascular development and function (Kitamura *et al*., 1993; Sakata *et al*., 1994; Hinson *et al*., 2000; Fernandez-Sauze *et al*., 2004; Zaho *et al*., 1998). These functions were discovered thanks to many physiological studies and are highlighted in knockout studies in which AM null mice die *in utero* from extreme hydrops fetalis and cardiovascular abnormalities (Caro *et al*., 2001; Shindo *et al*., 2001). AM is highly expressed in a variety of malignant tissues (Hata *et al*., 2000; Rocchi *et al*., 2001; Miller *et al*., 1996; Ouafik *et al*., 2002) and has been

demonstrated to be mitogenic for human lung, breast, colon, glioblastoma and prostate cancer cell lines (Rocchi *et al*., 2001; Miller *et al*., 1996; Ouafik *et al*., 2002). Incubation of U87 glioblastoma cultures with an AM neutralizing antibody decreased cell proliferation; chronic administration of the AM antibody into U87 subcutaneous xenografts dramatically decreased tumor size and weight. Similar to the studies for VEGF, the density of blood vessels in the AM antibody-treated tumors was also decreased, supporting the roles for AM in angiogenesis, vasculogenesis and/or vessel stabilization (Fernandez-Sauze *et al*., 2004; Zaho *et al*., 1998, Ouafik *et al*., 2002). In good agreement, Ishikawa *et al*. (Ishikawa *et al*., 2003) demonstrated that the AM antagonist (hAM (22-52)-NH_2) inhibited pancreatic cancer cell growth *in vivo* by suppressing tumor vascular development.

Many factors closely regulate AM expression including glucocorticoids, androgens, hyperthyroidism and hypoxia (Hinson *et al*., 2000; Murakami *et al*., 1998; Letizia *et al*., 1998; Pewitt *et al*., 1999; Garayoa *et al*., 2000). However, as for many peptidergic systems, the complex translational mechanism regulating AM production has not been well studied. Accordingly, given the relevance of AM in diverse biological systems, especially the vascular and tumor biology (Hinson *et al*., 2000; Shindo *et al*., 2001; Ouafik *et al*., 2002), and the important physiological consequences of altered AM expression, the present study evaluated the *cis*-acting sequences in the untranslated region of AM mRNA that might participate in the tight regulatory control of AM biosynthesis. Such control could be mediated by the 5' untranslated region (5' UTR) of AM mRNA, since the 5' UTRs of several eukaryotic mRNAs have recently been shown to influence the rate of their translation.

As part of a continuing study on AM function and regulation, we decided to investigate the role of the 5' UTR of the human AM mRNA in gene expression in different cell lines. Here we report the presence of stable a stem-loop structure in the 5' UTR of human AM mRNA and provide evidence that this structure plays a role in the regulation of translation of the AM message. Reporter assays using a chimeric mRNA that combined luciferase and the 5' UTR

of AM mRNA demonstrated a dramatic decrease of the reporter activity due to a decreased translation, while the deletion of the stem-loop structure localized between nt 31 to 95 from the cap site led to the recovery of activity. Using mRNA gel mobility shift assay, we showed that a cytoplasmic protein binds specifically to the stem-loop of the AM mRNA 5' UTR.

Results

Using a pair of primers spanning the full length region of the 5' UTR of AM mRNA for RT-PCR, we always observed the presence of two products, one being smaller than expected. The size discrepancy between products expected and found was consistently approximately ~60 bases. However, RT-PCR products using primers located within the coding region of the mRNA were always of the expected size. Sequencing of RT-PCR products from isolated RNA revealed that the size discrepancies of the products were, in fact, due to the absence of a central region of the 5' UTR consisting of 65 bases (Figure 1). Otherwise, the sequence corresponded to that reported for the 5' UTR of AM mRNA (Kitamura *et al*. 1993). The location of the missing segment was consistent among different RT-PCR amplifications of the same region. Furthermore, sequencing of the PCR fragment generated under low concentration of magnesium chloride resulted in the shift of the smaller major PCR product to the expected larger product.

The nucleotide sequences identified in the human AM cDNA are contained in four exons in the human gene for preproadrenomedullin (Ishimitsu *et al*. 1994). The analysis of AM gene sequence showed that the 65 bp sequence (nt 31 to 95) is included in exon 1 coding for nt 1-134 of the 5' UTR (Figure 1), ruling out the possibility that the 65 bp region is an exon and can be deleted by alternative splicing events. Taken together, these data suggest that the shortened PCR product is not due to the expression of AM mRNA isoform raised by alternative splicing, but rather, due to a secondary structure in the 5' UTR of AM mRNA. To confirm that the missing region was, in fact, present in the mRNA, a cDNA probe of 65 bp corresponding to the deleted region was prepared by PCR from pBShAM clone. Northern blots using this probe confirmed that the deleted region was indeed present in the isolated RNA used for RT-PCR (Figure 2).

Stem-loop structure at the 5' UTR of AM mRNA – Submission of the 5' UTR sequence to RNA folding software (*mfold*) predicted that this region could form extensive, stable secondary structures through internal base pairing (Figure 3B). Predicted structures of this region consistently indicated that ~72% of the bases were paired. This region of secondary structure included more than 50% of the 5' UTR of AM message.

To determine whether this 5' UTR region can in fact fold into higher-order structure, the RNA transcript was analyzed by limited RNase digestion. The 5'-end-labeled 71-nt RNA (nt 24 to 95) was transcribed and allowed to fold before digestion with double strand-specific RNase V1, or single-strand RNase T1, as described in Materials and methods (Figure 3A). The enzyme concentrations were empirically determined to produce on average a single cleavage hit per molecule. As anticipated, fully denatured transcripts were digested completely with RNase T1 (Figure 3A, lane 2). When this RNA was first allowed to fold, specific regions of the molecule showed resistance to digestion with nuclease T1 (lane 3), yet susceptible to RNase V1 (lane 5) attack, suggesting a double-stranded conformation. From these analyses, both weak and strong RNase cleavages were identified on the predicted stem loop structure (Figure 3B). Nuclease T1 cleaves RNA 3' to guanine nucleotides in single-stranded regions, and accordingly produced prevalent products corresponding to cleavages at two tandem guanine nucleotides (nt 40 and 41) within the predicted loop. As the region immediately proximal to the loop (nt 35 to 38) was cleaved by RNase V1, the results were congruent with those predicted for a stem loop structure (Figure 3B). The remaining segments (nt 6 to 20 and nt 57 to 71) were sensitive to RNase V1 double-stranded RNA digestion indicating a long stretch of nucleotide pairing to form the base of the stem.

Regulation of AM mRNA translation by 5' UTR using reporter assay - A prominent stem loop structure in the 5' UTR of the AM mRNA can introduce important RNA-based control elements for posttranscriptional regulation of AM expression. Accordingly, the functional

consequences of the 5' UTR on AM translational efficiency was evaluated using luciferase-based reporter assays and the results agreed with that hypothesis. Several SV40 - firefly luciferase expression constructs were prepared as shown in Figure 4A. The luciferase reporter gene combined with different sequences of 5' UTR of the AM mRNA was placed under the control of the simian virus 40 promoter (Figure 4A). The expression level of the reporter gene was quantified by assaying luciferase luminescence. The amount of firefly luciferase expression 24 h after transfection was expressed as a function of *Renilla* luciferase luminescence (20ng/transfection) to normalize transfection efficiency. Control cells transformed with vector alone showed high luciferase enzymatic activity, whereas the presence of the full-length AM mRNA 5' UTR resulted in a clear decrease of luciferase activity in all the cell lines used. The marked decrease (50% to 70%) in translational efficiency demonstrates that the response was independent of cell type (Figure 4B). In contrast, the enzymatic activity was recovered when the luciferase reporter gene with the truncated AM mRNA 5' UTRΔnt 31-95 was used to eliminate the secondary structure site (Figure 4B).

To ensure that the decrease in firefly luciferase activity reflected decreased translation rather than altered mRNA stability, transcription or RNA nuclear retention, total RNA was also isolated from parallel transfected cells and subjected to slot blot analyses using firefly and *Renilla* luciferase probes. Normalized to *Renilla* luciferase mRNA levels, the resulting analyses revealed that firefly luciferase mRNA expression was comparable between differents luciferase reporter constructs (Figure 4C). Accordingly, the differences in luciferase activity among the different transfectants were not due to changes in firefly luciferase expression levels, from mRNA stability or transfection efficiency, but most likely reflected altered translational efficiency.

Characterization of AM mRNA binding protein (AM mRNA-BP) – To determine whether cytoplasmic extracts might contain a protein(s) that binds to the AM mRNA, band-shift assays

were performed. Gel migration shift assays using riboprobe corresponding to the AM full length cDNA (AM FL) demonstrated the presence of a protein(s) in cytoplasmic extracts of U87 cells able to bind to AM mRNA (Figure 5). To estimate the mass of the AM mRNA binding protein(s) and characterize the composition of the RNA-protein complex, UV light was used to covalently cross-link radiolabeled AM RNA to proteins of U87 cytoplasmic extracts (Wilusz *et al*., 1988). Figure 5 illustrates the apparent migration of the RNA-protein complex on SDS-polyacrylamide gel (Laemmli, 1970). After the radiolabeled transcripts were incubated with cytoplasmic extracts and irradiated, two samples were retained for RNA band-shift analysis (Figure 5A, lanes 2 and 3), and the remaining samples were boiled in Laemmli buffer (Laemmli, 1970) (Figure 5A; lanes 4 and 5). Under these conditions, only a single stable complex migrating with a molecular mass of approximately 14 kDa was observed (Figure 5A), providing evidence for the existence of a single subunit in the AM mRNA-protein complex. No complex formation was observed when nuclear extracts were used (Figure 5A, lane 6), suggesting that the binding elements were derived entirely from the cytoplasm. In the absence of lysate, RNase T1 cleaved radiolabeled AM RNA, and no complex was observed (lane 1). No bands were detected in the absence of UV treatment (data not shown).

Incubation of ^{32}P-labeled AM transcripts with 30 μg cytoplasmic proteins, in the presence of 1- to 100-fold excess of unlabeled AM RNA in competition assays, completely blocked radiolabeled transcript-protein complex formation (Figure 5B; lanes 4 to 6). By contrast, the intensity of the band was not diminished by the addition of equal amounts of the unlabeled nonspecific hPAM RNA competitor (Figure 5B; lanes 7 to 9), suggesting that the U87 cytoplasmic protein(s) bound to the AM mRNA specifically.

To establish that the binding factor was indeed proteinaceous, the U87 cytoplasmic extracts were digested with proteinase K (100 μg/ml) or pronase (100 μg/ml) at 37°C for 15 min prior to RNA probe incubation. Digestion with proteases completely abolished the shifted band (Figure 4C, compare lane 1 with lanes 2 and 3). Heating the cytoplasmic extracts at

100°C for 10 min abolished complex formation (Figure 5C, lane 5). Denaturation of the cytoplasmic lysates with 0.02% SDS was also sufficient to suppress the RNA-protein complex formation (Figure 5C, lane 6). As the data demonstrated that the cytosolic material was heat- and protease-sensitive, the factor was termed AM mRNA-binding protein (AM mRNA-BP).

To map the binding region of AM mRNA to which binds AM mRNA-BP, additional band-shift analyses were performed using deletion variants of the AM transcripts. AM transcripts (AM1 to AM3) were transcribed *in vitro* as described in Materials and methods and assessed for their capacity to bind AM mRNA-BP using U87 cytoplasmic extracts. While the 5'-AM transcript (AM1) demonstrated the same capacity to bind AM mRNA-BP as the AM FL (Figure 5D), no binding was seen with riboprobes corresponding to either the coding region (AM2), or the 3' UTR (AM3) of AM mRNA. These data demonstrate that AM mRNA-BP interacts with a region common to full length AM mRNA and AM1, corresponding to the 5' UTR region of the AM mRNA. To refine the protein-binding site within the 150 nt of the 5' UTR, transcripts with 5' UTR truncations (AM4 to AM9) were radiolabeled and also tested for their abilities to bind AM mRNA-BP (Figure 6). From these studies, AM mRNA-BP binding was restricted to a site within +31 to +95 nt upstream of translation initiation site; very low binding was observed using transcripts (AM5, AM6 and AM8) that overlapped the identified region. Interestingly, no binding was seen with riboprobe of the 5' UTR lacking the region of secondary structure (AM9), suggesting that the region of secondary structure in the 5' UTR was required for binding of this cytosolic factor.

Discussion

This report reveals some novel aspects of posttranscriptional regulation of AM gene expression in different cell lines. We have investigated the role of the stem-loop structure found at the 5'-end of AM mRNA. Our data, using primers spanning the 5' UTR of AM mRNA, demonstrated that, in addition to the RT-PCR product with the expected size, a product shorter than expected by approximately ~65 bases was consistently observed. The sequence of this product clearly demonstrated that the smaller size was due to the deletion of a central region of the 5' UTR of the AM message. This deleted region contained 65 bases (nt 31 to 95) and was capable of forming stable secondary structures with ~70% of the bases paired. Northern blotting with probes specific to the missing region of the 5' UTR showed that the amplification of the smaller RT-PCR product was not due to the absence of the 65 bp region in the RNA used in the reaction. Furthermore, the 65 bp sequence is part of exon 1 sequence in AM gene and unlikely to be deleted by alternative splicing events. Mechanisms accounting for the ability of a polymerase copying a template to skip a region and then resume faithful transcription of the remainder of the sequence have been proposed by others to explain internal deletions occuring during DNA synthesis by DNA polymerase (Glickman *et al.,* 1984; Cariello *et al.,* 1991; Canceill et al., *1996;* Odelberg *et al.,* 1995). In all cases, these models require that the deleted sequence contains a region of stable secondary structure. Our observations were therefore consistent with the presence of a secondary structure in the 5' UTR of AM mRNA across which the polymerase or reverse transcriptase could skip in the PCR or RT-PCR reactions, continuing the replication of the sequence beyond the site of secondary structure. The role of secondary structure in this deletion event is supported by the fact that shortened PCR products were promoted by higher magnesium concentrations, since magnesium has been reported to promote base pairing into higher order structures (Laing *et al*., 1994).

Enzymatic probing has revealed a higher-order structure for the stem-loop in the 5' UTR of AM mRNA. Furthermore, the ability of RNase T1 to digest completely a riboprobe corresponding to the coding region (AM2) and the 3' UTR (AM3) of AM mRNA confirmed that this resistance of the 5' UTR to digestion was not a general characteristic of all regions of the AM message. Stem-loop structure negatively regulates expression of reporter mRNAs at the translational level, suggesting that the 5' stem-loop is sufficiently stable to prevent translation once ribosome scanning has started (Kozak, 1991). Using our constructs in transient transfections into U87, PC3, MCF7 and HTC116 cells, we demonstrated a three to six fold inhibition of the luciferase protein expression. The analysis of steady-state levels of mRNAs showed no destabilization of the mRNA containing the stem-loop compared to mRNA with deleted stem-loop. The stem-loop structure appears to be necessary to modulate the AM mRNA translation. These data provide good evidence for mRNA structure that regulate the expression of AM peptide, although alterations in AM protein stability could not be excluded. Finally, in cell extracts, we could demonstrate binding of a protein factor to the 5' stem-loop, which might be involved in the downregulation of translation.

There is a short, but growing, list of genes containing RNA *cis*-elements that modulate translation: the transcript for FMR protein (Fragile X mental retardation protein) encodes 5' UTR secondary structure which is sufficiently stable to suppress downstream FMR protein translation (Feng *et al*., 1995), tissue-specific regulation of fibroblast growth factor 2 translation appears to be dependent on its 5' UTR (Creancier *et al*., 2000), 15-lipoxygenase, fibronectin, lipoprotein lipase, folate receptor-α, interferon-γ, transforming growth factor-β1, *m-numb*, and neural nitric-oxide synthase (nNOS), among others (Galy *et al*., 2001; Rabganatham *et al*., 2000; Ben-Asouli *et al*., 2002; Ostareck *et al*., 1997; Imai *et al*., 2001; morrisey *et al*., 2001). Similarly, vascular endothelial growth factor regulation by hypoxia (Stein *et al*., 1998) and platelet-derived growth factor 2/*c-sis* expression that occurs during differentiation (Bernstein *et al*., 1997) are regulated in this way as well.

Evidence from gel mobility shift assays clearly demonstrates specific binding of a cytosolic protein(s) to riboprobes containing the 5' UTR of AM mRNA. Such binding is not seen to riboprobes corresponding to other areas of the AM mRNA and appears to require the presence of the region of secondary structure because a riboprobe containing the 5' UTR sequence but lacking the deleted region showed no binding. The RNA-protein binding seems specific to the AM 5' UTR sequences since only an excess of homologous unlabeled AM 5' UTR transcripts blocked RNA-protein complex assembly; unrelated nonspecific transcripts by contrast were unable to compete for protein binding. The proteinacious character of the binding element was verified as protease digestion or heat denaturation of the cell extracts abrogated complex formation, and UV crosslinking studies demonstrated that the binding protein had a mass of approximately 14 kDa. Furthermore, as the AM mRNA-BP was found only in cytosolic and not nuclear extracts, the binding protein was unlikely to participate in pre-AM mRNA splicing or nuclear shuttling.

Secondary structures in the 5' UTR of several mRNAs have been shown to repress translation of those mRNAs, presumably by stalling the "scanning" of ribosomal initiation complexes (Kozak, 1989). Studies involving insertion of synthetic oligonucleotides that form stable hairpin structures within the 5' UTR of preproinsulin (Kozak, 1986) and thymidine kinase (Pelletier, 1987) mRNAs and subsequent expression in COS cells demonstrated that excessive secondary structure in the 5' UTR represses translation. Furthermore, structures with predicted free energies greater than –50 kcal/mol were able to inhibit translation by 85-90%, whereas structures with predicted weaker secondary structure were readily translated (Kozak, 1986). We cannot exclude that the interaction of AM mRNA-BP with the stem-loop structure could be responsible of a steric hindrance impeding the fixation of the initiation complex and/or could better stabilizes the stem-loop structure to be higher than –30 kcal/mol and is thus sufficient to block scanning ribosomes to reach the start codon. Recently, it was demonstrated that binding of IRP-1 to the IRE located close to the cap structure of mRNAs

represses translation by precluding the recruitement and formation of the 43S preinitiation complex (Paraskeva *et al*., 1999).

In summary, this report reveals some novel aspects of posttranscriptional regulation of AM gene expression in various cell types and demonstrates the ability of the 5' UTR of AM mRNA to repress AM mRNA translation. However, our study poses several questions related to mechanisms of AM mRNA-BP actions in translation inhibition and the regulation of AM mRNA-BP itself. The 14 kDa AM mRNA-BP may interfere with ribosome binding and/or scanning to impede efficient translation. The ongoing purification and identification of the AM mRNA-BP will greatly contribute to our understanding of AM gene regulation and would permit examinations of its sites of actions and address whether initiation or downstream AM translational events are inhibited upon stem loop-AM mRNA-BP complex formation. This work also raises the question as to how this translational repression is overcome by inducing stimuli.

Materials and methods

Cell culture - All the cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). Human glioblastoma cell lines U87 and U138 were maintained in minimum essential medium (MEM); MCF-7 breast cancer cell line was maintained in DMEM/F-12 medium, and PC-3 prostate cancer cell line was maintained in RPMI 1640 medium. Among colon cancer cell lines, HCT 116 was cultured in Dulbecco's modified Eagle's Medium (DMEM). All of the culture media were supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Cergy Pontoise, France), penicillin (50 U/ml), streptomycin (50 μg/ml), glutamine (1 mg/ml), and the necessary associated factors for each cell line. The cells were cultured at 37°C under humidified 95% air/5% CO_2 atmosphere. The medium was replaced every two days.

Northern blot Analysis - Total RNA was prepared from U87 and U138 cell lines using the acid guanidinium isothiocyanate/phenol/chloroform procedure (Chomczynski *et al*. 1987). Northern blot analysis was performed essentially as described previously (El Meskini *et al*. 1997). Briefly, total RNA (10 μg) was resolved on 1% agarose-formaldehyde denaturing gel. The denatured RNAs were transferred to Hybond-N membranes (Amersham Biosciences, France) by capillary action in 10X SSC [1.5 M NaCl, 0.15 M sodium citrate (pH 7.0)], cross-linked by UV irradiation and hybidized to [α–^{32}P]-labeled human cDNA probes corresponding to the region of the AM 5' UTR nt 31 to 95 and 1.2-kb AM cDNA (Kitamura *et al.* 1993), respectively. Filters were prehybridized, hybridized, and washed as described previously (El Meskini *et al.* 1997).

Enzymatic mapping of the RNA structure - Radiolabeled 5' UTR stem-loop RNA was synthesized by *in vitro* transcription end-labeling with 30 μCi of [γ-^{32}P]GTP using T_7 RNA polymerase (Invitrogen Life Technologies). The precipitated labeled RNA was washed and dissolved at 5 x 10^4 cpm RNA/20 μl in 20 mM HEPES RNA renaturing buffer, pH 7.6, containing 200 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$. The RNA was heated to 85°C for 2 min, renatured by slow cooling to room temperature for 30 min, and placed on ice. Transfer RNA (0.15 mg/ml) was added to the tubes and the RNA aliquoted at 2 μl/ tube for RNase digestion. Twenty thousand counts per minute of the RNA in a total volume of 20 μl was digested with double-strand-specific RNase cobra venom nuclease V1 (RNase V1; 2.5 x 10^{-6} U/ml; Ambion) for 1 min at RT, or a single-strand-specific RNase T1 (1.5 x 10^{-4}; 6 x 10^{-5}; or 2.5 x 10^{-6}μg/ml; Roche Applied Science) for 1 min at RT. After the indicated times, the reactions were stopped by extraction with phenol-chloroform and the RNAs were precipitated with ethanol. As a control, the RNA was denatured and digested with 2.5 x 10^{-2} μg/ml of RNase T1 for 10 min at RT. All samples were analyzed on 8% denaturing polyacrylamide gels. Nucleotide assignments of the cleavage sites were made relative to the position of two G's in the top loop as they were resolved in the RNase T1 lane.

Preparation of luciferase constructs – The full-length human AM cDNA (1490 bp) was obtained by polymerase chain reaction (PCR) synthesis from total RNA prepared from U87 glioblastoma cell line and subcloned into *Srf* I site of the PCR-Script SK^+ plasmid (Stratagene Europe). The resultant plasmid, containing hAM cDNA in the sense orientation under the control of the T_3 polymerase promoter, was sequenced for fidelity and designated pBS-hAM. A cDNA containing the full length 5' UTR of AM was produced by PCR using pBS-hAM as a template. A cDNA containing the 5' UTR of AM, but lacking the region of secondary structure,

was produced by RT-PCR using total RNA prepared from U87 cells and previously reverse transcribed to cDNA. The upper primer AM5'sense(a) (AM $cDNA_{1-20}$; 5'-CGCCATGGCTTGGT GACACTGGATAGAA-3') was paired with lower primer AM5' antisense (b) (AM $cDNA_{150-130}$; 5'-CGCCATGGAGACCCTGCTAAGAGTGA-3') to produce 150 bp and 85 bp fragments, respectively. The PCRs were carried out for 35 cycles, with each cycle consisting of denaturation (94°C for 30 s), annealing (65°C for 30 s), and extension (72°C for 30 s) using *Taq* DNA polymerase (invitrogen life Technologies). The underlined sequences correspond to *Nco* I sites included for subsequent subcloning. The products were inserted into TOPO vector (Invitrogen Life Technologies) for sequencing to verify deletion and recloned into the *Nco* I site of the PGL3 vector (Promega, Lyon, France) to generate AM 5' UTR1-150/Luc (construct b) and 5' UTRΔ31-95 /Luc (construct c), respectively. All constructs were verified by direct sequencing.

Luciferase reporter assay and Slot blot analysis of reporter mRNA - A dual luciferase reporter assay system was employed. U87 Cells (2 x 10^5 cells/ml) were transfected with a mixture of firefly luciferase reporter vector (200 ng), *Renilla* luciferase control vector pRL-TK (20 ng, Promega, Lyon, France), and pBS SK^+ vector (780 ng, 1 μg vector total), using the Fugene 6 transfection reagent (Roche Applied Science). After 24 h, the cells were lysed with assay buffer (Promega) and both firefly (*Photinus pyralis*) (reporter) and sea pansy (*Renilla reniformis*) (control) luciferase activities were measured sequentially using the Dual-Luciferase Assay Reporter System (Promega) with a Berthold lumat LB9507 luminometer. The ratio of reporter luciferase activity in relative light units was divided by the control *Renilla* luciferase activity to give a normalized reporter luciferase value.

For RNA analysis, U87 cells were transfected as above for total RNA extraction using the acid guanidinium isothiocyanate/phenol/chloroform procedure (Chomczynski *et al*., 1987). Slot blot analysis was performed essentially as described previously (El Meskini *et al*. 1997)

to assess reporter luciferase mRNA and control *Renilla* luciferase mRNA expression. Briefly, total RNA (10 μg) was denatured by heating at 65°C for 15 min in 2.2 M formaldehyde, 6XSSC (1X SSC: 0.15 M NaCL, 0.015 M sodium citrate, pH 7.0) and cooled on ice before applying on a Hybond-N membrane (Amersham Biosciences) by using the Schleicher & Schuell (Keene, NH) Minifold II Slot-Blotter. After 1 x 10^6 cpm of the ^{32}P-labeled probe/1 ml of hybridization solution was added, filters were hybridized and washed as described (Brenet *et al*., 2005). The expression levels of luciferase/AM-5' UTR chimeric mRNA versus those of control *Renilla* mRNA in U87 cells were determined from the autoradiographic densitometry using NIH Image 1.54 software (National Institutes of Health, Bethesda, MD).

Cell extract preparation - Cells from confluent cultures (75-cm^2 flasks) were collected by centrifugation, washed once with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline and homogenized on ice in lysis buffer A containing 10 mM HEPES (pH 7.5), 40 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.2% Nonidet P40, 5% glycerol, 10 μg/ml leupeptin and 0.5 mM phenylmethylsulfonylfluoride (PMSF), using a teflon pestle. The homogenate was centrifuged at 800 x g for 10 min at 4°C to yield a P_1 pellet containing nuclei and a S_1 supernatant fraction. The S_1 fraction was centrifuged at 100,000 x g at 4°C for 30 min (Beckman Ti50 rotor) and the resulting supernatant retained as cytosol extract and stored at –70°C. The P_1 pellet was resuspended in 1 ml TKM buffer containing 0.25 M sucrose buffer (TKM; 50 mM Tris-HCl, pH 7.5, with 25 mM KCl, 5 mM $MgCl_2$, and inhibitors). The sample sucrose concentration was adjusted to approximately at 1.62 M by addition of 2 ml TKM buffer containing 2.3 M sucrose buffer; this material was layered onto 1 ml TKM 2.3 M sucrose buffer and centrifuged at 100,000 x g for 30 min at 4°C (Beckman SW60 rotor). The resulting nuclei pellet was resuspended in 100 μl of buffer A, homogenized and centrifuged at 12,000 x g for 10 min and nuclei protein extracts were frozen rapidly and stored at –70°C.

Sample protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce chemical Co., Interchim, Paris).

Plasmid constructions - The pBS-hAM plasmid was linearized with *Kpn* I to allow generation of the full-length 1400 nt hAM transcript (AM FL). PCR was used to generate 5' UTR (AM1; bp 1-150), open reading frame sequence (AM2; bp 157-714), and 3' UTR (AM3; bp 715-1400) truncated hAM cDNAs (see Figure 5D). To generate the described constructs the following primers were used. A 5' primer that included a T_7 RNA polymerase promoter sequence, which is underlined: (AM $cDNA_{1-20}$; 5'-AATACGACTCACTATAGGGCTTGGTGAC ACTGGATAGAA) was paired with 3' primer (AM $cDNA_{150-130;}$ 5'-AGACCCTGCTAAGAGTGA-3') to generate AM1. The 5'-AM $cDNA_{166-186}$; 5'-AATACGACTCACTATAGGGATGAAGCTGG TTTCCGTCGC-3' primer was paired with 3' primer (AM $cDNA_{724-700}$; 5'-CTAAAGAAAGTGGG GAGCAC-3') to generate AM2. For 3' UTR hAM cDNA, the sense primer (AM $cDNA_{725-745}$; 5'-AATACGACTCACTATAGGGGATTTAGGCGCCCATGGTAC-3') was paired with the antisense primer (AM $cDNA_{1400-1380}$; 5'-CCTTCTTCCACACAGGAGGT-3') to generate AM3 (Figure 5D). The amplified DNA fragments were excised after electrophoresis in low-melting-point agarose, precipitated and used for *in vitro* transcription.

Preparation of RNA transcripts - The *in vitro* transcription reaction mixture (20 μl), containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 20 μM DTT, 5 mM each rATP, rGTP, and rCTP (Invitrogen Life Technologies), 10 μM UTP, [α-^{32}P]UTP (50 μCi, 3,000 Ci/mmol; Amersham Biosciences), 20 U of T_7 RNA polymerase (invitrogen Life Technologies), 1 μg DNA template, and 20 U RNasin (Promega), was incubated at 37°C for 1 h. After template digestion, the radiolabeled RNA was precipitated and dissolved in 10 μl loading buffer (7M urea and 0.025% bromophenol blue) for electrophoresis on an 6%

polyacrylamide gel containing 7M urea. After autoradiography, the full-length radiolabeled RNA transcripts were identified and eluted from excised gel slices by incubation in 0.5 M ammonium acetate buffer, containing 1 mM EDTA, 1 mM DTT, 10 μg tRNA, and 5 U RNasin, overnight at 37°C. The radiolabeled probes were precipitated, reconstituted in water at a specific activity of 5 x 10^7 - 5 x 10^9 cpm/μg RNA, and stored at –80°C. Unlabeled RNA transcripts were synthesized as described above with 5 mM rUTP, processed, quantitated by absorbance at 260 nm, and stored at –80°C until use.

Gel mobility shift assay for RNA-protein interactions - RNA mobility shift assays were performed as described previously (Fraboulet *et al*., 1998; Brenet *et al*., 2005). Unless otherwise indicated, cytoplasmic extracts (30 μg) were incubated with radiolabeled transcript (2-3 x 10^5 cpm) in 20 μl of 15 mM HEPES (pH 8.0), containing 5 μg yeast tRNA, 10 mM KCl, 10% glycerol, and 1 mM DTT, for 10 min at 30°C prior to the addition of 0.1 mg/ml RNase A. For competitions studies, unlabeled AM RNAs were added 15 min before radiolabeled RNA to protein fraction in the buffer described above. Following a 20 min incubation with RNase A at 37°C, 4 μl of 6X native gel loading buffer (30% glycerol, 0.025% bromophenol blue and 0.025% xylene cyanol) were added, and the RNA-protein complexes were resolved on a 5% polyacrylamide gel. After RNase A digestion, UV cross-linking studies were performed by exposing the reaction mixtures on ice for 10 min to 254 nm UV light in a Stratalinker (Stratagene Europe) on the automatic settings. Subsequently, the cross-linked samples were either resolved directly on 10% polyacrylamide-SDS gels or previously boiled for 5 min in 1X SDS sample buffer. Gels were dried and exposed to X-ray film for 10 to 15h with intensifying screen at –70°C. All the experiments were performed at least three times.

RNA folding analysis - Mfold RNA/DNA folding program available through Rensselaer Polytechnic Institute webserver (bioinfo.math.rpi.edu/mfold/rna/) was used to predict AM 5' UTR secondary structure (Zuker *et al*., 2003).

Statistical analyses - Transfection and luciferase mRNA decay data are shown as mean ± SEM. Statistical analyses were performed using the one-way ANOVA followed by Fisher's protected least significant difference test (Statview 512, Brain Power Inc., Calabasas, CA). Statistical significance was assessed at $P < 0.05$.

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Figures legends

Figure 1. Comparison of the reported sequence with the sequences of the RT-PCR products. Only 5' UTR with sequence of coding region and exon 1 (nt 1 to 134) sequence of preproAM gene are shown. Bases are numbered as in Kitamura *et al*. (1993). The RT-PCR products were synthesized using primers a and b (see Materials and methods for primer sequences and sites), and total RNA isolated from U87 cells. PCR product was generated using primers a and b with pBS-hAM as the cDNA template that contains the entire 5' UTR of human AM mRNA. Upper and lower case letters indicate sequence of 5' UTR or exon 1, and intron 1 (Ishimitsu, *et al*. 1994), respectively. Translation signals are shown in bold.

Figure 2. Presence of the deleted sequence in total RNA preparations used for RT-PCR. Total RNA (10 μg) isolated from U87 and U138 cell lines were separated by 1% agarose-formaldehyde denaturing gel electrophoresis, transferred to Hybond-N membranes, and probed first (lanes 1 and 2) with a cDNA probe (nt 31 to 95) corresponding to the region of the AM 5' UTR deleted in the RT-PCR reaction. The membrane was then stripped until no radioactivity could be detected and was subsequently reprobed with a human AM cDNA (nt 200 to 1200) (lanes 3 and 4). Migrations positions of 28 and 18 S rRNAs, as determined using ethidium bromide staining, are indicated for reference.

Figure 3. AM 5' stem-loop structure. A, enzymatic probing of the synthetic 5' stem loop RNA. 5'-end-labeled human AM 5' RNA with the predicted stem loop sequence was subjected to digestion with RNase T1 under denaturing conditions (lane 2), or in its folded conformation (lanes 3-5) or with RNase V1 (lane 6) in its folded conformation. Cleavages after G nucleotides is indicated by open arrows. The cleavage products were separated on an 8% polyacrylamide

sequencing gel. B, Sequence of the human AM 5' stem-loop; nt 24 to 95 from the 5'-end are shown. Cleavages sites of RNase T1 are indicated by arrows.

Figure 4. Effect of the AM 5' UTR on the translational efficiency of chimeric RNA. A, Schematic representation of luciferase reporter constructs containing the 5' UTR of AM (b), the AM 5' UTRΔnt 31-95 (c), and pLuc vector (a). All the constructs were under the simian virus 40 (SV40) promoter. B, Luciferase reporter assay. U87, PC3, MCF7, and HTC116 cells were transiently cotransfected with the indicated luciferase reporter constructs and the *Renilla* reporter construct (pRL-TK; see Materials and methods) for 24 h. The results are presented as ratios of firefly luciferase reporter activity over sea pansy (*Renilla*) luciferase activity, the latter being used as a control. Each bar represents the mean ± SEM; asterisks indicate that the values are significantly different between a, c, and b (**, $p < 0.008$; ***, $p < 0.0001$). Similar data were obtained from three independent experiments. C, U87 cells were cotransfected with the indicated luciferase reporter constructs and the *Renilla* reporter construct. Total RNAs were prepared 24 h later. Slot blots containing 10 μg of each total RNA were hybridized to probe corresponding to firefly luciferase cDNA and exposed to X-ray film at –70°C with an intensifying screen. The blot was stripped and reprobed with ^{32}P-labeled *Renilla* luciferase probe to normalize data for quantification. Each bar represents the mean ± SEM of three independent experiments. The same results have been obtained with PC3, MCF7, and HTC 116 cells (not schown).

Figure 5. Formation of specific RNA-protein(s) complexes between AM mRNA and U87 cytoplasmic extracts. A, identification of a single cytosolic protein interacting with labeled hAM RNA. ^{32}P-labeled riboprobe hAM (2 x 10^5 cpm) was incubated at 30°C with U87 cytoplasmic extracts (30 μg) for 10 min before RNase A digestion. After incubation with RNase A, ^{32}P-labeled RNA-protein complexes were UV cross-linked as described in Materials and methods. The binding

mixtures were analyzed by electrophoresis on a SDS-10% PAGE. No RNA-protein complex was observed in the absence of protein extracts (lane 1). Band shift assays, performed in the presence of protein extracts without (lanes 2 and 3) or with (lanes 4 and 5) SDS sample buffer treatment, showed a 14 kDa protein complex formation. No RNA-protein complex was observed in the presence of nuclear extracts (lane 6). B, no RNA-protein complex formation can be observed when protein extracts were omitted (lane 1). Labeled RNA was incubated with protein extracts alone (lanes 2 and 3) or in the presence of increasing amounts of either unlabeled hAM transcript (specific competitor; 1-, 10-, and 100-fold molar excesses in lanes 4, 5, and 6, respectively) or unlabeled human Peptidylglycine α-amidating monooxygenase (hPAM) transcript (nonspecific competitor; lanes 7, 8, and 9). C, a complex at 14 kDa was observed using samples without proteases treatment (lanes 1 and 4). Treatment of protein extracts with proteinase K (PK; 100 μg/ml) or pronase (Pro; 100 μg/ml) at 37 ^{o}C for 15 min prior the addition to RNA-binding reaction mixtures containing ^{32}P-labeled RNA abolished complex formation (lanes 2 and 3). Heating the protein extracts for 10 min at 100 ^{o}C abolished the binding activity to form complex with labeled RNA (lane 5). Treatment of protein extracts with 0.02% SDS also prevented the complex formation (lane 6). D, Summary of RNA band shift analysis performed with deletion variants of the AM cDNA. Radiolabeled RNA probes containing the indicated regions of the AM cDNA were prepared as described in Materials and methods. Band shift assays were performed with 30 μg of U87 cytoplasmic extracts and each RNA. Each RNA probe either showed maximum binding (++), as illustrated in A for the full length AM riboprobe, or no binding (--).

Figure 6. AM mRNA core binding site maps within the 65-nt segment of nt 31-95 of the AM 5' UTR RNA. A, summary of deletion analysis of the AM 5' UTR band shift complex. The open bar is a representation of a complete 155-nt AM 5' UTR. RNA probes were prepared as described in

Materials and methods. The first and last nucleotides of each transcript are indicated. B, UV cross-linking was performed with U87 cytoplasmic extracts (30 μg) and each described RNA probe.