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The 5' untranslated region of the anti-apoptotic protein Survivin contains an inhibitory upstream AUG codon

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ABSTRACT

Survivin (BIRC5) is an anti-apoptotic protein that is important in cancer. Mechanisms responsible for controlling Survivin levels in cells include transcriptional regulation and modulation of protein stability via post-translational modifications; however to date, translational control has been poorly studied. Here, we focused particularly on the primary control elements present in the Survivin 5′ untranslated region (5′UTR). Bioinformatic analysis of ribosome occupancy on the Survivin 5′UTR revealed the presence of elongating ribosomes upstream of the canonical initiator AUG, suggesting an alternative upstream initiator AUG (uAUG) might exist. This uAUG was found out-of-frame at position −71 and appeared as a conserved element in mammals. RACE analysis revealed different transcriptional start sites for BIRC5, which indicated that translational control by this uAUG is restricted to longer 5′UTR variants. We studied the activity of the uAUG in different cell types by cloning the Survivin 5′UTR DNA sequence (wild-type and mutated variants) upstream of renilla luciferase (RLuc) into a pcDNA3 plasmid. Changes in RLuc activity were determined by luminescence assays and Western blotting. Results showed that when this uAUG was mutated to AUU or AGG in the cloned Survivin 5′UTR, RLuc activity was significantly increased. Similar results were obtained when uAUG was positioned inframe with the RLuc initiator AUG. Immunodetection of Renilla (35 kDa) by Western blotting revealed the presence of a second band (37 kDa approximately) in cells transfected with the Inframe reporter constructs, indicating that the uAUG was functional in our experimental conditions. In conclusion, our experimental data demonstrate the presence of an alternative and inhibitory initiator uAUG in the Survivin 5′ UTR. This inhibitory uAUG may help understanding how Survivin expression is downregulated under physiological or pathological conditions.

1. Introduction

The human protein Survivin (human BIRC5 gene) is a member of inhibitor-of-apoptosis protein (IAP) family that participates in many important processes, including reproduction, apoptosis, cell cycle, development, metabolism, cell communication, and angiogenesis [1]. Importantly, Survivin is strongly upregulated in most human cancer types where its presence promotes tumor growth, survival, metastasis, as well as neo-vascularization [2].

BIRC5 contains 4 exons and 3 introns spanning 14.796 base pairs at the chromosome location 17q2512 [3]. Of note, BIRC5 seems to be transcribed from different transcription start sites (TSS) in HeLa cells at −57/-61 and −72 [4]; however, the relevance of these sites in the control of Survivin expression has not been addressed. Presence of such alternative 5' ends may reveal control elements-including alternative start sites or secondary structures-that

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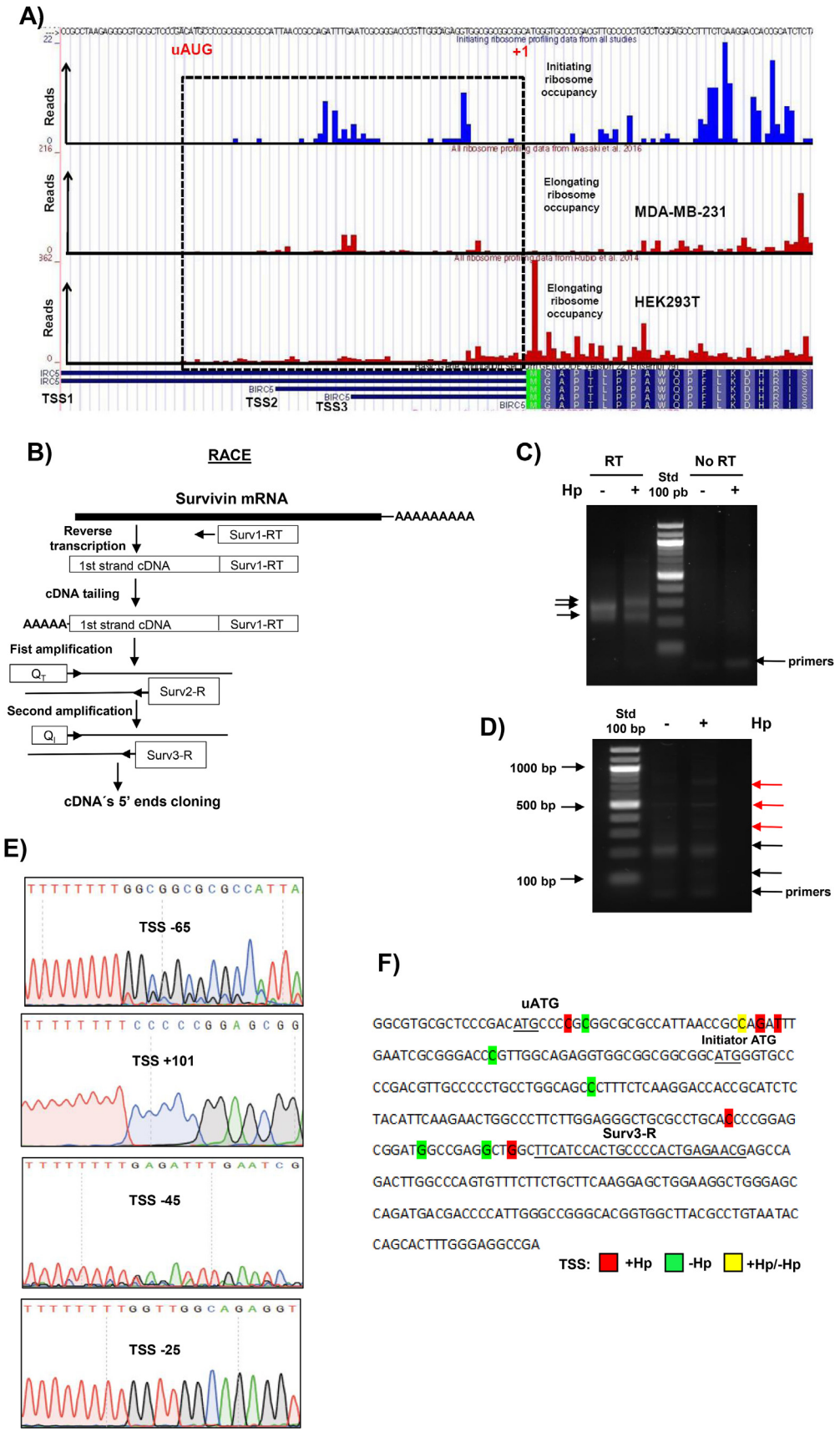

Fig. 1. Survivin 5′UTR presents a putative upstream initiator codon at -71. (A) Profile of initiating ribosome (Global data from several cell lines, blue columns) and elongating ribosome (specific data from MDA-MBA 231 and HEK293T cells, red columns) occupancy in the Survivin 5′UTR with nucleotide resolution. Data available at GWIPS page (http://gwips.ucc.ie/cgi-bin/hgGateway), showed initiating and elongating ribosome accumulation upstream of the canonical Survivin ORF following cycloheximide treatment [24], as

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determine specific translational regulation [5].

To date, the best-characterized mechanisms implicated in the control of Survivin expression include transcriptional activation/repression [2], modulation by miRNAs [6] and regulation of protein stability mediated by post-translational modifications, which also determine Survivin function and subcellular localization [7]. Survivin is poorly expressed in the G1 phase, increases by 6-fold in the S phase and by more than 40-fold in the G2/M phase of the cell cycle [8]. These changes are attributed to the presence of *cis*-regulatory elements in the promoter region [4]. In addition to this basal regulation, the Survivin promoter is activated by a wide variety of transcription factors [2]. Additionally, the presence of certain single nucleotide polymorphisms (SNPs) found in the Survivin promoter region, such as the −31G-C transversion (rs9904341) have been associated with elevated protein levels and increased cancer risk [9].

On the other hand, translational control mediated by 5′ untranslated region (5′UTR) is another possible mechanism that has essentially been ignored to date. Following nuclear splicing and polyadenylation, mature mRNAs are actively exported to the cytoplasm where they are translated into proteins [10]. Translation initiates when the eIF4E initiation translation factor recognizes the m7GTP cap modification present in the 5′ region of mature eukaryotic mRNAs [11]. Then, eIF4E binds eIF4G which recruits the 43S pre-initiation complex that scans the mRNA in a 5′ to 3′ direction until a favorable AUG initiation codon is detected. At that site, the large ribosomal subunit 60S attaches to form the catalytically competent 80S ribosome [11]. Additionally, the interaction between eIF4G and the poly (A)-binding protein (PABP) circularizes the mRNA to generate a spatial configuration required to allow 3′UTR-binding factors to regulate translation initiation, elongation and/or termination [12].

Bearing in mind these basic elements of translation, two different modes of regulation are described. Global control by which translation of most cellular mRNAs is regulated and mRNA-specific control, where a group of defined mRNAs is regulated without affecting general protein biosynthesis [11]. Global regulation is basically achieved by modifying the phosphorylation state of initiator factors, by disrupting the eIF4E-eIF4G interaction or by PABP cleavage by caspase-3 or viral proteases [13]. On the other hand, specific control of mRNA translation may involve also the untranslated terminal regions (UTRs). This particular mode of control is driven by regulatory proteins or RNA molecules that recognize particular unstructured primary or secondary elements present in the 5′- or 3′-untranslated terminal regions of the target mRNAs [14]. A relevant element in translational control is the presence of alternative upstream AUGs (uAUG) in the 5′UTRs. These uAUGs have different effects on the translation of a messenger RNA. For example, when found inframe with the initiator AUG, these generate proteins with different N-terminal ends that in turn have different functions [15] or produce peptides able to repress translation by interacting with the translational machinery or by generating aberrant peptides as a consequence of a shift in the reading frame [14,16]. Of note, these primary and structural control elements present in 5′UTRs are important to define translation during stress conditions, for instance, by recruiting specific stress-associated factors or modulating alternative translation from uAUGs [17].

In the present study we describe the presence of a novel primary translational control element present in the Survivin 5′UTR.

2. Material and methods

2.1. Cell culture

The human gastric AGS (ATCC CRL-1793) and the cervical Hela (ATCC CCL-2) cancer cell lines were cultured in RPMI-1640 medium (Gibco). The human gastric GES-1 (kindly gifted by Dr. Dawit Kidane, University of Texas, USA) and the embryonic kidney HEK293T (ATCC CRL-3216) immortalized cell lines were cultured in DMEM high glucose (Gibco), as reported elsewhere [18].

2.2. Construction of Survivin 5′UTR-renilla luciferase reporter plasmids

The Survivin 5′UTR long region was amplified using the Phusion High-Fidelity polymerase chain reaction (PCR) Master Mix with GC Buffer (Thermo Scientific) and the primers F-*Hpa*I 5′-ccgGTTAACCCCAGAAGGCCGCGGGGGGTGGAC-3′ and R-*Bam*HI 5′-ccgGGATCCCC**CAT**GCCGCCGCCGCCACCTCTGCC-3’ (Integrated DNA Technologies, IDT). Template DNA was obtained by combining two complementary ultramer^{R} DNA oligonucleotides (IDT), corresponding to the positive and negative strands of the Survivin 5′UTR sequence (121 bp). The resulting fragment was digested and cloned into the pcDNA3-renilla luciferase (pcDNA3-RLuc) previously described [19]. Survivin 5′UTR variants were obtained as detailed in supplementary material.

2.3. Rapid amplification of cDNA ends (RACE)

The specific acquisition of 5’ ends of Survivin cDNAs was performed as previously described by Scotto-Lavino et al. [20]. A more detailed protocol is presented in supplementary material.

2.4. Reporter assay

Cell transfection was performed using the reagent Viafect^{R}, following instructions provided by the manufacturer (Promega). After 24 h of transfection, cells were lysed, and protein extracts were used to determine FLuc and RLuc activities using the Dual-Glo® Luciferase Assay System (Promega). Luminescence readouts were quantified using a multiplate reader Infinite M200Pro (TECAN).

indicated by reads of occupancy in this region (area highlighted by the dashed square).**(B)** Diagram of RACE protocol. **(C and D)** Polyadenylated 3′ end single strand cDNAs prepared from AGS cells, infected or not with *H. pylori* (+/− *H. pylori* infection), were amplified by PCR using the primers Surv2-R and Q_T–F **(C)** or Surv3-R and Q_I **(D)**. PCR samples were resolved by agarose gel electrophoresis (2%). As a control, samples without reverse transcriptase addition were also amplified (No RT). Non consumed primers and amplicons are indicated by arrows to the left side of the image. A 100bp DNA ladder was also included **(E)** Representative sequencing profiles found for Survivin cDNA 5′ ends by RACE analysis in AGS cells (+/− *H. pylori* infection). **(F)** Schematic representation for Survivin alternative TSS revealed by RACE methodology in AGS cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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```
  Geospiza fortis            GGACTAAGGCTGTGT----------------------------TAGGCCTTCCCCTGCT        31
  Xenopus tropicals          -------CGAGTGCTATTCCCGTGATGCAG-----TG--CGCGAAAACGTCATAGGGGCG        46
  Danio rerio                ----TGTGGCGCTTTG----------------------CAGCCAATCAAAACCCGCGCT        33
  Rhincodon typus            ------------------CCCAC----------------CGGGAGCCGCC--ACCGAGAG        24
* Phascolarctos cinereus     --CCTGCGACACCCTCCTCCAGTTCCTTGGAGG--CCACGGATAGAAGAAAGCCCAGGTG        56
  Crocodylus porosus         -----ACGGCCGCTAACTACCACCCCGCGCTTCCTCCTACCTGCGCACGCGCACGGCGCC        55
* Mus musculus              ---------------------------------GCGGGCGAGGGCGTGGGGCCGGGGCT        26
* Desmodus rotundus         ----------------------GGCCGCGGGGGCCCGCTCACCCATTCAAAAGCGGGGCG        38
* Papio Anubis              -------------------------GGCTGCGGGGGGTGAACCTCCTAAGAGGGCGTGCG        35
* Chlorocebus sabaeus       ------------------------AAGGCTGCGGGGGGTGAACCTCCTAAGAGGGCGTGCG        37
* Mandrillus leucophaeus    --------CGCCTCTACTCCCAGAAGGCTGCGGGGTGTGAACCTCCTAAGAGGGCGTGCG        52
* Pan troglodytes           ------------------------AAGGCCGCGGGGGTGGACCGCCTAAGAGGGCGTGCG        36
* Macaca mulatta            ------------------CCCAGAAGGCCGCGGGGGGTGAACCGCCTAAGAGGGCGTGCG        42
* Homo sapiens              ------------------CCCAGAAGGCCGCGGGGGGTGGACCGCCTAAGAGGGCGTGCG        42
* Gorrilla gorilla          ------------------------------------------------------------         0
* Sus scrofa                ------------------------------------------------------GCCGCG         6
* Felis catus               ---------------------------------------------CTCAGGGACCGTGCG        15
* Equus caballus            ------------------------------------------------------GGGGCG         6
* Physeter catodon          ----------------------------------------------------GGCGGCGCG         9
* Delphinapterus leucas     -------------CATTTCCCAG-------------------AAGGCCGCGGGCGGCGCG        28
* Capra hircus              ------------------------------------------------------GCGCG         5


  Geospiza fortis            CTCCAGGTGCAATGTGTCACCAATT----AGCAAAGGCCTTAAA---TGTGAGAGCAGGA        84
  Xenopus tropicals          C--AT------ATTTTTGAATCCGGT-----------AACATAATGAGCGCTTCTTTGAT        87
  Danio rerio                TTCAATTTTAAACTTTTACCGGAAGAATCAACAAGCAAGCGAGACTCCTGCGGATTTATC        93
  Rhincodon typus            -----------TCTTCCCCCTCCAC-----------CGGGAGCCGTCACCGAGGTATCCT        62
* Phascolarctos cinereus     CTCCCG-----GCATGCCGCGCGGT----------------------GGCAGAATTTGAA        89
  Crocodylus porosus         CCGCC-------------CGGGGG-------------AGCCGCGACCCCGCCCCGTTCCC        89
* Mus musculus              CTCCCG-----GCATGCTCTGCGGC-----------GCGCCTCCGCCCGCGCGATTTGAA        70
* Desmodus rotundus         TTGCGG-----ACTTGTTTTTCGGC-----------CGCTGCCAACCGCCGGATTTGAA        81
* Papio Anubis              CTCCCG-----ACACGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        79
* Chlorocebus sabaeus       CTCCCG-----ACATGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        81
* Mandrillus leucophaeus    CTCCCG-----ACATGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        96
* Pan troglodytes           CTCCCG-----ACATGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        80
* Macaca mulatta            CTCCCG-----ACATGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        86
* Homo sapiens              CTCCCG-----ACATGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        86
* Gorrilla gorilla          -----G-----ACATGCCCCGCGGC-----------GCGCCATTAACCGCCAGATTTGAA        39
* Sus scrofa                CTCCCG-----ACATGCCCCGCAGC------------GGCCGCCCACCGCCGGATTTGAA        49
* Felis catus               TTCCCG-----ACATGCCCCGCGAC------------GGCCGCCAACCTCCGGATTTGAA        58
* Equus caballus            TTCCCG-----ACATGCCCCGCGGT------------GGCCGCCAACCGCCGGATTTGAA        49
* Physeter catodon          TTCCCG-----ACATGCCCCGCGGC------------GGCCGACAACCGCCGGATTTGAA        52
* Delphinapterus leucas     TTCCCG-----ACATGCCCCGCGGC------------GGCCGACAACCGCCGGATTTGAA        71
* Capra hircus              TTCCCG-----ACATGCCTCGCGGA------------GGCCGTCAACCGCCGGATTTGAA        48


  Geospiza fortis            GGGAGGCTCTGCCCTTCACTGACTGCAGG-------CTGCGTAGATG     124
  Xenopus tropicals          TAGC----GCTCTTCCTCCCTGCGGTAATG------AGCCGCCTATG     124
  Danio rerio                TCGGTTGTCTTTTGTTTAATTTCCACACC--AACCTCCCACAAAATG     138
  Rhincodon typus            TTTCTCGCCCT-----------------------------CACGATG     80
* Phascolarctos cinereus     ACGG-TCTGCA-------TTCCCG------------GTTGCAAAATG     116
  Crocodylus porosus         GTCAGCCCCCGCGCGGCCGCGGTC------------GTTTGAAGATG     124
* Mus musculus              TCCTGCGTTTGAGTCGTCTTGGCGGAGGTTGTGGTGACGCCATCATG     117
* Desmodus rotundus         TCGGGACGCTGCTTCCTGGCGCCGGCGGGGTGGCG-GTGAGATG---     124
* Papio Anubis              TCGCGGGACCCGTTGGCAGAGGTGGCGGCAGCGGC-ATG--------     117
* Chlorocebus sabaeus       TCGCGGGACTCGTTGGCAGAGGTGGCGGCGGCGGC-ATG--------     119
* Mandrillus leucophaeus    TCGCGGGACCCGTTGGCAGAGGTGGCGGCAGCGGC-ATG--------     134
* Pan troglodytes           TCGCGGGACCCGTTGGCAGAGGTGGCGGCGGCGGC-ATG--------     118
* Macaca mulatta            TCGCGGGACCCGTTGGCAGAGGTGGCGGCGGCGGC-ATG--------     124
* Homo sapiens              TCGCGGGACCCGTTGGCAGAGGTGGCGGCGGCGGC-ATG--------     124
* Gorrilla gorilla          TCGCGGGACCCGTTGGCAGAGGTGGCGGCGGCGGC-ATG--------     77
* Sus scrofa                TCGCAGCACCGCTTTGTGGCGGCGGCGGCGGCGGG-ATG--------     87
* Felis catus               TCGAGTCAGCGCTCGGCGGTTGCGGTGTCGGGATG-------------     93
* Equus caballus            TCGCGGCAGCGCTCGGCGGCCGCGGGATG------------------     78
* Physeter catodon          TCGCGGCACCGCTCGGCGGCGGCGGGATG------------------     81
* Delphinapterus leucas     TCGCGGCACCGCTCGGCGGCGGCGGGATG------------------     100
* Capra hircus              TCGCGGCACTGCTCGGCGGCGGCGGGATG------------------     77
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Fig. 2. BIRC5 uAUG is conserved in mammals. Multiple alignment of BIRC 5′UTR in vertebrates was performed with the Clustal O program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Survivin 5′UTR sequences of mammals are indicated by an asterisk to the left side of the figure. To the right, the extension of 5′UTR sequences are indicated. uAUG codon is highlighted in color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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2.5. Quantitative real-time PCR

After 24 h of transfection, cDNA was obtained as previously described [21,22] and amplified using specific primers for FLuc (FLuc-F 5′-cttcgaaatgtccgttcggt-3′ and FLuc-R 5′-taggctgcgaaatgcc-cata-3′), RLuc (RLuc-F 5′-aggtgaagttcgtcgtccaacattatc-3′ and RLuc-R 5′-gaaacttcttggcaccttcaacaatagc-3′). Changes in mRNA levels were evaluated by the $2^{-\Delta\Delta C}{}_{T}$ method [23].

2.6. Western blot analysis

Western blots were performed as previously described [18]. Primary antibodies used in this study are listed in supplementary material.

2.7. Infection of gastric cells

AGS cells were infected with *H. pylori* 26695 (ATCC 700392) at a multiplicity of infection (MOI) of 1:100, as previously described [18].

2.8. Online bioinformatic resources

Ribosome profiles of initiating and elongating ribosomes in the Survivin 5′UTR (chr17:78,214,219−78,214,422) were available at the GWIPS server http://gwips.ucc.ie/cgi-bin/hgGateway. DNA 5′UTR sequences were obtained from Genbank. Multiple sequence alignment was performed using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.9. Statistical analysis

Numerical data is shown as the means ± standard deviation (SD). Statistical significance of differences was determined using the Wilcoxon-Mann-Whitney test for non-parametric data and were considered significant at $p < 0.05$.

3. Results

3.1. Survivin 5' UTR presents a putative out-of-frame upstream AUG

In this study, we focused on primary elements of translational control present in the Survivin 5′UTR. Initially, we analyzed the ribosome occupancy at nucleotide resolution along the Survivin 5′UTR comprising the chromosomal region chr17:78,214,219−78,214,422 using an online tool for the analysis and visualization of ribo-seq data obtained with the ribosome profiling technique at the GWIPS server [24]. Interestingly, this region showed a notable occupancy of initiating and elongating ribosomes upstream of the initiator AUG of Survivin, suggesting the presence of an alternative uAUG in this region (Fig. 1A). In fact, this uAUG was found at the −71 position (Fig. 1A, highlighted in red in the upper DNA sequence). Of note, the usage of this uAUG is likely to be inhibitory given that it would promote a shift in the open reading frame. However,although the uAUG context does not represent the typical “Kozak consensus”, e.g., 5'-(A/G)CCAUGG-3' [25], this putative uAUG (5′-GACAUGC-3′) has been shown to be functional in the context of several initiator AUGs in vertebrates genes [26]. As mentioned above, several alternative transcriptional start sites have been described for Survivin mRNA isolated from HeLa cells [4]. Furthermore, alternative mRNA variants exist with similar 5′UTR extensions (GenBank accession: NM_001168.3, NM_001012271.2 and NM_001012270.2). In order to confirm the presence of such alternative TSS for BIRC5, we performed a rapid amplification of 5′ cDNA ends analysis (RACE), a methodology useful for detecting alternative 5′ ends for specific mRNAs, using total RNA isolated from AGS gastric cells. Also, we included RNA isolated from cells infected with *H. pylori* 26695, a model of infection previously described by our group [27], in order to determine if TSS selection could be altered in response to stress. As shown in Fig. 1B, the protocol consisted in a reverse transcription using a specific Survivin reverse primer (Surv1-RT), followed by a poly-adenylation modification of the first strand of cDNA at the 3′ end. Then, these polyadenylated cDNAs underwent two successive rounds of PCR amplification. The first amplification (with Q_T-F and Surv2-R primers), resulting in a diffuse pattern of electrophoretic bands (Fig. 1C, left side of the gel). No-RT reactions were included in order to discard DNA presence in the samples (Fig. 1C, right side of the gel). However, a second round of PCR (with Q_I-F and Surv3-R primers), increased specificity and generated more defined amplicons which ranged between 100 and 1000 bp as shown in Fig. 1D (indicated by arrows in the right side of the gel). It is important to mention here that the amplicons generated from *Hp*-infected cells appeared as more robust bands of higher molecular weight (MW) than the control situation (indicated by the red arrows in Fig. 1D). Then, the amplicons were purified, cloned and sequenced. As shown in Fig. 1E, sequencing profiles displayed different 5′ ends ligated to the poly(T) tails (+/− *H. pylori* infection). Interestingly, RACE performed with RNA isolated from *H. pylori*-infected AGS cells displayed essentially similar 5′UTR regions as those from the uninfected cells. Of note, longer 5′UTRs were not identified by this methodology despite that high MW amplicons were observed by agarose gel electrophoresis (Fig. 1D). A diagram that summarizes the TSS found is shown (Fig. 1F). In conclusion, these findings confirm that BIRC5 is transcribed from different TSS in AGS cells, which apparently are not affected by *H. pylori* infection.

3.2. Survivin uAUG is a conserved primary element in mammals

Since the presence of an out-of-frame uAUG would have an inhibitory effect on the translation of the Survivin mRNA, it could be a relevant translational control element. Therefore, we wondered if this codon could also be found in the Survivin 5′UTRs of other organisms, which would be indicative of a more conserved regulatory function. To address this question, we performed an alignment between some ortholog DNA sequences of BIRC5 5′UTR in vertebrates. As shown in Fig. 2, the multiple alignment compared 5′UTR sequences present in birds, reptiles, amphibians, fish and mammals, which ranged between 77 up to 188 nucleotides of extension. This analysis revealed that the uAUG was indeed conserved in mammals (14/16), but was not present in other vertebrates, which presented even divergent 5′UTR sequences. Moreover, although 5′UTRs in mammals varied in length, the out-of-frame position of this uAUG was conserved in all sequences.

3.3. Survivin uAUG (−71) is a functional start codon with an inhibitory effect on translation

In order to determine the impact of the Survivin uAUG on translation, we evaluate the effect of the insertion of the human Survivin 5′UTR DNA fragment (with a representative extension for mammals of 121 pb) immediately before the ORF of the renilla luciferase reporter gene (RLuc) in the pcDNA3-RLuc reporter plasmid (Fig. 3A). This methodology has been previously described in similar studies [19]. Initially, we cloned the wild-type 5′UTR sequence (WT) and the other three variants. Two of these variants included an insertion of one nucleotide between the uAUG and initiator AUG, with the purpose of creating an artificial inframe scenario (Inframe-1 and Inframe-2 variants, see UTR sequences with highlighted mutations in Fig. 3A). Additionally, a 5′UTR variant

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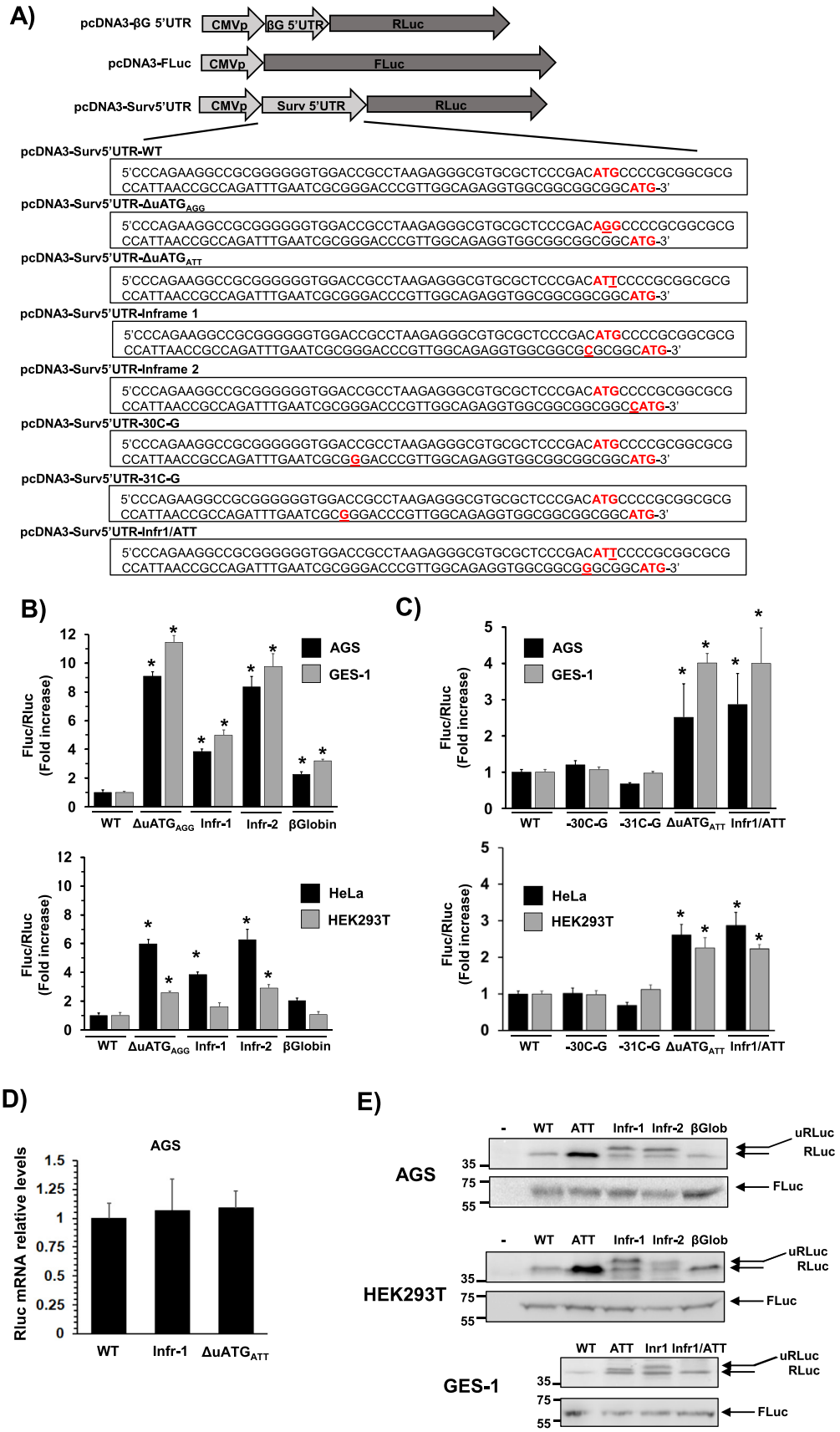

Fig. 3. Effect of Survivin 5′UTR variants on the renilla luciferase reporter activity. Different variants of Survivin 5′UTR were cloned into the pcDNA3-RLuc reporter plasmid to generate the pcDNA3-Surv5′UTR plasmids. **(A)** Schematic representation of reporter constructs. The wild-type Survivin 5′UTR sequence is shown and the putative uAUG and initiator AUG codons are highlighted in red. Point mutations in the sequence are shown in the corresponding panels of each variant. Also, pcDNA3-Fluc and human beta globin

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containing an AGG codon instead the uAUG (ΔATG_{AGG} variant) was included. Reporter assays in different cell backgrounds showed a significant increase in the RLuc luminescence activity when cells were transfected with either the Inframe-1, Inframe-2 or ΔATG_{AGG} plasmids as compared with the WT sequence harboring plasmid, although reporter activity was higher in the gastric cell lines (Fig. 3B). Additionally, a β-globin 5′UTR was included as a control given that structural aspects of this UTR have been characterized previously (Fig. 3A and 3B). As shown, the RLuc activity produced by these mutated versions of Survivin 5′UTR were notably higher than those produced by the β-globin 5′UTR sequence.

Three polymorphisms have been described in the promoter region of human BIRC5, including the C-to-G transversion at −31. This SNP is interesting because it has been associated with elevated Survivin protein levels in cancer and it is present in the Survivin 5′UTR region. However, this mutation had no impact on the RLuc reporter activity when compared with the WT sequence (Fig. 3C). We also mutated the nearby −30C nucleotide (C to G); however, as for the −31C-G mutation, no significant changes in the reporter activity were detected. This result excludes the possibility that C-to-G transversion at −31 affects translation under our experimental conditions. Additionally, we included another different point mutation in the uAUG, to generate the pcDNA3-Surv5′UTR-ΔATG_{ATT} plasmid. The Survivin 5′UTR structure was predicted to change its secondary structure when the uAUG codon was substituted by the AGG codon (analysis not shown). As shown in Fig. 3C, for the ΔATG_{ATT} variant the reporter activity increased compared to the WT sequence, albeit to a lesser extent (approximately 50−60% less) than observed with the ΔATG_{ATT} codon.

The reporter constructs were designed to reveal the impact of 5′UTR regions on translation, but not transcription (constitutive expression under the CMV promoter). In order to confirm this premise, we compared the RLuc mRNA levels produced by some of these plasmids. As shown in Fig. 3D, no differences in RLuc mRNA levels were observed when AGS cells were transfected with either the pcDNA3-Surv5′UTR plasmids harboring the WT, Inframe-1 or the ΔATG_{ATT} variants. Then, we analyzed by immunoblotting the levels of RLuc expression in cell lines transfected with the pcDNA3-Surv5′UTR plasmids. In protein extracts from AGS and HEK293T cells transfected with the pcDNA3-Surv5′UTR plasmids harboring either the Inframe-1 or -Inframe-2 variants (Fig. 3E), a double-band was detected by the anti-RLuc antibody (indicated as uRLuc and RLuc bands). This increase in the molecular weight was consistent with the addition of 24 amino acids to native RLuc due the alternative translation initiation from the Survivin uAUG codon. Since the AUG codon is not the unique initiator AUG in vertebrates [28], we constructed a double mutant variant including the Inframe-1 and ΔATG_{ATT} mutations (Infr1/ATT) in order to discard the possibility that the inframe mutations promote initiation from other alternative initiator codons. As expected, immunoblot analysis of protein extracts from GES-1 cells transfected with the pcDNA3-Surv5′UTR-Infr1/ATT plasmid did not express the uRLuc protein (Fig. 3E, inferior panel). Together, these results indicate that the Survivin uAUG is a functional inhibitory initiator codon in cancer and immortalized cell lines.

4. Discussion

In this study we have uncovered an upstream AUG codon at the position −71 as a novel primary control element present in the Survivin 5′UTR in mammals. Also, our results revealed its inhibitory effect on translation, since this uAUG was found to be out-of-frame with respect to the initiator AUG codon. As mentioned above, the canonical Survivin 5′UTR is a short sequence of 61 nucleotides, therefore, the relevance of the uAUG is restricted to longer 5′UTRs harboring this codon. However, a previous study showed that Survivin mRNA contains different 5′ ends as alternative TSS in Hela cells. In agreement with this observation, we also found different 5′ ends for the Survivin mRNA in AGS cells by RACE analysis. However, longer 5′UTRs were not detected by this methodology, probably due to a lower proportion of theses amplicons with respect to shorter 5′UTRs amplicons in the ligation with the vector (see Fig. 1D). It has been reported that AUG election can be altered following stress [17], so we performed reporter assays in cells infected with *H. pylori* (Supplementary Figure-1A). Notably, *H. pylori* infection of AGS cells did not decrease in RLuc activity when cells were transfected with the pcDNA3-Surv5′UTR-WT plasmid (neither with Infr-1 or Infr-1/ATT plasmids), indicating that this type of stress may alter mainly TSS election rather than uAUG/AUG usage. However, the specific contribution mRNAs with longer 5′UTRs that harbor the uAUG to the downregulation of Survivin protein levels needs to be confirmed with additional experiments beyond the scope of this study.

The Survivin uAUG represents a conserved primary element in mammals which suggests a physiological role, for instance, during developmental processes. The Survivin promoter region contains an elevated GC content, susceptible to methylation/demethylation as occurs in cancer [29]. This epigenetic mark could be responsible for alternative TSS election in a tissue-specific manner [30]. Of note, a single nucleotide polymorphism is present in the uAUG codon, where the SNP rs1177008518 changes U to A; however, the clinical significance of this polymorphism has not been reported. Another important point is that the secondary structure of the Survivin 5′UTR could influence the usage of the uAUG. For instance, as shown in reporter assays, mutation of the uAUG to AGG lead to increased reporter activity compared with the ATT mutation under similar conditions that was associated with greater changes in the 5′UTR structure (data not shown). Secondary structure plays an important role in translational control. This was demonstrated early on by Kozak and co-workers, who showed that secondary structures present in 5′ UTR regions of the α- and β-globin mRNAs have a major impact on translation efficiency [31].

5′UTR (pcDNA3-βG 5′UTR) reporter plasmids were included. **(B and C).** Cell lines cells were co-transfected with the reporter plasmids pcDNA-Surv5′UTR (WT or variants) or the pcDNA3-βG 5′UTR in combination with the control plasmid pcDNA3-FLuc (Firefly luciferase). After 24 h, protein extracts were prepared to determine luciferase reporter activities. Bars represent values of normalized luminescence (RLuc/FLuc), compared with the control condition (WT) (mean ± SD, n = 3, *p ≤ 0.05). **(D)** AGS cells were co-transfected with the pcDNA3-Surv5′UTR (WT, Inframe-1 or ΔAUG_{ATT} variants) and the pcDNA3-FLuc plasmids. After 24 h of transfection, mRNA levels of RLuc were evaluated by RT-qPCR and normalized to FLuc mRNA levels. Data were expressed relative to values obtained for the transfection with the pcDNA3-Surv5′UTR-WT plasmid (mean ± SD, n = 3; *p < 0.05). **(E)** Evaluation of Survivin 5′UTR-driven renilla luciferase expression by immunoblotting. Cell lines were transfected with the indicated pcDNA3-Surv5′UTR plasmids (WT, ΔATG_{ATT}, Inframe-1, Inframe-2 or Infr1/ATT) in combination with the pcDNA3-FLuc plasmid as a control. Following 24 h of transfection, total protein extracts were prepared; and RLuc and FLuc protein expression was evaluated by immunoblotting. Representative blots are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

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