HnRNP A1 is a Novel IRES *trans*-acting Factor that Modulates Alternative Initiation of Translation of the Fibroblast Growth Factor 2 mRNA

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ABSTRACT

Alternative initiation of translation of the human fibroblast growth factor 2 (FGF-2) mRNA at five in-frame CUG or AUG translation initiation codons requires various RNA *cis*-acting elements, including an internal ribosome entry site (IRES). Here we describe the purification of a *trans*-acting factor controlling FGF-2 mRNA translation achieved by several biochemical purification approaches. We have identified the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) as a factor that binds to the FGF-2 5' leader RNA and that also complements defective FGF-2 translation *in vitro* in rabbit reticulocyte lysate. Recombinant hnRNP A1 stimulates *in vitro* translation at the four IRES-dependent initiation codons, but has no effect on the cap-dependent initiation codon. Consistent with a role of hnRNP A1 in the control of alternative initiation of translation, siRNA-mediated knock down of hnRNP A1 specifically inhibits translation at the four IRES-dependent initiation codons. Furthermore, hnRNP A1 binds to the FGF-2 IRES, implicating this interaction in the control of alternative initiation of translation.

INTRODUCTION

In eukaryotes, alternative translation initiation at several start codons is one of the process by which a single mRNA gives rise to multiple proteins, and this contributes to the generation of protein diversity (1). According to the ribosome-scanning model of translation, protein synthesis in eukaryotes involves 40S ribosomal subunit recruitment to the 5'-end cap structure of the mRNA, followed by its ATP-dependent linear scanning until an initiation codon in a good sequence context is encountered (2). In mRNAs containing several alternative translation initiation codons, it is proposed that the cap-proximal initiation codon is used inefficiently because of its usual weak sequence context and that some of the 40S ribosomal subunits read through the site without recognising it by a so-called "leaky scanning" process to initiate translation at the downstream position (3). However, this mechanism cannot occur when the RNA structure between the two (or more) initiation codons is stable and cannot be easily unwound by the eIF4A RNA helicase. Moreover, recent data demonstrate that 40% of mRNAs contain at least one upstream AUG that is in a similar or more favourable context than the actual initiator (4). Thus, the « leaky scanning » mechanism cannot explain the selection of internal codons in all mRNAs.

The human FGF-2 mRNA provides a very interesting system to understand how internal codons are selected in a highly structured 5' leader. Indeed, the control of alternative initiation of translation is a crucial aspect of the regulation of the human fibroblast growth factor 2 (FGF-2) gene (1). The FGF-2 protein isoforms belong to a family of 23 structurally related FGF polypeptides that play key roles in morphogenesis, development, angiogenesis, and wound healing (5). The FGF-2

mRNA contains five in-frame translation initiation codons (4 CUGs and 1 AUG) that give rise to five FGF-2 isoforms with different amino terminal extensions. Regulation of the expression of these isoforms could have a strong impact on cell behavior, since the various FGF-2 isoforms have different subcellular localizations and functions. The largest isoform (34 kDa) is initiated at a CUG codon located 86 nucleotides (nts) from the mRNA 5'-end. This isoform is nuclear and is involved in a cell survival function. The 24, 22.5 and 22 kDa isoforms are initiated at three CUG codons located 320, 347 and 362 nts from the 5'-end. These isoforms have a nuclear localization and are involved in cell immortalization. Translation initiation of the 18 kDa FGF-2 isoform occurs at an AUG codon located 485 nucleotides from the 5'-end. Constitutive expression of this secreted low molecular weight FGF-2 isoform leads to cell transformation.

Alternative expression of the FGF-2 isoforms is translationally regulated depending on the cell type or cell status and is controlled by various *cis*-acting elements including an internal ribosome entry site (IRES) (6-9). The process of internal entry of ribosomes leads to the recruitment of the 40S ribosomal subunit to IRESs present on eukaryotic viral or cellular mRNAs (10). It is an alternative to the mechanism of translation initiation that results from the attachment of the 40S subunit of the ribosome to the 5'-end capped structure of mRNAs. IRESs are present in more than 30 viral mRNAs and 50 cellular mRNAs (11). Internal entry of ribosomes requires the intervention of specific RNA binding proteins, also known as ITAF (« IRES *trans* acting factor ») that stimulate translation of some IRES-containing cellular mRNAs (11).

The FGF-2 IRES could play a critical role in the choice of alternative translation initiation codons on this mRNA. Indeed, whereas the 34 kDa isoform is initiated by a

cap-dependent translation process, the other four isoforms are initiated by an internal entry of ribosome mechanism (7,9). Such a situation with a 5'-end proximal cap-dependent start codon and an internal IRES-dependent codon was also found in the Moloney MuLV gag mRNA (12), and the human PITSLRE protein kinases mRNA (13). However the precise mechanism by which internal entry of ribosomes is favored over cap-dependent translation to select internal start codons is not known.

To better understand how control of alternative initiation of translation on the FGF-2 mRNA is achieved, we used several biochemical approaches to identify a possible *trans*-acting factor modulating this process. Our data demonstrate that heterogeneous nuclear ribonucleoprotein (hnRNP) A1 is a factor that binds to the FGF-2 5' leader RNA. We also show, by using *in vitro* translation experiments and siRNA-dependent knock down of endogenous levels of hnRNP A1 in cultured cells, that hnRNP A1 controls alternative initiation of translation of the FGF-2 mRNA. The activity of hnRNP A1 in FGF-2 IRES-mediated translation contributes to the choice of the alternative start codons. We thus provide experimental evidence supporting the notion that selection of internal codons is not only achieved by a « leaky scanning » mechanism at an upstream initiation codon but by a mechanism involving a *trans*-acting factor controlling an IRES.

EXPERIMENTAL PROCEDURES

DNA manipulations

Detailed information about DNA cloning procedures can be obtained from the authors upon request.

RNA affinity chromatography

1 nmol of *in vitro* transcribed RNA was incubated with 1 nmol of a $(dT)_{25}$ oligonucleotide biotinylated at its 3'-end RNAs and 100 μl of packed streptavidin acrylamide resin (Pierce) in 500 μl TMK buffer (50 mM Tris HCl pH 7.5; 10 mM $MgCl_2$; 150 mM KCl) during 1 hour at 4°C. After extensive washing, Hela cells nuclear extract (150 μg) was loaded onto the RNA column in TMK buffer (200 μl final volume). After incubation for 1 h at 4°C, the beads were washed three times with TMK buffer (1 ml). The proteins retained on the RNA column were eluted in 50 μl SDS-PAGE sample buffer at 95°C during 2 min. The eluted proteins were separated by 12% SDS-PAGE and stained by brilliant blue G-colloidal (Sigma). Proteins were excised from the gel and identified by tandem MS/MS mass spectrometry.

HeLa cells biochemical fractionation procedure

HeLa S3 cells cytoplasmic and nuclear extracts were prepared according to (14). HeLa nuclear extract (8 ml at 20 mg/ml) was loaded onto a 5-ml HiTrap heparin sepharose

column (Amersham Pharmacia Biotech) on a FPLC system. Proteins were eluted with a linear 10 ml gradient (0.4 to 0.8 M NaCl). Fractions (1 ml) resulting from the heparin sepharose were pooled, dialysed against buffer A (20 mM Hepes-KOH pH 7.9, 150 mM NaCl) and loaded onto a Mono S cation-exchange chromatography equilibrated with buffer A on a FPLC system. Proteins were eluted with a linear 20 ml gradient (0.4 to 0.8 M NaCl). 1ml fractions were collected. Fraction 7 was eluted between 0.51 and 0.53 M NaCl while fraction 9 was eluted between 0.57 and 0.59 M NaCl. All fractions were subsequently dialyzed against buffer D (20mM Hepes-KOH pH 7.9; 20% glycerol; 100 mM KCl; 0.2 mM EDTA; 0,5 mM DTT).

hnRNP A1 depletion from purified fraction 9

Fraction 9 was depleted of hnRNP A1 with a 5' biotinylated DNA oligonucleotide (5'-CTAGTATGATAGGGACTTAGGGTG-3') corresponding to the hnRNP A1 RNA binding site identified by a SELEX procedure (15). Briefly 1 nmole of oligonucleotide was incubated with 100 μl of packed streptavidine acrylamide resin (Pierce) in 150 μl of buffer A. After extensive washing, fraction 9 (100 μg) was incubated with the resin during 60 min at 4°C. After centrifugation at 2000 rpm, the supernatant was kept. Depleted fraction 9 was checked by western blot. A mock control column was done with a mutated DNA oligonucleotide (5'-CTAGTATGAGATGGACTGATGGTG-3').

Expression and purification of recombinant proteins

The three GST-tagged hnRNP A1 (GST-A1), hnRNP I/PTB (GST-PTB) and La (GST-La) proteins were expressed in *E. Coli*, purified to homogeneity by glutathione agarose chromatography and dialyzed against buffer D.

UV cross-linking/Immunoprecipitation

Protein extracts (5 μg) were mixed with *in vitro* transcribed ^{32}P-labelled RNAs (150000 cpm) in buffer GS (5 mM Hepes-KOH pH 7.6; 30 mM KCl; 2 mM $MgCl_2$; 0.2 mM DTT and 4 % glycerol) for 10 min. The reaction mixtures were irradiated on ice with UV light (254 nm) in a Stratalinker (Stratagene) at 0.4 J/cm^2 at 10 cm distance. 5 units of RNAse ONE (Promega) were then added and the reaction mixtures were incubated for 45 min at 37°C. SDS gel loading buffer was added and the samples were boiled 2 min before fractionation on a 10% SDS-polyacrylamide gel. When competitions were performed, extracts were preincubated with cold competitors in GS buffer 10 min at room temperature before addition of the probe. For immunoprecipitation of UV cross-linked proteins, the RNAse ONE treated samples were diluted in 150 μl of IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X100), precleared and mixed with 1 μl of anti-hnRNP A1 monoclonal antibody (α4B10; Gift from G. Dreyfuss). The mixtures were allowed to rotate 1h at 4°C. 50 μl of protein A beads was added to the mixtures and incubation continued for 1h at 4°C. After extensive washing of the beads, the bound proteins were eluted in SDS-loading buffer.

Gel retardation assay

In vitro transcribed ^{32}P-labelled RNA (10000 cpm) was incubated for 15 min at 25°C in 10 µl of GS buffer with 200 ng of GST–A1. After addition of 5 µl of glycerol 80%, the mixture was loaded on a 6% native polyacrylamide gel (bisacrylamide:acrylamide 1:29 ; 0.5x TBE) and migration was performed for 4 h at 200 V at 4°C. The gel was then subjected to phosphoimaging and autoradiography. For competition experiments, unlabelled *in vitro* transcribed RNAs were preincubated with GST-A1 in GS buffer for 15 min.

Nitrocellulose filter binding assays

Increasing amount of a purified GST-A1 were added to an *in vitro* transcribed ^{32}P-labelled RNA corresponding to the FGF-2 IRES (1-177) in a total volume of 10 µl GS binding buffer containing 400 ng of yeast tRNA. The mixture was allowed to incubate 10 min at room temperature. 8 µl of each binding reaction were applied on a pre-soaked nitrocellulose membrane on a slot dot apparatus (hybrislot manifold, BRL) under moderate succion. Each slot dot was washed with 200 µl of room temperature GS buffer and the membranes were dried for 1 hour at room temperature. The filters were exposed in a phosphoimager cassette (Molecular Dynamics) for 3 hours and revealed. The quantifications were performed with the Image Quant v1.1 software and the data were corrected for the background (RNA retention without any added protein) which was < 2%. The fraction of RNA bound was plotted against the protein concentration. Binding curves of three independent experiments were fitted by using SigmaPlot to determine the apparent dissociation constants.

In vitro translation

In vitro transcribed RNAs (3 μg/ml except otherwise indicated) were translated in rabbit reticulocyte lysate (Promega) in presence of ^{35}S methionine (Amersham Pharmacia Biotech). When protein fractions or recombinant proteins were added to the reaction, preincubation with RNA for 5 min at room temperature was done before addition of RRL. The translation reaction was incubated 1 hour at 37°C, followed by RNAse A treatment. The reaction mixture was loaded on a 12% SDS-PAGE, followed by autoradiography of the gel.

RNA interference (RNAi)

SiRNA oligonucleotides (sequences in figure 7) were synthesized by Dharmacon Research. DsRNA were transfected in HeLa cells with Oligofectamine (Invitrogen) according to the manufacturer's recommendations. Western blot analysis was performed 72 hours post-transfection.

Western blot analysis

Protein analysis by western blot on whole cell lysates were performed with standard protocols and monoclonal antibody against hnRNP A1 (4B10; Gift of G. Dreyfuss) or polyclonal antibodies against PTB (done in our laboratory), FGF-2 (Santa Cruz) or actin (Sigma).

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

Expression of FGF-2 mRNA was determined by real-time PCR with a sequence Detection System model 7700 (Perkin Elmer) according to the instruction manual. Total RNA from HeLa cells was isolated by the SV Total RNA isolation system (Promega). The sequences of primers set for the human FGF-2 were as follows :sense 5'-TGTGTCTATCAAAGGAGTGTGTGCTA-3', reverse 5'-ATCCGTAACACATTTAGAAGCCAGTA-3'. Total RNA levels from each sample were normalized by the quantity of 18S rRNA in each sample.

RESULTS

A purified HeLa cell fraction containing hnRNP A1 promotes FGF-2 mediated *in vitro* translation

As a biochemical approach towards the identification of proteins that would promote FGF-2 mediated translation, we took advantage of the weak translation activity of the FGF-2 RNA in rabbit reticulocyte lysate (RRL) (6). We therefore attempted to complement this inefficient translation with a nuclear extract from HeLa S3 cells. The choice of a nuclear extract was determined by the results showing that efficient translation driven by several cellular IRESs required a nuclear event (16,17) and that several predominantly nuclear proteins such as hnRNPI/PTB or the La autoantigen were involved in IRES-mediated translation (e.g. (18-20). However, in our assay, addition of an HeLa nuclear extract to RRL had no effect on the translation of an FGF-2-CAT reporter mRNA RNA which consists of the first 539 nucleotides of the FGF-2 mRNA fused to the chloramphenicol acetyl transferase (CAT) ORF (6) (Fig. 1A and

data not shown). This suggested that this nuclear extract either did not contain proteins able to stimulate FGF-2 translation or contained proteins whose specific or non specific inhibitory action masked the effects of proteins potentially able to activate FGF-2 mediated translation. We favoured the second hypothesis and assumed that by fractionation of the extract, we should be able to separate these antagonistic activities. We therefore fractionated the extract by heparin sepharose chromatography followed by cation exchange chromatography to purify positively charged proteins (Fig. 1B). At each step of the purification procedure, fractions were tested for their ability to modulate FGF-2 mediated translation when added to RRL. Up to cation exchange chromatography, none of the fractions tested had an effect. Eighteen from the 20 fractions resulting from cation exchange chromatography had no or a slight inhibitory effect on FGF-2 translation (see for instance fraction 10 in Fig. 1C and data not shown). However, fractions 7 and 9 had a stimulatory effect on FGF-2 translation (Fig. 1C). Fraction 7 activated translation at all five FGF-2 initiation codons whereas fraction 9 activated FGF-2 translation at the CUG_1, CUG_2, CUG_3 and AUG initiation codons but inhibited translation at the CUG_0 (Fig. 1C). The second band migrating slightly faster than the CUG_0 band results from an initiation at an ACG codon located 122 nts from the 5'-end. This initiation only occurs in RRL but does not occur in transfected cells (9). As a specificity control, we tested the effect of these two fractions on translation of a CAT reporter RNA containing the EMCV 5' UTR instead of the FGF-2 5' leader. Whereas fraction 7 stimulated EMCV translation, fraction 9 had no effect (Fig. 1D). We concluded that the stimulatory effect of fraction 9 on translation initiation at the CUG_1, CUG_2, CUG_3 and AUG initiation codons is specific. Moreover fraction 9 was able to exert the effect we expected in controlling alternative initiation of translation on the FGF-2 mRNA.

It was therefore of interest to find out which RNA binding protein(s) present in fraction 9 exerted this effect. SDS-PAGE and Coomassie staining analysis revealed that this fraction, although still containing numerous proteins, was enriched in specific proteins (Fig. 1E). An ultraviolet crosslinking assay was employed to detect specific ribonucleoprotein complexes that could form between the FGF-2 5' leader and proteins present in fraction 9. A ^{32}P-labelled FGF-2 5' leader RNA (nts 1-539) was incubated with HeLa nuclear extracts or fractions 7 to 10, irradiated with ultraviolet light and digested with RNAse. In fraction 9, several proteins of 90, 66, 48 and 34 kDa were crosslinked to the FGF-2 RNA (Fig. 1F).

Identification of *trans*-acting factors bound to the FGF-2 5' leader RNA

Since the amount of material present in fraction 9 was not compatible with protein identification by mass spectrometry following an RNA affinity chromatography procedure, we performed this experiment with a total nuclear extract. Two *in vitro* transcribed RNAs containing a 3' terminal poly(A) tail were bound to a streptavidine acrylamide column through a biotinylated poly(dT) oligonucleotide (Fig. 2A). This strategy was used to avoid the possible steric hindrance of biotinylated groups in the RNA when biotinylation is performed during *in vitro* transcription. The FGF-2 5' leader sequence (from nucleotides 1 to 539) was chosen as an affinity reagent to purify FGF-2 RNA binding proteins, since it is sufficient to mediate FGF-2 translational control (8). An RNA corresponding to the EMCV IRES was also used to set up the proper chromatographic conditions to purify the known EMCV ITAFs, hnRNP I (PTB) and ITAF45 (21,22). We subjected HeLa S3 cell nuclear extracts to the various RNA columns and, after extensive washing, the retained proteins were eluted and analyzed

by SDS-PAGE. Colloidal blue staining of the gel revealed that several proteins were specifically retained by each RNA (Fig. 2B). The protein bands that appeared selectively purified on the EMCV RNA or FGF-2 RNA columns were excised from the gel and subjected to trypsin digestion with subsequent microsequencing of at least two tryptic peptides. From that analysis, we identified several ribosomal proteins and RNA binding proteins. The 57 kDa and 45 kDa proteins purified on the EMCV RNA column were identified as two known ITAFs, hnRNP I (PTB) and ITAF45 (Fig. 2B and table I), revealing that our purification procedure allowed the proper identification of ITAFs. The 64 kDa and 30 kDa proteins retained on the EMCV RNA column corresponded to the hnRNP K and hnRNP E1 proteins. These two proteins have never been described as EMCV IRES *trans*-acting factors but are involved in translation control of the lipoxygenase mRNA (23). Furthermore, hnRNP E1 interacts with the hepatitis C and the Bag-1 IRES (24,25). The role of these proteins in EMCV-mediated translation remains to be evaluated.

Among the ribosomal proteins purified on the FGF-2 RNA column (Table I), the 40S ribosomal protein S9 was shown to interact with the hepatitis C IRES (26). The possible function of this protein and of other ribosomal proteins in FGF-2 translation needs to be addressed in the future. Three of the other isolated proteins on the FGF-2 RNA column were identified as known RNA binding proteins. The 25 kDa protein was identified as the U1A protein. U1A is part of the U1 snRNP involved in splicing but can also act, independently of the U1 snRNP, to regulate pre-mRNA 3'-end processing (27). A role for the U1A protein in translational control has never been documented. The 30 kDa protein was identified as the ASF/SF2 splicing factor. Although predominantly nuclear and involved in splicing regulation, this protein was recently shown to be involved in several other steps of gene expression such as mRNA stability or translation (28,29).

Finally, we identified the protein migrating at 34 kDa as hnRNP A1 (Fig. 2B and Table I). HnRNP A1 belongs to the class of the heterogeneous nuclear ribonucleoproteins (hnRNP) and is involved in various aspects of mRNA metabolism (reviewed in (30). hnRNP A1 is a shuttling protein associated with poly(A)+ mRNA in the cytoplasm (31,32). The Chiromonus tentans hrp36 protein, that is similar to the mammalian hnRNP A1, becomes associated with the RNA concomitantly with transcription and remains bound with the RNA in polysomes (33). hnRNP A1 can act as a general RNA binding protein in mediating cap-dependent translation inhibition (34). However, since hnRNP A1 can also function as a sequence-specific RNA binding protein (15), it has been a long standing proposal that hnRNP A1 could be a translational regulator of specific mRNAs (31,34).

HnRNP A1 is present in fraction 9

We next examined the presence of the proteins identified in the RNA affinity procedure in fraction 9. A western blot analysis showed that neither U1A nor ASF/SF2 was detected in fraction 9 (data not shown). However, hnRNP A1 was enriched in fraction 9 (Fig. 3A). Fraction 9 was not enriched for hnRNP proteins in general. For instance, the hnRNP I/PTB protein was not present in fraction 9 but was enriched in fraction 7 (Fig. 3A).

We therefore examined whether the 34 kDa protein crosslinked to the FGF-2 5' leader in fraction 9 (Fig. 3B) was immunologically related to hnRNP A1 by immunoprecipitation of UV crosslinked proteins with the hnRNP A1 specific 4B10 monoclonal antibody (kindly provided by G. Dreyfuss). The 34 kDa protein was detected after immunoprecipitation of the UV cross-linked proteins from fraction 9,

showing that it is indeed hnRNP A1 (Fig. 3B, left panel). We also demonstrated by using a similar experimental approach that hnRNP A1 from a HeLa nuclear extract was able to bind to the FGF-2 RNA (Fig. 3B, right panel). Altogether, we concluded that hnRNP A1 from fraction 9 or from a HeLa nuclear extract was able to bind directly to the FGF-2 5' leader RNA.

To get more evidences on the possible role of hnRNP A1 in FGF-2 mediated translation, we attempted to deplete hnRNP A1 from fraction 9 on a hnRNP A1 SELEX DNA column. Specifically, RNA sequences containing one or more copies of the motif UAGGGA/U were identified through the SELEX procedure as having a high affinity for hnRNP A1 (15). Since hnRNP A1 utilizes similar principles for recognition of single-stranded RNA and DNA (35), we used the deoxy form of the A1 SELEX sequence. We first controlled the ability of the SELEX A1 DNA oligonucleotide to compete for the binding of hnRNP A1 to the FGF-2 5' leader RNA by UV crosslinking experiments. We added, prior to UV cross-linking, increasing amount of unlabelled competitor DNA oligonucleotides corresponding to the wild type (A1-WT) or mutated (A1-MUT) SELEX RNA sequence of hnRNP A1. The binding of hnRNP A1 was specifically reduced when the A1-WT oligonucleotide, but not the A1-MUT oligonucleotide, was used as a competitor (Fig. 4A). This showed that the A1-WT oligonucleotide specifically impaired the binding of hnRNP A1 to the FGF-2 5' leader RNA without affecting the binding of the other FGF-2 5' leader RNA binding proteins.

We then performed the depletion with the biotinylated A1-WT or A1-MUT oligonucleotides bound to streptavidine beads. The depletion of hnRNP A1 was efficient as judged by western blot analysis of the A1-WT depleted fraction 9 compared to the A1-MUT depleted fraction 9 since more than 95% of hnRNP A1 was depleted in the A1-WT depleted fraction 9 (Fig. 4B). The depletion of hnRNP A1 was not visible

after coomassie staining of the depleted fractions since several proteins are present in the band containing hnRNP A1 (data not shown). Analysis of the proteins retained on the beads revealed that several similar proteins were retained on the A1-WT and A1-MUT beads (Fig. 4C). However, hnRNP A1 was found only on the A1-WT beads (Fig. 4B and 4C). Therefore, the composition of the A1-WT depleted or A1-MUT depleted extract is expected to be similar except for hnRNP A1. When added to RRL, the A1-WT depleted fraction 9, but not the A1-MUT depleted fraction 9, had lost its ability to activate FGF-2 translation at the CUG_1, CUG_2, CUG_3 and AUG initiation codons and to inhibit translation at the CUG_0 codon (Fig. 4D). To control that the effect observed with the A1-WT depleted extract was not due to the depletion of other proteins than hnRNP A1, we tried to restore the activity of the A1-WT depleted fraction 9 with an *E. Coli* produced recombinant hnRNP A1 protein. Addition of hnRNP A1 to the A1-WT depleted fraction 9 restored the effect observed with fraction 9 (data not shown). However, since hnRNP A1 alone is able to exert an effect comparable to the one observed with fraction 9 in stimulating FGF-2 mRNA translation (see Fig. 6 below), the restoration experiment does not provide a proper control for the specificity of the depletion. Nevertheless, this series of experiments strongly indicated that the effect of fraction 9 on FGF-2 alternative initiation of translation might be due to the presence of hnRNP A1 in this fraction.

hnRNP A1 binds directly to the FGF-2 IRES

To examine the ability of hnRNP A1 to interact directly with the FGF-2 5' leader we carried out UV crosslinking and electrophoretic mobility shift assays (EMSA) together with competition with unlabelled RNAs.

UV cross-linking of purified GST-hnRNP A1 (GST-A1) (Fig. 5A) to the ^{32}P-labelled FGF-2 5' leader (nts 1 to 484) gave a single band migrating at the expected size of GST-A1 (Fig. 5B). Competition with unlabelled RNAs corresponding to the entire FGF-2 5' leader (1-484) or with RNAs containing the IRES region (1-318 or 1-177) (as shown in (5)) strongly impaired the binding of GST-A1 to the ^{32}P labelled RNA (Fig. 5B). In contrast, unlabelled RNAs corresponding to nts 176-318 or 318-484 only impaired binding when added in large excess (Fig. 5B). We concluded that hnRNP A1 binds specifically to the FGF-2 5' leader within its IRES domain. We also reported recently that a region located between nts 128 to 144 is required for IRES activity (36). Strikingly, a competitor RNA that has a non-functional IRES, due to the deletion of nucleotides 128 to 144, competed less efficiently for the binding of hnRNP A1 to the FGF-2 RNA.

In EMSA experiments, GST-A1 was able to retard the migration of a ^{32}P-labelled FGF-2 5' leader (nts 1 to 484) RNA on native polyacrylamide gel electrophoresis (Fig. 5C, lane 2). Competition with unlabelled RNAs corresponding to the entire FGF-2 5' leader (1-484) or with RNAs containing the IRES region (1-318) strongly impaired the formation of the complex between GST-A1 and the ^{32}P labelled RNA (Fig. 5C). In contrast, unlabelled RNAs corresponding to 318-484 only impaired binding when added in large excess (Fig. 5C). Again, a competitor RNA that has a non-functional IRES, due to the deletion of nucleotides 128 to 144, competed less efficiently for the binding of hnRNP A1 to the FGF-2 RNA. The defect in IRES function of this deletion mutant could therefore be caused by the lack of hnRNP A1 binding.

To confirm that the FGF-2 IRES contains a high affinity hnRNP A1 binding site, we measured the apparent equilibrium dissociation constant (K_d) of the recombinant GST-A1 protein for an RNA containing the minimal IRES sequence (1-177) (5) using a

nitrocellulose filter binding assay. Binding experiments were carried out by incubating variable amount of GST-A1 with constant amounts of RNA. The apparent K_d of the binding reaction was estimated by the concentration of proteins at which half maximum binding was observed and is about 200 nM (Fig. 5D).

Recombinant hnRNP A1 activates FGF-2 mediated translation in RRL

To investigate the role of hnRNP A1 in translation directed by the FGF-2 5' leader, we used the rabbit reticulocyte lysate as an *in vitro* translation system. Since RRL does not contain any detectable levels of hnRNP A1 (34), a direct effect of hnRNP A1 on FGF-2 mediated translation was assessed by adding *E. Coli* produced GST-tagged hnRNP A1 protein (GST-A1) to RRL. The experiments were performed with two different concentrations of capped FGF-2-CAT reporter RNAs, since it was previously described that at high concentration of RNA initiation at the CUG_0 can be detected whereas at low concentration of RNA initiation at CUG_0 is barely detectable (9). When the FGF-2-CAT RNA was present in RRL at a concentration of 3 μg/ml, addition of increasing amounts of GST-A1 to RRL activated FGF-2 mediated translation initiation at the CUG_1, CUG_2, CUG_3 and AUG codons up to 2.3 fold and inhibited translation initiation at the CUG_0 codon (Fig.6A and Table II). When the concentration of FGF-2-CAT RNA was reduced to 0.3 μg/ml, GST-A1 activated translation initiation at the CUG_1, CUG_2, CUG_3 and AUG codons up to 3.2 fold (Fig. 6A and Table II). This slight effect was nevertheless specific since GST-A1 had no effect on CAT or EMCV-CAT reporter RNAs (Fig. 6A and Table II). Importantly, GST-A1 also did not modulate translation of an FGF-2-CAT RNA that was unable to bind GST-A1 (Fig. 6A, FGF-2-CAT Δ128-144).

Furthermore, translation stimulation was not observed after addition of other *E. Coli* produced GST-tagged ITAF proteins to RRL (Fig. 6B, 6C and Table II). Specifically, whereas addition of increasing amounts of GST-PTB activated EMCV-mediated translation by about 2 fold, it had no effect on FGF-2-mediated translation (Fig. 6B and Table II). This result indicated that the stimulatory effect of fraction 7 on FGF-2 mediated translation was not due to the presence of PTB in this fraction. Moreover, addition of increasing amount of GST-La had a slight inhibitory effect on FGF-2-mediated translation whereas it activated Bip-mediated translation by about 2 fold (as expected from (19)) (Fig. 6C and Table II).

We finally addressed the role of hnRNP A1 on translation of other mRNAs containing a cellular IRES. Capped reporter RNAs containing the 5' UTR of the Bip, VEGF or c-myc mRNAs upstream of the CAT ORF were translated in RRL supplemented with GST-A1 (Fig. 6A and Table II). Translation of these RNAs decreased upon GST-A1 addition showing that hnRNP A1 is not a general translational enhancer of IRES-containing RNAs.

Since the addition of GST-A1 has an inhibitory effect on the translation of several mRNAs, the inhibitory effect of GST-A1 on the FGF-2 CUG_0 codon might be unspecific. We therefore concluded from this set of data that hnRNP A1 specifically stimulated translation initiation at four of the five FGF-2 initiation codons.

RNAi-mediated hnRNP A1 depletion impairs FGF-2 translation at the four IRES-dependent initiation codons

We used RNA interference (RNAi) (37) to knock down levels of endogenous hnRNP A1 in cultured cells. Two short interfering RNAs (siRNA) were designed against the

human hnRNP A1 mRNA (Fig. 7A). siRNA duplexes were transfected into HeLa cells. After 72 h, cell lysates were collected and analyzed for hnRNP A1 and FGF-2 endogenous protein expression by western immunoblotting. In HeLa cells transfected with the hnRNP A1 siRNAs, but not in untransfected cells or in cells transfected with a control siRNA, we observed a strong reduction in the hnRNP A1 levels while not in the expression levels of actin (Fig. 7B). siRNA-mediated knock downs of hnRNP A1 led to a 3-fold decrease in the expression of the FGF-2 isoforms initiated at the CUG_1, CUG_2, CUG_3 and AUG codons. No effect on the expression of the cap-dependent CUG_0 initiated isoform was detected after *ex vivo* hnRNP A1 depletions (Fig. 7B). This effect on FGF-2 isoforms expression was not due to a change in the level of endogenous FGF-2 mRNA, as judged by quantitative RT-PCR (Fig. 7C). Altogether, these results demonstrated that hnRNP A1 was able to control FGF-2 alternative translation initiation by specifically activating translation at four of the five FGF-2 initiation codons.

DISCUSSION

By using various biochemical approaches, we have identified several proteins that may function as *trans*-acting factors in FGF-2 mRNA translation. Specifically, the role of the 40S ribosomal protein S9 and the ASF/SF2 splicing factor, two proteins already show to be involved in translation (11) warrants further investigation. We have also obtained two purified fractions (called 7 and 9) from a HeLa nuclear extract that modulate *in vitro* FGF-2 translation. The presence of hnRNP I/PTB, an ITAF for EMCV-mediated translation in fraction 7 (Fig. 3A) may explain the stimulatory effect of this fraction on

EMCV-mediated translation (Fig. 1C). However, hnRNP I/PTB was never able to bind to the FGF-2 5' leader RNA (8) and to activate *in vitro* translation of the FGF-2 mRNA (Fig. 5). Thus, it cannot account for the stimulatory effect of fraction 7 on FGF-2 mediated translation. The identification of proteins from fraction 7 acting on FGF-2 mediated translation is currently under investigation.

We have focused our study on hnRNP A1, a protein that we have found in fraction 9 as well as following an RNA affinity chromatography procedure on the FGF-2 5' leader RNA. HnRNP A1 has been thought for a long time to be a translational regulator of specific mRNAs (31,34) and our study has demonstrated such a role for hnRNP A1 in translational control of the FGF-2 mRNA in both *in vitro* and *ex vivo* experiments.

It is noteworthy that a HeLa nuclear extract cannot stimulate FGF-2 translation when added to RRL although it contains hnRNP A1, a stimulatory protein for FGF-2 translation. This is consistent with our initial hypothesis that the role of stimulatory proteins may be masked by inhibitory proteins present in the extract.

Alternative initiation of translation controlled by a *trans*-acting factor

Selection of internal start codons in a mRNA containing several alternative initiation codons is known to be achieved by a "leaky scanning" process involving *cis*-acting elements of the mRNA in the vicinity of the cap-proximal start codon (2). We have however demonstrated here that a *trans*-acting factor, hnRNP A1, contributes to the selection of internal codons in the FGF-2 mRNA. This study therefore highlights a novel function for a specific RNA binding protein, in preferentially promoting translation initiation events at a given initiation codon of an mRNA containing several alternative

translation start sites. This is particularly striking for hnRNP A1 that is already described for its properties to control the selection of alternative splice sites (30). We have also shown that hnRNP A1 is able to promote translation initiation events at internal start codons through its binding to the IRES. The role of hnRNP A1 as an ITAF is not restricted to the FGF-2 mRNA. Indeed, we have recently found that at least one viral RNA requires hnRNP A1 for its IRES-mediated translation (S.B. and S.V., unpublished data). However, hnRNP A1 is not active in mediating IRES-dependent translation of the human bip, c-myc or VEGF-A mRNAs. hnRNP A1 could therefore be involved in specific translational control of a subset of IRES-containing cellular genes or viral RNA genomes.

Mechanism of hnRNP A1-dependent IRES-mediated translation

At least two models are proposed to explain the role of ITAFs. First, an ITAF may help to recruit the 40S ribosomal subunit to the mRNA through specific interactions with canonical translation initiation factors or ribosomal components. Second, the role of an ITAF may be to promote or to stabilize specific active conformations of the IRES (38). Since hnRNP A1 facilitates duplex formation by complementary single-stranded polynucleotides (39), it may promote intra-molecular annealing reactions in order to reach the proper FGF-2 mRNA structure for 40S ribosome binding**.**

hnRNP A1-dependent translational control of the FGF-2 mRNA

Since expression of hnRNP A1 is regulated during cell transformation and following cellular stress, it may constitute a key factor of FGF-2 translational control during these cellular processes. Specifically, it was observed that both hnRNP A1 and hnRNP I/PTB,

but not hnRNP C1/C2, mRNAs are loaded on polysomes in a growth-dependent manner (40). As a consequence, the intracellular levels of hnRNP A1 would increase very rapidly after a proliferation stimulus. Indeed, the level of hnRNP A1 increases after transformation of rat embryo fibroblasts (41), consistent with previous reports of increased hnRNP A1 expression in transformed and rapidly proliferating cells (42,43). In addition to the regulation of hnRNP A1 levels, its activity is regulated by its subcellular localization. The expression of a permanently active mutant of zeta-PKC promotes the cytoplasmic accumulation of hnRNP A1 in proliferating cells (44). Furthermore, the subcellular localization of hnRNP A1 can be modulated by the MKK3/6-p38 pathway in response to stress such as osmotic shock and irradiation with UV-C light (45). Since our study highlights a novel function of hnRNP A1 in translation, the deregulation of hnRNP A1 level or activity may have important consequences for the translational regulation of several genes.

Specifically, the role of FGF-2 in tumorigenesis has been largely described. FGF-2 promotes cancerous cell survival and proliferation as well as tumor angiogenesis (5). FGF-2 translational regulation was shown to be tissue-specific (46) and to depend on cell transformation and stress (8,47). Importantly, it has been demonstrated that the p53 tumor suppressor protein inhibits FGF-2 translation through its direct binding to the FGF-2 mRNA (48,49), revealing important links between tumorigenesis and FGF-2 mRNA translation. Determining the interplay between p53 and hnRNP A1 in controlling FGF-2 mRNA translation is a key issue of our future work. More generally, it will be of great value to link signal transduction pathways involving hnRNP A1 with FGF-2 translational control. However, much work remains needed to obtain a detailed characterization of the physiological or pathophysiological roles of hnRNP A1 in controlling FGF-2 alternative initiation of translation.

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FIGURE LEGENDS

Figure 1. Purification of proteins that modulate FGF-2 translation *in vitro* in rabbit reticulocyte lysate (RRL). (A) Schematic representation of the human FGF-2 5' leader with the five in-frame translation initiation codons. (B) Biochemical purification schema. (C) and (D) Translation assays of capped FGF-2-CAT (the first 539 nts of the FGF-2 mRNA fused to a chloramphenicol acetyl transferase (CAT) ORF) or EMCV-CAT (the 640 nts upstream of the EMCV AUG initiation codon fused to the CAT ORF) reporter RNA in RRL, supplemented with 0.5, 1, 2 or 4 μg of fractions 7, 9 or 10 resulting from cation exchange chromatography. A negative control reaction (lane RRL) contained reticulocyte lysate supplemented with buffer D. ^{35}S methionine-labelled translation products were visualized by SDS-PAGE and autoradiography. The asterisk (*****) corresponds to a translation product initiated at an ACG codon, located 122 nts from the 5'-end, that only occurs in RRL but is never detected in transfected cells (9). Mean values and standard deviation values resulting from at least three experiments are indicated below each lane. (E) Colloidal blue staining of fractions 7 to 10. (F) UV cross-linking of Hela cell nuclear extracts (NE) (4 μg) and chromatographic fractions 7 to 10 (2 μg each) with ^{32}P-labelled FGF-2 5' leader RNA. The positions of protein molecular weight markers in kDa are indicated at the left hand side.

Figure 2. Purification of the FGF-2 5' leader RNA binding proteins. (A) The RNA-affinity chromatographic support consisted of an *in vitro* transcribed RNA containing a 3' poly(A) tail (25 nts) bound to a streptavidine acrylamide resin (Pierce) through a

$(dT)_{25}$ oligonucleotide biotinylated at its 5' end. (B) HeLa nuclear extracts were loaded to three different RNA affinity chromatographic resins containing the FGF-2 5' leader RNA (nts 1-539) (lane FGF-2), an RNA corresponding to the 640 nts upstream of the EMCV AUG initiation codon (lane EMCV) or a 140 nts-long control RNA (lane Ctrl). Eluates of these different columns were analyzed by SDS-PAGE (12%) and the gel was stained with colloidal blue (Sigma). Protein molecular weight markers, of size indicated in kDa, are shown at the left hand side. Positions of identified proteins are shown together with an annotation that refers to table I.

Figure 3. hnRNP A1 is present in fraction 9 and binds directly to the FGF-2 mRNA. (A) Western immunoblot analysis of hnRNP A1 (αA1) or PTB (αPTB) in fractions 7 to 10 were done respectively with the 4B10 monoclonal antibody against hnRNP A1 (Gift of G. Dreyfuss) or a polyclonal antibody against PTB. (B) UV cross-linking of Hela cell nuclear extracts (NE) (4 μg) and chromatographic fraction 9 (2 μg) with ^{32}P-labelled FGF-2 5' leader RNA. The positions of protein molecular weight markers in kDa are indicated at the left hand side. The arrow on the left points to a band that corresponds to the size of the hnRNP A1 protein. Immunoprecipitation (IP) of crosslinked proteins from either fraction 9 (left hand panel) or HeLa NE (right hand panel) were performed with (αA1) or without (mock) the 4B10 monoclonal antibody.

Figure 4. Depletion of hnRNP A1 from fraction 9. (A) UV cross-linking of Hela cell nuclear extracts (NE) (4 μg) with the ^{32}P-labelled FGF-2 5' leader RNA and competition with an unlabelled DNA oligonucleotide corresponding to the hnRNP A1 binding site (A1-WT) or with a mutated DNA oligonucleotide unable to bind hnRNP A1 (A1-MUT). 0.3 (lanes 2 and 5), 0.6 (lanes 3 and 6) or 1.2 (lanes 4 and 7) picomoles

of oligonucleotides were added corresponding respectively to a 6, 12 or 24 fold excess. (B) Western immunoblot experiment with the 4B10 antibody on the A1-WT or A1-MUT depleted fraction 9 or on the proteins bound to the A1-WT or A1-MUT beads. (C) Coomassie staining of proteins bound to the A1-WT or A1-MUT columns. (D) *In vitro* translation of the FGF-2-CAT reporter RNA was performed in RRL (lane 1) or in RRL supplemented with 2 (lanes 2 and 4) or 4 μg (lanes 3 and 5) of A1-WT or A1-MUT depleted fraction 9. Mean values and standard deviation values resulting from three experiments are indicated below each lane.

Figure 5. hnRNP A1 binds directly to the FGF-2 IRES. (A) Coomassie staining of the purified *E.Coli* produced GST-hnRNP A1 (GST-A1) protein. BSA: Bovine Serum Albumin. (B) UV cross-linking experiments were performed with the ^{32}P labelled FGF-2 (nts 1-484) RNA and the GST-A1 recombinant protein. Competitions were performed with 25, 50, 100 and 200 fold excess of unlabelled FGF-2 RNAs corresponding to the various indicated regions. A representative experiment, that was repeated three times, is shown. (C) Electrophoretic mobility shift (EMSA) experiments were performed with a ^{32}P labelled FGF-2 (nts 1-484) RNA and the GST-A1 recombinant protein. Competitions were performed with 25, 50, 100 and 200 fold excess of unlabelled FGF-2 RNAs corresponding to the various indicated regions. A representative experiment, that was repeated three times, is shown. (D) hnRNP A1 binding curve to the FGF-2 IRES RNA (nts 1-177). Filter binding assays were carried out and evaluated as described under “Materials and Methods”. Filter-bound RNA is plotted as function of the protein concentration, corrected for the fraction of active protein. Curves were fitted to average data points of three independent experiments.

Figure 6. hnRNP A1 modulates FGF-2 alternative initiation of translation in RRL. Translation reactions (20 μl final) in RRL were performed with 3 μg/ml of RNA (except in panel A in which the FGF-2-CAT RNA was used at a concentration of 0.3 μg/ml). Either 75, 150 or 300 ng of *E. Coli* produced and purified GST-tagged hnRNP A1 (GST-A1; panel A), PTB (GST-PTB; panel B) or La (GST-La, panel C) were added to RRL. The FGF-2-CAT and EMCV-CAT RNAs are described in the legend to Fig.2. The CAT mRNA is from the pKSCAT-pA plasmid described in (9). It contains the CAT ORF and a 15 nts-long 5' UTR. The Bip-CAT, VEGF-CAT and Myc-CAT reporter RNAs are composed respectively of the first 220 nts of the human bip mRNA, the first 1360 nts of the human VEGF-A mRNA and the first 550 nts of the human c-myc mRNA initiated at the P2 promoter, each fused to the CAT ORF. The position of the CUG- or AUG- initiated isoforms are indicated at the left of each panel. Quantifications of the data are presented in Table II.

Figure 7. hnRNP A1 controls FGF-2 alternative initiation of translation in HeLa cells. (A) RNAi-mediated hnRNP A1 depletion in HeLa cells was performed with two different siRNAs targeting two sequences located at nucleotides 45-63 and 831-849 of the human hnRNP A1 ORF. (B) The consequences of siRNA treatments were analyzed 72 hours after siRNA transfection by western immunoblotting with the 4B10 monoclonal antibody (α A1), the actin polyclonal antibody (Sigma) (α actin) or the FGF-2 polyclonal antibody (Santa Cruz) (α FGF-2). The "Mock" lane corresponds to untransfected HeLa cells. The "Ctr sIRNA" lane corresponds to a treatment with a control siRNA (5'-CAGTCGCGTTTGCGACTGGdTdT-3'/5'-CCAGTCGCAAACGCGACTGdTdT-3'). Experiments were performed on three independent occasions. (C) Levels of endogenous FGF-2 mRNA was determined by real time RT-PCR analysis as described in Materials

and Methods. The ratio of the Ct value of reverse transcription real-time PCR reaction for FGF-2 mRNA to the Ct value for 18S rRNA is shown on the y-axis.

Figure 1, Bonnal et al.

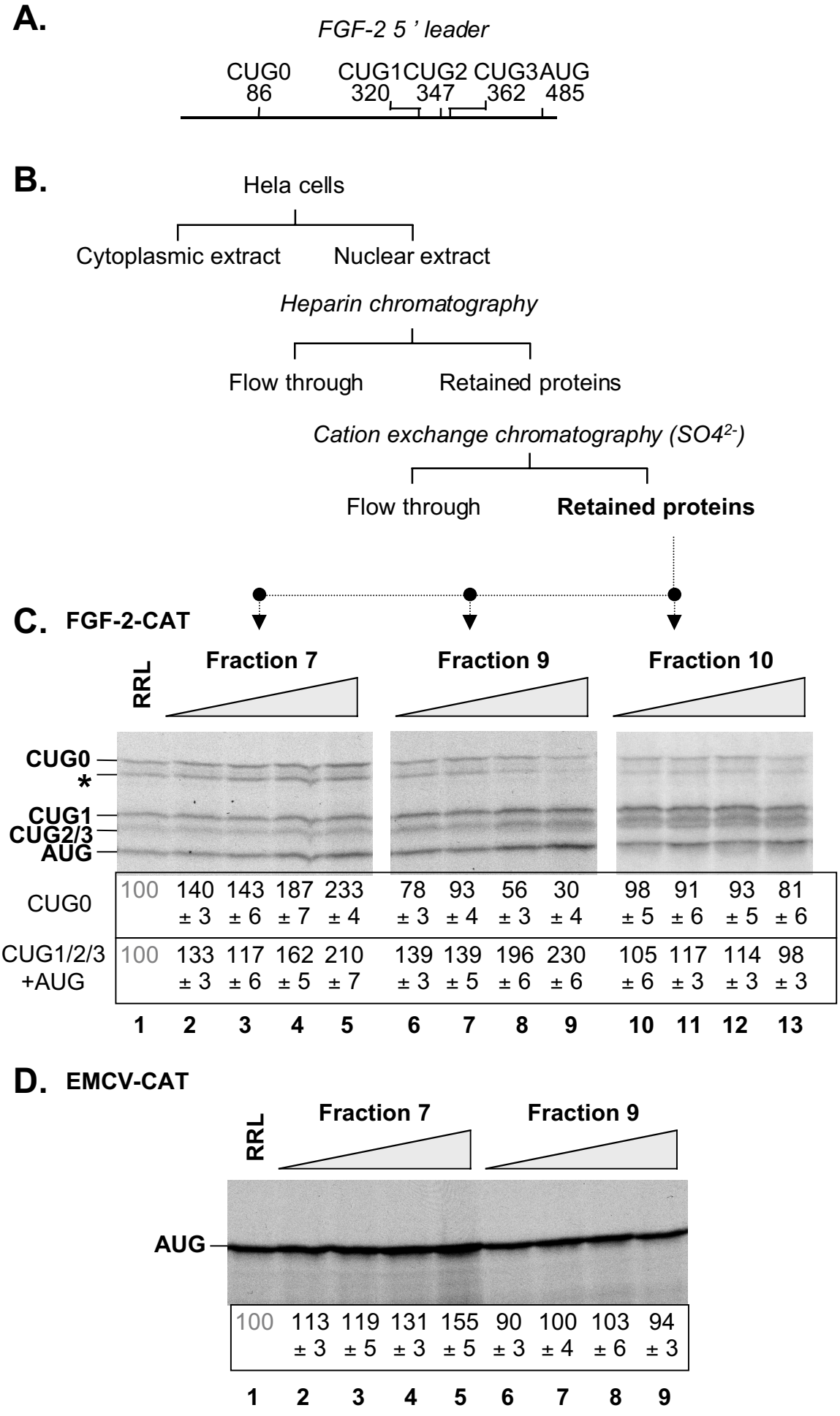


Figure 1, Bonnal et al.

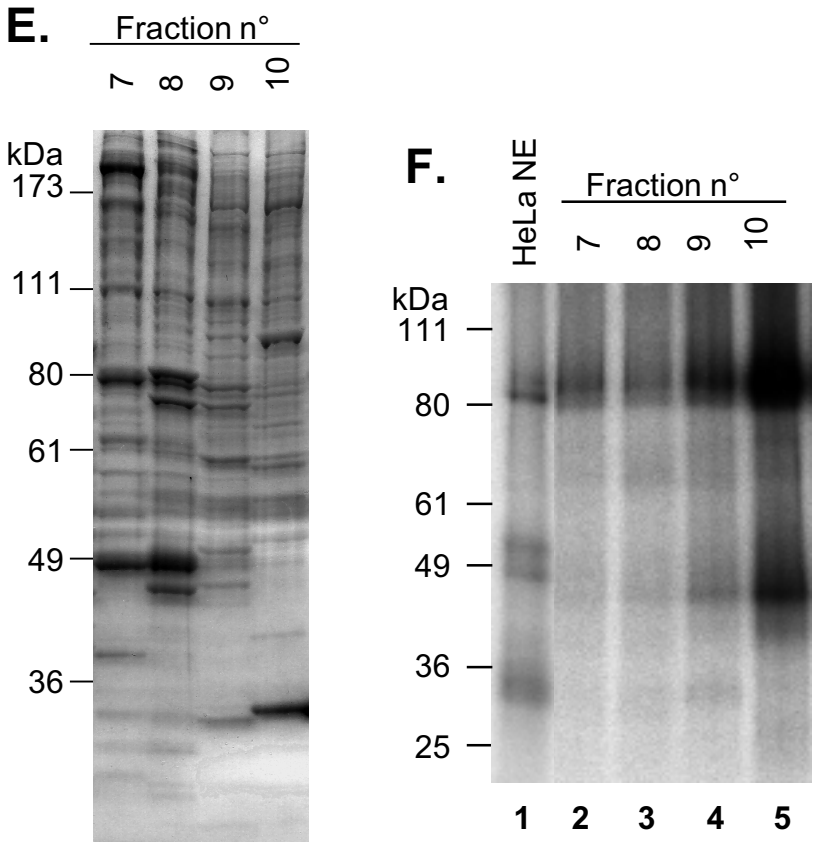


Figure 2, Bonnal et al.

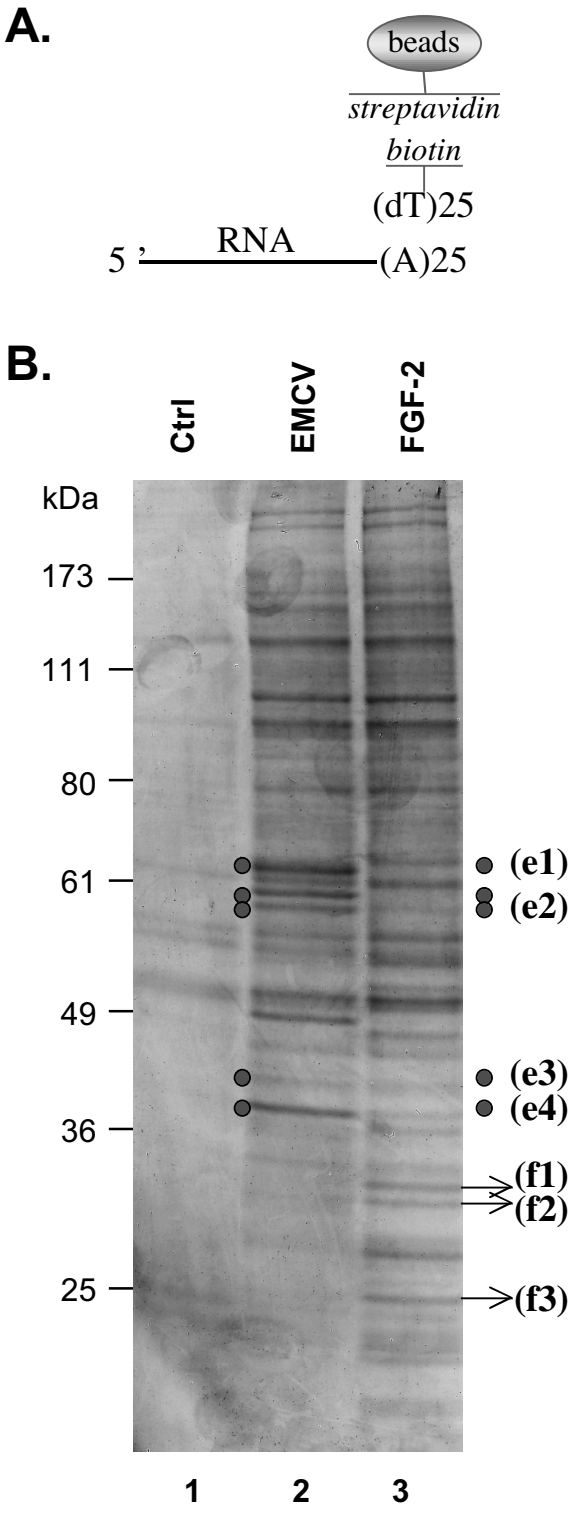


Figure 3, Bonnal et al.

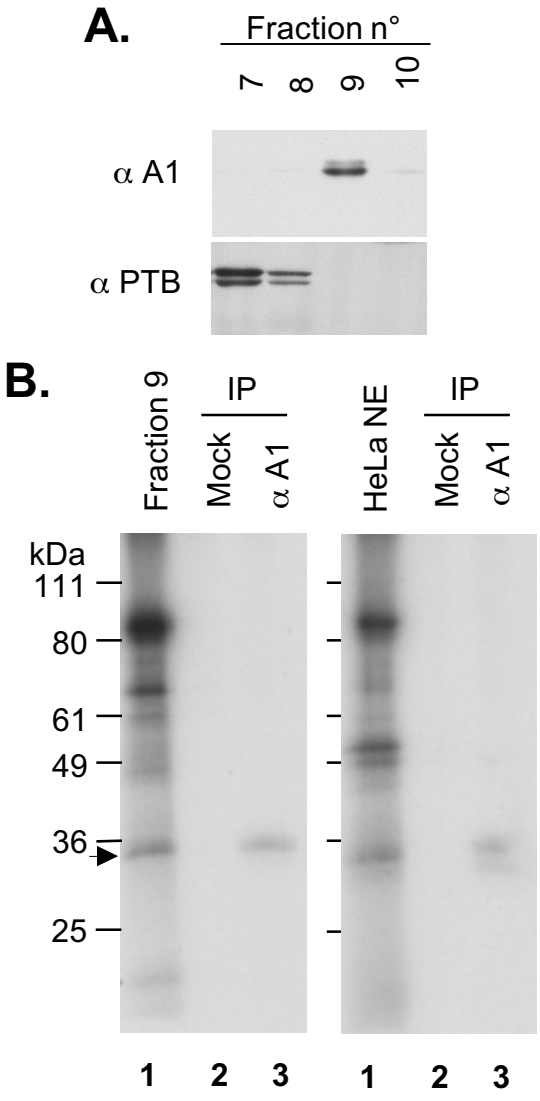


Figure 4, Bonnal et al.

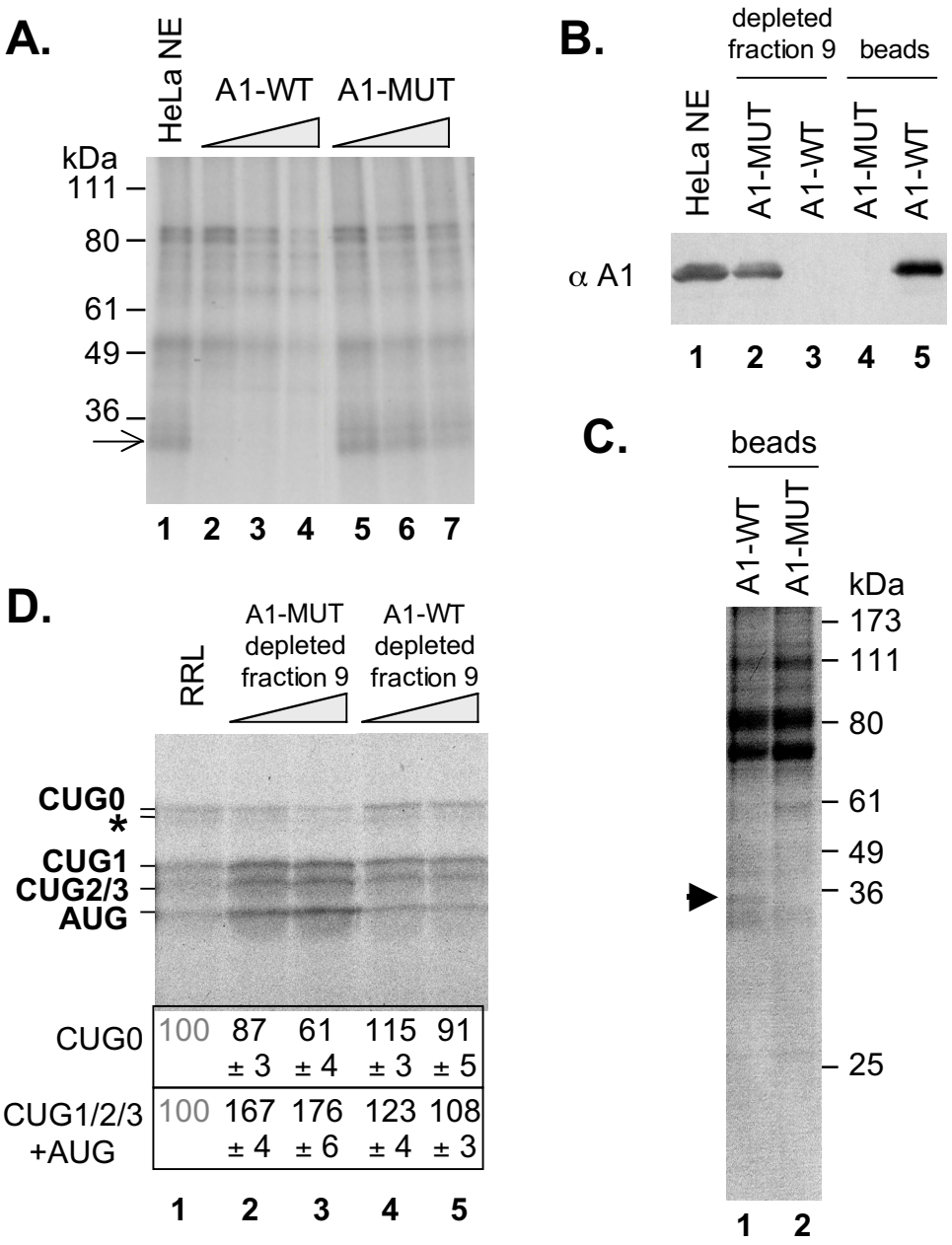


Figure 5, Bonnal et al.

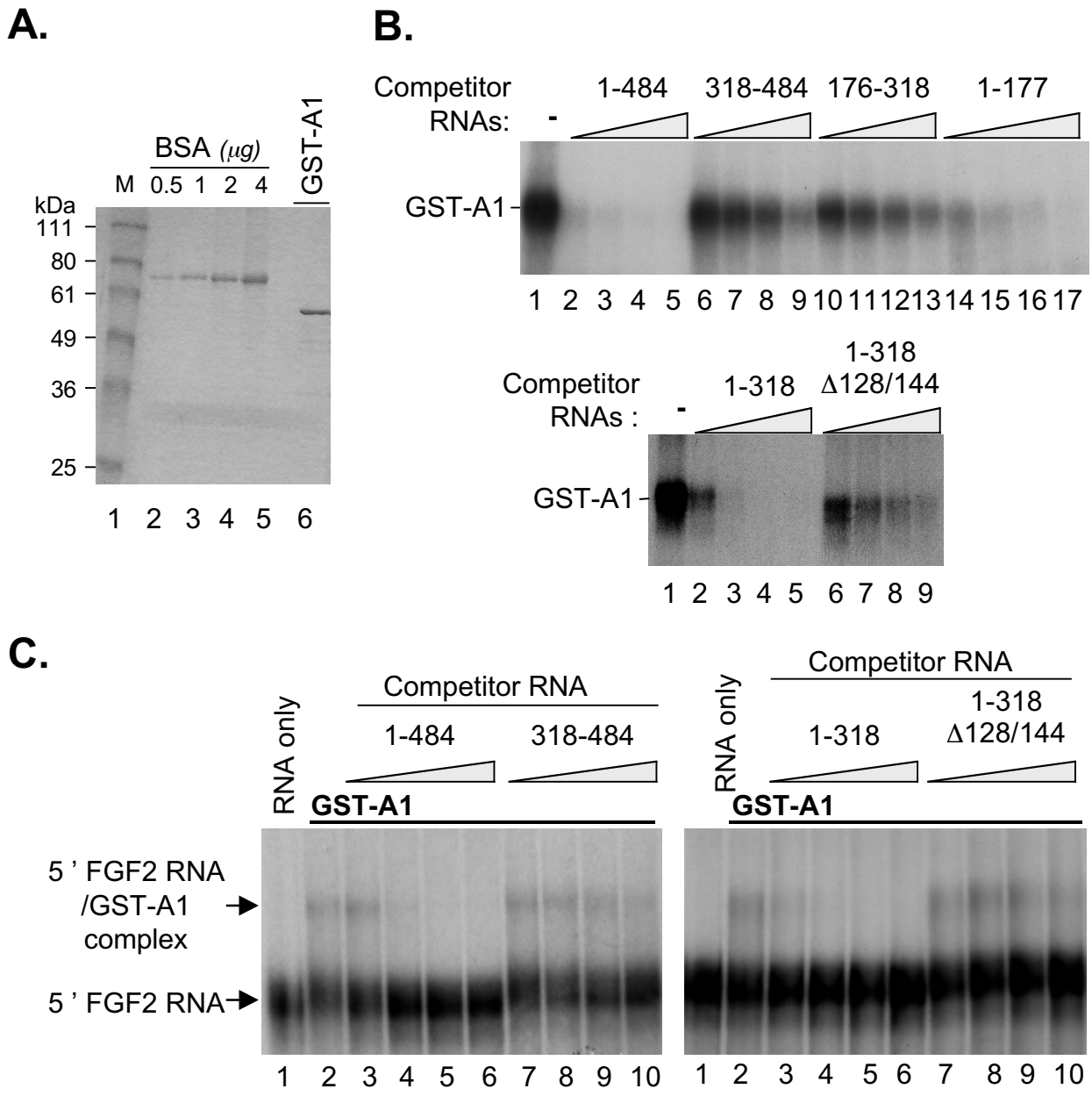


Figure 5, Bonnal et al.

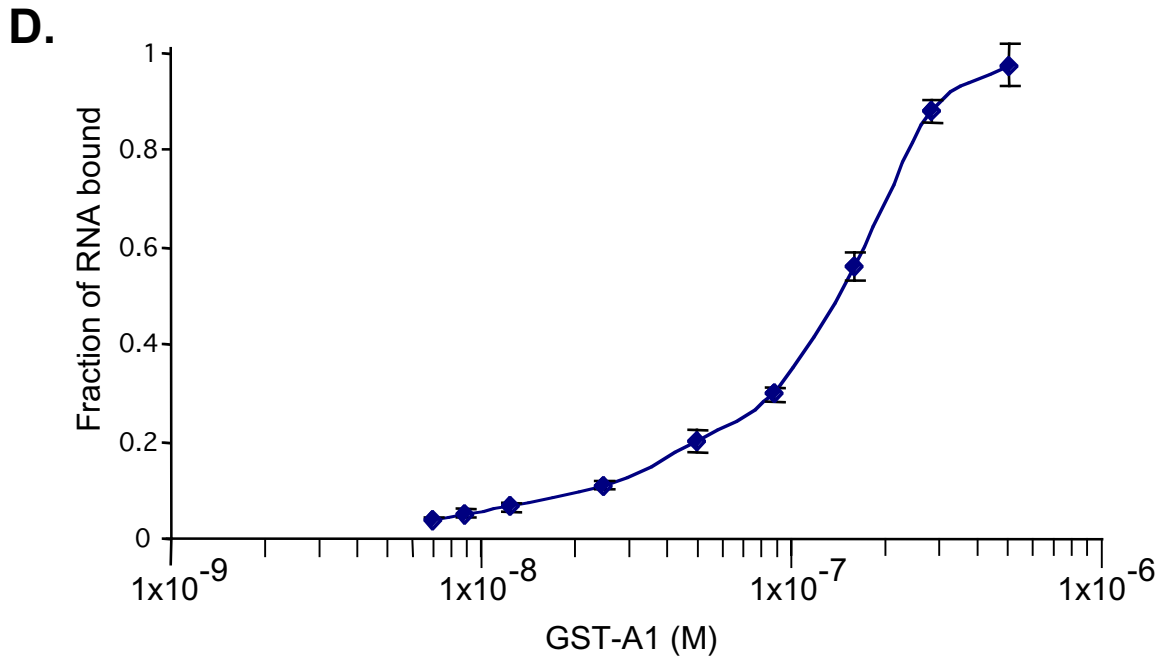


Figure 6, Bonnal et al.

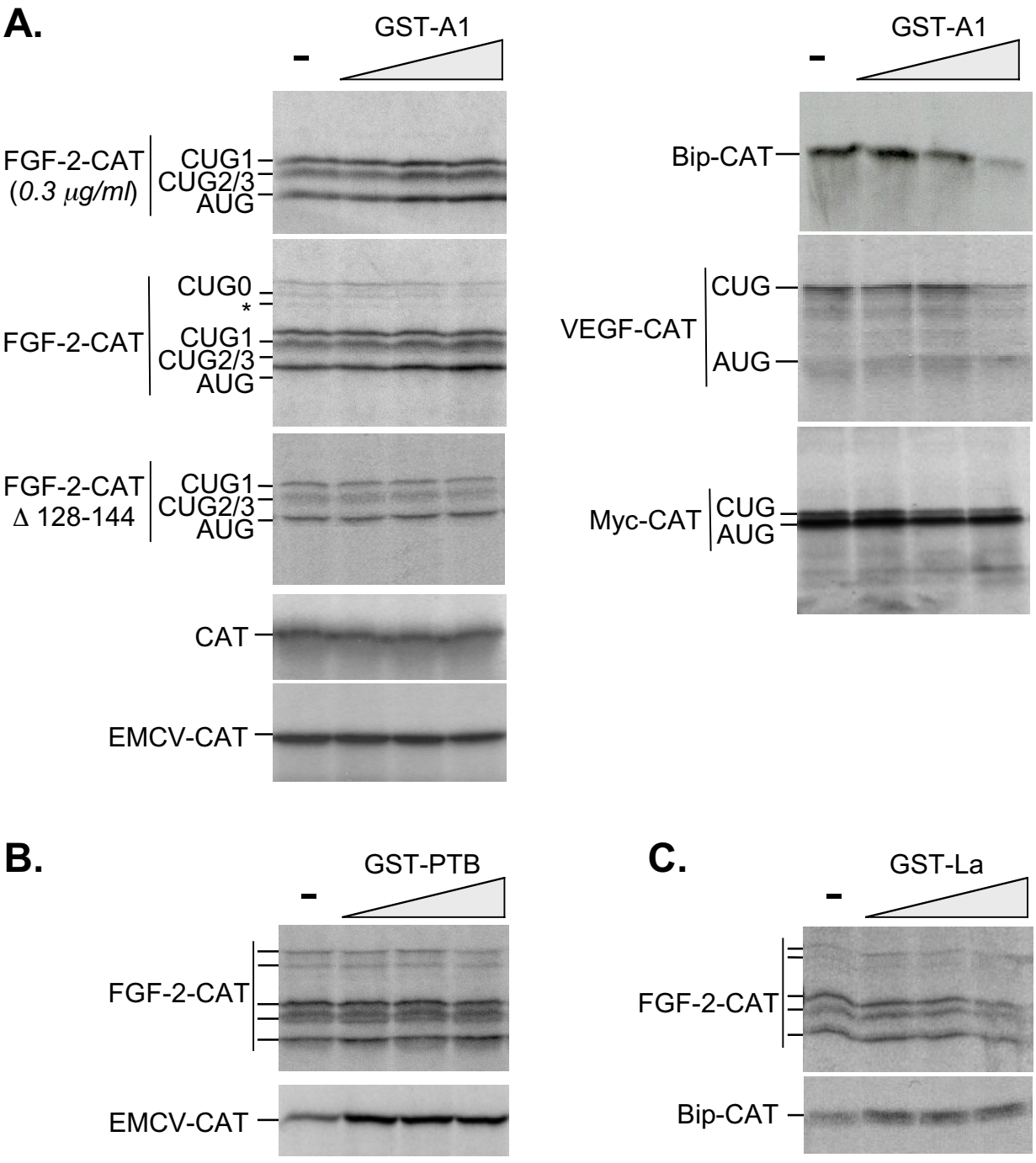


Figure 7, Bonnal et al.

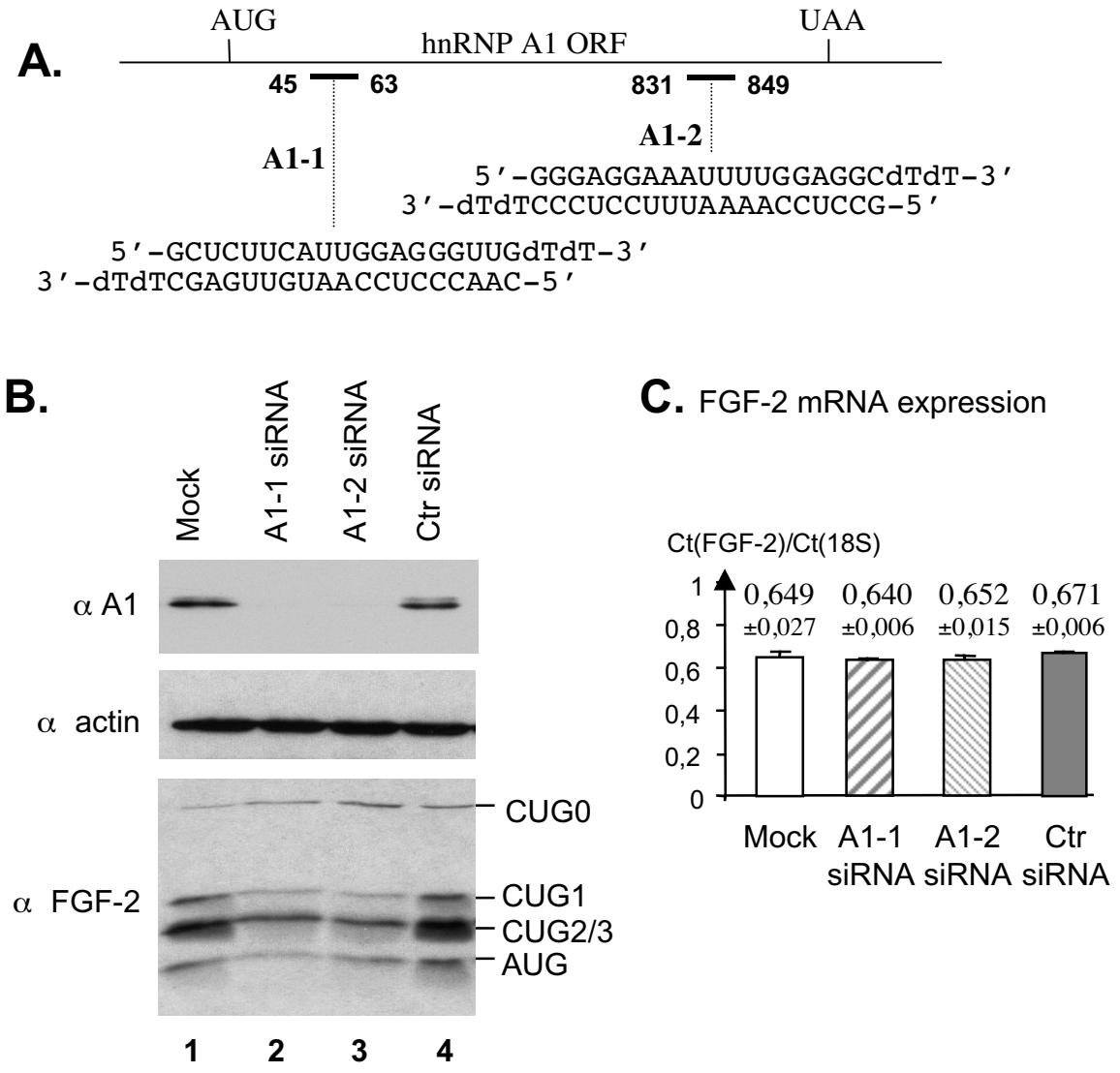


Table I. Proteins identified by tandem MS/MS following the purification procedure described in figure 1

Name	NCBI Accession number	Annotation on Fig.1
EMCV RNA column		
Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	QO7244	(e1)
Polypyrimidine tract-binding protein 1 (PTB/hnRNP I)	P26599	(e2)
IRES *trans*-acting factor 45 (ITAF45)/PA2G4/Mpp1	AAD05561	(e3)
Poly(rC)-binding protein 1 (Alpha-CP1) (hnRNP E1)	Q15365	(e4)
FGF-2 RNA column		
40S ribosomal protein S2	P15880	/
40S ribosomal protein S3A	P49241	/
40S ribosomal protein S3	P23396	/
40S ribosomal protein S6	P10660	/
40S ribosomal protein S9	P55935	/
Heterogeneous nuclear ribonucleoprotein A1(hnRNP A1)	P09651	(f1)
ASF/SF2 splicing factor	P09012	(f2)
U1 small nuclear ribonucleoprotein A (U1 snRNP A protein)	Q13809	(f3)

Table II, Bonnal et al.

Table II. Quantifications of the effects of adding various recombinant proteins on *in vitro* translation in RRL of the RNA substrates described in Fig. 5, measured as a percentage of translation efficiency in presence of the recombinant protein compared to its absence.

RNA (initiation codon)*	Recombinant protein								
	GST-A1			GST-PTB			GST-La		
	75 ng	150 ng	300 ng	75 ng	150 ng	300 ng	75 ng	150 ng	300 ng
FGF-2-CAT (CUG0)	90 ± 3	55 ± 3	22 ± 3	104 ± 5	104 ± 6	89 ± 3	106 ± 5	83 ± 3	71 ± 3
FGF-2-CAT (CUG1/2/3 + AUG)	92 ± 6	230 ± 6	215 ± 6	98 ± 6	100 ± 3	101 ± 6	108 ± 4	90 ± 3	69 ± 4
FGF-2-CAT (CUG1)	91 ± 3	214 ± 7	135 ± 3	ND	ND	ND	ND	ND	ND
FGF-2-CAT (CUG2/3)	94 ± 5	201 ± 6	185 ± 6	ND	ND	ND	ND	ND	ND
FGF-2-CAT (AUG)	91 ± 3	272 ± 7	318 ± 7	ND	ND	ND	ND	ND	ND
FGF-2-CAT (CUG1/2/3 + AUG)	106 ± 3	197 ± 11	320 ± 6	ND	ND	ND	ND	ND	ND
FGF-2-CAT (CUG1)	116 ± 7	203 ± 5	333 ± 8	ND	ND	ND	ND	ND	ND
FGF-2-CAT (CUG2/3)	101 ± 4	188 ± 5	292 ± 7	ND	ND	ND	ND	ND	ND
FGF-2-CAT (AUG)	107 ± 5	196 ± 6	352 ± 8	ND	ND	ND	ND	ND	ND
FGF-2-CAT Δ 128-144 (CUG1/2/3 + AUG)	108 ± 8	104 ± 8	99 ± 5	ND	ND	ND	ND	ND	ND
CAT (AUG)	104 ± 5	105 ± 8	106 ± 3	ND	ND	ND	ND	ND	ND
EMCV-CAT (AUG)	99 ± 7	104 ± 5	90 ± 2	218 ± 4	224 ± 5	203 ± 6	ND	ND	ND
Bip-CAT (AUG)	89 ± 6	75 ± 6	58 ± 8	ND	ND	ND	210 ± 6	197 ± 4	196 ± 5
VEGF-CAT (CUG + AUG)	98 ± 4	123 ± 8	70 ± 7	ND	ND	ND	ND	ND	ND
Myc-CAT (CUG + AUG)	104 ± 5	78 ± 4	77 ± 6	ND	ND	ND	ND	ND	ND

Each value is an average of three independent experiments and indicates the mean value and the standard deviation. Experiments were carried out as described in the legend to Fig. 5. *All RNAs were at a final concentration of 3 μg/ml in the translation reaction except FGF-2-CAT (CUG1/2/3 + AUG) annotated in italic that was at a final concentration of 0.3 μg/ml. ND: not determined.