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# RNA binding specificity of Unr, a protein with five cold shock domains

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## ABSTRACT

**The human *unr* gene encodes an 85 kDa protein which contains five cold shock domains (CSD). The capacity of Unr to interact *in vitro* with RNA and its intracellular localization suggest that Unr could be involved in some aspect of cytoplasmic mRNA metabolism. As a step towards identification of Unr mRNA targets, we investigated the RNA-binding specificity of Unr by an *in vitro* selection approach (SELEX). Purine-rich sequences were selected by Unr, leading to the identification of two related consensus sequences characterized by a conserved core motif AAGUA/G or AACG downstream of a purine stretch. These consensus sequences are 11–14 nt long and appear unstructured. RNAs containing a consensus sequence were bound specifically by Unr with an apparent dissociation constant of $1 \times 10^{-8}$ M and both elements, the 5′ purine stretch and the core motif, were shown to contribute to the high affinity. When the N-terminal and C-terminal CSD were analyzed individually, they exhibited a lower affinity than Unr for winner sequences (5- and 100-fold, respectively) but with similar binding specificity. Two combinations of CSDs, CSD1-2-3 and CSD1*2-3-4-5 were sufficient to achieve the high affinity of Unr, indicating some redundancy between the CSDs of Unr for RNA recognition. The SELEX-generated consensus motifs for Unr differ from the AACAUC motif selected by the *Xenopus* Y-box factor FRGY2, indicating that a diversity of RNA sequences could be recognized by CSD-containing proteins.**

## INTRODUCTION

The *unr* gene (upstream of N-*ras*) was identified as a transcription unit located immediately upstream of the N-*ras* gene in the genome of several mammalian species (1–3). The *unr* gene is ubiquitously expressed in cell lines and tissues (2,3). It encodes Unr, a highly conserved 85 kDa protein (99% amino acid identity between rat and human), which contains five cold shock domains (CSD). Unr is essential for mouse development, since embryos homozygous for a disrupted *unr* allele die at mid-gestation (O.Boussadia *et al*., manuscript in preparation).

The CSD is the most evolutionarily conserved nucleic acid-binding protein domain, found in bacteria and eukaryotes (4,5). This domain of ~70 amino acid residues mediates binding to single-stranded DNA and RNA (6,7). Recent studies have revealed similarities between the CSD and the RRM (RNA recognition motif), a motif found in a variety of functionally diverse RNA-binding proteins and which has been characterized in detail. The CSD contains the RNA-binding motifs RNP-1 and RNP-2, which are the hallmark of RRM (8,9). Moreover, the solution and crystal structure of a CSD determined from bacterial cold shock proteins revealed a striking similarity with the RRM, both domains folding in a compact β-barrel, with the basic and aromatic amino acids of RNP-1 and RNP-2 being solvent exposed (10–12). Those residues which make direct contacts with RNA in the RRM protein U1A (13,14) are also involved in nucleic acid-binding of the bacterial protein CspB (15).

CSD proteins, prokaryotic and eukaryotic, have been found up to now to be involved in two processes: transcriptional and translational control. In prokaryotes, most studies have been developed around CspA and CspB, the major cold shock proteins in *Escherichia coli* and *Bacillus subtilis*, respectively. CspA and CspB are massively and transiently induced after a temperature downshift and are involved in the adaptation to cold shock (16). These two homologous proteins consist of a unique 67 amino acid CSD and function as transcriptional inducers of genes involved in the cold shock response, most likely by binding to the open transcription complex (17–19). In addition, CspA has been shown to increase translation of its own mRNA, by promoting destabilization of secondary structures (20).

In eukaryotes, the Y-box factors (YB) have been described in vertebrates; they contain a single CSD and an auxiliary basic-aromatic tail domain. Initially, YB proteins were characterized as transcription factors, interacting with a variety of double-stranded or single-stranded DNA sequences in promoters (6). Subsequently, some members of the YB family were identified as major components of messenger ribonucleoprotein particles (mRNPs) in germ cells (FRGY2/mRNP4 and MSY1) and somatic cells (p50) (21–25). *In vitro*, Y-box factors are involved in translational control, a positive or negative effect depending on the protein

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concentration (26). Specifically, in *Xenopus*, FRGY2/mRNP4 plays an essential role in the regulation of translation during early embryonic development and is involved in coupling transcription and translational repression of certain mRNAs (27,28).

Besides the YB proteins, three other CSD proteins have been described in eukaryotes: *Drosophila* YPS which contains one CSD and one RGG box (29), *Caenorhabditis elegans* Lin-28 which contains one CSD and two zinc finger motifs (30) and Unr. Lin-28 and YPS are cytoplasmic proteins and are likely to function in post-transcriptional regulation. Unr is also mostly localized in the cytoplasm, in part associated with the endoplasmic reticulum (31), which suggests a role for Unr in cytoplasmic mRNA metabolism. Unr was recently shown to be required for internal initiation of translation of human rhinovirus RNA (32).

In our initial characterization of the human Unr protein, we have determined that it has the capacity to interact *in vitro* with single-stranded DNA and RNA. Competition analysis of Unr–RNA interaction indicated that among simple polymers poly(A/G) was the best competitor, providing a first indication of its sequence specificity. To further characterize the RNA-binding specificity of Unr and eventually identify RNA ligands, we have used an *in vitro* selection/amplification approach (SELEX; 33). As previous studies with Unr (31) and FRGY2/mRNP4 (34) indicated that their interaction with RNA was inhibited by $MgCl_2$, we performed two independent SELEX experiments, in the presence or absence of $MgCl_2$. In this study we isolated high affinity binding sites for Unr and through mutagenesis of selected RNAs, we analyzed sequence requirement for Unr–RNA interaction. We also determined that a single CSD exhibits the same sequence specificity as Unr, although with a 5-fold lower affinity.

## MATERIALS AND METHODS

### Oligonucleotides and DNA templates

Synthetic oligodeoxynucleotides (Oligo Express) used for SELEX experiments were identical (Rev) or similar (T7 and Random N20) to those used by Tsaï *et al*. (35): T7, 5′-TGCATGGATCCTAATACGACTCACTATAGGGGCCACCAACGACATT-3′; Random N20, 5′-CCCGGTGGTTGCTGTAA(N)$_{20}$CAACTATATTTATCACGGGT-3′; Rev, 5′-CAACTATATTTATCACGGGTACTTAAGCTGTC-3′. Restriction sites for *Bam*HI and *Eco*RI were introduced in oligos T7 and Rev, respectively (underlined), for cloning. T7 primer contained a T7 RNA polymerase promoter sequence.

The mutant DNA templates were synthesized by PCR as described for the SELEX pool C0, using T7 and Rev as amplification primers. The oligonucleotide templates for mutagenesis have the same fixed sequences as oligo N20, but the 20 nt internal sequence was substituted by the indicated sequences in Table 2. For the two control oligonucleotides, Ctr and Sc Pu, the 20 nt internal sequence was such as to minimize secondary structures, similarly to most selected RNAs: Ctr, 5′-CTTAGTCCGATTGCCACTCT-3′; ScPu, 5′-AACGATAATGCAATGGGCAA-3′. Before *in vitro* transcription, all PCR DNAs were *Eco*RI digested.

Sequencing primer: 5′-GCTATGACCATGATTACGCC-3′.

### Recombinant proteins

*Constructs*. The human cDNA encoding full-length Unr (767 amino acids), in which the ATG initiating codon was substituted by a *Bam*HI site, was subcloned as a *Bam*HI–*Sma*I fragment into the *Bam*HI and *Pvu*II sites of *E.coli* expression vector pRSETA (Invitrogen). The fusion protein derived from this plasmid contains a 36 amino acid tag that includes six consecutive histidine residues. Similarly, a cDNA with a stop codon at position +381 of the open reading frame, encoding a 127 amino acid protein (CSD1, amino acids 2–128), was subcloned in the same way in pRSETA (Invitrogen). The Unr derivatives CSD1-2-3 (amino acids 15–387) and CSD5 (amino acids 629–718) were generated by PCR using *Pfu* turbo DNA polymerase (Stratagene) and human *unr* cDNA as template. The PCR primers introduced an in-frame stop codon, as well as *Bam*HI and *Hin*dIII sites at the 5′- and 3′-ends of the amplified fragment, respectively. The Unr mutant protein CSD1*2345 was a gift from Dr R Jackson; this protein was purified from *E.coli* as a C-terminal histidine-tagged protein (32). In this mutant protein, the essential phenylalanine of the RNP-1 motif of the first CSD (amino acid 39) was mutated to alanine to prevent RNA-binding activity of this CSD.

*Protein expression and purification*. The His-tagged proteins were expressed in *E.coli* BL21 cells using the pET system (Novagen) and purified on a $Ni^{2+}$-NTA-agarose column. Briefly, ~0.5–1 mg of protein equilibrated in urea–Tris buffer (9 M urea, 20 mM Tris–HCl, pH 7.4) was incubated with 2 ml of $Ni^{2+}$-NTA-agarose (Qiagen) for 40 min. The beads were transferred into a column and successively washed with decreasing urea concentrations (from 9 M to none) in the same buffer, allowing renaturation of the proteins. Proteins were eluted with 2 ml of 500 mM imidazole and dialyzed against TNG buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol). For Unr, purification by SDS–polyacrylamide gel electrophoresis was performed as previously described (31) prior to loading on the $Ni^{2+}$-NTA column. The length and protein concentrations were estimated from SDS–PAGE electrophoresis after Coomassie Brilliant Blue staining, using protein molecular weight markers as standard. Protein fractions were stored at –70°C until use.

### *In vitro* selection

A degenerate double-stranded DNA template was synthesized by PCR using the three oligonucleotides T7, Rev and N20 (template for PCR reaction). PCR was performed with 0.8 µg of T7 and Rev primers and 120 ng of N20, with native *Pfu* polymerase (Stratagene) for nine cycles.The resulting DNA pool consisted of about $3 \times 10^{12}$ molecules ($10^{12}$ unique sequences). An aliquot of 1 µg of the DNA pool was *in vitro* transcribed with T7 RNA polymerase (Gibco BRL) to give the $^{32}$P-labeled RNA pool C0. Selection steps were performed using Unr protein immobilized on $Ni^{2+}$-NTA-agarose (five initial cycles) and by a gel mobility shift assay (five subsequent cycles). Selection of RNA on Unr-$Ni^{2+}$-agarose beads was carried out in a batchwise fashion as follows: 5 µg of purified His-Unr protein were mixed with 25 µl of $Ni^{2+}$-NTA-agarose equilibrated with RNA selection buffer [25 µg/ml BSA, 25 µg/ml tRNA, 5 U/ml RNAguard (Pharmacia), 1 mM PMSF, 1 mM β-mercaptoethanol and 1 µg/ml of leupeptin, antipain and aprotinin, in TNG buffer] in a final volume of 100 µl and incubated for 45 min at 25°C with shaking. After three washes with 0.5 ml of selection buffer, $Ni^{2+}$-agarose-bound His-Unr protein was mixed with 200 µl of the same buffer containing 0.25 µg of labeled RNA pool and 80 U of RNAguard. After a 30 min incubation at 30°C with occasional shaking, the resin was pelleted at 1000 *g* and free RNA was removed by

washing three times with 0.5 ml of ice-cold binding buffer. Bound RNA was eluted by two incubations with 0.4 ml of elution buffer (0.1 M glycine–HCl, pH 4.0, 25 µg/ml tRNA, 0.025% SDS, 5 mM $MgCl_2$), for 5 min at 35°C. The eluate was extracted with phenol/chloroform and chloroform and RNA was ethanol precipitated. Half of the selected RNA was reverse transcribed for 1 h at 42°C in 30 µl of RT buffer (Promega), with 10 U of avian myeloblastosis virus reverse transcriptase (Promega) and 100 ng of Rev primer. After phenol/chloroform and chloroform extractions, the cDNAs were ethanol precipitated with 1 µg of tRNA. The cDNAs were then diluted 2-fold and amplified by 15 cycles of PCR, with 2.5 U of native *Pfu* polymerase, 600 ng of T7 and Rev primers in 100 µl of PCR buffer. The PCR DNA product was then *in vitro* transcribed and utilized for the next round of selection. After the fifth cycle, the RNA pool was applied to Unr-less $Ni^{2+}$-NTA-agarose to remove non-specific RNA. Five additional selection/amplification cycles were performed using a band shift assay to separate Unr–RNA complexes. An aliquot of 50 ng of labeled RNA pool was incubated in 40 µl of selection buffer with 250 ng (70 nM) of Unr protein. Following a 30 min incubation at 30°C, Unr-bound RNAs were electrophoresed in an 8% polyacrylamide gel in 1× Tris–borate–EDTA buffer at 4°C overnight, at 2.5 V/cm. After a –70°C gel exposure to X-ray hyperfilm (Amersham Life Science), the bands corresponding to complexed RNAs were located and excised. Elution was carried out in 0.5 M $NH_4Ac$, 1 mM EDTA, overnight at 37°C. The eluate was extracted with phenol/chloroform and chloroform and ethanol precipited with 1 µg of tRNA. RT–PCR was carried out as described above. After the last PCR, the DNA was digested with *Eco*RI and *Bam*HI and cloned into the plasmid pUC13 and 50 individual clones were sequenced using a primer located 60 nt upstream of the random sequence. DNA sequencing was performed with Sequenase v.2.0 (US Biochemical Corp.)

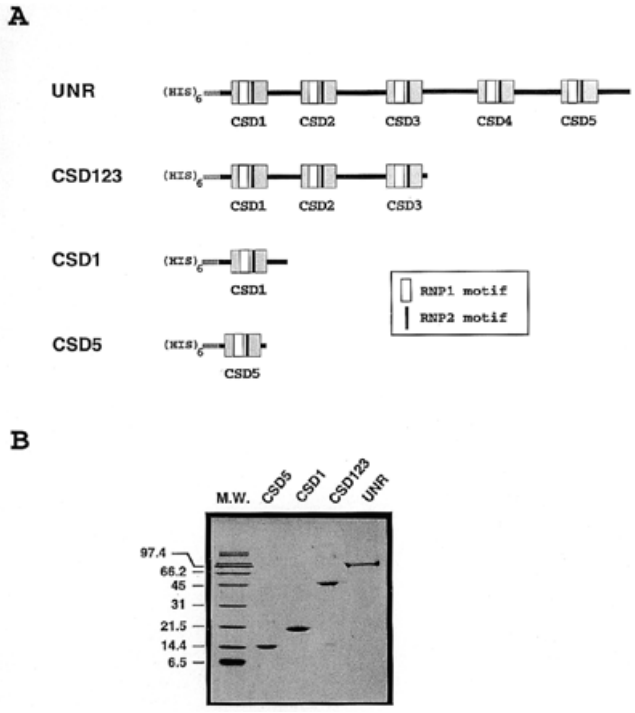


**Figure 1.** (**A**) Schematic representation of the Unr protein and of the mutants used in this study. The full-length human Unr protein and the CSD1, CSD5 and CSD1-2-3 derivatives contain a 36 amino acid histidine tag at their N-terminus. The CSDs are boxed and RNP1 and RNP2 motifs are indicated. (**B**) SDS–PAGE analysis of purified proteins. Aliquots of 2 µg of each protein were loaded on a 15% SDS–polyacrylamide gel that was stained with Coomassie Blue. The size of the molecular weight markers is indicated on the left.

### RNA transcription

*Eco*RI-linearized clones or *Eco*RI-digested PCR products were *in vitro* transcribed using 50 U of T7 RNA polymerase, with 20 µCi of [α-$^{32}$P]UTP (300 Ci/mmol) and 1 mM unlabeled nucleotides. Resulting labeled RNAs (62 nt long) were purified by phenol/chloroform and ethanol precipitation and their integrity was checked by electrophoresis on a urea–acrylamide gel. [α-$^{32}$P]UTP incorporation was quantified to estimate RNA concentration.

### Determination of RNA-binding affinities

*Nitrocellulose filter-binding assays*. Aliquots of 1–3 fmol of labeled RNA were incubated at 30°C for 20 min in 20 µl of RNA-binding buffer (25 µg/ml BSA, 10 ng of tRNA, 1 mM β-mercaptoethanol and 1 mM PMSF, in TNG buffer), with increasing amounts of protein as indicated. After cooling on ice for 5 min, the reaction was filtered through a wet 0.45 µm pore nitrocellulose filter (Schleicher & Schuell) under gentle suction and dried. Retention of labeled RNA was analyzed by liquid scintillation. Each assay included a control for RNA retention in the absence of protein. Percentage of RNA bound to filters was corrected by substracting the retention of free RNA (1–2%) from data points. Affinity constants were derived by plotting the fraction of complexed RNA as a function of protein concentration (36). Efficiencies of retention for the RNAs studied here ranged from 50 to 80%. The fraction of complexed RNA was taken from the plateau of binding and the plateau was assigned as 1.0. The reported $K_d$ values are an average of three binding curves. The variation in $K_d$ values obtained from different experiments was ~20–30%.

*Gel retardation assays*. RNA/protein incubations were performed as described for nitrocellulose assays, in a 40 µl final volume. After cooling on ice, the mixture was directly loaded on a 3.8% polyacrylamide gel (acrylamide:bisacrylamide, 40:1) containing 5% glycerol and 25 mM Tris–glycine, pH 8.0. The gel was electrophoresed at 4°C for 3–4 h at 20 V/cm and then dried. The amount of free and bound RNA was analyzed with a phosphor screen and a Storm 860 (Molecular Dynamics).

## RESULTS

### *In vitro* selection of Unr-binding RNA

To investigate the sequence requirements for Unr–RNA interaction, we used the selection amplification procedure (SELEX; 33). The RNA population used consisted of 62 nt long molecules, with 20 nt of randomized sequence (35). Selection of RNA–protein complexes was performed with hexahistidine-tagged protein. Recombinant His-Unr protein was purified to homogeneity as shown in Figure 1 and we checked that the N-terminus His tag did not alter the RNA-binding capacity of Unr (data not shown).

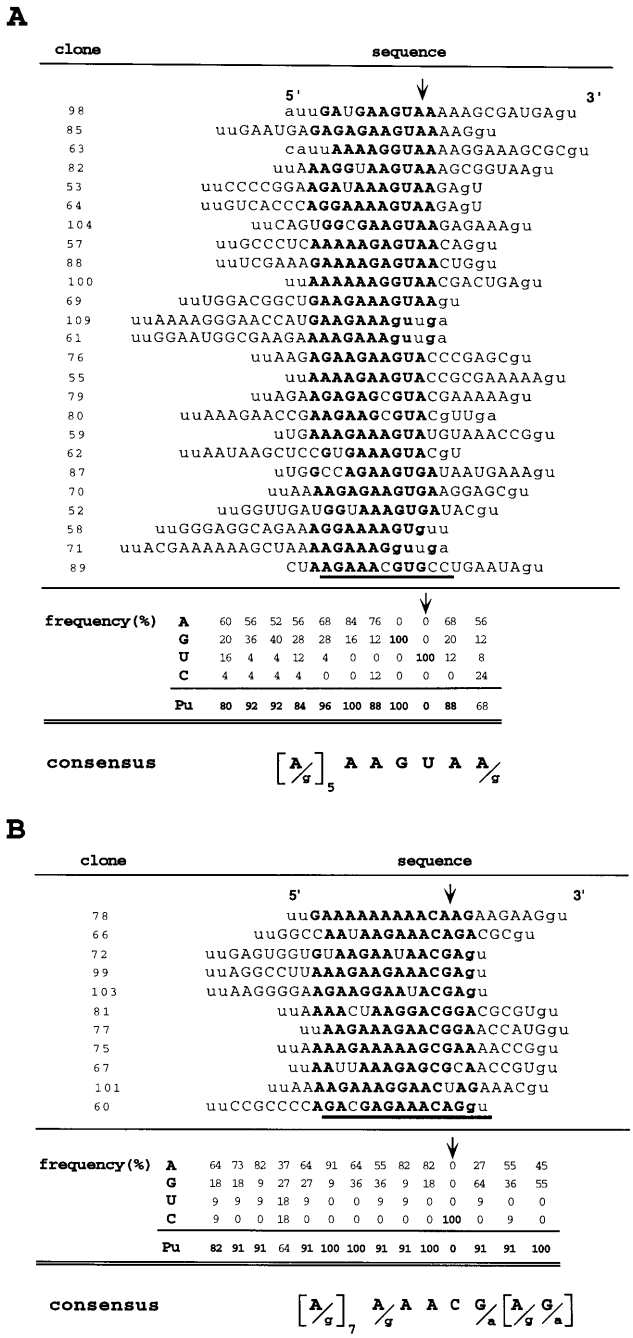


**Figure 2.** Nucleotide RNA sequences selected by Unr protein from a randomized pool. The names of the individual clones are given to the left of each selected sequence. Individual sequences are classified as the GU group (**A**) or the AC group (**B**) and aligned by shared sequence motif indicated by bold, underlined characters. The variable region is in capital letters and nucleotides belonging to the 5′ and 3′ flanking constant regions (two or more are shown) are in lower case. The frequencies of nucleotides selected at each position of the bolded underlined region and the two deduced consensus motifs are shown at the bottom of each group of selected sequences. Individual bases are specified when they appear with a frequency ≥66%.

Ten rounds of selection/amplification were performed. For the first five rounds of selection, His-Unr protein immobilized on a $Ni^{2+}$-NTA matrix was used, as described for the identification of specific RNA ligands for the splicing factors ASF/SF2, SC35 and RBP1 (37,38). To prevent selection of RNAs that interact with the $Ni^{2+}$ matrix (39), we used a band shift assay for the five subsequent rounds of selection to isolate Unr–RNA complexes.

The first SELEX experiment, SELEX A, was performed in the absence of $MgCl_2$. After a total of 10 cycles, 46 clones were sequenced.The sequences of 36 inserts of SELEX A are presented in Figure 2. They showed a marked enrichment in purines (76%), which was mostly due to adenine residues (49%). Visual inspection of the sequences revealed the presence of a purine-rich cluster of 11–14 nt containing a pyrimidine located near its 3′-end. The presence of this single pyrimidine residue provided the basis for a unique alignment of these sequences. This was further supported by an analysis with the polyphylogenetic alignment program Clustal W which yielded two similar albeit slightly different consensus sequences depending upon the nature of the pyrimidine (Fig. 2A and B). More precisely, a first group of 25 sequences contained a uridine while a second group of 11 sequences contained a cytosine. Using a threshold value of 75% to indicate a conserved position the following consensus sequences were derived $(Pu)_5$AAGUA(Pu) and $(Pu)_8$AAC-$^{G}/_{A}(Pu)_2$. In three clones of the first group, the aligned G and U residues were derived from the downstream primer. Ten sequences could not be aligned in either group, although the purine enrichment was in the same range (72% Pu, 55% A). As such ‘scrambled’ purine-rich sequences display a lower affinity for Unr, these sequences were not further analyzed.

A second SELEX (SELEX B) was carried out under the same conditions as SELEX A, except for the presence of 1 mM $MgCl_2$. After a total of 10 cycles, 50 clones were sequenced. The purine enrichment was similar to that observed in SELEX A and 27 sequences could be aligned in the two groups defined by the results of SELEX A (not shown). As for SELEX A, the 23 other sequences presented a high purine content, but the presence of several pyrimidines made possible multiple alignments with the consensus which did not provide additional information.

The presence of $Mg^{2+}$ cation in the selection therefore did not reveal other sequence motifs or structures in the Unr RNA targets. In the subsequent analysis some sequences obtained in SELEX B were included for comparison and were denoted ‘b’.

The high purine content of the selected sequences should prevent the formation of secondary structure by Watson–Crick base pairs. To further investigate the structural context of the purine clusters, we analyzed 15 sequences using the Mfold software (40). In 11 cases, and irrespective of the presence or absence of magnesium during the selection process, the selected sequence was clearly predicted to be located within a single-stranded domain of the molecule. These results thus agree with the previously observed lack of affinity of Unr for double-stranded RNA (31).

In summary, the same consensus motifs were obtained in the two independent SELEX experiments. These consensus sequences are characterized by the motif AAGUA or AAC$^{G}/_{A}$ located downstream of a 5–8 nt long purine stretch.

## Affinity of Unr for the selected RNAs

To further characterize the interaction of Unr with the selected sequences, we first used a gel mobility shift assay. Individual sequences were transcribed from the corresponding plasmids after linearization at their 3′-end, yielding molecules of the same size as those used in the SELEX experiments. Figure 3 presents the results obtained with three different Unr-selected sequences

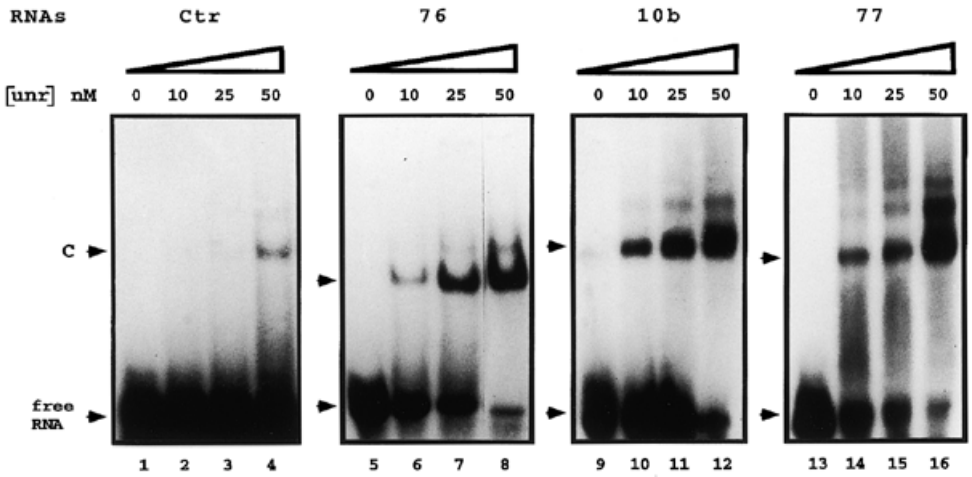


**Figure 3.** Gel mobility shift assay of His-Unr with several selected and unselected RNAs. Aliquots of 2 fmol of $^{32}$P-labeled RNAs were incubated with increasing protein concentrations and the products were resolved by native gel electrophoresis as described in Materials and Methods. The RNAs analyzed were from SELEX A (RNAs 76 and 77) or SELEX B (RNA 10b). A random RNA (Ctr) was used as a control. The RNAs are numbered on top and the concentrations of Unr are indicated above the lanes. Positions of free RNA (probe) and RNA–protein complexes (C) are indicated with arrows on the left. RNA insert sequences are presented in Figure 2 (RNAs 76 and 77) or in Table 1 (RNA 10b).

(76, 10b and 77) and a non-selected sequence (Ctr), in which 14 of the 20 nt variable region are pyrimidines. As shown in Figure 3A, Unr bound the three selected sequences efficiently (e.g. lanes 6–8, 10–12 and 14–16), 50% of the RNA being shifted at a Unr concentration of 10–25 nM. In contrast, a shift was detected only at the highest protein concentration tested with the non-selected sequence Ctr (50 nM, lane 4). These results indicate that Unr binds specifically and with high affinity to the selected sequences and with no apparent preference for sequences of the GU or AC consensus group (Fig. 2).

In these experiments, one major band was observed which could be accounted for by the formation of 1:1 RNA–Unr complexes. However, at the highest Unr concentration one or two minor species could also be observed (Fig. 3, lanes 8, 12 and 16). These more slowly migrating species suggest the presence of several Unr molecules within one complex, either as the result of an interaction of additional Unr molecules with the flanking RNA sequences or because of the formation of Unr multimers.

These band shift experiments were suggestive of an apparent dissociation constant for the selected sequences in the 20 nM range. To refine and extend this analysis to a larger set of selected sequences, we used a nitrocellulose filter-binding assay (Fig. 4). Figure 4A presents representative binding curves of five sequences of SELEX A. As is frequently observed in nitrocellulose-binding assays (41,42), the plateau did not reach 100%, even at higher Unr concentrations (data not shown). The dissociation constants were therefore derived from the Unr concentration yielding half-maximum retention. Of the five sequences analyzed (Fig. 4A), four (77, 58, 88 and 85) had dissociation constants of $10 \pm 2$ nM and therefore could not be distinguished by this assay (Table 1). The fifth one (98) had a 2-fold greater dissociation constant of 20 nM. Concordant with the shift assay, Unr binds the non-selected Ctr sequence with a much lower affinity (Fig. 4A).

Figure 4B presents the results of the nitrocellulose filter-binding assay for four sequences isolated in SELEX B. In these experiments the binding buffer was supplemented with 1 mM $Mg^{2+}$ in accordance with the SELEX conditions. Three sequences (4b, 36b and 10b) had a dissociation constant of 10–12 nM, the fourth one (28b) being 15 nM (Table 1). Thus, the sequences isolated in SELEX B had the same affinity for Unr as those obtained in SELEX A. To further investigate the role of magnesium, we used heterologous conditions to determine the dissociation constants of sequences from the SELEX A and B experiments (i.e. 1 mM $Mg^{2+}$ for the A sequences and no $Mg^{2+}$ for the B ones). The presence or absence of $Mg^{2+}$ had no effect on the affinity of Unr for these sequences. Further studies indicated that the presence of 5 mM $Mg^{2+}$ induced a 2-fold reduction in the affinity of Unr for sequences derived from both SELEX experiments (data not shown). Thus, the presence of magnesium modified neither the nature of the selected RNAs nor the affinity of Unr for these sequences. In the subsequent experiments, binding assays were performed in the absence of magnesium.

The dissociation constants of a total of 13 sequences are compiled in Table 1. Ten sequences had dissociation constants of $10 \pm 2$ nM and three had slightly higher $K_d$ values (16, 18 and 20, respectively). Thus a majority of the isolated sequences are high affinity binding sites with indistinguishable affinities for Unr, a few sequences having slightly lower affinities. As the SELEX procedure is known to converge very slowly in the presence of multiple targets with comparable affinities these results clearly indicate the efficiency of the selection.

More specifically, data presented in Table 1 indicate that: (i) high affinity binding sites were identified in the GU as well as the AC consensus group, confirming the results of the gel shift experiments; (ii) interruption of the upstream purine stretch as in sequences 98 and 29b slightly decreases the affinity (at most 2-fold); (iii) comparable dissociation constants of Unr for sequences 85, 88 and 76 which contain respectively five, two and one purines downstream of the pyrimidine indicate that the 3′ purines are dispensable for efficient binding.

In summary, all of the aligned selected RNA sequences, whether of the GU or AC consensus group, are bound with a high affinity by Unr. Moreover, single base changes (Pu→Py) have very limited effect on Unr–RNA recognition.

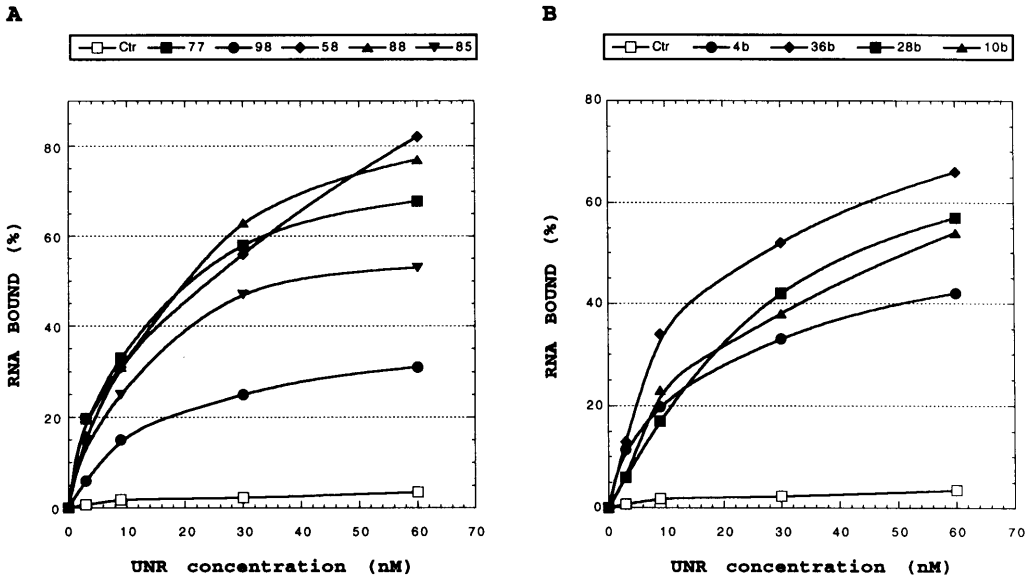


**Figure 4.** His-Unr binding curves on several selected RNAs. Graphs show some results of filter-binding experiments, in which the percentage of complexed labeled RNA retained on the filter is plotted against the amount of protein added to the binding reaction. The protein concentration is varied, whereas RNA (~$10^4$ c.p.m.) is held at a constant concentration of 0.15 nM. Binding reaction and filtration are described in Materials and Methods. (**A**) Incubation mixture does not contain $MgCl_2$; (**B**) incubation mixture contains 1 mM $MgCl_2$. Symbols used to represent selected RNAs and Ctr RNA (control) are indicated at the top.

**Table 1.** $K_d$ values of Unr for selected RNAs

| CONSENSUS TYPE | RNA [a] | SEQUENCES | Kd (nM) [b] |
|---|---|---|---|
| GU | 85 | GAGAGAA**GU**AAA | 11 |
| | 88 | GAAAAGA**GU**AAC | 10 |
| | 76 | AGAAGAA**GU**ACC | 10 |
| | 98 | uGAUGAA**GU**AAA | 21 |
| | 58 | AGGAAAA**GU**guu | 13 |
| | 10b | AAAAAAA**GU**AAG | 12 |
| | 36b | AAGAAAG**GU**AAC | 10 |
| | 23b | AAGAAAA**GU**GCA | 12 |
| | 28b | GGAAAAG**gu**uga | 16 |
| | 29b | AAAAUGA**GU**GAU | 18 |
| AC | 77 | AGAAAGA**AC**GGAA | 10 |
| | 78 | AAAAAAA**AC**AAGA | 8 |
| | 16b | UAAGAGA**AC**AAAA | 12 |

The two consensus sequences are indicated by their conserved GU or AC motifs.
[a]The name of each selected RNA tested is given on the left and the 'b' set of sequences were derived from the SELEX B experiment.
[b]$K_d$, apparent affinity of RNA ligands for His-Unr was determined from nitrocellulose-filter binding assays. Each $K_d$ value is the average of three independent experiments. Standard deviations are ~30%.

## RNA sequence requirements for binding

To assess the functional significance of the upstream purine stretch and of the adjacent conserved motif, mutations were introduced in two selected sequences, 85 and 78 (Table 2). Introduction of a single pyrimidine within the upstream purine stretch (mutant 85M2) decreased the affinity slightly, while the introduction of a pyrimidine at position +1 (mutant 85MT4) had no significant effect. These results therefore confirm the limited effect on Unr binding of point mutations within the consensus. In mutants M3 and M5, pyrimidine-rich sequences were introduced to preserve only the conserved core motif. These mutations reduced the interaction by 2.5- to 3-fold, revealing that the core motif surrounding the pyrimidine is not sufficient for a high affinity interaction. Eliminating the conserved core motif while preserving the upstream purine stretch (mutant MT3, AGUA→UUCU) decreased the affinity by 5-fold, demonstrating the importance of the core motif for Unr–RNA interaction.

These results underscore the importance of the observed organization of the consensus sequences and suggest that, by itself, a high purine content should not be sufficient to create an optimal binding site for Unr. To confirm this we generated an RNA target with a 70% purine content but containing no stretch of more than three purines (Sc Pu). Despite its high purine content, this sequence displayed a 10-fold lower affinity for Unr.

In summary, both the upstream purine stretch and the conserved environment of the pyrimidine contribute to the high affinity for Unr. The presence of only one of these elements reduces affinity by a factor of three and, in their absence, the affinity decreases by a factor of 10.

## RNA binding properties of Unr derivatives

Since Unr contains five CSDs and two consensus sequences were identified in the SELEX experiments, it was possible that different CSDs had selected these two sets of sequences. To assess the role of individual CSDs we constructed three proteins, which are schematically presented in Figure 1A. CSD1 and CSD5 contain the N-terminal and the C-terminal CSD of Unr, respectively; CSD1-2-3 contains the three N-terminal CSDs. These proteins were expressed with a His tag in *E.coli* and purified on a $Ni^{2+}$-NTA-agarose column to near homogeneity as shown in Figure 1B. We

**Table 2.** Mutational analysis of the consensus RNA binding sequences of Unr

| | RNA variants | Binding Ratios |
|---|---|---|
| **A** | | |
| | -14 0 +5 | |
| 85 | GAAUGAGAGAGAAGUAAAAG | 1 |
| M2 | GAAUGAGAGUGAAGUAAAAG | 0.6 |
| M3 | CCAUUAUCCGUAAGUAAUCA | 0.4 |
| MT3 | GAAUGAGAGAGAUUCUAAAG | 0.2 |
| MT4 | GAAUGAGAGAGAAGUUAAAG | 1 |
| **B** | | |
| | -10 0 +9 | |
| 78 | GAAAAAAAAACAAGAAGAAG | 1 |
| M5 | CUACGCUAAACAAGUCAUAU | 0.3 |
| Sc Pu | AACGAUAAUGCAAUGGGCAA | 0.1 |

Binding ratios were derived from binding curves, using a filter-binding assay at a Unr concentration of 10 nM using RNA 85 (A) or RNA 78 (B) as reference. Sc Pu was compared to RNA 85. RNA 85 and 78 belong to the GU and AC consensus groups, respectively. The values are the average of three determinations. Mutated nucleotides are indicated in outline.

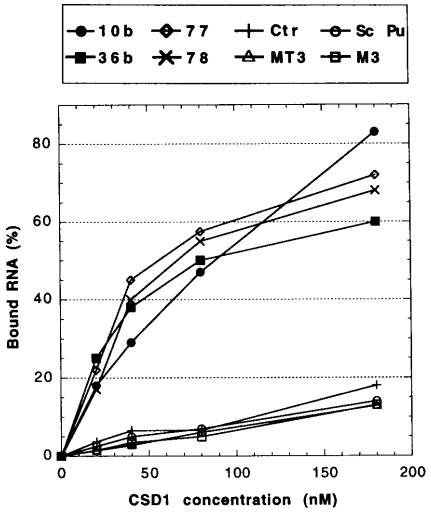


**Figure 5.** RNA binding specificity of CSD1. The binding of CSD1 to Unr-selected RNAs, mutant RNAs and random control RNAs was analyzed using a nitrocellulose filter-binding assay as in Figure 4. Each binding curve represents the results of three independent measurements. Symbols used to represent tested RNAs are indicated at the top.

first investigated the binding affinity and specificity of CSD1. As shown (Fig. 5), CSD1 had a 5-fold lower affinity for the winner sequences than Unr. However, the specificity of CSD1 was comparable with that of Unr, as the control RNAs Ctr and Sc Pu as well as the mutated sequences M3 and MT3 were poor substrates for CSD1. Importantly, CSD1 did specifically interact with both groups of selected sequences: GU (10b and 36b) and AC (77 and 78). We then analyzed the binding of the other Unr derivatives with two sequences of the GU group (10b and 36b), two of the AC group (77 and 78) and the MT3 mutant (Ctr RNA was also included and gave concordant results with those of MT3; data not shown). These results are summarized in Table 3. CSD5 had a much lower affinity for RNA than the other proteins ($K_d$ values in the micromolar range), but still retained some specificity. With its three CSDs, CSD1-2-3 was almost indistinguishable from the full-length Unr protein (Table 3) indicating that the interaction of Unr with the selected sequences does not require all the CSDs. We also investigated the behavior of a full-length Unr protein carrying a mutation within the first CSD (CSD1*-2-3-4-5; 32). Despite the lack of a functional CSD1, this protein exhibited both high affinity and selectivity for the winner sequences (Table 3)

These results indicate that a single CSD (CSD1) can account for a specific interaction with the two groups of winner sequences. This observation is also true for the C-terminal CSD (CSD5) although in this case, the overall affinity for RNA is reduced by 100-fold. The presence of three CSDs as in CSD1-2-3 is sufficient to confer the same affinity for the winner sequences as the full-length protein indicating that not all the CSDs are required. Intriguingly, the presence of a functional CSD1 is not required to achieve either affinity or specificity, suggesting the existence of redundancy between the CSDs of Unr.

**Table 3.** RNA binding affinity of Unr derivatives

| Protein | Binding affinities Kd (nM) | | MT3 Relative Binding |
|---|---|---|---|
| | GU Group | AC Group | |
| UNR | 10 | 10 | 0.2 |
| CSD1 | 55 | 50 | 0.08 |
| CSD1-2-3 | 10 | 12 | 0.2 |
| CSD1*2-3-4-5 | 16 | 15 | 0.2 |
| CSD 5 | ~1500 | ~1500 | 0.3 |

Apparent dissociation constants ($K_d$ values) were determined as in Table 1. Two RNAs of the GU group (10b and 36b) and of the AC group (77 and 78) were used. The binding affinities are the mean of four measurements (two with each RNA); standard deviations were 20–30%. The MT3 relative binding was determined with the filter assay at a protein concentration of 15 nM (300 nM for CSD5) and using sequence 77 as a reference.

## DISCUSSION

In this study, *in vitro* selection experiments were performed to determine the RNA-binding specificity of the human Unr protein. Two SELEX experiments were carried out, differing only by the presence or absence of $MgCl_2$. In both experiments, long purine-rich sequences (11–14 purines and a single pyrimidine) were recovered after 10 rounds of selection. Alignment of the sequences of 36 of 46 clones (SELEX $-Mg^{2+}$) and of 27 of 50 clones (SELEX $+Mg^{2+}$) identified two related consensus motifs: 5′-$(Pu)_5$AAG**U**A(Pu)-3′ and 5′-$(Pu)_8$AA**C**$^{G}/_{A}(Pu)_2$-3′ characterized by an upstream purine tract and an adjacent conserved motif surrounding the pyrimidine. Selected RNA

sequences from either consensus group bound Unr with a high affinity ($K_d$ values in the range of 10 nM), as determined by nitrocellulose and mobility shift assays. Stable secondary structures that rely on canonical base pairs do not exist for the two consensus sequences, suggesting that Unr discriminates between RNA via their primary sequences rather than their secondary structure. Interestingly, the affinity of Unr for the selected sequences shows little sensitivity to the presence of $MgCl_2$ in contrast to our previous observation with longer RNA substrates. One possibility, which we are currently investigating, is that on long RNA molecules multimeric Unr complexes are formed which are sensitive to the presence of $Mg^{2+}$. Alternatively, magnesium could act via an effect on RNA conformation, by stabilizing secondary structures, which are frequently extensive on long RNA molecules.

Unr binds specifically to the selected RNAs; its affinity for selected sequences is at least 10-fold higher than for non-selected control sequences. Independently of the nature of the pyrimidine, the interaction of Unr with selected RNAs is characterized by a high affinity ($K_d$ = 8–20 nM) which varies little between individual sequences. These data indicate that Unr–RNA recognition is not rigidly sequence dependent. To better understand the basis of Unr–RNA binding specificity, mutations were introduced in two of the selected RNAs. While a single base change interrupting the purine stretch decreases the affinity by less than 2-fold, eliminating one of the elements of the consensus, i.e. the upstream purine stretch or the pyrimidine-containing core motif, decreases the affinity by 3- to 4-fold. Finally, scrambling of a selected sequence leads to a 10-fold lower affinity. Together, these data eliminate the possibility that Unr affinity for RNA depends only on the purine content of the molecules; they support the notion that the two parts of the consensus sequences, the upstream purine stretch and the adjacent pyrimidine-containing core motif, are necessary to constitute an optimal substrate for Unr.

Two broad classes of RNA-binding proteins can be distinguished with respect to their RNA target structures. In the first class, RBPs interact with stem–loop structures. These proteins include U1A (43), U2B″ (44), nucleolin (45), iron regulatory factor IRF (46) and eIF-4B (47). Proteins from this class can bind to their RNA targets with high affinity ($K_d = 10^{-9}$–$10^{-11}$ M) and specificity. In the second class, RBPs interact specifically with single-stranded RNA sequences. These proteins include poly(A)-binding protein (48), the splicing factors ASF/SF2, SC35 and Sxl (37,49), the polypyrimidine tract-binding protein (49,50), and the polyadenylation factor CstF (51,52). SELEX analysis of the binding specificity of these proteins usually leads to degenerate consensus sequences reflecting the existence of several high affinity binding sites. Point mutations of the conserved nucleotides of these consensus sequences are often associated with limited variations in affinity *in vitro* (49). Unr, with its degenerate consensus and limited sensitivity to point mutations, clearly belongs to this class of proteins. In this class, it is interesting to note the analogy with PTB, a protein involved in regulation of splicing (49,50) and internal initiation of translation (53) for which the SELEX consensus is similarly organized with a core motif embedded in a pyrimidine-rich sequence PTB (50).

As Unr contains five CSDs, the pool of selected sequences could be the resultant of the sequences selected by individual CSDs. Specifically, the two consensus sequences, which differ by the nature of the pyrimidine, could have been selected by distinct CSDs. However, a protein which contains only the N-terminal CSD (CSD1) exhibits the same sequence specificity as Unr, albeit with a 5-fold lower affinity. Moreover, although a protein which contains only the C-terminal CSD (CSD5) has a much lower affinity for RNA than Unr ($K_d$ values in the micromolar range), it retained a similar sequence specificity. Thus, these studies indicate that a single CSD has the ability to interact with the two consensus sequences obtained in the SELEX experiments. In this respect, Unr differs from many of the RBPs with multiple RRM, as in several instances it has been shown that the observed sequence specificity requires the contribution of several RRMs: ASF/SF2, (37); PABP, (54,55); nucleolin, (56); Sxl, (57).

Within Unr several CSDs are required to achieve a high affinity for the selected sequences indicating that several CSDs can contribute to the binding energy. The properties of CSD1 suggest that the N-terminal CSD could be the major contributor to the binding energy, other CSDs, as in CSD1-2-3 and Unr, playing the role of auxiliary domains. However, analysis of CSD1*2-3-4-5 reveals that CSD1 is not absolutely required for high affinity binding, indicating that other CSDs can substitute for it. Taken together these results suggest that within Unr several CSDs can contribute to the binding energy, but that several combinations of CSDs can lead to similar RNA–protein interaction. This and the idea that a single CSD is sufficient to achieve the observed sequence specificity lead to the proposition that there is some redundancy between the CSDs of Unr. Further studies will be required to determine how many CSDs can simultaneously interact with a substrate.

Up to now, a characterization of RNA binding specificity for CSD proteins using the selection/amplification procedure has only been performed for Unr (this work) and for the two homologous Y-box proteins FRGY1 and FRGY2 (58). Unr and FRGY1/FRGY2 have the capacity to bind RNA with sequence specificity, but distinct consensus sequences were obtained. The consensus for FRGY1 and FRGY2 (AACAUC) clearly differs from the two related purine-rich consensus sequences for Unr. FRGY1 and FRGY2 contain only one CSD (almost identical in the two proteins) and one additional tail-binding domain. As shown by Bouvet *et al.* (58), it is the CSD that determines RNA-binding specificity of FRGY1/FRGY2, the tail domain contributing to the overall affinity. The bacterial cold shock proteins CspA and CspB, which consist of only a single CSD, bind RNA with a low affinity ($K_d = 3 \times 10^{-5}$–$5 \times 10^{-6}$ M) and a broad specificity (5,59). It thus appears that the CSD can mediate specific RNA interactions, as observed for Unr and FRGY1/FRGY2, or more relaxed ones, as reported for CspA and CspB. In this respect, the CSD behaves similarly to the RRM, which can bind to a variety of RNA sequences with various levels of specificity (60,61). According to the analysis of the human U1A protein (62,63), the high level of amino acid variation between CSDs (up to 70%) should allow a large range of sequence specificity.

Because of the sequence flexibility of the consensus, the results of the present study do not allow a direct identification of the mRNA targets for Unr. However, an ongoing study of cellular factors involved in internal initiation of translation of the HRV-2 rhinovirus has identified Unr as one of them (32). This raises the possibility that Unr could be involved in internal initiation of translation of cellular mRNAs. Indeed, an increasing number of cellular mRNAs, including those of some growth factors and developmental regulators, have been shown to be translatable by internal initiation (64–66). The consensus identified in this work will foster studies on the role of Unr in internal initiation of translation both *in vitro* and *in vivo*.

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