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Histone H3 N-Terminal Peptide Binds Directly to Its Own mRNA: A Possible Mode of Feedback Inhibition to Control Translation

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Messenger RNA (mRNA) delivers information from genes to proteins. Therefore, concentrations of mRNA in living cells must be tightly controlled at both transcriptional and translational stages.[1] One mode for translational control is feedback inhibition, which is caused by direct binding of metabolic product(s) to the corresponding mRNA. Most of the controllable RNA moieties are found as riboswitches in prokaryotes[2] and in some lower eukaryotes.[3] However, few cis-elements have been discovered that use their own proteins or peptides as ligands, especially among house keeping proteins in high eukaryotes.[4]

Histone is one of the most abundant proteins in cells and its concentration is closely governed by the amount of DNA present. When cells divide, large amounts of histone proteins are needed to pack DNA at the G1 and S phases.[5] The histone mRNA level at the end of the S phase is principally controlled[6] by the stability of mRNA,[7] which is mediated by way of interactions between a stem-loop in 3′-untranslated region (UTR) of histone and a stem-loop binding protein (SLBP).[8] The stem-loop RNAs are found in H2a 3′-UTR and other histone mRNA.[9]

Early reports describing histone autoregulation suggested that a complex of histone proteins and the cognate mRNA is involved in reducing histone levels.[10] A consideration of these early observations led us to propose several hypotheses. Firstly, several mechanisms might be operable for controlling histone concentrations at the translational level. One of these could involve binding of trans-elements directly to mRNA; this would affect histone levels. Finally, pairs of histone mRNA/histone protein (or peptide) might participate in feedback inhibition by prohibiting translation, as has been shown to take place in some RNA binding house-keeping proteins, such as in ribosomal proteins[4,11] and in p53.[12]

At this point in time, no binding motifs in histone mRNAs and histone proteins have been identified. In order to test the hypotheses suggested above, we have carried out a search to uncover cis-elements against the histone H3 N-terminal peptide. Because it is the most heavily post-translationally modified peptide among histone proteins, the H3 protein participates in many control mechanisms.[13] Furthermore, since the H3 N-terminal peptide contains a large amounts of Lys and Arg, it likely binds to a cis-element, if one exits.

Below, we report that the selection of aptamers against H3 peptide has provided specific hairpin RNAs that possess high homology with histone H3 mRNA. The identified H3 hairpin RNA specifically binds to the H3 peptide with low micromolar affinity. In addition, in vitro translation of the H3 protein is inhibited dose-dependently by the H3 N-terminal peptide. Consequently, the hairpin RNA and H3 peptide are one of the rare cis- and trans-elements on coding regions found among house keeping proteins in higher eukaryotes.

Since the most important sites of modification are located at the end of the H3 N terminus,[14] a peptide with 18 amino acids was thought to have a sufficient length to be recognized by a cis-element.[15] Standard Fmoc chemistry was utilized to synthesize a histone H3 peptide with 18 amino acids (N-ARTKQTARKSTGGKAPRK-biotin). RNA binders to the H3 peptide were obtained by using the H3 peptide as bait in the SELEX methodology.[16] Fifty long nucleotides were used as a random RNA library with a T7 promoter.[17] The selection cycle began with 20 μg of the RNA library (1.0×10^{14} molecules).

After six selection cycles, pools of RNA were subjected to a pull-down assay against the H3 peptide. As specific binding to the cognate peptide was observed (data not shown), the RNA pools were cloned to afford 20 different sequences.[17] Pull-down assays performed again on these clones led to the identification of two selective binding RNA clones (C1 and C2; Table 1). BLAST searches revealed that C1 and C2 have high homologies with H3 mRNA (Table 1). In order to confirm the above finding, we again tried a pull-down approach with total mRNA from HL60. Indeed, endogenous histone H3 mRNA was selectively captured by the H3 18-mer peptide (Figure 1).

Encouraged by high homologies to H3, a hairpin mRNA (31-nt) containing about 13-nt homology with C1 and C2 clones was chosen for further studies. The 31-nt hairpin RNA (Figure 2) was transcribed in vitro to measure its binding affinity against the H3 peptide by using a surface plasmon resonance technique. The 31-nt hairpin had approximately one micromolar binding affinity ($K_d = 1.2$ μM using BIAcore in Table 2, 8.2 ± 1.4 μM using pull-down method in Figure S3) with the cognate H3 peptide. As negative controls, measurements made with other well-known hairpin RNAs (RRE, pre-miR-24) showed that they do not bind to the peptide (Figure S4B, C). Several mutants, constructed in a manner to prevent drastic alteration of

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Table 1. Sequences of aptamers and BLAST-searched mRNAs.

RNA	Hairpin region (Random region for aptamer) [a]
C1	5′CTTTACTTTAGCGAGGCTTTGGTAGTCAATTGCGCAGG**TCCGTCT**CCTTC3′
C2	5′AATCCTTTGCTTACCTCTAAGGCCCACCGTCTTAA**TCCG**AGACGGTCTAT3′
H3[b]	5′(262)**GCGCAGGACTTTAAGACGGACCTGCGC**(288)3′
H1.4[c]	3′(1093)CGCGTCCTGAA*G*TTCTG*G*CTGGACGCG(1067)5′
A. Paf400[d]	5′(10486)CGC*T*TC*T*TGAAATT−TG*A*−TG− − − − −(10504)3′
N. Paf400[e]	5′(10252)**GC*C*CAGGACTTTAA−AC***T***−ACCTGC**CG(10276)3′
HAF01[f]	3′(416)CG*A*G*A*CCTGAAATT*T*GA*T*GG− − − − − −G(396)5′

[a] Gray regions indicate sequences homologous to those of the human histone cluster 2 H3 mRNA hairpin structure (sequences 262–288). The underlined sequences of C1 and C2 are complementary to H3 mRNA hairpin sequences 288–262. [b] *Homo sapiens* cluster 2 H3a, c mRNA. [c] *Bos taurus* histone H1.4 mRNA. [d] *Aspergillus fumigatus* Af293 histone acetylase complex subunit Paf400 mRNA. [e] *Neosartorya fischeri* NRRL 181 histone acetylase complex subunit Paf400 mRNA. [f] *Arabidopsis thaliana* HAF01 (Histone acetyltransferase TAFII250 family) mRNA (Bold, conserved hairpin region; Underlined, reversed order of hairpin sequence; Italic, substituted sequence).

Even though the most structured and controllable mRNAs normally form structured motifs in 5′- or 3′-UTR, the hairpin RNA identified in this effort is found in the coding region. This finding that the controllable hairpin follows a mechanism that is not mediated by a stem-loop in 3′UTR and SLBP.[18] Hydroxyl radical footprinting showed that the binding of the H3 peptide to 213–340 sequences of H3c mRNA induces a conformational change around the hairpin structure; this suggests that binding has effects on translation efficiency (Figure S5).

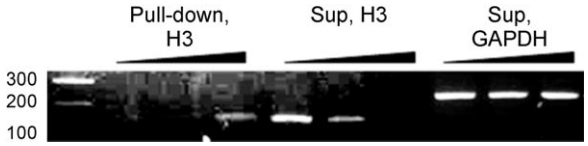

Figure 1. Pull-down assay with total cellular RNAs from HL60 cells. 50 μg of cellular RNAs were incubated with biotinylated histone H3 N-terminal peptide (0, 10, 100 μM) and pulled-down using streptavidin–sepharose resins. Each sample was subjected to RT-PCR using H3 (213–320) or GAPDH primers as a control (Pull-down, PCR with pulled-down RNAs; Sup, PCR with supernatant RNAs which were not pulled-down).

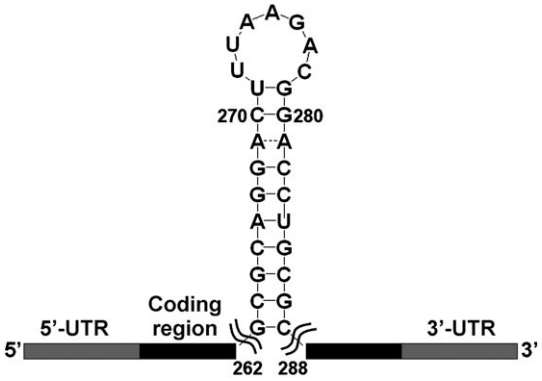

Figure 2. Predicted hairpin structure of H3c (cluster 2) mRNA from *H. sapiens*.

Table 2. Calculated values of binding affinities of the H3 hairpin RNAs using BIAcore 3000 instrument. Binding affinities of H3 hairpin RNAs to the H3 18-mer peptide were calculated by using BIAevaluation software (1:1 Langmuir binding model). N.C. means not calculable.

H3 hairpin	K_a [$M^{-1}s^{-1}$]	K_d [s^{-1}]	K_d [M]
wt	1.3×10^{4}	1.5×10^{-2}	1.2×10^{-6}
mt1	3.6×10^{4}	5.2×10^{-2}	1.4×10^{-6}
mt2	N.C.	N.C.	N.C.
mt3	N.C.	N.C.	$\geq1.0\times10^{-5}$
mt4	1.7×10^{4}	3.9×10^{-2}	2.4×10^{-6}
mt5	N.C.	N.C.	N.C.
mt6	3.8×10^{4}	8.3×10^{-2}	2.2×10^{-6}

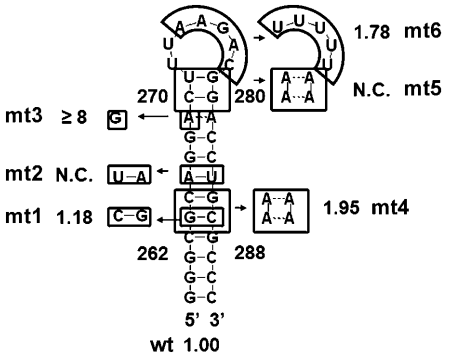

Figure 3. Binding specificity of H3 hairpin RNA to the H3 peptide using BIAcore. Relative binding affinities (mutant K_d/wild type K_d, which means reduced binding degree compared with that of wild type as value 1) of hairpin mutants (mt1 ~ 6) were calculated from BIAcore results. Values are shown in black boxes. N.C. means not calculable.

the secondary structure of the 31-nt hairpin, had reduced affinities or aborted binding to the H3 peptide compared with that of the wild type hairpin (Figure 3). The data described thus far suggest that the high affinity is associated with specific interactions between the N-terminal H3 peptide and its own mRNA, which has a specific hairpin motif. An upper stem of the hairpin seems to be the most plausible binding region of the peptide, judged by mutant experiments.

Next, we attempted to observe specific interactions between the hairpin in H3 mRNA and N terminus H3 peptide in an in vitro translation system. H3 mRNA of *Homo sapiens* was prepared by using the pCMV-SPORT6-H3c construct.[19] The peptide was then added to determine if translation of the H3 protein would be inhibited. As shown by the data displayed in

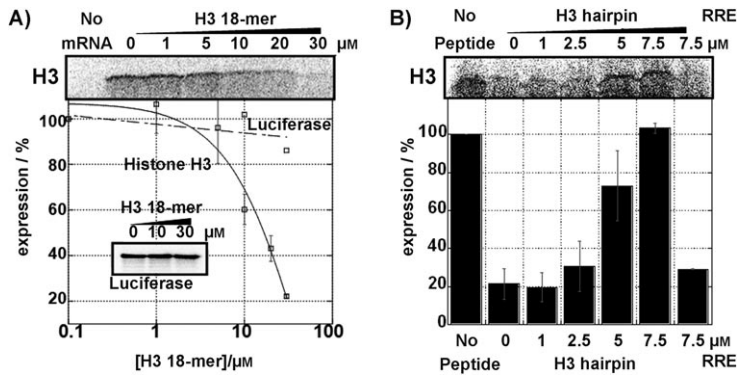

Figure 4. A) Inhibition of in vitro translation of H3 protein by addition of the H3 peptide. Indicated concentrations (1–30 μM) of H3 18-mer peptide and 1 μg of H3c mRNA (or luciferase mRNA) were used for each reaction. B) Regeneration of H3 protein expression in the presence of 30 μM H3 18-mer peptide by addition of indicated concentrations (1–7.5 μM) of H3 hairpin RNA (7.5 μM of RRE RNA as a control).

Figure 4A, the H3 peptide caused dose-dependent inhibition of H3 protein expression ($IC_{50} = 24 \pm 8$ μM). Full-length H3 protein showed the same does-dependent inhibition pattern as shown in Figure S7. In contrast, expression of luciferase was not inhibited by the H3 peptide in cases in which its gene was inserted into the same system (Figure 4A). The specificity of the interaction was again confirmed by observing a full recovery of H3 protein expression upon addition of the 31-nt hairpin RNA (Figure 4B) in the presence of the N-terminal peptide. Dose-dependent regeneration of the inhibited H3 protein expression was observed upon addition of the 31-nt hairpin RNA, but did not occur upon addition of a general stem-loop RNA molecule, RRE RNA, which was used as a negative control (Figure 4B). The findings demonstrate that the interaction between the hairpin RNA and H3 peptide is specific and the measured binding affinity is around micromolar. The observed IC_{50} value is reasonable when one takes into account the fact that the concentration of histone is in the micromolar range and the suggestion by Rose that 10 μM is close to the minimum histone concentration required for auto-regulation.[20] It is reasonable that the high concentration of histone would be maintained through the operation of a feedback inhibitor against its own mRNA; this would lead to a short-lived adjustment of histone levels.

Finally, the H3 hairpin, its mutants and the RRE hairpin were introduced into 5′-UTR of luciferase mRNA, which was transfected into 293A cells. The expression level of the reporter was reduced to around 60% with the H3 hairpin. However, no reduction was observed with the inserted RRE hairpin and mt2 (noncalculable affinity in BIAcore data) and a 65% reduction in expression was observed with mt3 (eight times weaker affinity relative to wild type in BIAcore data). RT-PCR products suggest that all transfected constructs have a similar expression level. The cell-based data above suggest that a specific interaction with the H3 hairpin is a key player in translation inhibition (Figures 5 and S8).

In summary, by using the SELEX method against the H3 peptide we have identified a 31-nt hairpin RNA that is located in the coding region of H3 mRNA and serves as a cis-element. The short hairpin RNA, which is transcribed in vitro, has micromolar affinity against H3 peptide. In vitro translation of the H3 protein was inhibited by the H3 peptide in a dose-dependent manner. Furthermore, addition of H3 hairpin RNA restored H3 protein expression. The expression level of transfected luciferase was reduced to around 60% with the H3 hairpin, while no reductions were observed with inserted RRE hairpin and other controls in a 293A cell-based assay. Thus, the pair comprised of hairpin RNA in the H3 coding region and the cognate peptide is one of the rare cis-elements on coding regions found in higher eukaryotes. A possibility exists that more hairpin RNAs are inhibited by their products and, as such, this might serve as general feedback regulation. This regulatory mechanism might be required for the control of highly abundant housekeeping proteins in cells.

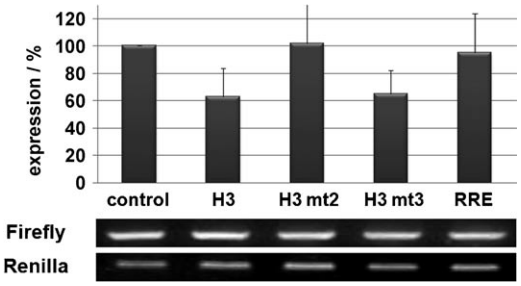

Figure 5. Inhibitory effect of the H3 hairpin in the luciferase reporter gene system. Reporter gene assays using pGL3-control construct containing H3 hairpin motif, its mutants and RRE hairpin on 5′-UTR in 293A cells. In order to observe transcription levels, firefly and renilla luciferase mRNA were detected by RT-PCR using gene specific primers.

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Keywords: aptamers · feedback inhibition · histone H3 · RNA · SELEX

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