A sensitive procedure for mapping the boundaries of RNA elements binding in vitro translated proteins defines a minimal hepatitis B virus encapsidation signal

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ABSTRACT

Using the structured RNA encapsidation signal (De) and the reverse transcriptase (P protein) of duck hepatitis B virus (DHBV) as an example, we devised a sensitive mapping procedure that yields accurate information on the minimal RNA sequence required for interaction with a few nanograms of an RNA-binding protein. RNAs from pools of end-labeled, partially hydrolyzed transcripts that bound to in vitro translated His-tagged P protein were isolated using immobilized Ni²⁺-ions. Size analysis by PAGE is consistent with a gradual gain in binding-competence from a minimum of 5 to a maximum of 8 base pairs in the basal stem of De. The procedure should be generally applicable to the convenient and precise fine mapping of RNA–protein interactions.

The fate of cellular and viral RNAs is regulated by the interaction of multiple cis elements with a variety of trans-acting partners, often proteins. Conventionally, the borders of a cis element are mapped by individually testing a limited number of differently sized transcripts, obtained from 5'-terminal deletion variants of an RNA expression plasmid linearized at varying 3′-proximal restriction sites, for binding to the isolated protein of interest. However, a complete set of shortened molecules can easily be obtained from the RNA itself by partial alkaline hydrolysis. Using RNA labeled at its 5′- or 3′-end, two pools are generated encompassing all possible deletions from one or the other terminus. Incubation with the protein, separation of protein bound from free RNA, and sizing by PAGE should reveal an overlapping set of nucleotides representing the minimal binding-competent sequence (Fig. 1). Using purified bacteriophage proteins, this principle has recently been used to determine the minimal size of binding-competent RNAs after their separation from free RNA on nitrocellulose filters (1).

Frequently, however, the protein of interest is neither available in pure form nor in sufficient amounts. In vitro translation, on the other hand, is a straightforward procedure to express almost any protein, although the yields are low both in absolute terms, and in

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DHBV and its cognate RNA, the structured encapsidation signal, are essential for forming an interaction-competent system (2); the apparent affinity to immobilized Ni²⁺-ions (3), and hence allow for efficient protein immobilization even at very low concentrations. The tagged P protein has wild-type activity in an in vitro priming assay which measures the D-dependent covalent transfer to the protein of a dNMP from the corresponding [ε-3²P]dNTP (data not shown).

In vitro transcribed De RNA (Fig. 3), either 5'- or 3'-terminally labeled with ³²P, was subjected to partial alkaline hydrolysis. The RNA pools were allowed to bind to in vitro translated P protein, and bound RNAs were separated via immobilization of the protein to Ni²⁺-NTA agarose beads (Fig. 1) that should exhibit a very high affinity to immobilized Ni²⁺-ions (Kₐ = 10⁻¹³ M; ref. 17), and hence allow for efficient protein immobilization even at very low concentrations. The tagged P protein has wild-type activity in an in vitro translation systems. For the mapping experiments described below, we used the reverse transcriptase, P protein, of duck hepatitis B virus (DHBV) and its cognate RNA, the structured encapsidation signal, De (Fig. 3). Here, in fact, in vitro translation is the only source for active, authentic protein (2), probably because the enzyme requires cellular chaperones for activity (3). DHBV is an avian representative of the hepadnavirus family which includes the medically important human hepatitis B virus (HBV). These small enveloped DNA-containing viruses replicate by reverse transcription of an RNA intermediate, the RNA pregenome (for review see 4,5). As shown by transfection experiments, encapsidation of the HBV pregenome into nucleocapsids (6) and initiation of reverse transcription (7) are mediated by the interaction of P protein (8) with the 5'-proximal ε-signal. ε forms a characteristic secondary structure with a lower and an upper stem, separated by a bulge of probably 6 nt (9–11). DHBV provides the distinct advantage that the interaction of its P protein with DHBV RNA (Fig. 3), either 5'- or 3'-terminally labeled with ³²P, was subjected to partial alkaline hydrolysis. The RNA pools were allowed to bind to in vitro translated P protein, and bound RNAs were separated via immobilization of the protein to Ni²⁺-NTA agarose beads (see legend of Fig. 1 for details). After 90 min at 30°C, 400 µl binding buffer (0.1 M sodium phosphate pH 7.5, 150 mM NaCl, 0.1% Nonidet-P40, 20 mM imidazole, 100 µg/ml yeast total RNA) containing 25 µl Ni²⁺-NTA–agarose beads (Qiagen, Hilden, Germany) were added. The mixture was shaken for 2 h at 4°C, then the beads were washed twice each with 1 ml binding buffer and 1 ml TMK buffer (50 mM Tris–HCl pH 7.5, 40 mM KCl, 10 mM MgCl₂, 100 µg/ml yeast total RNA). Phenol extracted, ethanol precipitated RNAs were analyzed on denaturing gels containing 8% polyacrylamide. RNase treatments for assigning band positions were performed with 50 000 c.p.m. end-labeled RNA in 50 µl TMK buffer at 4°C for 30 min using 2.5 U Benzonase (Merck, Darmstadt, Germany) for the 5'-labeled, and 2 ng RNase A (Boehringer, Mannheim, Germany) for the 3'-labeled RNA. Lanes 1, 3, 5 and 6, input RNA pools at two different concentrations; lanes 2 and 7, protein-bound RNAs; lane 4, 5'-labeled RNA treated with Benzonase; lane 8, 3'-labeled RNA treated with RNase A; lane 9, shorter exposure of lane 8. Sequence assignments are shown at the left and right side. °C indicates the position of a major Benzonase cut at C2609; due to the 3'-heterogeneity of the pregenome transcripts, three signals rather than one were generated from the 3'-labeled RNA by RNase A (e.g. A2575 in lane 9). (B) Semi-quantitative determination of protein binding-competence for 5' and 3'-labeled De RNAs. Band intensities of input and protein-bound RNAs were quantified using a PhosphorImager (Molecular Dynamics, Krefeld, Germany), and plotted, on a logarithmic scale, as percent intensity of the intact full-length RNA from the same lane versus the positions of the corresponding 3'- or 5'-terminal nucleotides. Note the gradual drop in binding-competence over a stretch of 4–5 nt.

Comparison with the total protein content of the cell lysate-based translation systems. For the mapping experiments described below, we used the reverse transcriptase, P protein, of duck hepatitis B virus (DHBV) and its cognate RNA, the structured encapsidation signal, De (Fig. 3). Here, in fact, in vitro translation is the only source for active, authentic protein (2), probably because the enzyme requires cellular chaperones for activity (3). DHBV is an avian representative of the hepadnavirus family which includes the medically important human hepatitis B virus (HBV). These small enveloped DNA-containing viruses replicate by reverse transcription of an RNA intermediate, the RNA pregenome (for review see 4,5). As shown by transfection experiments, encapsidation of the HBV pregenome into nucleocapsids (6) and initiation of reverse transcription (7) are mediated by the interaction of P protein (8) with the 5'-proximal ε-signal. ε forms a characteristic secondary structure with a lower and an upper stem, separated by a bulge of probably 6 nt (9–11). DHBV provides the distinct advantage that the interaction of its P protein with DHBV RNA (Fig. 3), either 5'- or 3'-terminally labeled with ³²P, was subjected to partial alkaline hydrolysis. The RNA pools were allowed to bind to in vitro translated P protein, and bound RNAs were separated via immobilization of the protein to Ni²⁺-NTA agarose beads (see legend of Fig. 1 for details). After 90 min at 30°C, 400 µl binding buffer (0.1 M sodium phosphate pH 7.5, 150 mM NaCl, 0.1% Nonidet-P40, 20 mM imidazole, 100 µg/ml yeast total RNA) containing 25 µl Ni²⁺-NTA–agarose beads (Qiagen, Hilden, Germany) were added. The mixture was shaken for 2 h at 4°C, then the beads were washed twice each with 1 ml binding buffer and 1 ml TMK buffer (50 mM Tris–HCl pH 7.5, 40 mM KCl, 10 mM MgCl₂, 100 µg/ml yeast total RNA). Phenol extracted, ethanol precipitated RNAs were analyzed on denaturing gels containing 8% polyacrylamide. RNase treatments for assigning band positions were performed with 50 000 c.p.m. end-labeled RNA in 50 µl TMK buffer at 4°C for 30 min using 2.5 U Benzonase (Merck, Darmstadt, Germany) for the 5'-labeled, and 2 ng RNase A (Boehringer, Mannheim, Germany) for the 3'-labeled RNA. Lanes 1, 3, 5 and 6, input RNA pools at two different concentrations; lanes 2 and 7, protein-bound RNAs; lane 4, 5'-labeled RNA treated with Benzonase; lane 8, 3'-labeled RNA treated with RNase A; lane 9, shorter exposure of lane 8. Sequence assignments are shown at the left and right side. °C indicates the position of a major Benzonase cut at C2609; due to the 3'-heterogeneity of the pregenome transcripts, three signals rather than one were generated from the 3'-labeled RNA by RNase A (e.g. A2575 in lane 9). (B) Semi-quantitative determination of protein binding-competence for 5' and 3'-labeled De RNAs. Band intensities of input and protein-bound RNAs were quantified using a PhosphorImager (Molecular Dynamics, Krefeld, Germany), and plotted, on a logarithmic scale, as percent intensity of the intact full-length RNA from the same lane versus the positions of the corresponding 3'- or 5'-terminal nucleotides. Note the gradual drop in binding-competence over a stretch of 4–5 nt.

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About 25 ng (~250 fmol) of the 87 kDa DHBV P protein can be produced per 25 µl of reticulocyte lysate programmed with a recombinant P mRNA obtained from plasmid pT7AMVpol16 (15), as estimated by [³⁵S]Met incorporation (16). Many smaller proteins are more efficiently expressed, but here the low amounts of P protein provided a rigorous test for the sensitivity of the method. For efficient separation of protein bound from free RNA, we modified the parental plasmid such that an N-proximal (His)₆-tag was introduced (Fig. 1) that should exhibit a very high affinity to immobilized Ni²⁺-ions (Kₐ = 10⁻¹³ M; ref. 17), and hence allow for efficient protein immobilization even at very low concentrations. The tagged P protein has wild-type activity in an in vitro priming assay which measures the D-dependent covalent transfer to the protein of a dNMP from the corresponding [ε-³²P]dNTP (data not shown).

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transcripts (18), three bands rather than one were observed in the control reaction with RNase A used for calibration (Fig. 2A, lanes 8 and 9). Binding-competence was gradually established (Fig. 2B, lower panel) with fragments having from 9 to 13 more nt at their 5′-end than the most prominent RNase A product starting with A2575 (Fig. 3). This corresponds to the left side of essentially the same lower stem region as defined by the 5′-labeled RNAs. Both datasets are hence consistent with a minimal requirement for 5 bp in the lower stem to produce detectable binding; provision of further base pairs enhances binding up to a plateau once the stem contains 8 bp (Fig. 3). This gradual increase probably reflects the increasing stability of the lower stem necessary to maintain the bulge-and-loop shape of the apical part. Secondary structure calculations using the program m-fold (19) support this view: terminal deletions such that <5 bp could form the lower stem strongly favor alternative structures. In turn, these data strongly support the importance of the bulge-and-loop structure in De.

That both De pools indicate essentially the same boundary suggests that the flanking 5′- and 3′-sequences have no major impact on P protein binding.

In summary, the method described here allows the simple, sensitive and accurate determination of the functional borders of protein-binding RNA elements using tagged, in vitro translated protein. Even nanogram amounts of the relatively poorly expressed hepadnaviral P protein proved sufficient for rapid data acquisition approaching single nucleotide resolution; a few thousand counts per minute of protein-bound RNA allowed for a clear distinction between binders and non-binders. Hence the procedure should work well with a whole variety of RNA binding proteins, many of which will conveniently, and in higher yield than P protein, be available by in vitro translation. The only requirement is an efficient method for immobilizing the protein, e.g. by specific antibodies, or an artificially introduced tag to which a high affinity capture reagent is available. With DHBV F protein, we have not observed major truncated in vitro translation products which could obscure the results. However, N-terminal tags, as in our case, discriminate against shortened proteins arising from internal translation initiation; potential problems due to C-terminal truncations will be circumvented by placing the tag at the C-terminus.

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