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Template-Directed Interference Footprinting for RNA Based on Inosine-Specific Cleavage

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Abstract—We report here the development of a Template-directed Interference (TDI) footprinting assay for RNA. The TDI nucleotide analogue inosine (I) lacks the exocyclic amine of G and is a suitable probe for the role of this group in RNA structure and function. Using an I-specific cleavage protocol we identified three functionally significant G residues in the *Tetrahymena* ribozyme. These residues are proximal to the active site of the folded intron and likely contribute to the positioning of substrates at the catalytic core.

Recent advances in X-ray crystallography and NMR have provided significant insights into basic elements of RNA structure and the means by which these contribute to both formation of higher structures in RNA and sequence-specific recognition by RNA binding proteins.[1] There is a pressing need for structure-probing chemical methodologies to complement the insights gained from such studies and to provide information where no high-resolution structural data is available.

In the RNA molecule, double-stranded RNA (dsRNA) is a basic structural motif as evidenced by the high-resolution structures of tRNA, rRNA, and both large and small ribozymes. The major groove of the A-form RNA helix is much deeper and narrower than that of the B-form helix. Thus, recognition of RNA involves interactions with a distorted dsRNA helix, the phosphodiester backbone, 2′-hydroxyl groups, or minor groove base functionalities in addition to interactions with single-stranded regions. One of the most important structural elements in dsRNA is the exocyclic amine of guanosine (G), which is presented as a recognition element protruding from the minor groove. Because of the critical role of this exocyclic amine in RNA,[2] we were interested in the possibility of using a Template-directed Interference (TDI) footprinting analysis[3,4] as a direct probe of its importance. Here we report the development of a TDI footprinting assay based on interference by inosine and its application to the study of the *Tetrahymena* RNA intron.

Inosine (I) has similar base-pairing properties to G (Fig. 1) and can be incorporated into an RNA in place of G by T7 transcription using inosine triphosphate (ITP).[5] Since I differs from G only in the replacement of the exocyclic amine by hydrogen (Fig. 1), it is an excellent probe for the importance of this amine in RNA structure and function.[2] In addition, RNAs containing inosine can be selectively cleaved at this position by a combined chemical/enzymatic procedure (Fig. 2).[6] RNase T1 cleaves RNA specifically after both G and I. Prior treatment of an RNA containing both G and I with glyoxal masks the G base by reaction at the N1 and N2 positions to form a stable adduct — the glyoxylated RNA can then be cleaved specifically after I residues with RNase T1 generating an I cleavage ladder.

We chose the *Tetrahymena* intron as a model to test TDI-I footprinting because it has been characterized by mutational and interference analysis[7,8] and because of

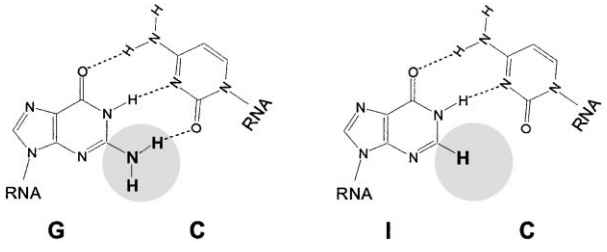

Figure 1. (a) Comparison of guanosine (G) and inosine (I) in double-stranded RNA showing the position of the hydrogen-bond donating exocyclic amine in a G–C base-pair and its hydrogen replacement in an I–C base-pair.

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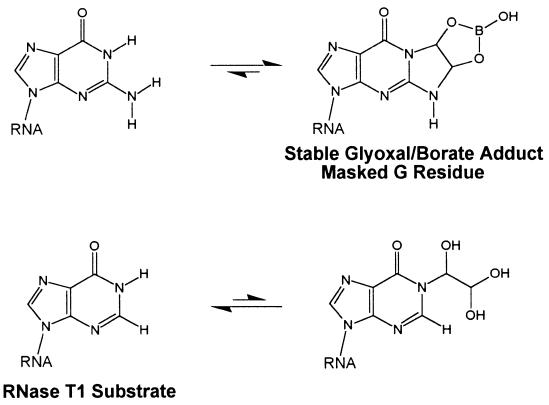

Figure 2. Differentiation between G and I in an RNA based on G-specific reaction with glyoxal. RNase T1 cleaves the RNA after unmodified I residues but not after the glyoxal/borate modified G residues.

the significant advances in terms of understanding its structure through X-ray crystallography.[9,10] The L-21 *Sca*I *Tetrahymena* ribozyme is a 385 nucleotide sequence-specific RNA endonuclease: a modified version of this RNA containing a G at its 3′ end performs a reaction that mimics the reverse of the second step of the *Tetrahymena* self-splicing reaction: attack of the 3′ G of the intron on an eleven nucleotide RNA results in cleavage of the short RNA and transfer of its five terminal nucleotides to the intron.[8,11] Radio-labeling of the short RNA at the 3′ end in such a reaction permits discrimination between active and inactive mutants of the larger RNA since only those molecules undergoing ligation become labeled.[8]

We synthesized the extended *Tetrahymena* intron by T7 transcription using a template generated by PCR from plasmid pT7L-21 and confirmed that it performed the cleavage/ligation reaction with a 3′ end-labeled eleven nucleotide RNA.[11] We next prepared a pool of RNA in which G was randomly replaced with I — incorporation of approximately one inosine per RNA was achieved using a 1:40 mixture of ITP to GTP in the transcription. In order to confirm the specificity of I incorporation, the RNA was 3′ end-labeled, glyoxylated, and treated with RNase T1 to produce an I cleavage ladder indistinguishable from an RNase T1 G ladder generated from unmodified RNA.

In order to carry out the TDI-I assay with the *Tetrahymena* RNA we incubated the pool of I-doped RNA with 3′-end labeled substrate oligonucleotide. Following glyoxylation and RNase T1 treatment, the reactions were analyzed by denaturing PAGE alongside an I cleavage ladder generated from 3′ end-labeled input pool RNA (Fig. 3). A densitometric analysis and comparison of these lanes allowed the immediate identification of several interfering substitutions.

The TDI-I footprint for the *Tetrahymena* ribozyme shows significant interference at three positions: G303 (~3-fold; Fig. 3b), G111 and G112 (~2-fold at each position). The observed interferences are consistent with the conservation of these nucleotides in the *Tetrahymena* subclass of intron sequences[12] and also with the results of

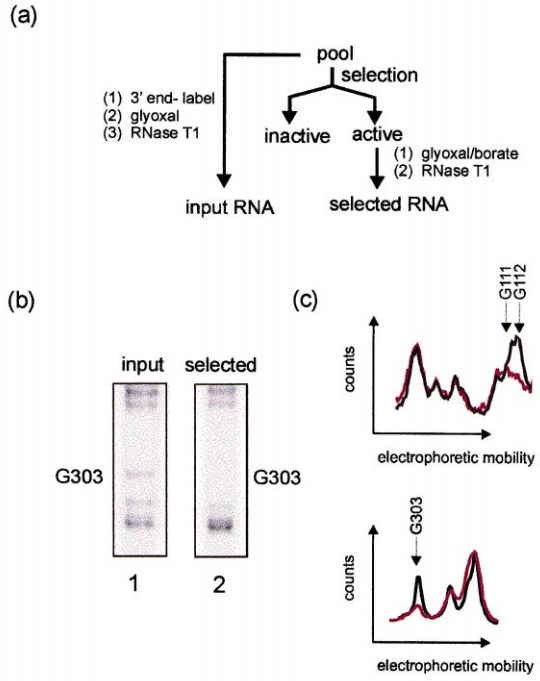

Figure 3. (a) Scheme for Inosine interference analysis of the *Tetrahymena* ligation reaction. (b) TDI-I footprinting of the *Tetrahymena* intron showing strong interference by the G303-I303 substitution. Lane 1: control; inosine specific cleavage of input pool RNA; Lane 2: inosine-specific cleavage of selected RNA showing strong intereference at G303. (c) Densitometric analysis of intreferences observed in this study showing input RNA (black) and selected RNA (red).

phosphorthioate-based[13] nucleotide interference mapping.[8] Because the only modification on the interfering nucleotide is the substitution of hydrogen for the exocyclic amino group, the magnitude of the interference is a direct reflection of the importance of this group in RNA structure or function.

The recent crystallographic analysis[10] of a large fragment of the *Tetrahymena* ribozyme shows the exocyclic amines of all three interfering nucleotides positioned to interact with the catalytic core- G111 and G112 are base-paired as part of the P4 helix: the exocyclic amines are potential hydrogen bond donors protruding from the minor groove into a cleft opposite from the amine functionality of G303 (Fig. 4). Although the cleavage/ligation assay is based on reactivity, it is improbable that the three amino groups are directly involved in catalysis since the actual catalytic site (containing the binding site for the G nucleophile and leaving group of the first and second self-splicing steps, respectively)[14] is located around ~20 Å away from these groups (Fig. 4). Rather, it is likely that the three guanosines play an important role in stabilizing the P1 helix, which is formed at the active site, positioning it in order to allow both cleavage and ligation events to occur.

The substitution of I for G is a conservative modification which permits the direct assessment of the function of the exocylic amino group of G in RNA. The methodology described here should be readily applicable to the analysis of the importance of this group in other RNA systems. In addition, the availability of several other RNA

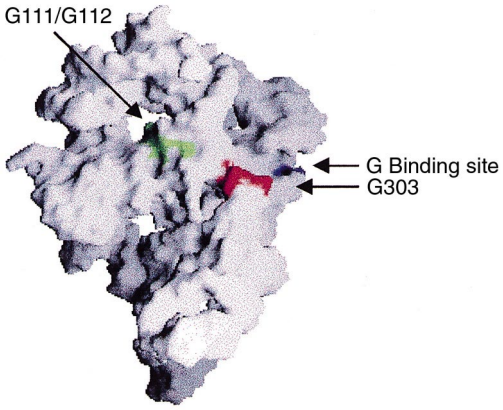

Figure 4. X-ray of a large fragment of the *Tetrahymena* intron[9] showing location of the interfering G residues (green and red) proximal to the G binding site (blue) at the catalytic core.

interference analogues, which may be detected by direct cleavage protocols, suggests that this general approach may be extended to the analysis of the importance of other functionalities within RNA.[15,16]

Acknowledgements

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15. It should be possible, in analogy to TDI-DNA footprinting,[3] to probe for contacts to other functionalities in RNA using the appropriate analogue (all of which are commercially available as either nucleosides or triphosphates): N4 of cytidine (5-azacytidine), N7/O6 of guanosine (7-methylguanosine), and N7 of adenosine (7 deazaadenosine). These analogues may all be detected, within oligomers, by direct or indirect chemical cleavage protocols.[3]
16. Full experimental details for the work described here are available directly from the authors by FAX or e-mail.