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Isolation of Oligoribonucleotides Containing Intramolecular Cross-Links

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Recently we developed a cross-linking methodology for probing the tertiary structure of RNA and used it to assess the catalytic competence of two different three-dimensional models of the hammerhead ribozyme (1). In essence, our strategy was to introduce conformational constraints into two ribozyme constructs by the means of a disulfide cross-link between helices I and II and to use the catalytic efficiencies of these two cross-linked ribozymes for evaluation of the structural models. We prepared two ribozymes (Fig. 1), each carrying two 2′-amino-modifications which were reacted with an aromatic isothiocyanate containing a protected mercaptan functionality. Deprotection of the thiols, followed by air-oxidation yielded a mixture of cross-linked and non-cross-linked material. Since the catalytic efficiencies of the cross-linked ribozymes could be affected by contamination of non-cross-linked material we needed to completely separate the two species. Denaturing polyacrylamide gel electrophoresis (DPAGE)[2] has previously been used for the isolation of intramolecular cross-links (2–4) in spite of the fact that they have the same nucleotide sequence, the same charge, and nearly the same mass as the corresponding non-cross-linked material. Surprisingly, in a preliminary attempt to isolate the two cross-linked ribozymes by 20% DPAGE we could only separate one adequately from the non-cross-linked material. However, we found that the relative mobility of cross-linked to non-cross-linked material varied greatly with the percentage of acrylamide in the denaturing gels and this facilitated the separation of the other sample. We report here a systematic study of this interesting phenomenon which suggests DPAGE as a general method for the analysis and isolation of intramolecularly cross-linked oligoribonucleotides.

The two cross-linked ribozymes, ribozyme A (Rz A) and ribozyme B (Rz B), were prepared and purified as previously described (1). Rz A and B as well as the corresponding non-cross-linked ribozymes, obtained by the reduction of the cross-linked samples with dithiothreitol (1), were subjected to DPAGE containing 8, 12, 16, 20, and 24% acrylamide (0.04 × 20 × 40-cm gel; acrylamide:bisacrylamide 19:1). The distance that the cross-linked and non-cross-linked material migrated in the gels was subsequently measured. To facilitate a comparison of results from all the gel analyses, the mobilities of Rz A and B were normalized, assuming that the non-cross-linked samples had migrated 30 cm in all the gels. The results are presented in the simulated gel shown in Fig. 2.

Inspection of Fig. 2 reveals an enormous difference in the relative mobility of Rz A to the non-cross-linked material, from 22.7 cm in a 24% gel to 32.9 cm in an 8% gel, a difference of 10.2 cm. The same effect is also observed for Rz B, although the difference in relative mobility is less than for Rz A. To our knowledge, there is only one other report in the literature that describes this phenomenon; Grabowski *et al.* (5) analyzed a ca. 230-nucleotide-long "lariat" intermediate in messenger RNA splicing by DPAGE and found that the apparent length was dependent upon the percentage acrylamide used and that it migrated as a longer oligomer under all conditions tested (4–10% acrylamide). By the same token,

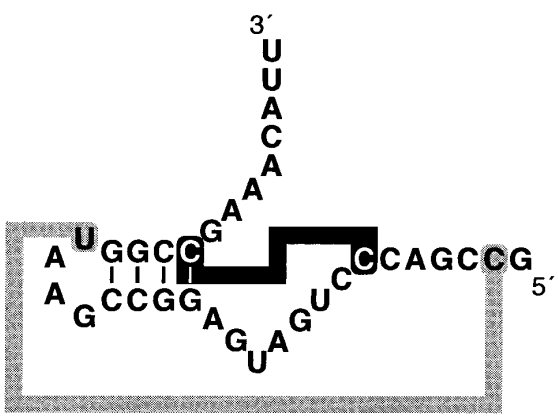

FIG. 1. The sequence and secondary structure of the two hammerhead ribozyme constructs used in this study, shown here without the substrate. Each ribozyme contained two 2′-amino-groups that were subsequently modified and cross-linked. The position of the cross-link in Rz A and Rz B is indicated by black and gray lines, respectively.

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[2] Abbreviation used: DPAGE, denaturing polyacrylamide gel electrophoresis.

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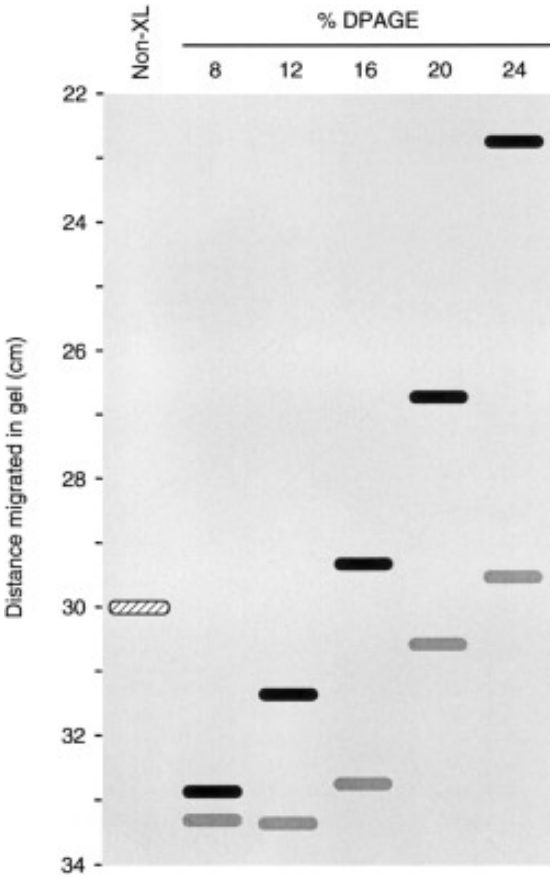

FIG. 2. A normalized representation of the distances migrated by the cross-linked oligomers during DPAGE as a function of percentage acrylamide, assuming that the non-cross-linked (non-XL) material migrated 30 cm under all conditions. The black and the gray bands represent Rz A and Rz B, respectively.

we observe that Rz A and Rz B are retarded relative to the non-cross-linked material in gels containing high percentage acrylamide. This is presumably due to the increased interaction of the branched RNA with the high percentage gels, relative to the non-cross-linked material which can more easily "snake" through the gel. However, Rz A and Rz B migrate faster than the non-cross-linked oligomers in low percentage gels. The same effect has recently been observed with an intramolecularly cross-linked RNA-hairpin (6). One interpretation of this phenomenon is that in the lower percentage gels the compactness of the cross-linked material causes the oligomers to migrate faster through the gel and is in agreement with previous findings for the relative mobilities of supercoiled and nicked circular DNA in agarose gels (7).

The position of the cross-link within the oligomer plays an important role in determining its electrophoretic properties. In previous reports (2, 3) the electrophoretic mobilities of cross-linked transcripts, a few hundred nucleotides long, were correlated with the number of nucleotides comprising the loop. Here the loop sizes for Rz A and B are 20 and 21, respectively, and would thus be expected to have similar mobilities. However, Rz A clearly has a greater range of mobilities and it is possible that loop size determines not only the electrophoretic properties of cross-linked oligomers, but also their general shape. The cross-link in Rz A is between residues relatively close to the center, giving rise to a highly branched structure, while Rz B is not as branched, having a lariat structure where a residue close to the end is linked to a central residue. An alternate explanation is that the position of the cross-link in Rz A stabilizes the hairpin structure of helix II to such an extent that it does not completely denature during electrophoresis and thus has aberrant mobilities at higher percentage acrylamide (3).

Our results emphasize the choice of percentage acrylamide in DPAGE gels for optimal separation of RNA containing intramolecular cross-links from non-cross-linked material. Although our study was performed with cross-linked RNA, this method will presumably also be applicable to the analysis and isolation of intramolecularly cross-linked DNA. Structural engineering of cross-links into nucleic acids is becoming an important tool in the elucidation of structure and function of nucleic acids (1, 6, 8, 9) and our observations should increase the scope of this approach even further.

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