Rapid, High Precision Analysis of DNA and RNA Footprinting Reactions Utilising the WAVE® Nucleic Acid Fragment Analysis System

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Abstract

The analysis of footprinting reactions is a highly involved process, normally requiring the use of a radioactive label, polyacrylamide gel electrophoresis and autoradiography. This method of analysis may take two to three days before results are available and requires skilled interpretation to avoid errors. By utilising the WAVE® Nucleic Acid Fragment Analysis System we developed an automated protocol for DNA and RNA footprinting reactions with a 15 minute run-time without the requirement for radioactive labels. Comparison of previously published analysis of an RuvA/Holliday junction complex using conventional polyacrylamide gel electrophoresis and X-ray crystallography with the WAVE® System gave predictions of a protected region extending over 13 bases, 8 bases and 9 to 10 bases, respectively. We demonstrate that the greater accuracy and precision of the WAVE® System in comparison to gels in separating the footprinting reaction products results in a more accurate prediction of the protected region. Footprinting of a hairpin ribozyme complex gave results in agreement with previously published data. This analysis is quantitative and interpretation is assisted by automatic integration of peak areas with graphical display to assist in identification of protected bases.

DNA Footprinting Analysis

Standard footprinting reactions involve the binding of a protein to radioactively labeled DNA containing the sequence that the protein recognizes. This complex is then digested, either enzymatically using DNase I or chemically using hydroxyl radicals. The regions of the DNA molecule covered by the bound protein are protected from digestion, whilst the rest of the DNA backbone undergoes cleavage. Modifications of the standard footprinting reaction that allow the reaction products to be analyzed on the WAVE® include the use of fluorescently labeled DNA. This allows the analysis of the cleavage products with the aid of a fluorescence detector. The analysis of a protein:DNA interaction using hydroxyl radical cleavage is exemplified here by the interaction between the RuvA component of the bacterial resolvasome and a synthetic Holliday junction.

RNA Footprinting Analysis

RNA has the ability to catalyse biological reactions (catalytic RNA). This catalytic function is dependent on the 3-dimensional shape of the RNA molecule. One technique that has been employed to obtain RNA structural information is "RNA footprinting". To differentiate between the internal and external regions of the folded RNA molecules, the solvent accessibility of the C5'-(deoxyribose) and also the C4'-position of the ribose moiety can be monitored by the addition of an Fe (II)-EDTA complex together with hydrogen peroxide to the RNA in solution. The hydroxyl radicals generated, primarily attack the C5'/C4'-position of the sugar and result in cleavage of the phosphodiester bond. The cleavage products are then directly analysed to identify those sites that show altered solvent accessibility. The use of a fluorescent label allows the reaction products to be analysed on the WAVE® using ion pair reversed phase liquid chromatography (IP RP HPLC). Analysis of the footprinting products is rapid with run-times of approximately 30 minutes for each sample, with direct quantification of the cleavage products.

Materials and Methods

Separation of fluorescent cleavage products – The use of Tetrabutylammonium bromide (TBAB) as the ion pairing reagent for DNASep® chromatography, is essential for the size dependent separation of fluorescently labeled nucleic acids. This regime removes the influence of the hydrophobic fluorescent group and sequence specific effects.

GA sequencing of the RuvA Holliday junction – Using a modified Maxam & Gilbert GA sequencing reaction the fluorescent oligonucleotide is cleaved at every G and A residue. This allows the phasing of the hydroxyl radical cleavage fragments and subsequent identification of the cleavage products.

Base catalysed hydrolysis of RNA – Base catalysed hydrolysis of the fluorescently labelled RNA was used to prepare a sequence ladder allowing alignment of the sequence of hydroxyl radical generated cleavage products.

Materials and Methods (Cont.)

Hydroxyl radical footprinting of DNA – footprinting was performed as described by Tullius & Dombroski (PNAS, 83, 1986, pp5469-5473) utilising 1-5 pmoles of fluorescently labelled Holliday junction mixed with 0-10 μM RuvA. The resulting reaction products were first purified by RP-IP-chromatography, then loaded into the WAVE® System for analysis by denaturing HPLC. Samples were analysed at 75°C under the following conditions: Buffer A, 2.5 mM TBAB, 0.1% (v/v) acetonitrile. Buffer B, 2.5 mM TBAB, 70% (v/v) acetonitrile. The elution gradient was initiated at 30% buffer B and extended to 50% buffer B over 12 minutes at a flow rate of 0.9 ml per minute, followed by an extension to 60% buffer B over 18 minutes at a flow rate of 0.9 ml per minute.

Hydroxyl radical footprinting of RNA – footprinting was performed as described by Hampel et al. (Biochemistry, 37, 1998, pp14672-14682) utilising 20 pmoles of fluorescently labelled substrate strand mixed with 100 pM each of unlabelled loop A & B RNA. The resulting reaction products were loaded into the WAVE® System for analysis by denaturing HPLC. Samples were analysed at 75°C under the following conditions: Buffer A, 2.5 mM TBAB, 0.1% (v/v) acetonitrile, 1mM (Na4) EDTA. Buffer B, 2.5 mM TBAB, 70% (v/v) acetonitrile, 1mM (Na4) EDTA. The elution gradient was initiated at 25% buffer B and extended to 42% buffer B over 10 minutes at a flow rate of 0.9 ml per minute, followed by an extension to 50% buffer B over 15 minutes at a flow rate of 0.9 ml per minute.

Results & Discussion

DNA footprinting of RuvA-Holliday junction complex – The results from the hydroxyl radical cleavage of the Holliday junction in the absence of RuvA are shown in figure 1A, and in the presence of RuvA in figure 1B. Sequence allocations were made by reference to fragments from a GA sequencing ladder (figure 1C). Subtraction of the traces with RuvA from those without indicated the protected region (underlined in figure 1C). Comparison with published footprinting and structural determinations of the RuvA-Holliday junction complex showed full agreement with the expected 8 base protected region extending from the crossover point of the Holliday junction (Hargreaves et al. (1998) Nat. Struc. Biol. 6 pp441-446, Ariyoshi et al. (2000) PNAS 97 pp8257-8262).

RNA footprinting of the hairpin ribozyme – The footprinting experiment was performed both in the presence and absence of $Co(NH_3)_6^{3+}$ (cobalt hexamine is required for folding of the ribozyme into an active conformation) (Figure 2A). Protection of the substrate was observed in the presence of $Co(NH_3)_6^{3+}$ spanning the substrate cleavage site (a-1, g+1,u+2 and c+3). These results are consistent with those obtained by Hampel *et al.* (Biochemistry (1998) 37 pp14672-14682) who demonstrated that the c-2, a-1, g+1 and u+2 are protected, thereby demonstrating that the C5'/C4'-atoms surrounding the cleavage site ribonucleotides are internalised upon folding of the hairpin ribozyme. These results are also in agreement with a tertiary structure model of the hairpin ribozyme, proposed by Earnshaw et al. (Biochemistry (2000) 39 pp6410-6421). Analysis of the accessibility of the C4'/C5'-positions of the ribonucleotides in the predicted model were compared to the experimentally observed sites of protection in the substrate strand. The protection seen at a-1 and u+2 is in agreement with this model.

Figure 1 – Hydroxyl Radical Footprint of the RuvA Holliday Junction Complex in the Absence (A) and Presence (B) of RuvA

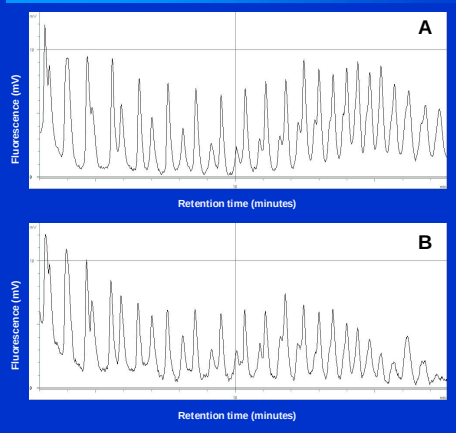


Figure 2 – GA Sequence Ladder used for Peak Allocation. The protected region is underlined.

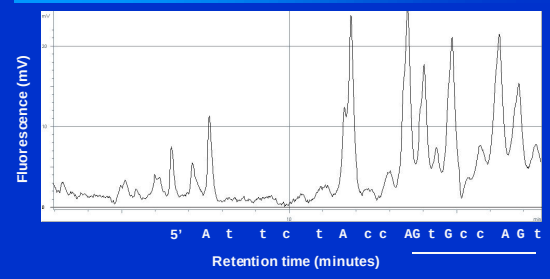


Figure 3 – Hydroxyl Radical Footprint of the Fluorescently Labelled Substrate Strand in the Presence (dashed line) and Absence (fixed line) of Cobalt.

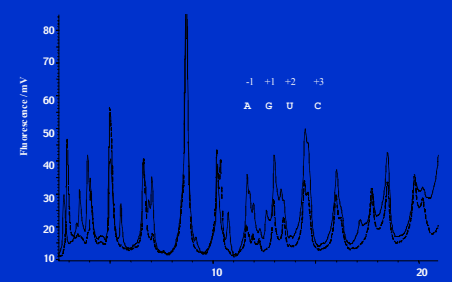


Figure 4 – (A) Schematic diagram of the hairpin ribozyme complex, (B) Tertiary structure model of the hairpin ribozyme substrate complex. The substrate strand is shown in blue, substrate binding strand in green and the B strand in red. The g+1 residue is highlighted in yellow. (Adapted from Earnshaw et al., 2000)

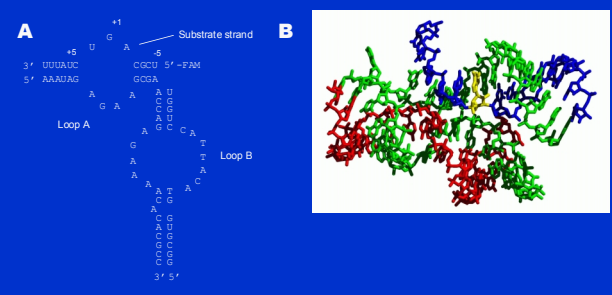
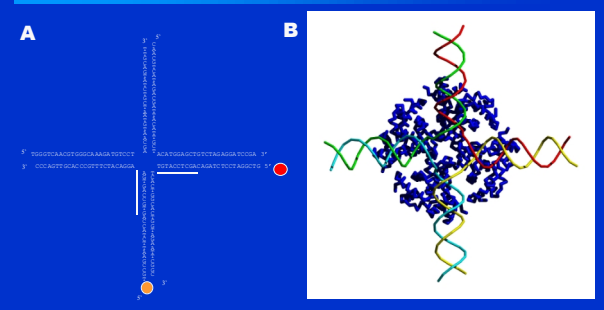


Figure 5 – (A) Schematic view of the RuvA-Holliday junction. White lines indicate protected bases and the locations of fluorescent labels are shown by coloured circles. (B) Structure of the RuvA Holliday junction complex obtained by X-ray crystallography. The RuvA tetramer is shown as a backbone worm trace in blue and the DNA is shown as a backbone (from Hargreaves et al, 2000)



Conclusions

The WAVE system has been used to analyse the products of DNA and RNA footprinting reactions. This analysis gave results equivalent to those obtained using conventional methodologies but with a dramatic saving in time (a reduction from 2 days to 20 minutes) and without the need to use radioactivity. Direct analysis of the cleavage products can be performed using proprietary software tools allowing the rapid analysis of cleavage products and automated analysis of multiple chromatograms, providing the potential for high throughput analysis of DNA/protein and RNA/RNA interactions.