Introduction

The introduction of a double-stranded DNA copy of the viral RNA genome into the host genome is an important part of the life cycle of a retrovirus such as HIV.\(^1\) Integrase has been shown to be the only enzyme required to mediate this integration event in vitro and proceeds in 3 distinct steps. The first stage involves the specific cleavage of two nucleotides from the 3' end of the viral DNA. The enzyme then makes a staggered cut in the host DNA to which the recessed viral 3' ends are joined in a concerted cleavage/ligation reaction. Finally, the gaps in the host DNA are presumably repaired by host enzymes resulting in an integrated proviral genome\(^2\).

The cleavage and ligation reactions have also been shown to be reversible in vitro, termed the disintegration reaction\(^3\). We have used this activity to produce a robust, high throughput screening assay for efficient measurement of integrase inhibition using scintillation proximity detection. The input of Dr. Pat Brown and co-workers (Stanford University Medical Center) in the design of this substrate is acknowledged.

The specificity and role of integrase in the retroviral life cycle has made it an important therapeutic target with recent evidence demonstrating that integrase mutants deficient in the forward and disintegration activities can have deleterious effects on viral replication in vivo.\(^4\) This assay therefore provides a valid mechanism and format for the screening of large numbers of compounds in the discovery of putative integrase inhibitors.

Substrate Design

The double stranded DNA disintegration substrate (Figure 1) consists of 2 oligonucleotides, one containing biotin, the other containing radiolabeled nucleotides. They both also contain inter and intra sequence complementary sequences which produce the secondary structure shown and are required for efficient integrase activity. This enzyme activity is observed when the DNA strands are cleaved and ligated such that the radiolabel is now on the same strand as the biotin. This reaction product is detected under conditions shown and are required for efficient integrase activity. Enzyme mutants with amino acid substitutions in the conserved active site lack both forward and disintegration activities. While other mutants have exhibited disintegration activity only in vivo, it is postulated that these proteins have amino acid changes which reside in the DNA binding or recognition sites of the protein. This assay is therefore most likely to discover putative inhibitors of disintegration which interact at or near the active site, or molecules which prevent its multimerization, rather than binding domains associated with the enzyme.

Assay Validation

Standard assay protocol per tube or well

1. Pipette 10µl of pre-mixed solutions containing DNA substrate, assay buffer and manganese chloride (replaced with water for control tubes).
2. Pipette 1µl of inhibitor sample, inhibitor solvent or water for control tubes.
3. Start the reaction with 10µl of integrase diluted in enzyme dilution buffer.
4. Incubate at 30-33°C for 30 minutes.
5. Add 10µl stop solution.
6. Pipette 110µl of a pre-mixed solution containing streptavidin SPA beads and denaturing reagent.
7. Incubate at room temperature for 30 minutes.
8. Count in a β-scintillation counter.

The integrase enzyme used in these studies was kindly supplied by Parke-Davis\(^5\).

Gel Validation

The nature of the [H]DNA substrate and products were studied by polyacrylamide gel electrophoresis. Using 2x the amounts in the standard protocol, SPA bound counts were determined in enzyme reactions (including controls) as shown in Table 1.

<table>
<thead>
<tr>
<th>Table 1. SPA analysis of gel separation experiment</th>
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<tr>
<td><strong>Assay</strong></td>
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<tr>
<td>+ Enz, + Mn</td>
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<tr>
<td>+ Enz, -Mn</td>
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<tr>
<td>-Enz, -Mn</td>
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<td>-Enz, + Mn</td>
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The beads were settled by centrifugation and the DNA oligonucleotides in the supernatant phosphorylated with γ-ATP. After desalting, the labelled DNA was analysed on a 15% sequencing gel run under denaturing conditions. This revealed a band by autoradiography present only in positive test wells containing manganese and enzyme which co-migrated with molecular weight markers at 16 bases, and was consistent with one of the expected products (Figures 2 and 3).

The bands shown between 26 and 33 bases represent intermediates of the labelling reaction incorporating H' in the substrate.

Inhibition Studies

A known topoisomerase 2 inhibitor, doxorubicin had previously been identified as an inhibitor of integrase activity in cleavage and strand transfer reactions giving IC\(_50\) of 0.9 and 2.4µM respectively.\(^8\) We have used the hydrochloride salt to determine the IC\(_50\) in a disintegration reaction. Values were obtained using a colour quench standardisation curve based on tariazine and settled beads, the SPA counts obtained were normalized and adjusted to give % inhibition of a control disintegration assay with no inhibitor present. Assays were performed using the standard protocol with 5µlols of enzyme and final concentrations of inhibitor as indicated. Doxorubicin hydrochloride was diluted in water giving an orange-red colour.

Samples were measured in a Wallac 1450 MicroBeta scintillation counter and colour quenched.

References