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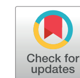
Research paper

The sequence preference of DNA cleavage by T4 endonuclease VII



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ABSTRACT

The enzyme T4 endonuclease VII is a resolvase that acts on branched DNA intermediates during genetic recombination, by cleaving DNA with staggered cuts approximately 3–6 bp apart. In this paper, we investigated the sequence preference of this cleavage reaction utilising two different DNA sequences. For the first time, the DNA sequence preference of T4 endonuclease VII cleavage sites has been examined without the presence of a known DNA substrate to mask any inherent nucleotide preference. The use of the ABI3730 platform enables the cleavage site to be determined at nucleotide resolution. We found that T4 endonuclease VII cleaves DNA with a sequence preference. We calculated the frequency of nucleotides surrounding the cleavage sites and found that following nucleotides had the highest incidence: AWTAN*STC, where N* indicates the cleavage site between positions 0 and 1, N is any base, W is A or T, and S is G or C. An A at position −1 and T at position +2 were the most predominant nucleotides at the cleavage site. Using a Sequence Logo method, the sequence TATTAN*CT was derived at the cleavage site. Note that A and T nucleotides were highly preferred 5′ to the cleavage sites in both methods of analysis. It was proposed that the enzyme recognises the narrower minor groove of these consecutive AT base pairs and cleaves DNA 3′ to this feature.

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1. Introduction

T4 endonuclease VII (EC 3.1.22.4) is a resolvase that can convert branched DNA intermediates into duplex structures during genetic recombination. It is a double-stranded endonuclease that recognises distortions in DNA and cleaves at these perturbations to produce staggered cuts approximately 3–6 bp apart [1]. T4 endonuclease VII has been shown to interact with a number of substrates, including: Holliday structures [2], supercoiled and hybrid cruciforms [3], heteroduplex loops [4,5], mismatches in double-stranded DNA [4–6], bulky adducts including cisplatin [7,8] and abasic sites [9]. This variety of substrates for T4 endonuclease VII suggests that it may be involved in DNA repair. The enzyme can be utilised for mismatch and mutation identification, due to its ability to detect all possible mismatch combinations [10,11].

Under native conditions, T4 endonuclease VII exists as dimer, with a tendency to aggregate into multiples of 18 kDa [12,13]. The crystal structure reveals a domain-swapped dimer architecture, which helps stabilise the open fold of the monomer subunits [14,15]. The subunits act independently on the DNA duplex [16].

T4 endonuclease VII recognises DNA kinks and perturbations, and it has been proposed that its mechanism of action involves interaction with the minor groove side of the junction [13], where it can cleave both strands of the duplex [17]. The failure of T4 endonuclease VII to cleave a bulged molecule, constructed as a DNA-RNA hybrid, suggests that the geometry of the double helix is critical to the recognition process [17]. Substrates that cause defects in DNA have the potential to locally distort the duplex. However, the features of the DNA helices that are recognised by the enzyme are not known [9,18]. The bend angle of the duplex is dependent on the number and binding of the unpaired bases that comprise the bulged substrate [17]. Axial kinking may play a role in the recognition of bulges and bulky adducts [8,19]. It has been proposed that the structural distortion at the crossover site is the recognition target [20], but neither the total number, nor arrangements, of intermediate forms of the branched DNA substrate impact the action of T4 endonuclease VII [21]. Although it has been hypothesised that the inclination of the DNA helix segments is important for recognition by T4 endonuclease VII [17], the fact that single base mismatches and abasic sites are efficiently cleaved questions this [9]. The cleavage of single base mismatches has not been explained, as they do not display the significant bending of other substrates [9].

The resolvase action of T4 endonuclease VII occurs via the placement of nicks within several nucleotides 3′ from the substrate

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Abbreviations

CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
TE	Tris-HCl, pH 8.8, 0.1 mM EDTA

site, achieved by hydrolysing internal phosphodiester bonds, yielding ligatable 3′–OH and 5′–PO_4 termini [2,8,18,22]. The cleavage itself is known as nick and counter-nick, where the enzyme nicks both strands in a divalent cation-catalysed reaction, with two independent, yet closely correlated, reactions [21]. The nicks occur immediately 3′ of the substrate site on the same strand [12,23], and generally 3–6 bp 5′ to the cleavage site on the opposite strand. There is some variation in the cleavage site on the opposite strand, but it will be within 9 bp of the recognition site [10].

It has been proposed that the sequence preference of T4 endonuclease VII cleavage may be caused by indirect effects of the base sequence on structural parameters of the DNA [24], or by direct contacts to the bases in both the minor and major grooves [9]. Major cleavage sites were accompanied by a series of minor sites, which mapped out further 3′ of the mismatch.

Early studies on the endonuclease revealed that it prefers to cleave 3′ of a T nucleotide in Holliday structures [25]. Mismatch repair studies reveal varying nucleotide efficiencies, at the site of cleavage [1,4,21,26]. Studies have also demonstrated that the cleavage position is not affected by changing the type of branched substrate, but is strongly influenced by the local nucleotide sequence close to the recognition site [21]. The relative efficiency of cleavage fell to 16% when the substrate nucleotides were replaced with consecutive Gs; fell to 26% when replaced with As; fell to 65% with Cs; and replacement with T nucleotides had no effect [21]. Various branched DNA junctions were cleaved by T4 endonuclease VII primarily at the first C of a GGGGCTGC sequence [17]. The enzyme also primarily cleaves between T and C residues in the recognition of short cruciform structures [22]. Single-strand overhangs were used to examine the local nucleotide sequence context on the efficiency of cleavage. The most prominent cleavage site was found to be between the first T and C nucleotides, in the sequence T*CTCCC [21]. However, the addition of nucleotides 5′ of the cleavage site dramatically changed the cleavage efficiency. A single A nucleotide, which gives a cleavage site of AT*CTCCC, resulted in a two-fold increase in the level of cleavage. However, the addition of further A nucleotides to the recognised single-strand substrate sequence resulted in a reduction in T4 endonuclease VII cleavage efficiency [21]. Based on the above, we can expect the preferred cleavage sequence to be a T*C dinucleotide and T nucleotides to be favoured at the cleavage site. When single-base mismatches are examined as a substrate, pyrimidine insertions are generally preferred over purine [4]. This agrees with the aforementioned expected cleavage efficiencies.

Studies investigating T4 endonuclease VII have previously focused primarily on the cleavage site and the nucleotides immediately surrounding the cleavage site of a known substrate e.g. cruciforms, mismatched bases, etc. This current study is the first time that the DNA sequence preference of T4 endonuclease VII cleavage sites has been examined without the presence of a known substrate that could mask any inherent nucleotide preference. We utilised two DNA plasmid sequences to determine the DNA sequence preference of T4 endonuclease VII with the ABI3730 DNA sequencing platform that enables the cleavage site to be quantitatively determined at nucleotide resolution. This data was then examined to determine the influence of the surrounding sequences on the degree of cleavage.

2. Materials and methods

Two random DNA sequences were utilised in this study (Fig. 1a and b). The plasmids, Mito 15 and Mito 69, were generated by cloning PCR products into a pUC19 vector at the *Sma*I site (New England Biolabs) and verified by DNA sequencing both strands [27]. The Mito 15 mitochondrial insert sequence corresponds to bp 11,851 - 12,097 and the Mito 69 mitochondrial insert sequence corresponds to bp 8 – 432 in the hg19 human mitochondrial sequence (http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/chromFa.tar.gz).

Non-labelled oligonucleotides and oligonucleotides fluorescently-labelled with 6-carboxyfluorescein (FAM) were obtained from Invitrogen. Deoxynucleotide triphosphates were from Thermo Fisher Scientific, *Taq* DNA polymerase from Applied Biosystems and T4 endonuclease VII from USB (In Vitro Technologies).

2.1. Production of 5′-end labelled PCR products

The polymerase chain reaction (PCR) was used with primers 5′-ATGTGCTGCAAGGCGA-3′ (SEQ2) and 5′-ATTGTGAGCGGATAAC-3′ (REV2) to amplify the region of interest within the plasmid. In order to fluorescently-label the top and bottom strands, each fluorescently-labelled primer was utilised with the non-fluorescent counterpart: FAM-SEQ2 with REV2 fluorescently labels the top strand, and FAM-REV2 with SEQ2 fluorescently labels the bottom strand. In each 20 μl reaction, approximately 30 ng of plasmid DNA was added to 10 pmol of each primer, 1 μl 20× PCR buffer (16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$), 0.3 mM deoxynucleotide triphosphates and 0.1 U *Taq* DNA polymerase, with the remaining volume made up with MilliQ water. The thermal cycling protocol consisted of: incubation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min, followed by a single cycle of 72 °C for 10 min.

The labelled PCR products were then purified on a 6% (w/v) native polyacrylamide gel. The PCR products were excised from the gel and incubated with 500 μl 0.3 M sodium acetate, at 37 °C overnight with constant agitation. The supernatant was removed, ethanol precipitated, and finally dissolved in 20 μl 10 mM Tris-HCl (pH 8.8), 0.1 mM EDTA (TE).

2.2. The T4 endonuclease VII cleavage reaction

In each 20 μl reaction, 3 μl of 5′-labelled PCR product was added to 2 μl 10× reaction buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA), 4 μl T4 endonuclease VII (diluted to 100 U/μl), with the remaining volume made up with MilliQ water. Enzyme dilution was performed using the provided dilution buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and for each enzymatic reaction performed, a control was run that substituted the enzyme for an equivalent volume of dilution buffer. All samples were incubated at 37 °C for 1 h, followed by heat inactivation at 65 °C for 30 min. Samples were then ethanol precipitated and dissolved in 20 μl TE.

2.3. Dideoxy sequencing reactions

For dideoxy sequencing size standard reactions, a 20 μl reaction consisted of either; 4 μl 1 mM dideoxy ATP, 1 μl 1 mM dideoxy CTP, 1.5 μl 1 mM dideoxy GTP or 2 μl 1 mM TTP. Each dideoxynucleotide was combined with: 1 pmol of FAM-labelled primer, 1 μl 20× PCR

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