Primary and secondary structure specificity of the cleavage of 'single-stranded' DNA by endonuclease HinfI

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Received 30 March 1982; Accepted 22 April 1982

ABSTRACT

The interaction of endonuclease Hinf I with single-stranded fd DNA was examined. The sizes of the cleavage products indicate that the enzyme cuts this substrate at the same sequences as double-stranded DNA (GANTC). To determine whether or not the recognition sites in single-stranded DNA have to be present in double-stranded form in order to be cleaved, DNA fragments containing complementary or non-complementary Hinf I sequences were prepared and tested as substrates. The results suggest that completely base-paired recognition sites are necessary for cleavage. Sequences surrounding the Hinf I pentanucleotides significantly modulate the reaction rates.

INTRODUCTION

A few years ago it was detected that ss¹ DNAs are substrates for endonuclease Hae III (2). Since then a number of restriction enzyme preparations were found to possess the same property (3 - 6). For only a few of these enzymes have the sites of cleavage and features of their interaction with ss DNA been investigated. In two publications it was briefly noted that also preparations of endonuclease Hinf I were able to cleave ϕ X 174 plus-strand DNA (4, 5). However, it could not be ruled out that the fragments observed were produced by contaminating enzymes. Using ss DNA fragments from bacteriophage fd we investigated the interaction between Hinf I and ss DNA with respect to primary and secondary structure specificity.

MATERIALS AND METHODS

Enzymes. Endonuclease Taq I was obtained from PL Biochemicals. Endonuclease Hinf I and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories. Alkaline phosphatase from calf intestine was obtained from Boehringer Mannheim (grade I).

Substrates. Isolation of ss and synthesis of ds fd DNA have been described previously (7, 8). γ - 32 P-ATP was purchased from New England Nuclear.

Preparation of ss 5'- 32 P-labeled DNA fragments. 1 μ g (0.25 pmol) of 3 H-labeled ds fd DNA was incubated with an appropriate amount of Taq I in 50 μ l of 6 mM Tris·HCl, pH 7.5; 6 mM MgCl₂; 6 mM NaCl; 6 mM ME and 0.1 mg/ml gelatine at 65°C for 4 h.

The pH was adjusted to about 8.5 by addition of Tris base, and the mixture was incubated at 55°C for 5 min with about 1 unit of alkaline phosphatase. The reaction was stopped with EDTA and the solution was deproteinized by two successive phenol extractions. The aqueous phase was twice extracted with ether.

The fragment mixture or selected fragments isolated by preparative polyacrylamide gel electrophoresis were subjected to hybridization with a 20-fold excess of ss DNA as described by Horiuchi and Zinder (2).

After renaturation the solution was concentrated and applied to a small column of Sephadex G-50. Elution was done with 0.5 mM Tris·HCl, pH 7.5; 0.01 mM EDTA. Peak fractions were pooled and concentrated by lyophilization.

An aliquot corresponding to about 0.07 μ g of ss Taq I-fragments was 5'-phosphorylated with γ - 32 P-ATP by the standard procedure (9).

The labeled fragments were separated by electrophoresis in a 30 cm long 5% polyacrylamide / 7 M urea slab gel (for further details see ref. 7). Elution of individual fragments was essentially done as described by Maxam and Gilbert (9). Ethanol precipitation was replaced by concentration, passage through Sephadex G-50, and lyophilization.

Incubation of ss DNA with Hinf I. Taq I-fragments (0.5 - 3.5 fmol) were incubated with 0.8 - 14 enzyme units in 10 - 20 μ l of 6 mM Tris·HCl, pH 7.5; 6 mM MgCl₂; 50 mM NaCl; 6 mM ME; 0.1 mg/ml BSA for 0.5 - 4 h at 37°C. The products were separated in 5% polyacrylamide / 7 M urea slab gels (7) and visualized by autoradiography.

RESULTS

The sequence specificity of the interaction between Hinf I and ss DNA was deduced from the lengths of the digestion products. As will be shown below, it is identical in ss and ds DNA. Therefore, the strategy to investigate the secondary structure specificity of the enzyme could be based on the property that its recognition site contains an odd number of nucleotides. Hence, two types of ss DNA fragments can be isolated and tested as substrates:

Type 1 contains either only one or several Hinf I sites with non-matching central nucleotides. It should never be cleaved if only a completely and correctly base-paired recognition sequence is accepted as substrate by the enzyme.

Type 2 contains Hinf I sites with matching central nucleotides. It should always be cleaved if a duplex recognition sequence can really be formed and if the formation of this structure is not only a necessary but also a sufficient prerequisite for the activity of the enzyme.

The genome of bacteriophage fd served as source of the DNA fragments. As it is not too large, but contains 24 sites for Hinf I, can easily be isolated in the ss form, and has been sequenced completely (10), it provides an appropriate substrate for the generation, selection, and separation of DNA fragments which meet the specifications just mentioned. In the present work they were obtained by digestion of ds fd DNA with Taq I, hybridization of the fragments with viral plus-strands, and separation of the resulting ss fragments by polyacrylamide gel electrophoresis. A schematic representation of the steps is given in figure 1. For experimental details see MATERIALS AND METHODS. The ten fragments generated by Taq I were 5'-labeled with ^{32}P . Eight of them were selected for incubation with Hinf I. In the following their chain lengths (in nucleotides) are used to designate them.

Fragments 139 and 441 contain no recognition sequence for Hinf I. They were not found to be cleaved (see fig. 2a, lanes 2 and 5).

Four sites¹¹ exist on the different fragments, which have no matching partner: 1403-C on fragment 381, 2011-G and 2497-T on fragment 579, and 5330-C on fragment 850. They were all cleaved

Figure 1: Scheme of experimental design. $\circ$ and $\bullet$ denote unlabeled and labeled 5'-phosphate groups, respectively. Arrows indicate Hinf I cleavage sites. Note that only those products carrying labeled ends are detectable.

Figure 2: Incubation of ss DNA fragments with Hinf I. The products were analyzed in 5% polyacrylamide / 7 M urea slab gels. Migration is from top to bottom. Lanes a1 and b9 contain ss marker fragments. Their chain lengths (in nucleotides) are indicated. In the other lanes the chain lengths of the starting products and of Hinf I-specific sub-fragments are given (assuming cleavage between G and A within the Hinf I recognition site). In part b of this figure control incubations without Hinf I are shown in the odd-numbered lanes (except lane b9).

in the ds state (not shown). However, none was found to be cut on the ss fragments (see fig. 2a, lane 3; fig. 2b, lanes 2 and 8). The band seen with fragment 850 corresponds to cleavage at a recognition site which can pair with a matching partner (see below). Control assays were carried out with aliquots of fragments 381 and 579. They were mixed with ss fragment 357 (see below) and incubated with Hinf I. In both cases only fragment 357 was found to be cleaved. This indicates that the negative results obtained with fragments 381 and 579 were not due to inhibition of the enzyme.

The remaining twelve Hinf I sequences on the different fragments potentially can form perfect duplex structures. Eleven of them could be examined. (Cleavage at site 6043-T on fragment 703 would produce a dinucleotide which would have run off the gel.) Seven Hinf I-specific bands were found: two produced from fragment 357 (fig. 2a, lane 4), two from fragment 652 (fig. 2b, lane 4), two from fragment 703 (fig. 2b, lane 6), and one from fragment 850 (fig. 2b, lane 8). The sizes of the products were deduced from a plot of chain length versus reciprocal mobility as proposed by Southern (12). Within the limits of experimental error all bands generated by Hinf I correspond to cuts at the conventional recognition sites of this enzyme.

A survey on the sub-fragments produced and the recognition sites involved is given in table 1.

DISCUSSION

The results obtained indicate that Hinf I recognizes the same pentanucleotide in ss and in ds DNA. The ss Taq I fragments 441 and 139, which contain no GATC, were not cut by Hinf I, and the lengths of all digestion products obtained agree with cleavages of ss DNA at the conventional recognition sites.

Studies performed with other restriction enzymes which cleave ss DNA or oligonucleotides also indicate that the target sequences are identical in ds and ss substrates (2, 3, 6).

The following results point to the secondary structure requirement in the regions of cleavage. 1) At all four sites without matching partners no cleavage could be detected. 2) At seven of eleven sites, which potentially can base pair with

Table 1: Results of the incubation of ss DNA fragments with endonuclease Hinf I.

Taq I fragments ^a	Hinf I site ¹¹	matching partner	cleavage observed	potential 5'-labeled cleavage products ^a
357	5767-G	+	+	83
	5789-C	+	+	105
381	1403-C	-	-	276
579	2011-G	-	-	62
	2497-T	-	-	548
652	490-T	+	+	154
	511-T	+	+	175
	723-A	+	-	387
703	6043-T	+	not determined	2
	6199-A	+	+	158
	136-A	+	+	503
	216-T	+	-	583
850	5121-A	+	+	287
	5330-C	-	-	496
	5376-T	+	-	542
	5439-T	+	-	605
139	-	-	-	-
441	-	-	-	-

^a chain length in nucleotides.

matching partners, cleavage was found to occur. 3) Fragment 357 contains two Hinf I sites (5767-G and 5789-C). They can form a base-paired duplex. A potential structure is shown in figure 3. According to results on hairpin stability recently obtained by Haasnoot et al. (14) this duplex should be thermodynamically favoured at 37°C even without any stabilizing influence of the enzyme. Cleavage at one of the target sites would yield a labeled sub-fragment of chain length 105 or 83 (if cleavage between G and A in the recognition site is assumed). Both species were in fact obtained. At different enzyme concentrations or reaction times their ratio was found to be constant. This indicates that sub-fragment 105, which is a potential precursor of sub-fragment 83, is not further digested. The straightforward explanation is that on this fragment site 5767-G is no longer part of a ds structure.

These findings indicate that Hinf I needs a duplex recognition site in order to cleave 'ss' DNA. With all other enzymes studied so far (Eco RI (15), Hae III (5), Hpa II (16), Mno I (16), Msp I (6)) the results also support the duplex model.

It is not surprising that not all of the sites which potentially can form ds structures were found to be cut. The nature of the variable central nucleotide does not seem to play a role (compare table 1). But several other reasons could account for this phenomenon. 1) The fragments could fold up into stable secondary and tertiary structures which impede or prevent the formation of ds Hinf I sequences and/or the access of the protein to these sites. The result obtained with fragment 357 is in keeping with such an explanation. Correct binding of the enzyme to site 5767-G rather than to site 5789-C of the 'branched' structure shown in figure 3 should be sterically hindered. In fact cleavage occurs predominantly within site 5789-C. 2) Regions adjacent to a recognition sequence may significantly influence the stability of its ds form and/or the stability of the enzyme-DNA complex. Potential duplex structures of the Hinf I sites of fragments 652, 703, and 850 are shown in figure 4. It is a well-known phenomenon with ds DNA that sequences surrounding

Figure 3: Duplex structure around Hinf I sites 5767-G and 5789-C on fragment 357. The recognition sequences are boxed. Arrows indicate cleavage positions for Hinf I. The numbers for the 5'- and 3'-terminal nucleotides of the fragment are also given. Note that the loop contains the origin of viral strand replication, a nick (marked by a triangle) introduced by the fd gene II - protein (13).

Figure 4: Potential duplex structures of complementary Hinf I sites and their adjacent regions on fragments 652 (a), 703 (b), and 850 (c). The Hinf I pentanucleotides are boxed. Potential cleavage positions are marked by arrows. Designations of the Hinf I sites involved are on the 5'-sides of the strands.

the recognition sites exert remarkable effects on the rates of cleavage (17 - 19). It might be expected that their influence is even more pronounced in ss DNA.

Effects of this kind could lead to a 'strand preference' of the enzyme, i.e. one of the two sites in a duplex target is cleaved faster. Such phenomena have in fact been observed (16, 6). A strand preference is amplified, if the ds structure at the target site is so unstable that the introduction of the first nick crucially enhances its dissociation rate. Its re-association now has to be an intermolecular reaction. Therefore, especially at low DNA concentrations, the rate of this event will drop substantially. Consequently cleavage of the 'faster' site of a 'ss' substrate might completely abolish cleavage of the 'slower'.

ACKNOWLEDGMENT

We wish to thank H. Mayer for a gift of Hinf I, D. Müller for a gift of DNA fragments, and the Deutsche Forschungsgemeinschaft and the Ministry for Science and Technology (project PTB 8178) for financial support.

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