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Protection of particular cleavage sites of restriction endonucleases by distamycin A and actinomycin D

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ABSTRACT

It is shown here that distamycin A and actinomycin D can protect the recognition sites of endo R.EcoRI, EcoRII, HindII, HindIII, HpaI and HpaII from the attack of these restriction endonucleases. At proper distamycin concentrations only two endo R.EcoRI sites of phage lambda DNA are available for the restriction enzyme - sRI1 and sRI4. This phenomenon results in the appearance of larger DNA fragments comprising several consecutive fragments of endo R.EcoRI complete cleavage. The distamycin fragments isolated from the agarose gels can be subsequently cleaved by endo R.EcoRI with the yield of the fragments of complete digestion. We have compared the effect of distamycin A and actinomycin D on a number of restriction endonucleases having different nucleotide sequences in the recognition sites and established that antibiotic action depends on the nucleotide sequences of the recognition sites and their closest environment.

INTRODUCTION

Restriction endonucleases have proved to be extremely useful for the analysis of DNA structure and function. These highly specific endonucleases, each recognizing a small number of unique sites per DNA molecule, permit to obtain larger fragments of genome.

In the preliminary report it has been shown /I/ that lambda phage DNA at high concentration of distamycin A and actinomycin D cannot be cleaved by certain restriction endonucleases due to specific interaction of these antibiotics with double-stranded DNA /2,3/. The experiments reported here describe the cleavage of phage lambda DNA by endo R.EcoRI at particular endo R.EcoRI sites in the presence of antibiotics*.

*The nomenclature of restriction endonucleases according to Smith and Nathans /4/.

Proper concentrations of distamycin A give rise to larger fragments that we are going to term hereafter "distamycin protected fragments". The present paper describes isolation of the distamycin fragments from the agarose gels and investigation of their composition.

MATERIALS AND METHODS

Bacteriophage DNA. Lambda cI857s7 DNA was isolated from the purified phage particles by phenol extraction /5/.

Restriction endonucleases. Endo R.EcoRI and EcoRII were prepared as described previously /6/. Endo R.HindII and HindIII were purified according to R.J.Roberts unpublished procedure with insignificant modifications. Endo R.HpaI and HpaII were purified according to P.A.Sharp et al. /7/. The column with w-aminoalkyl agarose (n=5) /8/ was used to purify Endo R.HpaI from exoactivity. The used enzymes were free of nonspecific nucleases.

Digestion with restriction enzyme. The standard incubation mixture contained IO mM tris-HCl, pH 7.5, IO mM $MgCl_2$, IO mM 2-mercaptoethanol, I-2 µg of lambda DNA, I-2 µl or restriction endonucleases, distamycin A and actinomycin D in IO µl volume. The reaction mixture was incubated for I-3 hrs at 37°C. The reaction was terminated by addition of EDTA. The samples digested with EcoRI were heated for 5 min at 65°C and then rapidly cooled in ice to prevent interaction of cohesive ends.

Agarose gel electrophoresis. 0.5-I.0% agarose gels (Sigma) were used for the separation of DNA fragments. The gels were prepared according to Helling et al. /9/ and electrophoresis was carried in a tube-type apparatus at 2.0 V/cm for about I4-I7 hrs or at 4.5 V/cm for 3 hrs. The gels and the buffer contained 0.5 µg/ml ethidium bromide. The DNA fragments were visualized by short wave ultraviolet light (C50 Transilluminator, Ultraviolet Product, San Gabriel, Calif.). The size of distamycin protected fragments was calculated by their electrophoretic mobility. We used lambda DNA fragments generated by endo R.EcoRI and HindIII as inside molecular

weight standards according to the formerly described procedure /I/.

Isolation of DNA fragments from agarose gel. Various methods are described for DNA recovery after electrophoresis /IO-I4/. The modification of method /I2/ has given satisfactory results. The DNA bands stained with ethidium bromide were visualized by fluorescence, then cut out and put into the electrophoretic tubes supplied with a dialysis membrane. The DNA fragments were run out the gel by electrophoresis for 2 hrs at IO V/cm in electrode buffer containing ethidium bromide (0.5 mg/ml). DNA collected on the membrane was visualized with the aid of ultraviolet light and repeatedly extracted from the membrane (50 µl of tris-buffer). The residual agarose fragments were removed by centrifugation for 20 min at 4000 g. DNA from supernatant was precipitated with ethanol and the sedimented DNA was dissolved with 50 µl of buffer at 65°C (0.I5 M sodium citrate, pH 7.5). The samples were concentrated by evaporation at reduced pressure.

Distamycin and actinomycin. For the present experiments we have used distamycin A (Calbiochem) and actinomycin D (Reanal). The concentrations of these antibiotics in solution were calculated by their optical density. The molar absorption coefficient of distamycin A is 30,000 at 302 nm /2/ and that of actinomycin D is 24,000 at 445 nm /3/.

RESULTS AND DISCUSSION

The antibiotics distamycin A and actinomycin D interact with the template DNA with the formation of noncovalently bound complexes. The interaction is rather selective: distamycin forms complexes preferentially with d(A/T) rich regions /2/ and actinomycin does it with d(G/C) rich regions /3/ of double-stranded DNA. This property permitted to use these antibiotics for effective and highly selective protection of the cleavage sites recognized by the restriction endonucleases. Table I gives concentrations of these antibiotics producing partial and complete protection of the recognition sites. Selectivity of the antibiotics has been confirmed by the compa-

Table I

Protection of Recognition Sites of Various Restriction Endonucleases of Lambda DNA by Distamycin A and Actinomycin D

Restriction endonuclease	Recognition site	Distamycin A (M)		Actinomycin D (M)		Relation of (3) to (5)
		Partial protection	Complete protection	Partial protection	Complete protection	
(I)	(2)	(3)	(4)	(5)	(6)	(7)
EcoRI	5' GAATTC 3' CTTAAG	$2 \cdot 10^{-5}$	$2 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	No	0.04
EcoRII	5' CCTGG 3' GGACC	$3 \cdot 10^{-5}$	$1.5 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$0.6 \cdot 10^{-4}$	1.5
HpaI	5' GTTAAC 3' CAATTG	$0.5 \cdot 10^{-5}$	10^{-3}	$3 \cdot 10^{-5}$		0.15
HpaII	5' CCGG 3' GGCC	10^{-3}-10^{-2}	$1.5 \cdot 10^{-2}$	$1 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	100
HindII	5' GTPyPuAC 3' CAPuPyTG	$0.5 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	$1 \cdot 10^{-5}$	$5 \cdot 10^{-4}$	5
HindIII	5' AAGCTT 3' TTCGAA	$0.5 \cdot 10^{-4}$	10^{-3}	$3 \cdot 10^{-5}$	No	1.5

rison of their action on the same endonuclease. These data expressed as a relation of effective concentrations producing partial protection are presented in Table I. It is seen that sensitivity to distamycin depends on the content of d(A/T) pairs in the recognition site. All the enzymes which have the recognition sites containing at least one d(A/T) pair are more or less sensitive to distamycin. Restriction endonuclease HpaII is almost nonsensitive to distamycin. This fact is in agreement with the structure of Hpa-site containing exclusively dCdG pairs. On the other hand, actinomycin D protects the recognition sites of endo R.HpaII, EcoRII and HindIII since these sites contain GC and GG sequences most favourable for actinomycin binding. Endo R.HpaI is partially sensitive and endo R.EcoRI is non-sensitive to actinomycin. In both cases d(G/C) pairs are located at the ends of the recognition sites. We suppose that different sensitivity of these two enzymes to actinomycin may be explained by the peculiarities of the environments of the recognition site. Indeed, all EcoRI recognition sites in lambda DNA have shown to border on dA or dT /15/. Thus, either TGT or TGA are G-containing sequences in the region of EcoRI sites in lambda DNA. These sequences, as it was shown /3/, are not satisfactory for actinomycin binding. In the case of Hpa it may be supposed that HpaI sites sensitive to actinomycin have d(G/C) pairs in their environ-

ment and the nonsensitive sites include d(A/T) pairs.

A nonpredicted observation in the course of the work was that different recognition sites of the same restriction endonuclease may be effectively blocked by different antibiotic concentrations. We have used this phenomenon to protect certain endo R.EcoRI cleavage sites (see Fig.I) on phage lambda DNA by distamycin A. The electrophoretic pattern of the fragments generated by endo R.EcoRI of phage lambda DNA in the presence of distamycin A is given in Fig.2. It is clearly seen that the increase of the distamycin concentration has induced disappearance of original fragments B,C,D,E and F and formation of new distamycin protected fragments termed as BCD and EF.

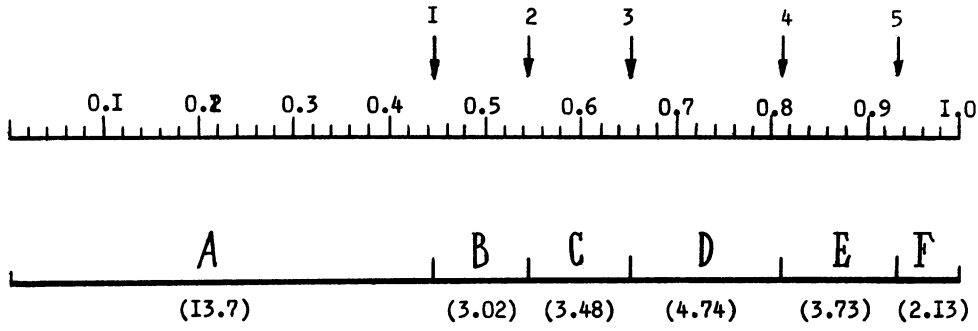

Fig.1. EcoRI cleavage map of DNA of λc 1857s7 phage (by Thomas and Davis /16/). The recognition sites are indicated with arrows. The DNA fragments are designated by letters A-F in order of their size of decrease /16/; molecular weights of the fragments are given in brackets

The goal of further experiments was to establish the composition of the distamycin protected fragments and the order of the protection of the recognition sites by distamycin. The sizes and appropriate molecular weights of these fragments have been determined from their mobilities relative to original EcoRI fragments included in the same gel. Knowing the molecular weights of the distamycin protected fragments and the order of disappearance of the original fragments we could deduce which original fragments composed the distamycin protected fragments.

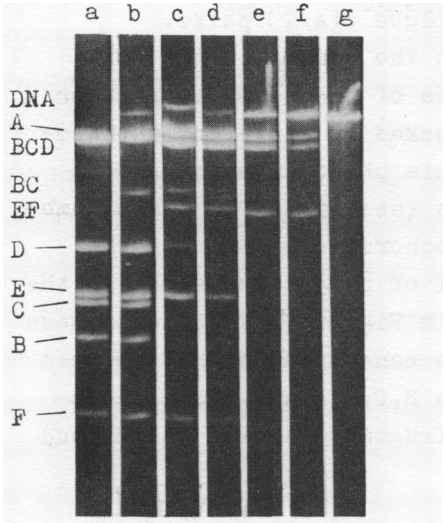

Fig.2. EcoRI digestion of DNA of λc 1857s7 in the presence of different concentrations of distamycin (10^{-4} M):
(a) - none; (b) - 0.15; (c) - 0.3; (d) - 0.5; (e) - 0.8; (f) - 1.2; (g) - 1.5.
Positions of EcoRI fragments are indicated on the left side of the plate

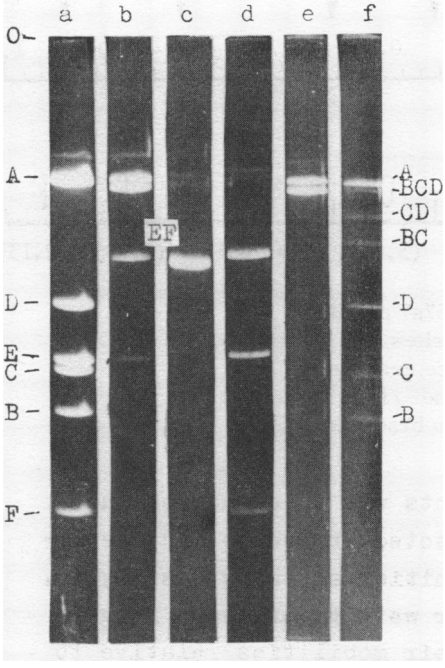

Fig.3. EcoRI digestion of DNA fragments obtained in the presence of distamycin.
(a) EcoRI cleavage pattern of λc DNA obtained in the absence of distamycin; (b) the same as (a) in the presence of distamycin ($I \cdot 10^{-4}$ M); (c) EF fragment cleaved with EcoRI; (e) fragments A and BCD isolated from agarose gel; (f) the same after cleavage with endonuclease EcoRI. Positions of original EcoRI and distamycin protected fragments are indicated on the left of the plate. "O" represents the origin. Conditions of the experiments and isolation of DNA fragments are described in Materials and Methods.

In further work the composition of the distamycin protected fragments was established by direct method. The fragments have been isolated from agarose gel after electrophoresis and digested by endonuclease EcoRI. Fig.3 shows that the recovered from gel EF fragment (molecular weight 6.0 ± 0.3 mln) possesses

sufficient purity. After the treatment by endonuclease EcoRI it has been splitted into E and F fragments. The fraction containing fragments A and BCD (molecular weight about II.4± 0.5 mln) has been also isolated from the gel. When this fraction had been digested with EcoRI five bands appeared (see Fig.3) which were identified by their electrophoretic mobilities and appropriate molecular weights as B,C,D,BC and CD. The molecular weights and composition of the distamycin protected fragments are shown in Table 2.

Table 2

Molecular Weights and Composition of Distamycin Protected Fragments (DNA of lambda c I857s7 phage cleaved by endo R.EcoRI in the presence of distamycin A ($0.7\text{-}I.0\cdot10^{-4}$ M). (See Fig.2 and 3)

Fragment	Molecular weight		Composition
	experimental	calculated (see Fig.I)	
BCD	II.4±0.5	II.24	B,C,D
EF	6.0±0.3	5.86	E,F
BC	6.45±0.3	6.50	B,C
CD	8.2±0.4	8.22	C,D

The order of disappearance of the original fragments and appearance of the distamycin protected fragments at increasing concentrations of distamycin allow to conclude that in lambda DNA the antibiotic first of all protects sRI2 which is proved by the appearance of fragment BC (Fig.2b). At further increase of distamycin concentration sRI3 and sRI5 are protected whereas fragments B,C,D,E and F completely disappear giving rise to BCD and EF fragments. Thus, at $I.0\cdot10^{-4}$ M distamycin concentration only two sites - sRI1 and sRI4 - are available for endo R.EcoRI, and correspondingly three fragments - A,BCD and EF - are formed.

The possibility of the site cleavage with restriction endonuclease in the presence of the antibiotic depends on two major parametres - on the affinity of the site for distamycin

and on the affinity of the same site for the restriction endonuclease. Therefore, a relative availability of the sites in the presence and absence of the antibiotic may differ significantly, especially, if the affinity of the site for antibiotic is higher than that for endonuclease. We expect this to occur in the case of the cleavage of lambda DNA with endo R.EcoRI. According to the data obtained by Thomas and Davis /I6/ this enzyme splits sRI5 from 5 to I0 times faster than sRI1 and sRI2 whereas the presence of distamycin rearranges the order of the sites in terms of their availability for endo R.EcoRI.

The different affinity of distamycin for EcoRI-sites in lambda DNA may be mainly dependent on the content of T-residues neighbouring the 3'-ends of EcoRI-sites for the reason that dT·dT sequence is shifted to 3'-end of EcoRI site (see Table I) and distamycin may interact with four T-residues situated one by one in the same strand of DNA (G.Gursky, personal communication).

We have also shown that the protection of the particular sites of restriction endonucleases and formation of the distamycin protected fragments occur for a number of enzymes and DNA like endo R.HindIII, endo R.HpaI (DNA of phage lambda, R6K plasmid) and endo R.HindII (DNA of phage fX174 and f1). Actinomycin D was shown to protect certain endo R.HpaI and endo R.HindIII cleavage sites giving rise to actinomycin protected fragments (the data are not given).

The data reported hear lead to a conclusion that the antibiotics distamycin A and actinomycin D may be useful for protection of particular cleavage sites of restriction endonucleases and producing of larger DNA fragments with the view to map the DNA and to obtain new vector molecules.

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