Structure at restriction endonuclease *Mbo*I cleavage sites protected by actinomycin D or distamycin A

Maj-Greth Nilsson, Catharina Skarped and Göran Magnusson

Department of Biochemistry I, Medical Nobel Institute, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Received 15 June 1982

Restriction endonuclease *Mbo*I cleavage of DNA was inhibited by actinomycin D and distamycin A. The two inhibitors protected different subsets of the 8 cleavage sites in polyoma DNA. The cleavage reactions were analyzed both in the presence of minimal inhibitory concentrations of the compounds and at higher concentrations, allowing cleavage at only 1 site/DNA molecule. The experiments showed that cleavage sites most efficiently protected by actinomycin D had putative inhibitor binding sites at a distance of 1–2 base pairs from the *Mbo*I recognition sequence. Distamycin A, in contrast, apparently has to bind immediately adjacent to the *Mbo*I recognition sequence to protect from cleavage.

Polyoma DNA Mbo*I cleavage* *DNA binding compound* *Enzyme inhibition*
Selective protection, of cleavage site *Inhibitor binding, adjacent to cleavage site*

1. INTRODUCTION

Restriction endonucleases recognize and cleave double-stranded DNA at specific base sequences [1]. Nosikov et al. reported [2] that the DNA binding compounds actinomycin D and distamycin A inhibit cleavage of DNA by a number of different restriction endonucleases. Actinomycin D intercalates between the bases of dG–dC dinucleotide residues in duplex DNA [3], whereas distamycin A binds by both electrostatic and non-ionic forces to runs of dT residues [4,5]. Digestion of phage lambda DNA showed that the two inhibitors specifically protected subsets of restriction endonuclease cleavage sites with the same recognition sequence, suggesting that base sequences contiguous to the recognition sites are important for the inhibition [5]. In these cases, however, the binding site of the inhibitor formed part of the recognition sequence of the enzyme.

We investigated the effect of actinomycin D and distamycin A on *Mbo*I cleavage of polyoma DNA. The *Mbo*I recognition sequence, GATC [1], cannot form part of a binding site of the two inhibitors. Since the base sequence of polyoma DNA has been determined [6], and the *Mbo*I cleavage site occurs eight times (fig.1) we could correlate the base sequence at the cleavage sites to the degree of protection by the inhibitors. The best protected

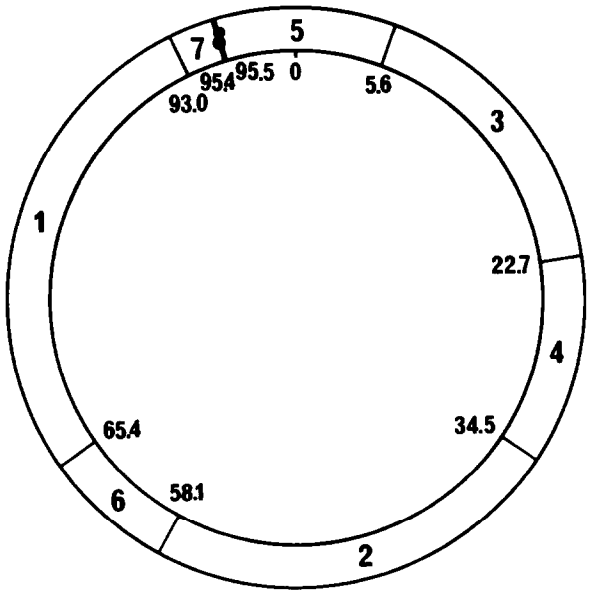

Fig.1. Physical map of polyoma DNA. The positions and coordinates of the *Mbo*I recognition sites relative to the single *Eco*RI site [6] are indicated.

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

cleavage sites had putative actinomycin D or distamycin A binding sites within two base pairs from, or immediately adjacent to, the GATC sequences, respectively, whereas poorly protected sites had inhibitor binding sequences at a distance of 4–7 base pairs.

2. MATERIALS AND METHODS

Covalently closed circular polyoma DNA [7] at a concentration of 15 μg/ml was digested with *Mbo*I restriction endonuclease (New England Bio-Labs) in a medium consisting of 0.01 M Tris–HCl (pH 7.6), 0.1 M NaCl, 0.01 M $MgCl_2$, 0.001 M β-mercaptoethanol and 0.1 mg/ml of gelatin. Incubations were for 1 h using an amount of enzyme sufficient for the complete digestion of the DNA in the uninhibited reactions. Actinomycin D (Sigma Chemical Co.) or distamycin A (Boehringer Mannheim GmbH) was added to the reaction mixtures before the enzyme. The reactions were terminated by the addition of phenol. Labeling of 5′-ends from [γ-^{32}P]ATP (The Radiochemical Centre, Amersham) using T4 polynucleotide kinase (a gift from M.-L. Hammarskjöld) was done as described by Berkner and Folk [8].

Gel electrophoresis was performed as described earlier [9].

Table 1

Analysis by gel electrophoresis of polyoma DNA digested with *Mbo*I in the presence of inhibitors

Inhibitor (M)	% Yield of *Mbo*I fragment					
	-1	-2	-3	-4	-5	-6
Actinomycin D						
–	100	100	100	100	100	100
1×10^{-6}	97	85	96	93	99	81
3×10^{-6}	97	63	89	69	86	53
1×10^{-5}	93	36	87	55	77	36
3×10^{-5}	35	0	35	14	41	0
1×10^{-4}	0	0	0	0	0	0
Distamycin A						
–	100	100	100	100	100	100
1×10^{-6}	107	105	101	85	86	78
3×10^{-6}	110	107	94	99	95	76
1×10^{-5}	43	104	83	86	76	0
3×10^{-5}	24	62	25	23	36	0
1×10^{-4}	9	26	0	0	12	0

Covalently closed circular polyoma DNA cleaved with *Mbo*I was analyzed by gel electrophoresis in 1.5% agarose gels. Photographs of ethidium bromide stained gels were analyzed by densitometer tracing to quantitate DNA in the fragments

3. RESULTS AND DISCUSSION

The effect of actinomycin D and distamycin A on *Mbo*I cleavage was tested by two methods. In the first type of experiment the cleavage reactions were performed in the presence of 10^{-6}–10^{-4} M concentrations of the two compounds. The cleavage products were then analyzed by gel electrophoresis (table 1). The patterns of partial cleavage products accumulating at increasing inhibitor concentrations were reproducible. However, the lowest inhibitory concentration of the compounds showed variations between experiments. The interpretation of these experiments is complicated by difficulties in the identification of partial cleavage products.

In the second type of experiment polyoma DNA was digested with *Mbo*I in the presence of actinomycin D or distamycin A at concentrations which allowed cleavage at, in average, only one site per DNA molecule. As a control, DNA was partially digested in the absence of inhibitors with a small amount of enzyme. The ends of linear DNA molecules of full length were labeled with ^{32}P. After complete *Mbo*I digestion, the resulting fragments were separated by gel electrophoresis and the amount of radioactivity of each fragment was determined. In the uninhibited reaction (fig.2A) all fragments were labeled to a similar extent, suggesting that *Mbo*I cleaved all sites of polyoma DNA with the same efficiency. In contrast, both actinomycin D (fig.2B) and distamycin A (fig.2C) preferentially protected certain cleavage sites which was reflected by the unequal labeling of the *Mbo*I generated fragments. The conclusions that can be drawn from this experiment alone are limited, since the radioactivity of each fragment represents the sum of label at the two ends.

Partial inhibition of *Mbo*I cleavage by actinomycin D primarily resulted in decreased molar yields of fragments *Mbo*I-2, -4, and-6. In contrast, the generation of *Mbo*I-1, -3 and -5 was much

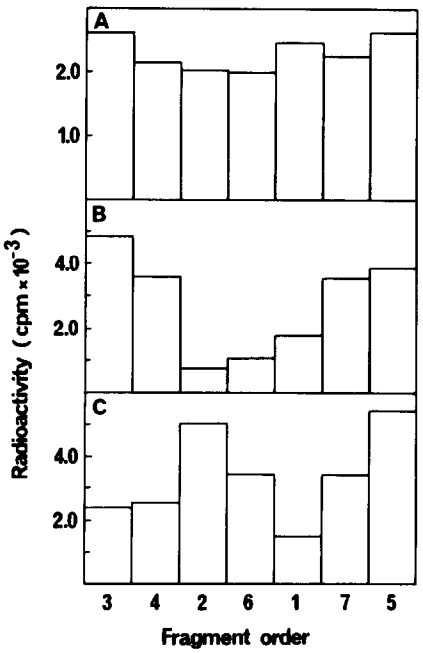

Fig.2. Distribution of cleaved recognition sites after partial digestion of polyoma DNA with *Mbo*I. A permuted set of linear polyoma DNA molecules was generated by partial *Mbo*I cleavage, either with a small amount of enzyme (A), or in the presence of inhibitors. Actinomycin D (B) and distamycin A (C) were used at a concentration of 2×10^{-5} M. In the reaction only trace amounts of cleavage products shorter than full length linear DNA were formed. The ends of the linear DNA molecules were ^{32}P-labeled before cleavage to completion with *Mbo*I. The resulting fragments were separated on polyacrylamide gels and the radioactivity of each fragment was determined. In the figure the fragments are ordered on a linearized map of polyoma.

more resistant to inhibition (table 1). Inspection of the original photographs of the gels also showed that the formation of *Mbo*I-7 was quite resistant to inhibition.

Linear DNA molecules formed by *Mbo*I cleavage in the presence of actinomycin D predominantly ended with *Mbo*I-3, -4, -5 and -7. Relatively few molecules appeared to be cleaved at the sites determining *Mbo*I-1, -2 and -6 (fig.2B).

Together, the two experiments show that the cleavage sites at 34.5 and 58.1 map units, limiting *Mbo*I-2 from *Mbo*I-4 and -6, were well protected by actinomycin D. Conversely, the sites at 5.6, 22.7, 93.0, 95.4 and 95.5 map units, limiting *Mbo*I-3, -7 and -8, respectively, were poorly protected. Conflicting results were obtained for the formation of *Mbo*I-1 and -4. The apparently high yield of *Mbo*I-1 shown in table 1 could result from the accumulation of partially cleaved *Mbo*I-3 plus -5. It is therefore probable that the site at 65.4 units was better protected than the site at 93.0 map units, as suggested from the data of fig.2B. The relatively high radioactivity of *Mbo*I-4 seen in fig.2B probably resulted from effective labeling of the fragment at 22.7 map units.

Distamycin A inhibition primarily decreased the yield of the two neighbouring fragments *Mbo*I-1 and -6. At a slightly increased concentration (3×10^{-5} M) of the compound also the generation of the two contiguous fragments *Mbo*I-3 and -4 was reduced. In contrast, the formation of the other fragments was more resistant to inhibition. Again, the amount of *Mbo*I-1 probably was overestimated (table 1).

End labeling of DNA molecules showed (fig.2C) that circular DNA molecules were most frequently cleaved at sites determining *Mbo*I-2 and -5, at least frequently at sites determining *Mbo*I-1.

From the two sets of data we conclude that *Mbo*I cleavage at 65.4 map units, between *Mbo*I-1 and -6, was most sensitive to distamycin A, and cleavage of the site at 22.7 map units slightly less sensitive. At the other end of the scale, the sites at 34.5, 58.1, 95.5 and 5.6 map units, limiting *Mbo*I-2 and -5, respectively, were least efficiently protected. The two remaining cleavage sites at 93.0 and 95.4 map units were probably protected at intermediate levels.

The base sequences at the *Mbo*I cleavage sites (table 2) show that the three sites best protected by actinomycin D all have a base paired dG–dC dinucleotide residue within two base pairs from the GATC recognition sequence. The remaining five *Mbo*I sites all have other GC rich sequences within a distance of a few base pairs, and the site at 95.5 map units has a dG–dC residue separated by six nucleotides from the recognition sequence.

The two *Mbo*I sites protected by distamycin A have two or three contiguous dT residues next to the point of cleavage. Of the relatively unprotected sites, one at 95.4 map units has a potential binding site separated by 1 base pair from the GATC sequence, whereas another site at 34.5 map units has

Table 2

Base sequences at recognition sites and inhibition of *Mbo*I cleavage by actinomycin D and distamycin A

Map position	Base sequence	Protection by	
		Actinomycin D	Distamycin A
5.6	CAACCTTGATGATCCCCTGTTAAT GTTGGAACTACTAGGGGACAATTA	–	–
22.7	AGATGTATTTGATCCTGATATTCA TCTACATAACTAGGACTATAAGT	–	+
34.5	TTTTTTGCTGGATCTGGATGCCAG AAAAAACGACCTAGACCTACGGTC	+ +	–
58.1	AGAAGGGCTGGATCCCGCCATGGT TCTTCCCGACCTAGGGCGGTACCA	+ +	–
65.4	TCACTTACTTGATCAGCTTCAGAA AGTGAATGAACTAGTCGAAGTCTT	+ +	+ +
93.0	GCCAATGGAGGATCTGTATCCGAT CGGTTACCTCCTAGACATAGGGCTA	–	–
95.4	GGCTCATTTCGATCCGATCCTAGA CCGAGTAAAGCTAGGCTAGGATCT	–	–
95.5	ATTTCGATCCGATCCTAGATGCGA TAAAGCTAGGCTAGGATCTACGCT	–	–

The base sequences at the eight *Mbo*I sites [6] are shown with the recognition site of the enzyme underlined. The symbols + +, + and – denote the efficiency of protection against *Mbo*I cleavage by actinomycin D and distamycin A

a dT_6 tract at four base pairs from the recognition sequence. Hence it appears that distamycin A molecules must bind immediately adjacent to the point of cleavage to protect from the *Mbo*I enzyme.

The mechanism of actinomycin D and distamycin A inhibition of *Mbo*I cleavage is unknown. It is conceivable that the binding of the compounds at the restriction endonuclease recognition sites prevents binding of the enzyme or the cleavage reaction by steric hindrance. Another possible mechanism of the inhibition is the structural change of the DNA, such as a local unwinding or increased melting temperature, which is induced by the binding of the two agents [3,4]. Actinomycin D and distamycin A present at high concentrations inhibited *Mbo*I cleavage of polyoma DNA completely. Under conditions of partial cleavage, however, no cleavage site was completely protected. Instead, the relative efficiency of protection of the individual sites was similar at high and low degrees of inhibition.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Cancer Society and Magn. Bergvall's Foundation.

REFERENCES

[1] Roberts, R.J. (1981) Nucleic Acids Res. 9, 75–96.
[2] Nosikov, V.V., Braga, E.A., Karlishev, A.V., Zhuze, A.L. and Polyanovsky, O.L. (1976) Nucleic Acids Res. 3, 2293–2301.
[3] Sobell, H.M. (1973) in: Progress in Nucleic Acid Research and Molecular Biology (Cohn, W.E. ed) Vol. 13, pp. 153–190. Academic Press, New York.

[4] Zimmer, C. (1975) in: Progress in Nucleic Acid Research and Molecular Biology (Cohn, W.E. ed) Vol. 15, pp. 285–318, Academic Press, New York.
[5] Nosikov, V.V. and Sain, B. (1977) Nucleic Acids Res. 4, 2263–2273.
[6] Griffin, B.E., Soeda, E., Barrell, B.G. and Staden, R. (1980) in: Molecular Biology of Tumor Viruses (Tooze, J., ed) 2nd edn. Part 2, pp. 831–896, Cold Spring Harbor, New York.
[7] Magnusson, G. and Nilsson, M.-G. (1977) J. Virol. 22, 646–653.
[8] Berkner, K.L. and Folk, W.R. (1977) J. Biol. Chem. 252, 3176–3184.
[9] Nilsson, S.V. and Magnusson, G. (1982) Nucleic Acids Res. 10, 1425–1437.