Volume 228, number 2, 223–227 FEB 05563 February 1988

Sizing of single-stranded regions in double-stranded DNA by preparative benzoylated DEAE-cellulose chromatography

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Received 20 October 1987; revised version received 17 December 1987

The concentration of caffeine required to elute wholly single-stranded DNA from benzoylated DEAE-cellulose is proportional to the polynucleotide length. The use of benzoylated DEAE-cellulose chromatography for isolating and sizing single-stranded regions in double-stranded DNA has been examined using a series of hybrid molecules. Restriction fragments of the replicating form of bacteriophage φX174 were hybridized to the intact 'plus' strand, thereby forming hybrids having single- and/or double-stranded regions in the kilobase range. A series of such hybrid preparations were subject to caffeine concentration gradient elution from benzoylated DEAE-cellulose. After logarithmic transformation, a linear relationship ($R=0.94$) could be demonstrated between eluting caffeine concentration and single-stranded length, irrespective of the length of associated double-stranded regions or the location, within a given fragment, of unpaired nucleotides. Benzoylated DEAE-cellulose chromatography may therefore be used to separate and characterize, on a preparative scale, double-stranded DNA containing single-stranded regions.

DNA structure; Benzoylated DEAE-cellulose; Single-stranded region; Chromatography; Nucleotide length; DNA hybrid

1. INTRODUCTION

Single-stranded regions in DNA are central to a range of biological processes. Such regions are generated during transcription, de novo replication and repair of DNA and also in the course of DNA degradation in tissue necrosis [1–4]. Despite this significance, methods for measuring and analysing single-stranded regions in DNA are extremely limited. Virtually the only means of sizing unpaired regions in DNA involves determination of molecular size following digestion with appropriate nucleases and hence destruction of the macromolecules [5]. For preparative purposes, BD-cellulose and BND-cellulose chromatography may be used to separate DNA exhibiting structural discontinuity from its double-stranded counterpart.

DNA containing single-stranded regions binds to derivatized DEAE-cellulose in the presence of 1.0 M NaCl which elutes totally double-stranded DNA from the column. Subsequent elution with caffeine (or formamide) solution results in recovery of the bound fraction, be it totally single-stranded DNA, or double-stranded DNA containing single-stranded regions [6,7]. The concentration of caffeine required to elute single-stranded DNA from either BND-cellulose or BD-cellulose is, in the first instance, proportional to the polynucleotide length. With respect to BND-cellulose, the relationship between caffeine concentration and single-stranded length has been characterized for strand lengths up to 1000 residues [8,9]. However, as single-strand length exceeds 1000–2000 nucleotides, DNA is recovered from BND-cellulose with decreasing caffeine concentrations [9]. By comparison, a consistent relationship between the binding characteristics of totally single-stranded DNA molecules up to 50

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Abbreviations: BND-cellulose, benzoylated naphthoylated DEAE-cellulose; BD-cellulose, benzoylated DEAE-cellulose

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50 © 1988 Federation of European Biochemical Societies

kilobases in length and the eluting caffeine concentration has been demonstrated for BD-cellulose [10].

The use of BD-cellulose chromatography for characterization and preparation of structural defects in DNA is constrained by the unknown contribution which associated double-stranded regions might make to the binding of DNA fragments. To assess this situation, we have examined the binding characteristics of a series of hybrid DNA molecules to BD-cellulose. Hybrids were prepared containing known lengths of paired and unpaired nucleotides in various configurations. It was desirable to establish whether the relationship between caffeine concentration and totally single-stranded polynucleotide length [10] was applicable to double-stranded DNA containing single-stranded regions or whether anomalous binding characteristics could be anticipated due to the association of a double-stranded region with structural lesions responsible for binding to BD-cellulose.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes, T_4 DNA ligase, ϕX174 DNA (RF1) and ϕX174 virion DNA were purchased from New England Biolabs (MA, USA); nick-translation kit and deoxy[1′,2′,5′-^{3}H]CTP (62 Ci/mmol) from Amersham International (England); NACS minicolumns from BRL (MD, USA); centricon 30 microconcentrators from Amicon (MA, USA); DNA grade low gel temperature agarose from Biorad (CA, USA); and BD-cellulose and ATP from Boehringer Mannheim (Mannheim, FRG).

2.2. *Radiolabelling of DNA strands*

ϕX174 DNA (replicating form, RF1) was digested with either *Hae*III or *Acc*I restriction enzyme in the recommended buffers and electrophoresed in a 1% agarose gel. The major bands were excised and extracted using the NACS minicolumns according to the protocol recommended by the manufacturer. After either ethanol precipitation or microconcentration, individual fragments (1 μg) were nick-translated at 15°C for 90 min in a final volume of 100 μl, using 12.5 μCi [^{3}H]dCTP. The specific activities obtained were greater than 5×10^6 dpm/μg.

2.3. *Ligation*

The reaction mixture from section 2.2 was heated to 70°C for 5 min in order to inactivate the DNase I and DNA polymerase I. Ligation was performed by bringing the reaction mixture to a total volume of 200 μl containing 66 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 4 mM mercaptoethanol, 5 mM ATP, and 5 units of T_4 DNA ligase. Incubation was for 2 h at room temperature, following which a further purification was done using the NACS minicolumns. Ligation was necessary in order to catalyse the formation of phosphodiester bonds at sites where 'nicking' had occurred.

2.4. *Generation of hybrid molecules*

Hybridization was performed in sealed siliconized glass capillaries containing the individual radiolabelled fragments with a 50–100-fold excess of ϕX174 virion DNA. The total volume was 15–20 μl with a hybridization buffer containing 50 mM NaCl, 6 mM Tris-HCl (pH 7.4) and 6 mM $MgCl_2$. After boiling for 5 min, the capillaries were transferred to a 65°C water bath where hybridization was allowed to proceed for at least 30 min. Under such conditions the 'negative' strand of the denatured radiolabelled fragment was more likely to hybridize with its complementary region on the virion ('plus' strand) DNA, rather than renature with its partner. The hybrid mixture was subjected to electrophoresis in agarose and linear and circular hybrid forms were detected upon UV visualization. These hybrid bands were excised and extracted as before. Where necessary circular hybrids were linearized by digestion with the restriction enzyme *Nru*I in the recommended buffer.

2.5. *BD-cellulose chromatography*

The hybrid molecules were individually fractionated on BD-cellulose columns, using a biphasic linear caffeine gradient for elution as described [11]. For the purposes of standardization denatured preparations of the original *Hae*III and *Acc*I derived fragments were also individually fractionated.

3. RESULTS

The hybrid molecules generated for the purpose of the present study are shown schematically in fig.1. Each of these molecules was generated by permitting radiolabelled restriction fragments of ϕX174, obtained by digestion of the double-stranded replicating form of the virion, to hybridize with the complete 'plus' strand of the bacteriophage. As commercially supplied, ϕX174 DNA ('plus' strand) contained a high proportion of linear molecules. These virion molecules were presumably linear from the origin of replication [12]. After hybridization, linear and circular forms were separated. As indicated (fig.1) circular hybrids 1 and 4 were linearized using *Nru*I. Included amongst the hybrids prepared were molecules having single-stranded regions of the same length, but either internally or terminally located (hybrids 1 and 2, respectively, fig.1). Each of the hybrid preparations, as well as 3 of the single-stranded precursors, were subjected to caffeine gradient elution from BD-cellulose. With the exception of preparation 5, all chromatograms consisted of a single peak, the general character of

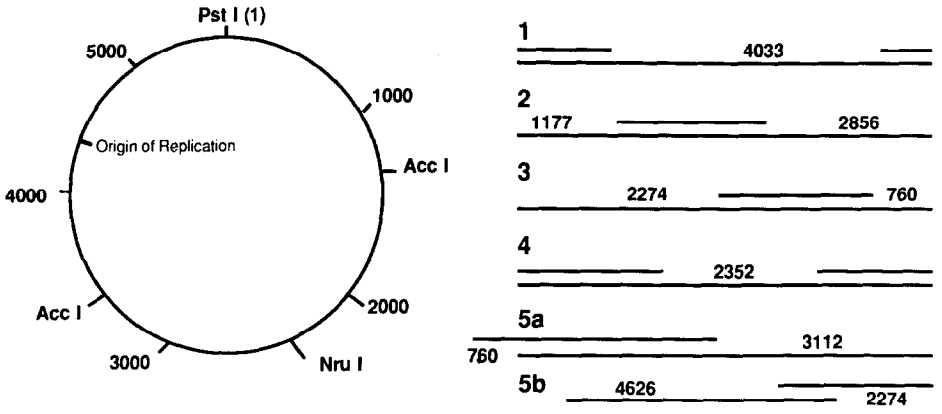

Fig.1. Schematic diagram of bacteriophage ϕX174 (left). Numbering the 5386 bases begins by convention at the unique *Pst*I site [12]. Short strands for hybrid molecules (right) were obtained by digesting the replicating (double-stranded) form of ϕX174 with restriction endonucleases. *Hae*III digestion was used to obtain a 1353 residue fragment (source of the short strand in hybrid preparations 1 and 2) and *Acc*I to obtain either a 2352 (the short strand in hybrid 3) or a 3034 residue fragment (the short strand in hybrids 4 and 5). These fragments were then hybridized to the single (plus) strand of ϕX174 DNA. Hybridization to the linear molecule generated preparations 2, 3 and 5 (the latter giving rise to two structures, 5a and 5b), whilst bonding to the circular form and linearization with *Nru*I generated hybrids 1 and 4.

elution profiles being as described in [10]. Chromatography of preparation 5 resulted in a double peak which could be rationalized in terms of two products being generated during hybridization of these fragments (fig.1).

For analysis of the relationship between single-stranded length and eluting caffeine concentration, each chromatogram was expressed in terms of the individual fraction at which peak recovery of radioactivity occurred, this procedure having been used in a study involving wholly single-stranded DNA [10]. Then, for each hybrid, as well as for the totally single-stranded standards, total single-stranded length and peak eluting caffeine concentration were subjected to logarithmic transformation (table 1). Similar calculations (not shown) were made on the basis of double-stranded nucleotide length of respective hybrids. No rela-

Table 1

Elution of hybrid and single-strand DNA from BD-cellulose

DNA	Total single-strand length (nucleotides)	Log(single-strand length)	Total double-strand length (nucleotides)	Peak eluting [caffeine] (%)	Log(peak [caffeine]
Hybrid no.					
1	4033	3.6056	1353	0.33	−0.4815
2	4033	3.6056	1353	0.34	−0.4685
3	3034	3.4820	2352	0.30	−0.5229
4	2352	3.3715	3034	0.32	−0.4948
5a	3872	3.5879	2274	0.39	−0.4089
5b	6400	3.8388	760	0.47	−0.3279
Standard no.					
1	603	2.7803	–	0.16	−0.8013
2	1078	3.0327	–	0.24	−0.6198
3	1353	3.1313	–	0.29	−0.5406

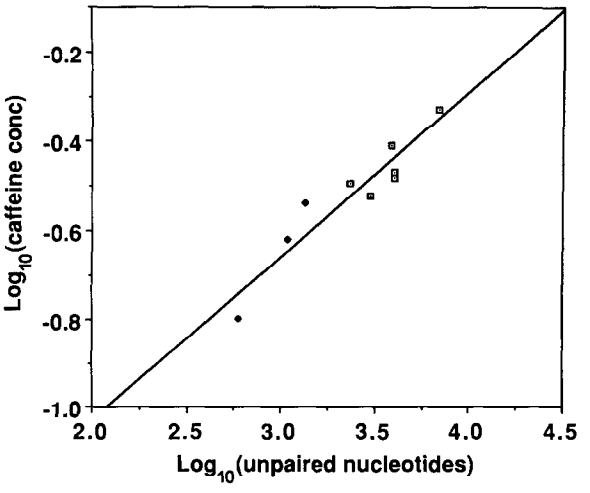

Fig.2. The relationship (after logarithmic transformation) between peak eluting caffeine concentration and single-stranded nucleotide length for hybrid (open symbols) and single-stranded (closed symbols) nucleotide molecules as listed in table 1.

tionship was apparent between double-stranded length and eluting caffeine concentration.

After logarithmic conversion, a linear relationship (R = 0.94) could be demonstrated between single-stranded polynucleotide length and eluting caffeine concentration (fig.2). With reference to this relationship, no distinction was apparent between data derived from hybrid molecules and that from totally single-stranded polynucleotides: the respective points were distributed uniformly about the same straight line.

4. DISCUSSION

Discontinuities in the otherwise double-stranded structure of DNA are usually inferred by degradative analysis. Thus, single-strand breaks are apparent following strand separation in alkaline sucrose gradients [13] whilst single-stranded regions may be evidenced by the activity of strand-specific nucleases [1,14]. Chromatographic options for characterizing structural defects are limited. Although totally single-stranded DNA may be separated from double-stranded DNA using hydroxyapatite [15], double-stranded DNA containing single-stranded regions is eluted at high phosphate concentrations and hence not separated from totally double-stranded material [11,15]. In contrast, such a separation is affected by BD-cellulose or BND-cellulose.

Single-stranded DNA was found to bind irreversibly to naphthoylated DEAE-cellulose, whilst the introduction of some benzoyl residues (i.e. BND-cellulose) afforded fractionation depending upon secondary structure [16]. The basis of fractionation is believed to be interaction between aromatic rings of the column and nucleotides in DNA [6,7]. The contribution of naphthoyl groups in determining the affinity of DNA to derivatized DEAE-celluloses is evidenced by the observation that twice the caffeine concentration (0.35% compared with 0.16% for 600 nucleotides, for example) is required to elute single-stranded DNA from BND-cellulose [9] compared with BD-cellulose (table 1).

The present study includes chromatographic analysis of two classes of molecules: totally single-stranded DNA and double-stranded DNA containing single-stranded regions. Chromatograms obtained with hybrid molecules (fig.2) exhibited the same general characteristics (efficiency of recovery, sharpness of peak, level of background) as those for single-stranded polynucletides both in the present study and as described [10]. No distinction could be made between the binding to BD-cellulose of hybrids and single-stranded polynucleotides (fig.2). The data imply that, at least for a certain range of molecular configurations, the binding of single-stranded polynucleotides to BD-cellulose is not affected either by associated regions of double-stranded DNA or by the configuration of single-stranded regions within a hybrid molecule (fig.1, table 1).

The linear relationship between log(single-stranded polynucleotide length), x, and log(eluting caffeine concentration), y (fig.2), is defined by $x = 2.71y + 4.78$ which lies within one standard deviation (see [10]) of the standard curve for BD-cellulose ($x = 3.09y + 4.63$) determined using 20 single-stranded standards ranging in length from 72 to 49230 residues. These two studies involved different preparations of BD-cellulose which were otherwise handled identically to maintain consistent polynucleotide binding [17]. Taken together, the data suggest little variation in the binding characteristics of the media, thereby reducing the necessity to individually characterise different preparations of BD-cellulose. Again, this contrasts with BND-cellulose where, in the usable DNA size

range, considerable variation of binding affinities have been recorded [8,9,18].

It is possible that DNA molecules whose double-stranded length exceeds by orders of magnitude that of associated single-stranded regions may not bind to BD-cellulose according to fig.2. Nonetheless, the present findings extend the confidence with which BD-cellulose chromatography data may be used to infer the secondary structure of DNA. Binding characteristics of DNA during replication [19], repair [20] or degradation [4] suggestive of single-stranded regions of up to 10 kilobases may be more reasonably attributed to such structural change. More importantly, this media offers the possibility separating such structurally modified DNA on a preparative scale.

Acknowledgements: The authors are grateful to Dr Michelle Haber for helpful discussions. The Children's Leukaemia and Cancer Research Unit is supported by the Children's Leukaemia and Cancer Foundation (Australia).

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