The isolation of duplex DNA fragments containing (dG.dC) clusters by chromatography on poly(rC)-Sephadex

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Received 20 March 1978

ABSTRACT

Duplex DNA containing oligo(dG.dC)-rich clusters can be isolated by specific binding to poly(rC)-Sephadex. This binding, probably mediated by the formation of an oligo(dG.dC)rc⁺ triple helix, is optimal at pH 5 in 50% formamide, 2 M LiCl; the bound DNA is recovered by elution at pH 7.5.

Using this method we find that the viral DNAs PM2, lambda and SV40 contain at least 1, 1 and 2 sites for binding to poly(rC)-Sephadex, respectively. These binding sites have been mapped in the case of SV40; the binding sites can in turn be used for physical mapping studies of DNAs containing (dG.dC) clusters. Inspection of the sequence of the bound fragments of SV40 DNA shows that a (dG.dC)₆₋₇ tract is required for the binding of duplex DNA to poly(rC)-Sephadex. Although about 60% of rabbit DNA cleaved with restriction endonuclease KpnI binds to poly(rC)-Sephadex, no binding is observed for the 5.1 kb DNA fragment generated by KpnI digestion, which contains the rabbit β -globin gene. This indicates that oligo(dG.dC) clusters are not found close to the rabbit β -globin gene.

INTRODUCTION

We have recently described a method for the isolation of DNA containing oligo(dA.dT) clusters (1). DNA is chromatographed on a column of poly(rU)-Sephadex: DNA molecules carrying a (dA.dT) cluster are bound to the column by the formation of a triple helix (dA.dT)rU with the Sephadex-bound poly(rU), whereas molecules lacking these clusters are not bound. This method has made it possible to study the distribution of oligo(dA.dT) clusters in the rabbit genome (2) and to localize oligo(dA.dT) clusters in the 3' extragenic region adjoining the rabbit β -globin gene (3).

Shenkin and Burdon (4) showed that oligo(dG.dC)-rich clusters are ubiquitous in the DNA of higher organisms although nothing is known about the significance of these sequences. To study this further it would obviously be useful to isolate DNA containing

these oligo(dG.dC) clusters. Since poly(rC) can form a (rG.rC)-rC⁺ triple helix with oligo(rG.rC) at low pH (5), we have used this property to isolate (dG.dC)-rich DNA.

MATERIALS AND METHODS

DNA preparations

³H-labelled rabbit DNA was prepared as described (1) and rabbit-liver DNA as described in Flavell *et al.* (2). Phage lambda DNA was prepared as described (6). ³H-labelled phage lambda DNA was prepared in the same way except that 1.3 μ Ci (³H)thymidine (The Radiochemical Center, Amersham, UK) per ml was added to the growth medium.

Homopolymer columns

Poly(rC)-Sephadex was prepared as described for poly(rU)-Sephadex (1). Control CNBr-activated Sephadex G10 was prepared as for poly(rC)-Sephadex except that poly(rC) was not added during the coupling step. The columns were packed in siliconised (Siliclad, Clay Adams) pasteur pipettes (0.28 x 1.8 cm) and washed with 4 bed volumes of the buffer (usually 2 M LiCl, 50% formamide, 50 mM lithium acetate, 0.25% sodium dodecylsulphate (pH 5.0)). DNA samples were applied in 0.25 x column volume of binding buffer. After an incubation of 45 min (unless stated otherwise) at room temperature, the columns were eluted with 16 volumes of binding buffer followed by 12 volumes of 1 M LiCl, 0.25% sodium dodecylsulphate, 10 mM Tris-HCl (pH 7.5) to recover the bound DNA, followed by precipitation with 2 volumes of ethanol in the presence of 25 μ g Dextran 500 per ml (Pharmacia) and 0.2 M sodium acetate (pH 5.6). Gel electrophoresis and cleavage of DNA with restriction endonucleases were performed as described in Jeffreys and Flavell (7). Digestion with endonuclease PvuII or XhoI was in 10 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 1 mM dithiothreitol, 100 μ g gelatin per ml for 2 h at 37°C.

Radioactivity was assayed by mixing the sample (6 ml) with 10 ml of a mixture of 14.4 g Omnifluor (NEN Chemicals), 1 liter toluene (Analar) and 1 liter Triton X-100 (BDH).

RESULTS

Model experiments with synthetic polynucleotides have shown that the oligo(rG.rC)rC⁺ triple helix is stable at pH 5 and destabilized at pH >6 (5).

We therefore applied ³H-labelled rabbit DNA to a poly(rC)-Sephadex column in 2 M LiCl at pH 5 and indeed found significant binding (10-25% of input). A potential complication with the use of poly(rC)-Sephadex at pH 5, however, is that poly(rC) can form an rC-rC⁺ duplex (8). Such a duplex could reduce the efficiency of binding of DNA to the poly(rC) column, but should be eliminated by applying the DNA under conditions in which the rC-rC⁺ duplex is denatured. The addition of formamide to the binding buffer increased the binding of rabbit DNA and phage lambda DNA to about 60-80%; optimal binding was found in 50% formamide, 2 M LiCl, 0.25% sodium dodecylsulphate (pH 5.0) (Fig. 1). Optimal binding of rabbit DNA to the poly(rC) column required a preincubation of DNA with the column of about 45 min (Fig. 2, see also Fig. 4) as was previously found for the binding of DNA to poly-(rU)-Sephadex (2).

If the binding of duplex DNA to poly(rC)-Sephadex is mediated by a (dG.dC)rC⁺ triple helix, then the binding should be pH dependent. Fig. 3 shows that binding is optimal at pH 4.5-5.0 and drops sharply at higher pH. Moreover, the specificity of binding is the same in the pH range 4.5-5.5 for the phage lambda DNA fragments generated by restriction endonuclease EcoRI (data not shown). The pH dependence suggests that a wash at neutral pH should be a simple procedure for the elution of DNA from poly-(rC)-Sephadex. Approximately 75% of DNA bound is recovered by a wash with 1 M LiCl, 0.25% sodium dodecylsulphate, 10 mM Tris-HCl (pH 7.5): the remainder of bound DNA can be recovered by a wash with 1 M NH₄OH and this DNA contains the same specific bound fragments as the pH 7.5 wash.

Specificity of the binding of DNA to poly(rC)-Sephadex

The binding of DNA to poly(rC)-Sephadex at pH 5 is specific for DNA containing (dG.dC)-rich clusters as shown by the following:

a) No binding of rabbit DNA or phage lambda DNA is found to either poly(rI)-Sephadex (0.3% and 0.1% of input, respectively)

Fig. 1. The effect of formamide concentration on the binding of duplex DNA to poly(rC)-Sephadex. ^3H -labelled rabbit DNA (2.2×10^6 cpm/ μg) was applied to a 0.5-ml poly(rC)-Sephadex column in 2 M LiCl, 0.25% sodium dodecylsulphate, 50 mM Li-acetate (pH 5) containing the formamide concentration indicated. After a 45-min incubation at room temperature, the column was eluted with the binding buffer (6 column volumes) followed by 1 M LiCl, 0.25% sodium dodecylsulphate, 10 mM Tris-HCl (pH 7.5) (16 column volumes). Samples were assayed for radioactivity as described in Methods.

Fig. 2. Time course of binding of DNA to poly(rC)-Sephadex. 0.005 μg of ^3H -labelled rabbit DNA (2.2×10^6 cpm/ μg) plus 3 μg yeast RNA in 120 μl 2 M LiCl, 0.25% sodium dodecylsulphate, 50% formamide, 50 mM Li-acetate was applied to a poly(rC)-Sephadex column equilibrated with the same buffer. The loaded columns were incubated for the times indicated and then eluted with binding buffer (6 column volumes) followed by 1 M NH_4OH . Samples were assayed for radioactivity as described in Methods.

or to a control batch of Sephadex G10 activated with CNBr but not carrying poly(rC) (Table I).

b) Control DNAs not carrying (dG.dC)-rich clusters such as poly(dT) and alternating poly(dGC)(dGC) do not bind to the

Fig. 3. Effect of pH on the binding of duplex DNA to poly(rC)-Sephadex. 0.005 μg ^3H -labelled rabbit DNA (2.2×10^6 cpm/ μg) plus 3 μg yeast RNA in 120 μl of 2 M LiCl, 0.25% sodium dodecylsulphate, 50% formamide, 50 mM Li-acetate of the pH indicated was applied to a 0.5-ml poly(rC)-Sephadex column equilibrated with the same buffer. Columns were eluted as described in the legend to Fig.2.

column. Some natural DNAs do not bind to the column (e.g. phage $\phi 29$ DNA), presumably because these DNAs do not contain (dG.dC) clusters long enough to bind to the column.

TABLE I

THE BINDING OF DUPLEX DNA TO POLY(rC)-SEPHADEX

DNA was applied to poly(rC)-Sephadex in 50% formamide, 2 M LiCl, 0.25% sodium dodecylsulphate, 50 mM Li-acetate (pH 5) and incubated at room temperature for 45 min. The column was washed with 6 column volumes of 2 M LiCl (pH 5) buffer and then bound DNA was recovered by a 1 M NH_4OH wash (12 column volumes).

DNA	Poly(rC)-Sephadex	Control CNBr-activated Sephadex G10
	% of input DNA bound	
Rabbit	60-80*	1.2
Lambda	82	1.2
PM2	25	-
$\phi 29$	1	-
SV40	21	-
T ₇	10-25*	0.8
Poly (dT)	1	-
Poly (dG.dC)	96	-
Poly (dG.C) (dG.C)	4	-

*Range found in a number of experiments.

c) The binding of duplex phage PM2 and rabbit DNA to poly(rC)-Sephadex is prevented by incubation of the DNA with poly(rC) in solution at pH 5 prior to the application of the DNA to the column (Table II). Incubation with control poly(rU) or yeast RNA has no effect.

d) DNA with known (dG.dC) tracts binds to the column. Binding is observed for poly(dG.dC) (Table I) and for SV40 DNA which contains two oligo(dG.dC) clusters (see below; ref. 9).

e) The binding "site" on a given DNA can be mapped in restriction enzyme digests of the DNA in question. To demonstrate this, phage PM2 DNA cleaved with endonuclease HindIII was applied to a poly(rC) column. The bound and unbound fractions were recovered by ethanol precipitation and analysed by agarose gel electrophoresis (Fig. 4). The 5.0 kb phage PM2 x HindIII fragment binds to the column, whereas the remainder of the fragments does not.

Capacity of poly(rC)-Sephadex for duplex DNA

We determined the capacity of poly(rC)-Sephadex for duplex rabbit DNA by binding ^3H -labelled rabbit DNA in the presence of increasing amounts of unlabelled rabbit DNA of the same molecular weight (approximately 40 kb). Fig. 5 shows that the column can bind up to 50 μg of rabbit DNA per 0.5 ml of poly(rC)-Sephadex.

Restriction mapping of (dG.dC) clusters in viral DNAs

The experiment shown in Fig. 4 suggests that it should be pos-

TABLE II

PREINCUBATION OF DNA WITH POLY(rC) PREVENTS ITS SUBSEQUENT BINDING TO POLY(rC)-SEPHADEX

^3H -labelled DNA (0.005-0.1 μg) was incubated in 120 μl of 50% formamide, 2 M LiCl, 0.25% sodium dodecylsulphate, 50 mM Li-acetate (pH 5) with 3 μg of the respective competitor RNAs for 45 min at room temperature. The sample was then applied to a 0.5-ml (0.28 x 1.8 cm) column of poly(rC)-Sephadex and the column eluted as described in Table I.

DNA	% of DNA bound to column after incubation with			
	-	Poly(rC)	Poly(rU)	Yeast RNA
PM2	25	11	26	22
Rabbit	82	12	82	82
T ₇	10	0	-	12

Fig. 4. Specificity of binding of phage PM2 DNA x HindIII to poly(rC)-Sephadex. 1 μ g phage PM2 DNA cleaved with restriction endonuclease HindIII was applied to a poly(rC)-Sephadex column in 2 M LiCl, 0.25% sodium dodecylsulphate, 50% formamide, 50 mM Li-acetate (pH 5) and the column operated as described in the legend to Fig. 2, except that the bound DNA was recovered by a wash with 16 volumes of 1 M LiCl, 0.25% sodium dodecylsulphate, 10 mM Tris-HCl (pH 7.5). The bound and unbound fractions were recovered by ethanol precipitation in the presence of 25 μ g Dextran 500 (Pharmacia) per ml and analysed by electrophoresis in a 0.7% agarose gel in the presence of 0.5 μ g ethidium bromide per ml. The bands were detected by UV fluorescence photography (see Materials and Methods). From left to right, unbound (U) and bound (B) fractions for $t = 0, 5, 10, 20, 40$ and 60 min incubation of DNA with the column prior to elution. The last lane contains a marker of phage PM2 DNA x HindIII (I).

sible to map the (dG.dC) clusters relative to the known cleavage sites of various restriction enzymes of a given DNA. To test this we applied SV40 DNA, cleaved with various restriction endonucleases, to poly(rC)-Sephadex. The C and I fragments of a HindII+III digest of SV40 DNA bound to poly(rC)-Sephadex and either the B or C fragments of the HindIII digest (these are not resolved in our gel system) and also the D fragment of the HindIII digest bound to the column.

Inspection of the physical map of SV40 DNA (Fig. 6) shows that these data suggest the localization of two oligo(dG.dC) clusters in SV40 DNA in the C and I HindII+III fragments, respectively. This is confirmed by the fact that the 3.9 kb PstI

Fig. 5. Capacity of poly(rC)-Sephadex for duplex DNA. $0.005 \mu\text{g}$ ^3H -labelled rabbit DNA (2.2×10^6 cpm/ μg) plus $3 \mu\text{g}$ yeast RNA was applied to a 0.5-ml poly-(rC)-Sephadex column in the presence of unlabelled rabbit DNA as indicated. The columns were operated as described in the legend to Fig. 2.

Fig. 6. Map of cleavage sites on SV40 DNA for the restriction endonucleases used. The hatched areas show the positions of the (dG.dC) clusters (see ref. 9).

fragment binds to poly(rC)-Sephadex. To localize the binding sites within the HindII+III C and I fragments, SV40 DNA digested with HindIII and PvuII was applied to poly(rC)-Sephadex and the fractions analysed by agarose gel electrophoresis. PvuII cleaves the HindIII fragment B (which contains the HindII+III C fragment) within the HindII+III C fragment region to yield a 0.33 kb double digest fragment which binds to poly(rC)-Sephadex. This localizes this binding site for poly(rC)-Sephadex in the lower half of the HindII+III C fragment. The D fragment of the HindIII di-

gest (which contains the HindII+III I fragment) no longer binds to poly(rC)-Sephadex after digestion with PvuII. The DNA sequence of the HindII+III I fragment (9) contains a PvuII site 33 bp from the terminus of this fragment. Thus cleavage of the HindIII D fragment with PvuII generates two fragments, a 0.49 kb band which does not bind to poly(rC)-Sephadex and a 33 bp fragment that contains the sequence GTG₆ which mediates the binding of the HindIII D fragment to poly(rC)-Sephadex. Unfortunately, this PvuII x HindIII fragment is so small that it cannot be visualized in the agarose gel so that this could not be verified. Weak binding of the HindIII A fragment and the HindII+III B fragment (which maps within the HindIII A fragment) also occurs to poly(rC)-Sephadex, which suggests the presence of a shorter (dG.dC) cluster in the HindII+III B fragment. This experiment shows that it is possible to map (dG.dC) clusters on a DNA with a known physical map. It should also be possible to map unknown cleavage sites of new restriction enzymes using this technique with a DNA (such as SV40) containing a localized (dG.dC) cluster.

Use of chromatography on poly(rC)-Sephadex to map recognition sites for restriction endonucleases on DNA

To test the feasibility of this approach we analysed the cleavage of lambda DNA with a series of restriction endonucleases. The 20.8 kb phage lambda x EcoRI fragment binds strongly to poly(rC)-Sephadex. So too does the 5 kb lambda DNA x BamHI fragment. Application of phage lambda DNA cleaved with KpnI shows that the 16 kb fragment binds to the column, whereas the 30 kb and the 1 kb fragment do not. Hence, the 16 kb KpnI fragment should contain sequences present in the 20.8 kb EcoRI fragment. This was verified by cleavage of the bound EcoRI fragment with KpnI; this generates the 16 kb KpnI band.

As a second example phage lambda DNA was cleaved with XhoI to generate 14 kb and 33 kb fragments. The latter fragment binds to poly(rC)-Sephadex. This places the XhoI site at 71% on the phage lambda DNA physical map. The purified XhoI band also gave the 16 kb KpnI band on digestion with KpnI confirming the map position.

Fractionation of DNA containing (dG.dC) clusters

DNA containing long (dG.dC) clusters will show a higher affi-

nity for poly(rC)-Sephadex than DNA containing shorter clusters because the T_m of a long triple helix is higher than that of a short triple helix. To test whether it is possible to fractionate DNA on the basis of this property, we applied a mixture of phage lambda DNA x EcoRI, SV40 DNA x HindIII and phage PM2 DNA x Hind-III to a poly(rC) column and eluted the column with a linear gradient from pH 5 to pH 7.5. The fractions were recovered and analysed by gel electrophoresis. Some fractionation is evident: the 1.1 kb and 0.52 kb SV40 DNA x HindIII bands are eluted as a bound peak earlier than the sharp peaks of the 20.8 kb bands of phage lambda DNA and 5.0 kb band of HindIII x phage PM2 DNA. The separation is not sufficient to be of practical use, however.

Oligo(dG.dC) clusters in the vicinity of the rabbit β -globin gene

Oligo(dG.dC)-rich clusters are widespread in eukaryotic DNA. For example, 80% of high-molecular-weight rabbit DNA (40 kb) binds to poly(rC)-Sephadex. The (dG.dC)-rich clusters may be present at a random level and distribution in the genome. Alternatively, it is possible that such sequences are close to structural genes. Short oligo(dA.dT) clusters are found at transcription termination sites in prokaryotes (10) and the localization of oligo(dA.dT) clusters at the 3' terminus of the rabbit β -globin gene (3) is consistent with similar roles for these clusters in eukaryotes. It has also been suggested that (G+C)-rich regions may be involved in the termination of transcription in prokaryotes (10).

We have recently constructed a physical map of restriction endonuclease cleavage sites in and around the rabbit globin gene (7,11). To determine whether oligo(dG.dC) clusters are present close to this gene, total rabbit DNA was cleaved by KpnI (which gives a 5 kb fragment containing the β -globin gene) or KpnI plus BamHI (BamHI cuts the 5 kb KpnI fragment within the sequence coding for β -globin to give a 1.9 kb and a 3.1 kb fragment). The restricted rabbit DNA was applied to poly(rC)-Sephadex, the fractions recovered and the fragments containing the β -globin gene detected as described (7,11). Neither the 5 kb KpnI β -globin DNA fragment, nor the 1.9 kb and 3.1 kb double digest fragments bound to poly(rC)-Sephadex (Fig. 7). We conclude that oligo-

Fig. 7. Oligo(dG.dC)-rich clusters in the vicinity of the rabbit β -globin gene. Rabbit DNA (50 μ g) cleaved with endonucleases KpnI or KpnI + BamHI was applied to poly(rC)-Sephadex and eluted as described in the legend to Fig. 4. The DNA fractions were recovered, denatured, electrophoresed in a 1.2% agarose gel, transferred to a nitrocellulose filter by blotting and hybridized with 32 P-labelled P β G1 DNA as described; the labelled bands were detected by autoradiography (7,11). I is input; U is unbound fraction; B is bound fraction; M is a marker containing 10 pg P β G1 DNA x HindIII (5.6 kb fragment) plus 25 pg P β G1 DNA x EcoRI x AvaI x HindIII (2.3, 1.5, 1.2 and 0.65 kb fragments; see ref. 7).

(dG.dC) clusters are not found close to the rabbit β -globin gene.

DISCUSSION

In this article we show that DNA containing (dG.dC)-rich clusters can be bound specifically to poly(rC)-Sephadex at pH 5 and recovered from the poly(rC) column by a pH 7.5 wash. The evidence suggests that binding is probably mediated by the (dG.dC)rC⁺ triple helix which is only stable at pH <6.

No major technical problems have been encountered with this method. However, short fragments tend to be bound more efficient-

ly than longer fragments carrying the same binding site (80% binding for a 0.5 kb fragment against 50% binding for a 1.1 kb fragment containing the same cluster) as was seen for poly(rU)-Sephadex (3).

To determine the length of the oligo(dG.dC)_n tract required to bind duplex DNA to poly(rC)-Sephadex, we have examined the binding of DNA fragments with a known sequence. Thus, the Hind II+III I fragment of SV40 DNA, which contains the sequence GTG₆, binds efficiently to poly(rC)-Sephadex. Binding is also observed for the SV40 DNA HindII+III C fragment which contains two closely-linked G₄CG₃ tracts. We have also observed that the DNA fragments of a TaqYI digest of phage ØX174 RFI DNA show little binding. However, about 5-10% binding is seen for the largest TaqYI fragment (2.9 kb) of phage ØX174 DNA which contains a CCCTCCC residue closely linked to a CCTCCCCGTACGCCCGGC residue (14). Taken together these data suggest that the threshold for binding to poly(rC)-Sephadex is between 6 and 7 (dG.dC) base pairs.

The binding of DNA to poly(rC)-Sephadex can be used for the rapid isolation of a single, pure DNA fragment: this is especially useful when the fragment of interest is part of a mixture of fragments of the same molecular weight. Thus, the B HindIII fragment of SV40 DNA is bound to the poly(rC) column while the C HindIII fragment - poorly resolved from the B fragment by gel electrophoresis - does not bind. Moreover, this method can be applied to the mapping of restriction fragments in a DNA for which a physical map of other restriction enzyme cleavage sites is available and on which a (dG.dC)-rich cluster is localized as shown by the model experiments with SV40 DNA and phage lambda DNA.

Finally, the fact that it is possible to isolate DNA which contains (dG.dC)-rich clusters makes it possible to study the arrangement of these sequences in the eukaryotic genome. The experiments described here show that the region immediately surrounding the rabbit β globin gene is devoid of (dG.dC) clusters.

ACKNOWLEDGEMENTS

We thank Professor P.Borst for helpful discussions. We are also grateful to Mrs F.Fase-Fowler for gifts of phage Ø29 DNA, phage PM2 DNA and phage T₇ DNA, to Mr C.J.A.Sol (Laboratorium voor Gezondheidsleer, University of Amster-

dam) for a gift of SV40 DNA and to Dr. P.D.Baas (Laboratorium voor Fysiologische Chemie, State University Utrecht) for a gift of phage ϕ X174 RFI DNA. We are especially grateful to Professor W.Fiers for making available his sequence of SV40 DNA prior to its publication. This work was supported in part by grants to P.Borst and R.A.F. from The Netherlands Foundation for Chemical Research (SON) with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO). The electrophoretograms of SV40 DNA and λ DNA have been omitted, at the request of the editors, to save space. These data are available on request.

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