THE ISOLATION OF DUPLEX DNA CONTAINING (dA·dT) CLUSTERS BY AFFINITY CHROMATOGRAPHY ON POLY(U) SEPHADEX

Richard A. FLAVELL and Frank M. VAN DEN BERG

Section for Medical Enzymology and Molecular Biology, Laboratory of Biochemistry, University of Amsterdam, Eerste Constantijn Huygensstraat 20, Amsterdam, The Netherlands

Received 21 July 1975

1. Introduction

The genome of higher eukaryotes contains numerous short (dA·dT) tracts of unknown function [1,2]. At least some of these segments are transcribed into Hn-RNA, the candidate precursor for mRNA, which contains oligo(U) [3] and oligo(A) [4] clusters. Our understanding of the function of homopolymer sequences, and their flanking DNA regions, could be increased if we had a means of isolating them, preferably in a native form. This is not possible by standard methodology such as CsCl gradients, because these (dA·dT) tracts are short and, therefore, contribute little to the buoyant density of long DNA molecules. Duplex DNA containing (dA·dT) clusters can be hybridized to poly(U) [2] to form the well-characterized (dA·dT·rU) triple helix [5]. In this letter we show that by using poly(U) coupled to Sephadex beads this property can be exploited to specifically isolate DNA containing (dA·dT) clusters in native form (fig.1).

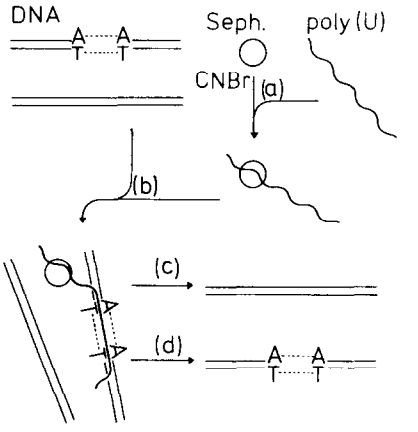

Fig.1. Scheme for the binding of duplex DNA containing (dA·dT) clusters to poly(U) Sephadex. (a) Coupling of poly(U) to Sephadex; (b) binding of DNA to poly(U) Sephadex; (c) elution of column with high salt to remove unbound DNA; (d) elution of column with low salt yielding duplex DNA containing (dA·dT) clusters.

2. Materials and methods

2.1. *DNA preparations*

^{3}H-labelled poly (dA·dT) was prepared by synthesizing ^{3}H-labelled poly(dT) on a poly(rA) template using AMV reverse transcriptase [6]. After removal of triphosphates by passage over a Sephadex G-50 column (0.5 × 7 cm), the poly(rA) was degraded with alkali (0.3 M NaOH, 20°C, 24 h), neutralized, 2 μg poly(dT) and 2.5 μg poly(dA) added and the sample incubated for 5 min at 20°C. The hybrid obtained was incubated with S_1 nuclease (50 units [7], 30 min, 37°C), phenol extracted and passed over a Sephadex G-50 column to remove degradation products, which comprised approximately 30% of input ^{3}H. *Dictyostelium* was grown on ^{32}P-labelled *E. coli*, and DNA prepared from isolated nuclei by a method [7] involving phenol extraction and two cycles of sodium iodide density gradient centrifugation, ribonuclease treatment and finally hydroxyapatite chromatography. Total rabbit DNA was prepared from primary kidney cells by sodium dodecyl sulphate lysis, pronase incubation (1 mg/ml, 16 h, 37°C), deproteinization with chloro-

form-isoamylalcohol, and precipitation with 1 vol of isopropanol. The precipitate was dissolved, treated with ribonuclease (20 μg/ml, 30 min, 37°C), then digested with proteinase K (0.5 mg/ml, 1% sodium dodecyl sulphate, 6 h, 37°C), extracted with phenol and the aqueous phase passed over Sephadex G-50. The preparation of T_7 [7], *E. coli* [7], SV40 [8] and ϕ29 [9] DNAs have been described. The specific activities of the preparations were: ^{3}H-labelled rabbit DNA, 1.8×10^5 cpm/μg; ^{3}H-labelled poly(dA·dT), 4.7×10^4 cpm/μg; ^{3}H-labelled T_7 DNA, 3.4×10^4 cpm/μg; ^{3}H-labelled SV40 DNA, 2.9×10^5 cpm/μg; ^{32}P-labelled *Dictyostelium* DNA, 3×10^4 cpm/μg; ^{32}P-labelled *E. coli* DNA, 3.5×10^3 cpm/μg; ^{32}P-labelled ϕ29 DNA, 1.2×10^4 cpm/μg. The mol. wts of eukaryotic and *E. coli* DNAs were $\geq 30 \times 10^6$; the viral DNAs were intact.

2.2. *Binding of DNA to homopolymer columns*

DNA samples were applied to homopolymer columns (column vol ≥ 4 times sample vol) in 2 M LiCl–0.5% sodium dodecyl sulphate, 10 mM Tris, pH 7.5, and incubated for at least 15 min (see Results). Unbound DNA was eluted with the same buffer and the remainder, constituting the bound fraction, eluted stepwise with: (a) 0.1 M LiCl, 0.5% sodium dodecyl sulphate, 10 mM Tris, pH 7.5; (b) 0.02 M LiCl, 0.5% sodium dodecyl sulphate, 10 mM Tris, pH 7.5; (c) 1 M NH_4OH. ^{32}P-labelled samples were counted by the Cerenkov method, and ^{3}H-labelled samples by trichloroacetic acid precipitation after adjusting the samples to 2 M LiCl. Recovery from the columns was ≥ 90%. Homopolymers were coupled to Sephadex G-10 as described [10].

3. Results and discussion

To test the feasibility of the approach schematized in fig.1 we synthesized ^{3}H-labelled poly(dA·dT). To avoid concatenation of free dA or dT tails to the homopolymer columns, single-stranded regions were degraded by an exhaustive digestion with S_1 nuclease, yielding a product ≥ 95% duplex as judged by further incubations with S_1 nuclease. About 95% of this poly(dA·dT) bound specifically to poly(U) Sephadex in 2 M LiCl; no binding was observed to control poly(C) or poly(A) Sephadex (table 1). In addition, preformation of a triple helix in solution by incubation of ^{3}H-labelled poly(dA·dT) with high molecular weight poly(U) resulted in the abolition of binding to poly(U) Sephadex and the concomitant ability of this triple helix to bind to poly(A) Sephadex by virtue of its poly(U) tail. The binding of the triple helix to poly(A) Sephadex is, however, less efficient than direct hybridization of the poly(dA·dT) duplex to poly(U) Sephadex (table 1) and, therefore, less suitable as a method for isolating DNA-containing $(dA \cdot dT)_n$ clusters.

Table 1
Binding of duplex DNAs to homopolymer columns

DNA	DNA bound to column (% of input)			
	Poly(U)	Poly(A)	Poly(I)	Poly(C)
Poly(dA·dT)	95	3.5	–	0.1
Poly(dA·dT), preincubated with 1 μg poly(U) for 2 min	0.5	53	–	0.5
Rabbit	85	3.9	0.4	–
Rabbit, preincubation with 1 μg poly(U)	2.7	–	–	–
Dictyostelium	88	4.9	–	–
T_7	0.5	3	–	0.5
ϕ29	0.8	–	–	–
E. coli	0.9	–	–	–
SV40	0.5	–	–	–

DNAs were applied to homopolymer columns as described in Materials and methods.

To see if the poly(U) column would specifically bind eukaryotic DNAs containing (dA·dT) clusters we tested *Dictyostelium discoideum* and rabbit DNAs; these contain (dA·dT) tracts of 25 [11] and about 20 base pairs [12], respectively. As can be seen from table 1, about 80–90% of high molecular weight ^{32}P-labelled *Dictyostelium* DNA, and ^{32}P- or ^{3}H-labelled rabbit DNA is retained by poly(U) Sephadex in 2 M LiCl. ^{3}H-labelled T_7 DNA, added as an internal control, did not bind under these conditions nor did *Escherichia coli* DNA, ϕ29 DNA or SV40 DNA measured in separate experiments. Thus, only DNAs containing (dA·dT) clusters bind to poly(U) Sephadex. There are two additional facts which demonstrate the specificity of binding of the eukaryotic DNA (table 1):

(a) The binding to poly(U) Sephadex is eliminated by a prior incubation with poly(U) in solution.

(b) No binding is observed to poly(A) or poly(I) Sephadex columns although control experiments demonstrate that these columns were capable of binding their complementary polymers (data not shown).

Although the binding of duplex DNA to poly(U) Sephadex is analogous to the binding of poly(A)-containing RNAs, it differs in detail. Whereas no prolonged incubation of mRNAs with the column is necessary, maximum binding of *Dictyostelium* DNA requires a 3–5 min incubation, and rabbit DNA about 45 min. The (dA·dT) cluster-poly(U) Sephadex complex is also less stable than that of poly(A) with poly(U) Sephadex. Poly (dA·dT) is eluted from poly(U) Sephadex in 20 mM LiCl (unpublished observations), but poly(A) requires elution with formamide solutions [10]. The complexes formed by natural duplex DNAs with poly(U) Sephadex are even less stable. Development of the column with a LiCl gradient elutes *Dictyostelium* DNA at 0.15 M and rabbit DNA at about 0.25 M LiCl (fig.2). We assume that the kinetics of binding and the stability of the triplexes are a function of the length of the (dA·dT) clusters, where poly(dA·dT) > *Dictyostelium* clusters > rabbit clusters. Finally, the rabbit DNA shows a much more heterogeneous elution profile than *Dictyostelium* DNA (fig.2). This reflects the heterogeneity of mammalian (dA·dT) clusters [1] and the homogeneity of those of *Dictyostelium* [11] as already reported.

An important question which remains is how long a (dA·dT) cluster must be to form a (dA·dT·rU) triple helix. Inspection of data from homopolymer model

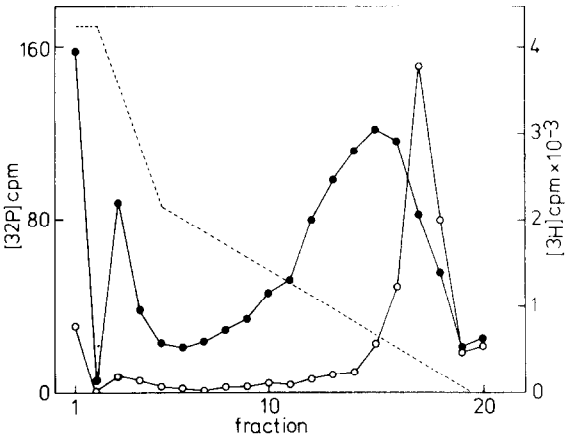

Fig.2. Gradient elution of duplex DNA bound to poly(U) Sephadex. A mixture of ^{3}H-labelled rabbit DNA (0.07 μg) and ^{32}P-labelled *Dictyostelium* DNA (0.05 μg) was applied to a column of poly(U) Sephadex (0.5 ml) in 2 M LiCl, 0.5% sodium dodecyl sulphate, 10 mM Tris, pH 7.5. The sample was left to hybridize for 45 min and then eluted with the same buffer to remove unbound DNA. The remaining DNA was eluted with a linear gradient (total vol 17 ml) of 1 M LiCl, 0.5% sodium dodecyl sulphate, 10 mM Tris, pH 7.5 to 0.5% sodium dodecyl sulphate, 10 mM Tris, pH 7.5. The column was finally eluted with 1 M ammonia (fraction 20). Fractions of 1 ml were collected. (○—○)*Dictyostelium* DNA; (●——●) Rabbit DNA; (- - - -) LiCl concentration.

systems suggests that a $(dA\cdot dT)_n rU$ helix has a T_m of about 20°C in 2 M LiCl when $n \simeq 10$ (Mol, J.N.M., personal communication). This suggests that clusters larger than 10 will bind under these conditions. The failure of T_7 DNA (which contains a dT_6 pyrimidine tract [13]) to bind to the column, even in salt concentrations up to 5 M, is consistent with this estimate.

We are now in a position to isolate eukaryotic DNA containing (dA·dT) clusters, to fractionate them on the basis of the length of the clusters and to use the DNA segments obtained as a probe to analyse their role in gene expression. The fact that the DNA is isolated as a duplex greatly simplifies analysis, not only because both strands of the DNA of interest are obtained, but also because the complications introduced by intra-strand interactions, found with denatured DNA, do not occur. We are at present examining the relationship of these clusters to structural genes in mammalian DNA. The fact that essentially all of the two DNAs

tested binds to the poly(U) column shows that the clusters are widely distributed in the genome, as already concluded from other approaches in the case of *Dictyostelium* [11]. As expected, shearing of the DNA reduces the overall percentage of DNA bound to poly(U) Sephadex (unpublished data), enabling us to determine the distribution of the clusters in the genome, and to purify the DNA segments proximal to (dA·dT) regions.

Acknowledgements

We thank Mrs Marian A. L. P. Ummels-Kremer for expert technical assistance, Mr J. N. M. Mol, Professor P. Borst and Dr. H. F. Tabak for fruitful discussions, Mrs Francesca Fase-Fowler for a gift of T_7 DNA, Dr J. Ter Schegget for SV40 DNA, Dr G. Sheep and Professor C. Weissmann for ϕ29 DNA, Dr P. Curtis for reverse transcriptase and Mr J. N. M. Mol for *Dictyostelium* DNA. We are also indebted to Ms A. Van Strien for a generous gift of primary rabbit kidney cells and Mrs Marijke A. Tilanus for help with the manuscript.

References

[1] Shenkin, A. and Burdon, R. H. (1974) J. Mol. Biol. 85, 19–39.
[2] Bishop, J. O., Rosbash, M. and Evans, D. (1974) J. Mol. Biol. 85, 75–86.
[3] Molloy, G. R., Thomas, W. L. and Darnell, J. E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3684–3688.
[4] Nakazato, H., Kopp, D. W. and Edmonds, M. (1973) J. Biol. Chem. 248, 1472–1476.
[5] Riley, M., Maling, B. and Chamberlin, M. J. (1966) J. Mol. Biol. 20, 359–389.
[6] Verma, I. M., Temple, G. F., Fan, H. and Baltimore, D. (1972) Nature New Biol. 235, 163–167.
[7] Sanders, J. P. M., Flavell, R. A., Borst, P. and Mol, J. N. M. (1973) Biochim. Biophys. Acta 312, 441–457.
[8] Sol, C. J. A., Walig, C., Ter Schegget, J. and Van der Noordaa, J. (1975) J. Gen. Virol., in press.
[9] Aaij, C. and Borst, P. (1972) Biochim. Biophys. Acta 269, 192–200.
[10] Coffin, J. M., Parsons, J. T., Rymo, L., Haroz, R. K. and Weissmann, C. (1974) J. Mol. Biol. 86, 373–396.
[11] Jacobson, A., Firtel, R. A. and Lodish, H. F. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1607–1611.
[12] Mol, J. N. M. and Borst, P. (1975) Biochim. Biophys. Acta, submitted.
[13] Mushynski, W. E. and Spencer, J. H. (1970) J. Mol. Biol. 52, 91–106.