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The binding of poly(rA) and poly(rU) to denatured DNA. II. Studies with natural DNAs.

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ABSTRACT

We have studied the interaction of poly(rA) and poly(rU) with natural DNAs containing $(dA.dT)_n$ sequences. The results indicate that hybridization of poly(rA) to denatured DNA can be used to estimate the size and frequency of large $(dA.dT)_n$ tracts, whereas hybridization with poly(rU) does not give reliable information on these points.

In 6.6 M CsCl, poly(rU) can form stable complexes with denatured DNA containing short $(dA)_n$ tracts ($n \leqslant 6$), whereas binding of poly(rA) to denatured DNA under these conditions requires much larger $(dT)_n$ tracts (estimated $n > 13$). Moreover, binding of poly(rA) requires pre-hybridization in low salt, because free poly(rA) precipitates in 6.6 M CsCl.

INTRODUCTION

In the preceding paper we have presented an analysis of the effects of oligomer chain length and salt concentration on the thermal stability of homopolymer hybrids of the poly(rA).oligo-$(dT)_n$ and 2poly(rU).oligo$(dA)_n$ series. In this paper these data are compared with the T_m's of the complexes of poly(rA) and poly(rU) with natural DNAs containing $(dA.dT)_n$ tracts of known and unknown lengths. The results of this comparison suggest that the stability of poly(rA).DNA complexes is a good measure of the length of $(dA.dT)_n$ tracts in DNA, whereas the stability of poly-(rU).DNA complexes is not.

METHODS AND MATERIALS

Preparation of DNAs

Dictyostelium discoideum nuclear DNA: *Escherichia coli* B cells were grown in 1-litre flasks into the stationary phase as described [1]. The cells were concentrated by centrifugation to a density of 10^{10} cells per ml, in a medium containing 6 mg NaCl,

Abbreviations: L-strand, light strand; H-strand, heavy strand; SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0).

7.5 mg KCl and 4 mg $CaCl.2H_2O$ per litre [2]. Amoebae were grown in 100 ml *E. coli* suspensions to a final density of 10^7 cells per ml and collected by centrifugation at 200 g. Nuclei, prepared according to Horgen and O'Day [3], were lysed in 20 ml 2% sodium dodecyl sulphate, 0.1 M Tris (pH 7.5), 0.1 M EDTA and the DNA was extracted and purified by a procedure involving phenol extraction, two cycles of NaI equilibrium centrifugation, ribonuclease treatment, pronase treatment and hydroxyapatite chromatography as described [4].

If ^{32}P-labelled DNA was required, the *E. coli* cells were grown in 100 ml dephosphorylated medium [5] containing 1-10 mCi ^{32}P. This resulted in a specific activity of the slime-mold DNA of 3000-30 000 cpm/μg.

Yeast RD1A mtDNA: The preparation [4] and strand separation [6] of RD1A mtDNA have been described previously.

Yeast RD1A mtDNA L-strand repeat fragment: The L-strand repeat fragment was prepared by endonuclease IV digestion of ^{32}P-labelled RD1A mtDNA L-strand as described in ref. [7]. The fragment was purified by electrophoresis through 10% polyacrylamide gels containing 8 M urea at 60°C and eluted from the gel in a small volume of 10 mM Tris (pH 7.4), 1 M NaCl [8]. The gel was removed by centrifugation at 10 000 g and the supernatant dialysed against 50 mM sodium phosphate (pH 6.8).

Ribopolymers

Unlabelled poly(rA) (6.7 S) and poly(rU) (molecular weight $>10^5$ daltons) were purchased from Miles and Sigma, respectively. ^{32}P-labelled poly(rU) was synthesized with *E. coli* RNA polymerase in a poly(dA)-directed polymerization of ^{32}P-labelled UTP (purchased from The Radiochemical Centre, Amersham, Great Britain; specific activity 6.6 Ci/mmol) as described [9]; the UTP concentration was 50 μM. The labelled ribopolymer was purified as described by Tabak and Borst [10]. To remove contaminating oligodeoxyribonucleotides, the preparation was boiled for 10 min in 10 mM Tris (pH 7.4) in the presence of 10 μg poly(dT) and centrifuged to equilibrium in a Cs_2SO_4 gradient as described by Szybalski [11]. The peak fraction was dialysed against 10 mM Tris (pH 7.5). ^3H-labelled poly(rU) (specific activity 0.2 μCi

per μg) was a product of Schwarz. ^{32}P-labelled poly(rA) was synthesized with E. coli RNA polymerase in a poly(dT)-directed, oligo(rA)$_5$-primed polymerization of ^{32}P-labelled ATP (purchased from The Radiochemical Centre, Amersham, Great Britain; specific activity 5 Ci/mmol) as described [9]; the ATP concentration was 50 μM. The ribopolymer was purified as described in ref. [10].

Poly(rU) glass fibre filters

Poly(rU) was coupled to glass fibre filters as described by Sheldon et al. [12].

Ribonucleases

Ribonuclease A was purchased from Sigma and dissolved in 50 mM Tris (pH 7.5) to a concentration of 10 mg/ml. The solution was heated for 10 min at 80°C to inactivate contaminating deoxyribonucleases.

Crude ribonuclease T_2 was prepared from Taka Diastase (purchased from Sigma) as described [13].

Hybridization of labelled poly(rA) to filter-bound denatured DNAs

0.1 μm Sartorius nitrocellulose filters containing 0.1-1 μg denatured DNA were prepared according to Denhardt's [14] procedure as described [15]. The filters were placed in a counting vial and shaken for 17 h at 22°C in 2 ml 5 mM Tris (pH 7.5), 0.1 M CsCl, 0.05% sodium dodecyl sulphate, containing 2-20 ng ^{32}P-labelled or ^{3}H-labelled poly(rA). The filters were extensively washed with 5 mM Tris (pH 7.5), 0.1 M CsCl on a sintered glass support and incubated in 0.5 M sodium acetate, 1 mM EDTA (pH 7.0) containing 1 Unit crude ribonuclease T_2 per ml (specific activity 0.1 Unit/μg protein) for 2 h at 25°C. [1 Unit ribonuclease T_2 solubilizes 1 pmol DNA-bound single-stranded poly(rA) in 1 h at 25°C.] After air-drying the filters were counted by standard liquid scintillation counting.

Hybridization of labelled poly(rU) to filter-bound denatured DNA

The same procedure was used as for poly(rA) except for the

higher CsCl concentration of 0.5 M. The filters were incubated in 2 x SSC containing 20 μg ribonuclease A per ml for 1 h at 0°C according to Bishop et al. [16]. After air-drying at room temperature, the filters were counted by standard liquid scintillation counting.

Hybridization of labelled poly(rA) to denatured Dictyostelium DNA in solution

100 ng heat-denatured Dictyostelium DNA was incubated with 2 pmoles ^{32}P-labelled poly(rA) in 25 μl 5 mM Tris (pH 7.5), 0.1 M CsCl for 17 h at 0°C. The extent of hybridization was monitored as radioactivity (cpm) precipitable by 5% trichloroacetic acid after digestion with ribonuclease T_2.

Thermal stability of ribopolymer.DNA hybrids

Filter-bound hybrids: Ribopolymer.DNA hybrids bound to mitrocellulose filters were melted as described [17]. The solvent used is indicated in each legend. The filter washings were either counted directly by their Cerenkov radiation (in the case of ^{32}P-labelled ribopolymer) or as 5% trichloroacetic acid-insoluble material collected on Sartorius nitrocellulose filters (pore size, 0.45 μm).

Poly(rA).Dictyostelium DNA hybrid in 6.6 M CsCl solution: 2.5 μg heat-denatured Dictyostelium DNA was hybridized to 50 pmoles of ^{32}P-labelled poly(rA) as described above. 25-μl samples were made 6.6 M in CsCl by addition of solid CsCl and were kept at different temperatures for 10 min. The samples were immediately quenched in a 50-fold excess of 0.5 M sodium acetate, 1 mM EDTA (pH 7.0) containing 1 μg unlabelled poly(rA) per ml, followed by ribonuclease T_2 digestion. The T_2-resistant, ^{32}P-labelled material was precipitated with 5% trichloroacetic acid in the presence of 100 μg bovine serum albumin and collected on nitrocellulose filters (pore size, 0.45 μm).

Equilibrium centrifugation of ribopolymer.DNA hybrids in 6.6 M CsCl

^{32}P-labelled DNA (2000-5000 cpm) was denatured in 3 or 4

ml 5 mM Tris (pH 7.4), 0.1 M CsCl in the presence of 10 μg poly-(rU) per ml or 50 μg poly(rA) per ml for 10 min at 100°C. The solution was made 6.6 M in CsCl either directly or after an annealing period of 17 h at 0°C. ^{3}H-labelled marker T_7 DNA was added (0.2 μg; 22 000 cpm/μg) and the resulting solution was centrifuged for 45 h either in a Beckman Spinco SW-50 rotor or in a 50-angle rotor at 44 000 rev./min. The temperature was 22°C unless otherwise stated. The gradients were fractionated by dripping or siphoning. The refractive index of every 5th fraction was measured and the distribution of radioactivity was determined by direct Cerenkov counting and by trichloroacetic acid precipitation of an aliquot of each fraction.

RESULTS

The hybridization of poly(rA) to Dictyostelium nuclear DNA

The only natural DNA, which has been proven to contain oligo$(dT)_n$ tracts larger than 13, is the nuclear DNA of the slime-mold Dictyostelium discoideum; 0.3% of this DNA consists of pure $(dT)_{25}$ tracts [18]. The properties of the complex of this DNA with poly(rA), studied by standard filter hybridization in 0.1 M CsCl, using ^{32}P-labelled poly(rA), are presented in Table I and Fig. 1. 0.3% of the DNA formed a stable hybrid with poly(rA) (not shown) confirming published results [18]. The T_m of this hybrid is identical to that of the poly(rA).-oligo$(dT)_{25}$ hybrid both in 0.1 and 1.0 M CsCl irrespective of whether the poly(rA) "tails" are removed with ribonuclease T_2 or not. We conclude from these results that the poly(rA) hybridizes to the $(dT)_{25}$ tract in the DNA by standard Watson-Crick base pairing and that the hybridization of the $(dT)_{25}$ to poly-(A) is the same whether this tract is free or covalently linked to DNA.

The behaviour of the poly(rA).Dictyostelium DNA hybrid in 6.6 M CsCl

The T_m of this hybrid cannot be determined on a filter since poly(rA) binds to such a filter in high CsCl concentrations (not shown). A method was, therefore, worked out to follow

TABLE I - FILTER HYBRIDIZATION OF POLY(rA) TO DENATURED *Dictyostelium discoideum* NUCLEAR DNA

Poly(rA) was hybridized to denatured nuclear DNA in 0.1 M CsCl and the T_m of the hybrid was determined as described in Methods.

Hybrid	Ribonuclease T_2	T_m (°C) in 0.1 M CsCl	1.0 M CsCl	6.6 M CsCl
Poly(rA).DNA	+	38	56	-
	-	38	55	42*
Poly(rA).oligo(dT)$_{25}$	-	39*	55*	-
Poly(rA).2oligo(dT)$_{25}$	-	-	-	43*

* T_m in solution.

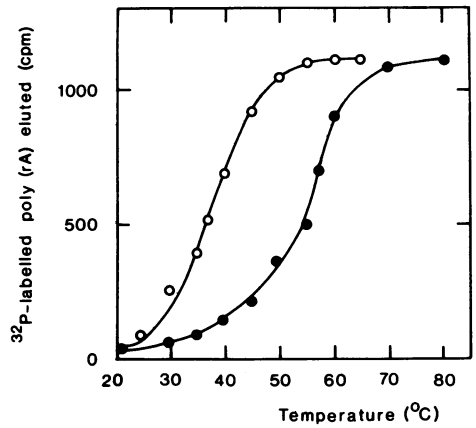

FIG. 1 - Thermal melting curves of filter-bound hybrids of *Dictyostelium* nuclear DNA and poly(rA). 10 ng ^{32}P-labelled poly-(rA) (specific activity 270 cpm/pmol) was hybridized to 0.5 µg filter-bound *Dictyostelium* nuclear DNA under standard conditions. The hybrids were melted in 5 mM Tris (pH 7.5), 0.05% sodium dodecyl sulphate containing 0.1 M CsCl (O—O) or 1.0 M CsCl (●—●).

melting of the hybrid in solution, using dilution with excess cold poly(rA) and ribonuclease T_2 digestion to determine the poly(rA) released from the hybrid. The melting curve obtained, shown in Fig. 2, gives a T_m of 42°C which is identical to the extrapolated T_m of triple-stranded poly(rA).2oligo(dT)$_{25}$ (see Table I).

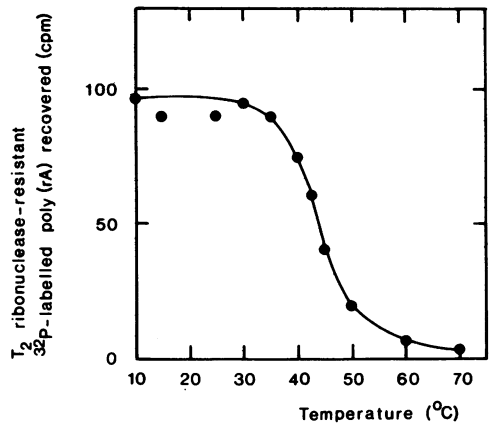

FIG. 2 - Melting curve of the poly(rA).Dictyostelium DNA hybrid in a 6.6 M CsCl solution. For details see Methods.

In 6.6 M CsCl, double-stranded poly(rA).oligo$(dT)_n$ is unstable and undergoes a rapid disproportionation reaction to yield poly(rA).2oligo$(dT)_n$ + free poly(rA). If a similar disproportionation reaction would occur with the poly(rA).DNA hybrid, the amount of poly(rA) in hybrid should decrease by a factor 2 when the DNA is transferred from 0.1 to 6.6 M CsCl. Table II shows that this is not the case. This experiment does not exclude the unlikely possibility, however, that intramolecular disproportionation occurs. The resulting hybrid would be expected to have near-maximal stability with two antiparallel stretches of $(dT)_9$ linked by a $(dT)_7$ loop [19]. Since the T_m of poly(rA).2oligo$(dT)_9$ is 11°C in 6.6 M CsCl, the loop would have to stabilize the molecule by 32°C, which is impossible for a loop low in base stacking. We therefore conclude that in 6.6 M CsCl the poly(rA).DNA hybrid contains double-stranded poly(rA).oligo$(dT)_{25}$ stretches and this implies that poly(rA).oligo$(dT)_n$ has about the same T_m as poly(rA).2oligo-$(dT)_n$ for n = 25. This is not so surprising since the slope of the T_m versus log$[Cs^+]$ graph for triple-stranded poly(rA).-2oligo$(dT)_n$ in CsCl concentrations $\gtrsim$ 2 M is mainly determined by the solubility of poly(rA) (see foregoing paper). It is not unreasonable to suppose that the same holds for the double-stranded helix and, therefore, poly(rA).oligo$(dT)_n$ and poly-(rA).2oligo$(dT)_n$ are expected to differ only marginally in T_m above 2 M CsCl.

TABLE II - RIBONUCLEASE T_2 RESISTANCE OF POLY(rA) HYBRIDIZED TO *Dictyostelium discoideum* NUCLEAR DNA IN SOLUTION

0.5 µg heat-denatured *Dictyostelium* DNA was hybridized to 10 pmoles of ^{32}P-labelled poly(rA) (300 cpm/pmole) as described in Methods. 25-µl samples were digested with ribonuclease T_2 in the presence or absence of 1 µg cold poly(rA) per ml. Another 25-µl sample was made 6.6 M in CsCl by addition of solid CsCl, incubated for 10 min at 22°C, quenched in 1.5 ml 0.5 M sodium acetate (pH 7.0), containing 1 µg cold poly(rA) per ml and incubated with ribonuclease T_2 as described.

Dictyostelium DNA present	Preincubation hybrid in 6.6 M CsCl	Cold poly(rA) added	Ribonuclease T_2 digestion	Poly(rA) in hybrid (cpm)
+	-	-	-	300
+	-	-	+	140
+	-	+	+	150
+	+	+	+	150
-	+	+	+	15

The hybridization of poly(rU) to Dictyostelium nuclear DNA

Whereas poly(rA) hybridizes to denatured *Dictyostelium* DNA in 0.1 M CsCl at 22°C, we failed to detect any hybridization with poly(rU) under these conditions. This is rather surprising in view of the fact that the hybrids poly(rA).oligo(dT)$_n$ and 2poly(rU).oligo(dA)$_n$ have equal T_m's in 0.1 M CsCl for n = 23 (Fig. 3). When the hybridization was performed in 0.5 M CsCl, however, a hybridization plateau of 0.5% was obtained (not shown), confirming the results of Jacobson *et al.* [18]. Two lines of evidence indicate that poly(rU) forms a triple-stranded helix with the $(dA)_{25}$ tracts present in *Dictyostelium* DNA:

(i) Denatured DNA binds twice as much poly(rU) as poly(rA) in a ribonuclease-resistant form;

(ii) It can be seen from Table III and Fig. 4 that the increase in T_m in going from 0.1 M to 1.0 M CsCl is about 30°C for both the ribonuclease A-digested poly(rU).*Dictyostelium* DNA hybrid and the homopolymer complex 2poly(rU).oligo(dA)$_{25}$. If double-stranded helices were formed between poly(rU) and (dA)$_n$ tracts in DNA, the increase in T_m would have been 16-18°C rather than the 30°C observed (compare the data for poly(rA)-

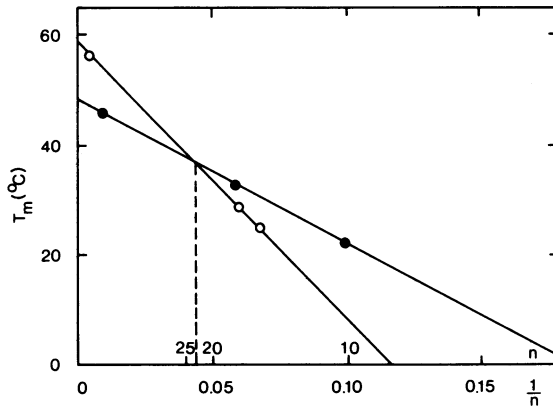

FIG. 3 - T_m versus reciprocal chain length plots for the hybrid complexes poly(rA).oligo(dT)$_n$ and 2poly(rU).oligo(dA)$_n$ in 0.1 M CsCl. O—O, poly(rA).oligo(dT)$_n$; ●—●, 2poly(rU).oligo(dA)$_n$.

TABLE III - FILTER HYBRIDIZATION OF POLY(rU) TO DENATURED *Dictyostelium discoideum* NUCLEAR DNA

Poly(rU) was hybridized to denatured filter-bound DNA in 0.5 M CsCl at 22°C. For comparison the "in solution" T_m's of 2poly(rU).oligo(dA)$_{25}$ are given.

Hybrid	Ribonuclease A treatment	T_m (°C) in 0.1 M CsCl	1.0 M CsCl	6.6 M CsCl
Poly(rU).DNA	+	21	53	71
	-	19	56	76
2Poly(rU).oligo(dA)$_{25}$	-	38*	63*	82*

* See preceding paper.

containing helices in Table I).

The hybrid between poly(rU) and denatured *Dictyostelium* DNA is much less stable than 2poly(rU).oligo(dA)$_{25}$ at all salt concentrations tested. The T_m of the hybrid in 1.0 M CsCl is equal to that of 2poly(rU).oligo(dA)$_{13}$, *i.e.* the estimated tract size is off by a factor 2. This discrepancy is taken up in the Discussion. Another unexpected finding is that the poly(rU).-DNA hybrid is destabilized in high salt by ribonuclease A treatment, an effect not observed with the poly(rA).DNA hybrid. This effect could be due to the existence of intramolecular

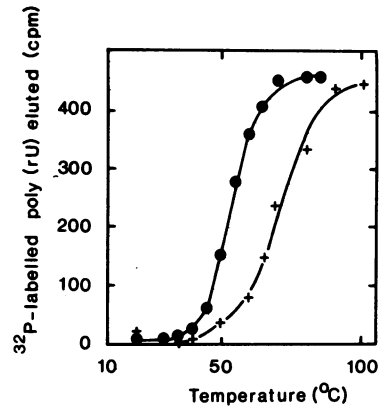

FIG. 4 - Thermal melting curves of filter-bound hybrids of Dictyostelium nuclear DNA and poly(rU). 10 ng ^{32}P-labelled poly-(rU) (specific activity 200 cpm/pmol) was hybridized to filters containing 0.5 µg Dictyostelium nuclear DNA under standard conditions. The hybrids were incubated with ribonuclease A and melted in 5 mM Tris (pH 7.4), 0.05% sodium dodecyl sulphate containing 1.0 M CsCl (●—●) or 6.6 M CsCl (+—+) as described in Methods.

triple-stranded complexes of DNA and poly(rU) at low poly(rU) concentrations, as shown in Fig. 5. Since a hairpin loop is stabilizing the base-paired regions to which it is attached [19], its removal would lower the T_m of the complex.

The binding of poly(rU) to denatured Dictyostelium DNA in 6.6 M CsCl

As poly(rU) is soluble in 6.6 M CsCl, its hybrids with DNA can be analysed in this solvent without a pre-hybridization step in low salt. Since ribonuclease A cannot be used to remove unhybridized poly(rU) in high salt, we used filtration over nitrocellulose filters to separate the hybrids from the remainder of the poly(rU). Table IV shows that the amount of poly(rU) bound to Dictyostelium DNA is the same in 6.6 M and in 0.5 M CsCl, suggesting that even in high salt only $(dA)_{25}$ tracts participate in the binding of poly(rU).

The binding of poly(rA) and poly(rU) to RD1A DNA and phage T_7 DNA

To get further information on the requirements for poly-(rA) and poly(rU) binding, two additional DNAs containing

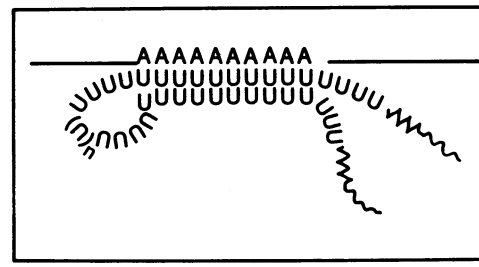

FIG. 5 - Schematic model of the intramolecular poly(rU).DNA triple-stranded complex.

TABLE IV - HYBRIDIZATION IN SOLUTION OF POLY(rU) TO DENATURED *Dictyostelium discoideum* NUCLEAR DNA

0.3-µg *Dictyostelium* DNA samples were heat-denatured in 100-µl 5 mM Tris (pH 7.5). The volume was raised to 1.0 ml by addition of buffered CsCl solutions to the salt concentrations indicated. 60 pmoles ^{3}H-labelled poly(rU) (specific activity 47 cpm/pmole) were added and the mixture was kept at 22°C for 17 h. Finally, all samples were made 6.6 M in CsCl followed by immediate filtration through nitrocellulose filters (pore size, 0.1 µm). After extensive washing with 6.6 M CsCl the filters were dried and counted.

Dictyostelium DNA present	CsCl concentration during annealing (M)	Poly(rU) retained (cpm)
+	0.5	769
+	1.0	862
+	6.6	767
+[a)]	-	36
-	-	56

a) Annealing omitted.

$(dA.dT)_n$ tracts of known composition were studied: mtDNA of the yeast petite mutant RD1A and phage T_7 DNA. RD1A mtDNA consists of a perfect repetition of a 69 nucleotide sequence of wild-type yeast mtDNA, containing 67 consecutive AT base pairs [4,6,7,20]. The complementary strands of this DNA can be separated in alkaline CsCl [6] and the light strand (L-strand) can be cut into segments of repeat unit length (L-strand repeat fragment) with endonuclease IV induced by phage T_4 [7]. The tract compositions of these DNAs are presented in Table V together with our hybridization results. Neither of the DNAs hybridizes with poly(rA)

in 0.1 M CsCl at 22°C. This is according to expectation, because only $(dT)_n$ tracts ≥ 14 should bind poly(rA) under these conditions, whereas both DNAs contain only pure $(dT)_n$ tracts with n <7 [6,7,21]. Neither DNA binds poly(rU) in 0.5 M CsCl.

In 6.6 M CsCl poly(rU) hybridizes only to the L-strand of RD1A mtDNA; the resulting complex is nearly as stable as the poly(rU).Dictyostelium DNA hybrid (Table III). It is obvious that the unexpectedly high stability of the poly(rU).L-strand mtDNA complex cannot be due merely to binding of separate poly-(rU) molecules to each $(dA)_6$ tract, because the T_m of such a complex should be 47°C (Table V). If the $(dA)_6$ tracts from adjacent repeating units manage to align along a single poly(rU) strand, the remaining 63 bases from each repeating unit loop out and are expected to stabilize the complex, because duplex block-copolymers are stabilized if they are coupled by loops that contain non-complementary bases [22,23]. This linkage introduces cooperativity of melting between different blocks and, therefore, elevates T_m. If this is true for the poly(rU).-L-strand mtDNA complex, endonuclease IV digestion of the L-strand should abolish this effect. Table V shows that a reduction in T_m has indeed taken place after endonuclease digestion

TABLE V - HYBRIDIZATION OF POLY(rA) AND POLY(rU) TO YEAST RD1A mtDNA AND PHAGE T_7 DNA

0.1-µg single-stranded RD1A mtDNA aliquots or 1.0 µg phage T_7 DNA were fixed to Sartorius nitrocellulose filters (pore size, 0.01 µm) and hybridized to 1 µg ^{3}H-labelled poly(rU) (specific activity 47 cpm/pmole) or 1 µg ^{32}P-labelled poly(rA) (specific activity 500 cpm/pmole) as described in Methods. 0.05 µg ^{32}P-labelled RD1A mtDNA L-strand or its fragment (specific activity 20 000 cpm/µg) were hybridized to a glass fibre filter containing >1 mg poly(rU) under identical conditions. Hybrids were melted as described in Methods. The tract composition of RD1A mtDNA is from ref. [6] and that of phage T_7 DNA from ref. [21].

DNA	Purine tracts per repeating unit	% Hybridization* to			T_m poly(rU) hybrid in 6.6 M CsCl (°C)
		Poly(rA) in 0.1 M CsCl	Poly(rU) in 0.5 M CsCl	Poly(rU) in 6.6 M CsCl	
RD1A H-strand	3 dA_2; 1 $dA_3G_2A_2$	<0.01	-	<0.3	-
RD1A L-strand	5 dA_2; 2 dA_3; 1 dA_6	<0.01	<0.5	150	62
RD1A L-strand fragment	as L-strand	-	-	-	55
Phage T_7	contains only $(dA)_n$ tracts with n <7	<0.001	<0.01	<0.01	-
Oligo$(dA)_6$		-	-	-	47

* Ribonuclease omitted.

of the L-strand but the T_m of the resulting complex still exceeds the T_m of 2poly(rU).oligo$(dA)_6$ by 8°C. We therefore think that smaller $(dA)_n$ tracts (n = 2 or 3) are also involved in the poly(rU) binding. In this context it is of interest that all $(dA)_n$ tracts with $n > 1$ are clustered in one half of the L-strand repeating unit (Van Kreijl, C.F., personal communication).

We conclude from these results that (A.T)-rich DNA stretches can form extremely stable complexes with poly(rU) in a CsCl gradient and that the T_m of such a complex is not a good measure for the size distribution of the $(dA)_n$ tracts present in this DNA.

The presence of long $(dA.dT)_n$ tracts in other eukaryotic DNAs

It has been shown that poly(rU) hybridizes to many denatured eukaryotic DNAs [16,24-26]; some preliminary poly(rA) hybridizations have been reported as well [24,27]. We have hybridized poly(rA) and poly(rU) to three denatured eukaryotic DNAs and compared the T_m's of the hybrids with the appropriate model system. The results of this analysis are presented in Table VI. Two discrepancies between the poly(rA) and poly(rU) data emerge: First, the hybridization plateau with poly(rU) is up to a factor 10 higher than the plateau obtained with poly-(rA) rather than the factor 2 expected (and also found with Dictyostelium DNA). Second, the tract lengths calculated from the T_m of the poly(rU).DNA hybrids are either much lower (calf, rabbit) or significantly higher (Physarum) than the lengths calculated from the poly(rA).DNA hybrids.

The tract lengths calculated from the T_m's of the poly(rA) hybrids are consistent with the finding that HnRNA in higher animals contains oligo(rA) [30] and oligo(rU) [31] tracts of 20-30 residues in length. The results with poly(rU) suggest that smaller $(dA)_n$ tracts are also involved in the binding of this ribopolymer and confirm that poly(rU) is unsuitable as probe for long $(dA)_n$ tracts.

Behaviour of poly(rA).DNA and poly(rU).DNA complexes in CsCl equilibrium gradients

The density of DNA.RNA hybrids in neutral cesium salts is

TABLE VI - THE PRESENCE OF LARGE $(dA.dT)_n$ TRACTS IN EUKARYOTIC DNAs

Nitrocellulose filters containing 1 μg DNA were incubated either with ^{32}P-labelled poly(rA) or ^{3}H-labelled poly(rU) as described in Methods. The hybridization was complete within 3 h and the ribopolymer concentration and incubation temperature were checked to be optimal. The hybrids were incubated with ribonuclease, melted in 0.1 M CsCl (poly(rA) hybrids) or in 1.0 M CsCl (poly(rU) hybrids) and their T_m's were used to calculate the tract size via comparison with the appropriate model system. Similar experiments using ^{14}C-labelled rabbit-liver DNA have shown that more than 90% of the DNA binds to the filter and remains bound to it during the hybridization procedure.

	DNA		
	Calf thymus	Rabbit liver	Physarum (nuclear)
DNA hybridized to poly(rA) (%)	0.006	0.015	0.014
n calculated from ΔT_m x n = 500	19	21	26
Analytical complexity of DNA*	2×10^{12} [28]	2×10^{12} [28]	4×10^{11} [29]
Number of tracts per genome	21 000	48 000	7000
DNA hybridized to poly(rU) (%)	0.063	0.071	0.049
n calculated from:			
ΔT_m x n = 270	10	12	33
ΔT_m x n = 500	19	23	62

* The amount of DNA per cell (daltons).

mainly determined by the density and the weight ratio of both constituents and by the hydration of the complex. When a poly-(rA).DNA hybrid is transferred to 6.6 M CsCl in the presence of a large excess of free poly(rA), the poly(rA) tails should aggregate (see preceding paper) and the density of the hybrid should approach that of poly(rA) at infinite poly(rA)/DNA ratio. In the absence of free poly(rA) the density of the hybrid should be much lower. To verify if all DNA containing $(dT)_{25}$ tracts can be displaced by poly(rA), we selected long DNA molecules with a calculated average of more than 4 $(dT)_{25}$ tracts per strand from an alkaline sucrose gradient (material larger than phage T_7 DNA), pre-hybridized these to poly(rA) in 0.1 M CsCl and centrifuged the complex to equilibrium in 6.6 M CsCl. The outcome of

this experiment is presented in Table VII. In the absence of a large excess of free poly(rA) there is no density shift detectable, whereas in the presence of excess poly(rA) about 70% of the material banded at ρ = 1.710 g/cm^3, the density of denatured Dictyostelium DNA; the remainder was on the bottom of the tube in the poly(rA) aggregate. The material banding at ρ = 1.710 g/cm^3 was assayed for the presence of $(dT)_{25}$ tracts by standard filter hybridization. The results in Table VII show that most of the $(dT)_{25}$-containing DNA is displaced to the bottom, but that a small fraction remains at ρ = 1.710 g/cm^3. The presence of this fraction can be explained by assuming that either the efficiency of poly(rA)-poly(rA) aggregation was less than 100% or that during the 17-h pre-annealing period part of the $(dT)_{25}$ tracts have been shielded by renaturation of repetitive DNA (cf. [32]). We conclude from these results that a stoicheiometric amount of poly(rA) cannot induce a density shift in this case, but that excess poly(rA) can because of poly(rA)-poly(rA) aggregation.

CsCl gradients of hybrids of poly(rU) with Dictyostelium DNA are presented in Fig. 6. The poly(rU).Dictyostelium DNA hybrid shows only one peak at ρ = 1.740 g/cm^3 ($\Delta\rho$ = 30 mg/cm^3),

TABLE VII - DISTRIBUTION OF Dictyostelium discoideum NUCLEAR DNA IN A CsCl EQUILIBRIUM GRADIENT IN THE PRESENCE OR ABSENCE OF POLY(rA)

100 ng ^{32}P-labelled Dictyostelium DNA (specific activity 30 000 cpm/µg) was processed as indicated in the table and centrifuged to equilibrium in 6.6 M CsCl in a Beckman type 50-angle rotor at 5°C. Peak positions were determined as described in Methods. The entire ^{32}P peak in each gradient was pooled and digested with alkali overnight (0.3 N NaOH at 37°C). After extensive dialysis the material was fixed to nitrocellulose filters and assayed for the presence of $(dT)_{25}$ tracts. Digestion with ribonuclease T_2 was omitted in this experiment (with this particular labelled poly(rA) preparation, the hybrids with Dictyostelium DNA are always 50% ribonuclease T_2 resistant).

DNA	Preannealing to poly(rA)	Poly(rA) added (µg)	Density of DNA (g/cm^3)	Hybridization of non-displaced DNA to poly(rA) (%)	Recovery of DNA (% of total)	
					in gradient	on bottom
Dictyostelium						
Duplex	-	-	1.685	0.6	>99	0
Denatured	-	-	1.710	0.5	>99	0
Denatured	-	200	1.710	0.5	96	4
Denatured	+	0.001	1.710	-	>99	0
Denatured	+	200	1.710	0.12	70	30
Phage T_7						
Duplex	-	-	1.710	<0.001	>99	0

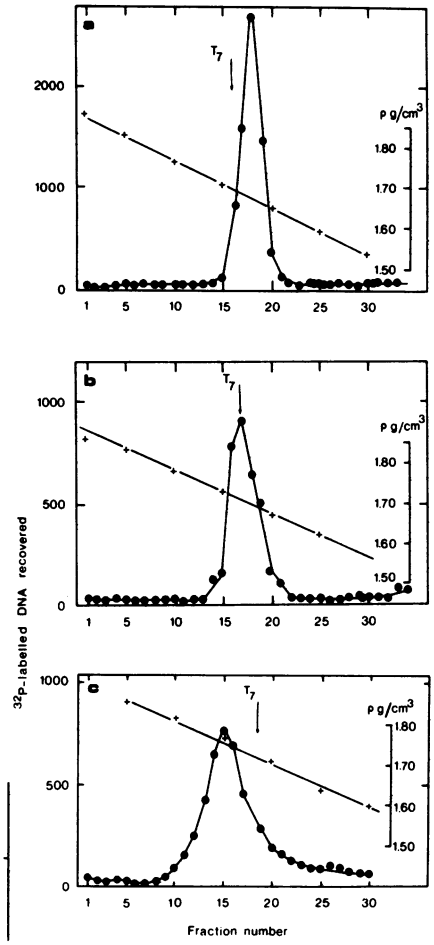

FIG. 6 - CsCl equilibrium gradients of denatured Dictyostelium nuclear DNA in the presence or absence of poly(rU). All SW50 tubes contained 2000-5000 cpm ^{32}P-labelled DNA (specific activity 10 000-30 000 cpm/μg) supplemented with 0.2 μg ^{3}H-labelled bacteriophage T_7 DNA (specific activity 22 000 cpm/μg). a) Duplex Dictyostelium nuclear DNA; b) Dictyostelium nuclear DNA denatured for 10 min at 95°C in 0.1 M CsCl; c) as b) but in the presence of 30 μg poly(rU).

whether a pre-annealing step is included or not (not shown). This differs from the result obtained with poly(rA) where only 30% of the DNA was displaced. Since DNA.DNA renaturation in the gradient is very unlikely with such minute amounts of DNA and since there is no evidence for shorter poly(rU) binding sequences ($n < 25$), the displacement of all of the DNA by poly-(rU) suggests that the $(dA)_{25}$ tracts are randomly distributed

in the DNA.

Table VIII lists the densities of the separated strands of RD1A mtDNA in the presence and absence of poly(rU). The density of the complex of poly(rU) with the L-strand of RD1A mtDNA is about 100 mg/cm^3 higher than the density of the L-strand itself. Such a large poly(rU)-induced density shift has only been reported for mouse satellite DNA [33], which is composed of the basic repeating unit $(dA)_5dC$ [34]. Since the H-strand of RD1A mtDNA does not react with poly(rU), the strands of RD1A mtDNA can be separated on the basis of differential poly(rU) binding (Table VIII, section f). It is remarkable, however, that the H-strand does not anneal to the poly(rU).L-strand complex in the gradient. This can be explained by the finding that the separated strands of RD1A mtDNA have a high degree of internal base pairing (Mol, J.N.M. and Borst, P., unpublished observation).

TABLE VIII - DENSITY IN CsCl OF RD1A mtDNA IN THE PRESENCE OR ABSENCE OF POLY(rU)

^{32}P-labelled RD1A mtDNA (0.2-0.4 µg; specific activity 10 000-30 000 cpm/µg) was supplemented with 0.2 µg ^{3}H-labelled native bacteriophage T$_7$ DNA (specific activity 22 000 cpm/µg) and centrifuged to equilibrium in a SW50 rotor in the presence or absence of 10 µg poly(rU) per ml as described in Methods. [In run f) RD1A mtDNA was denatured in 0.1 M CsCl, 10 mM Tris (pH 7.5) for 10 min at 100°C in the presence of poly(rU).]

RD1A mtDNA	Poly(rU) added	Density in CsCl (g/cm^3)
a) Duplex	-	1.675
b) H-strand	-	1.690
c) H-strand	+	1.690
d) L-strand	-	1.670
e) L-strand	+	1.770
f) Denatured	+	1.690; 1.770*

* Two bands observed containing equal amounts of DNA. These bands represent the separated strands of RD1A mtDNA (not shown).

DISCUSSION

Three main conclusions can be drawn from our results:

1. The extent of hybridization of poly(rA) to denatured

DNA estimates the number of long $(dT)_n$ tracts in this DNA and the T_m of the hybrid reflects the average size of the tracts.

2. Hybridization of poly(rU) to DNA cannot be used either to estimate the number of $(dA)_n$ tracts in DNA or their size.

3. Poly(rU) can form a stable complex in 6.6 M CsCl with DNA stretches composed of short $(dA)_n$ tracts ($n \leqslant 6$), whereas the formation of poly(rA).DNA complexes requires much larger (probably $n > 13$) $(dT)_n$ tracts. Therefore, poly(rU) is potentially useful for the strand separation in CsCl gradients of DNAs with short (dA)-rich stretches, whereas poly(rA) could be used for DNAs with long asymmetrically distributed $(dT)_n$ tracts.

Ad 1: We have shown that the T_m in 0.1 M and 1.0 M CsCl of a poly(rA).DNA hybrid containing poly(rA) binding sites of 25 (dT) residues is identical to the T_m of the poly(rA).$(dT)_{25}$ complex, calculated from the T_m versus 1/n graph for poly(rA).-oligo$(dT)_n$ hybrids, presented in the foregoing paper. We infer from this result that the poly(rA) binds to the $(dT)_{25}$ tract in DNA by standard Watson-Crick base pairing and that the longer oligo$(dT)_n$ tracts in denatured DNA behave in their binding to poly(rA) essentially as free $(dT)_n$ tracts. If this is correct, it follows from the T_m versus 1/n graph (Fig. 6 of the preceding paper) that poly(rA).DNA hybrids can only exist in 0.1 M CsCl at 22°C if the poly(rA) binding site exceeds 14 (dT) residues, whereas in 1.0 M CsCl it should exceed 10 (dT) residues. Although no natural DNAs with $(dT)_n$ tracts in this size range were available to test this, our other results are compatible with it: DNAs that are known to contain $(dT)_n$ tracts with $n < 7$ did not bind poly(rA), whereas mammalian DNAs that should contain the $(dA.dT)_{20}$ tracts transcribed into $(rA)_{20}$* [30], found in HnRNA, did bind poly(rA). Moreover, the fact that the effect of chain length on the T_m of DNA.DNA duplexes [35] is about the same as on the poly(rA).oligo$(dT)_n$ duplex (in both cases $\Delta T_m \times n$ = about 500), supports the idea that the $(dT)_n$ tracts in poly(rA).oligo$(dT)_n$ are a suitable model for $(dT)_n$ tracts in natural DNAs.

* From the base analysis presented in ref. [30] we infer that these tracts contain at most one internal C, G or U.

Although we, therefore, think that the poly(rA).DNA hybrid can be used to estimate the size of long $(dT)_n$ tracts, further experiments are required to determine if reliable values are obtained with $n < 20$, because the T_m of polymer.$(oligomer)_n$ helices depends on the oligomer concentration (see Discussion previous paper). This effect is apparently insignificant for $(dT)_{25}$ tracts, but it may become important for small $(dT)_n$ tracts in natural DNAs.

If hybridization with poly(rA) is used to estimate the size of $(dT)_n$ tracts in DNA, it is desirable to treat the hybrid with ribonuclease T_2 to remove non-base-paired nucleotides, that can be expected to destabilize the helix [36]. With long tracts this effect is insignificant (cf. Table I), but with shorter tracts it could lead to an under-estimation of n.

Ad 2: It is clear from our results that hybridization with poly(rU) gives erroneous values for the size and number of $(dA)_n$ tracts in most of the DNAs that we have analysed. Several explanations can be considered for this result:

a) The effect of oligomer length on T_m is different for poly(rU).DNA complexes and the 2poly(rU).oligo$(dA)_n$ model system. Whereas the $\Delta T_m \times n$ for natural DNA.DNA helices is reported to be about 500 [35], the value for poly(pyr).oligo$(pur)_n$ complexes is much lower [37]. These low values can in part be attributed to the presence of oligomer-oligomer interactions such as stacking. In the poly(rU).DNA hybrid in which the oligo-$(dA)_n$ tracts are separated by non-base-paired sections, these extra interactions are presumably absent. The calculated size of the poly(rU) binding site in Dictyostelium DNA using the relation $\Delta T_m \times n = 270$, found for 2poly(rU).oligo$(dA)_n$, equals 13 (dA) residues. The correct size of the binding site is obtained if the relation $\Delta T_m \times n = 500$ is adopted. Although this suggests that the factors leading to low $\Delta T_m \times n$ values in the model system do not operate in the poly(rU).DNA helix, this explanation does not account for the high ratio hybridized poly(rU)/hybridized poly(rA) found for the other eukaryotic DNAs. This high ratio indicates that shorter tracts ($n < 20$) are also contributing to the binding of poly(rU). Although the correct tract size is found for calf and rabbit DNA, using the relation $\Delta T_m \times n =$

500, the value for Physarum DNA is grossly over-estimated. This makes it difficult if not impossible to interpret the T_m of the poly(rU).DNA complex in terms of tract size.

b) A complication which apparently does not operate in Dictyostelium DNA is that (dG.rU) base pairs can be accomodated in a helix without disturbing the helical framework [38]. Since triple helical 2poly(rU).poly(rA,G) is well known [39], poly-(rU) may form triple helices with (dA,G)-rich regions in DNA. If these survive the ribonuclease digestion, a poly(rU)/poly-(rA) ratio > 2 will be found and the size of the pure $(dA)_n$ tracts involved in the poly(rU) binding will be over-estimated. This complication may operate in the eukaryotic DNAs tested for poly(rA) and poly(rU) binding in Table VI.

In view of these complications, we conclude that $(dA.dT)_n$ tract distributions and sizes calculated from the T_m of the poly(rU).DNA hybrid, using any ΔT_m x n value, can be in error. Therefore, results based on such an analysis (e.g. [24]) should be interpreted with caution.

Ad 3: We have compared the T_m of poly(rA).DNA and poly(rU).-DNA complexes in 6.6 M CsCl with the T_m of the appropriate model system (described in the foregoing paper) to get insight in the requirements for stable ribopolymer.DNA binding in a CsCl gradient.

In 6.6 M CsCl the T_m of the hybrid between poly(rA) and denatured Dictyostelium DNA containing double-stranded poly(rA).-oligo$(dT)_{25}$ stretches, equals the T_m of triple-stranded poly-(rA).2oligo$(dT)_{25}$. If this holds for other n values as well, it follows from Fig. 6 of the preceding paper that complexes between poly(rA) and denatured DNA can only exist in 6.6 M CsCl at 22°C if the size of the poly(rA) binding site exceeds 13 (dT) residues. It should be stressed that the size of the $(dT)_n$ tracts involved in the poly(rA).DNA complex in 6.6 M CsCl only depends on the preannealing conditions, since transfer of the hybrid to 6.6 M CsCl immediately stops the annealing reaction if excess poly(rA) is used. Preannealing at 22°C in CsCl concentrations exceeding 0.1 M in principle allows $(dT)_n$ tracts shorter than 13 residues to form a stable complex with poly(rA) as long as the ribopolymer is soluble. After transfer to 6.6 M

CsCl the hybrids involving $(dT)_n$ tracts <13 become unstable and melt. If the preannealing step is carried out in CsCl concentrations lower than 0.1 M, only $(dT)_n$ tracts larger than 13 residues will hybridize to poly(rA) and the resulting hybrids will remain stable after transfer to 6.6 M CsCl. In this way it should be possible to specifically select for DNA sections containing long $(dT)_n$ tracts with $n > 20$ or $n > 30$, etc.

Our experiments with RD1A mtDNA have shown that the $(dA)_n$ tract size required for stable poly(rU) binding in CsCl gradients is low. Stable binding of poly(rU) to short $(dA)_n$ tracts has been observed earlier with the L-strand of mouse satellite DNA, which contains $(dA)_5dG$ as basic repeat [34]. In this case an ordered helix can be formed, however, because the dG-residue does not destabilize the helix. This is not possible with the L-strand of RD1A mtDNA and in this case poly(rU) binding must involve $(dA)_n$ tracts with $n \leqslant 6$, separated by $(dT)_n$ segments. Tracts with n = 2 or 3 in (A.T)-rich DNA in themselves are not sufficient, because the H-strand of RD1A mtDNA, which contains such tracts, does not bind poly(rU) at all. Moreover, on a statistical basis $(dA)_n$ tracts with $n \leqslant 6$ can be expected to be present in all natural DNAs, but nevertheless many bacterial and phage DNAs do not bind poly(rU) at all. It seems likely, therefore, that short $(dA)_n$ tracts will only bind poly(rU) if they are in close proximity and if DNA intra-strand secondary structure does not successfully compete with the poly(rU) for the tracts. Further experiments with simple sequence DNAs are required to fully define the size and spacing of $(dA)_n$ tracts minimally required to bind poly(rU) in CsCl.

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REFERENCES

1 Hohl, H.R. and Raper, K.B. (1962) J.Bacteriol. 85, 191-198
2 Bonner, J. (1947) J.Exptl.Zool. 106, 1-26
3 Horgen, P.A. and O'Day, D.H. (1973) Cytobios 8, 119-123
4 Sanders, J.P.M., Flavell, R.A., Borst, P. and Mol, J.N.M. (1973) Biochim.Biophys.Acta 312, 441-457
5 Hollenberg, C.P. (1971) Ph.D.Thesis, Mitochondriaal DNA in wildtype bakkersgist en enige cytoplasmatische petite-mutanten, Mondeel-Offsetdrukkerij, Amsterdam
6 Van Kreijl, C.F., Borst, P., Flavell, R.A. and Hollenberg, C.P. (1972) Biochim.Biophys.Acta 277, 61-70
7 Mol, J.N.M., Borst, P., Grosveld, F.G. and Spencer, J.H. (1974) Biochim.Biophys.Acta 374, 115-128
8 De Wachter, R. and Fiers, W. (1972) Anal.Biochem. 49, 184-197
9 Van Kreijl, C.F. and Borst, P. (1973) Biochem.Biophys.Res. Commun. 54, 17-24
10 Tabak, H.F. and Borst, P. (1970) Biochim.Biophys.Acta 217, 356-364
11 Szybalski, W. (1968) in Methods in Enzymology (Grossman, L. and Moldave, K., Eds), Vol. 12B, pp. 330-360, Academic Press, New York
12 Sheldon, R., Jurale, C. and Kates, J. (1972) Proc.Natl. Acad.Sci.U.S. 69, 417-421
13 Hiramaru, M., Uchida, T. and Egami, F. (1966) Anal.Biochem. 17, 135-142
14 Denhardt, D.T. (1966) Biochem.Biophys.Res.Commun. 23, 641-646
15 Flavell, R.A., Birfelder, E.J., Sanders, J.P.M. and Borst, P. (1974) Europ.J.Biochem. 47, 535-543
16 Bishop, J.O., Rosbash, M. and Evans, D. (1974) J.Mol.Biol. 85, 75-86
17 Tabak, H.F., Borst, P. and Tabak, A.J.H. (1973) Biochim. Biophys.Acta 294, 184-191
18 Jacobson, A., Firtel, R.A. and Lodish, H.F. (1974) Proc. Natl.Acad.Sci.U.S. 71, 1607-1611
19 Uhlenbeck, O.C., Borer, Ph.N., Dengler, B. and Tinoco, Jr., I. (1973) J.Mol.Biol. 73, 483-496
20 Hollenberg, C.P., Borst, P., Flavell, R.A., Van Kreijl, C. F., Van Bruggen, E.F.J. and Arnberg, A.C. (1972) Biochim. Biophys.Acta 277, 44-58
21 Mushynski, W.E. and Spencer, J.H. (1970) J.Mol.Biol. 52, 91-106
22 Martin, F.H., Uhlenbeck, O.C. and Doty, P. (1971) J.Mol. Biol. 57, 201-215
23 Uhlenbeck, O.C., Martin, F.H. and Doty, P. (1971) J.Mol. Biol. 57, 217-229
24 Shenkin, A. and Burdon, R.H. (1974) J.Mol.Biol. 85, 19-39
25 Fitzmaurice, L.H. and Baker, R.F. (1974) Cell Diff. 3, 117-126
26 Pero, R.W., Bryngelsson, T., Bryngelsson, C., Deutsch, A., Nordén, Å. and Norgren, A. (1975) Nucl.Acids Res. 2, 1163-1176
27 Marshall, S. and Gillespie, D. (1972) Nature New Biol. 240, 43-45

28 Davidson, J.N. (Ed.) (1972) The Biochemistry of Nucleic Acids, 7th edn, p. 13, Chapman and Hall, London
29 Fouquet, H., Bierweiler, B. and Sauer, H.M. (1974) Europ. J.Biochem. 44, 407-410
30 Nakazato, H., Kopp, D.W. and Edmonds, M. (1973) J.Biol. Chem. 248, 1472-1476
31 Molloy, G.R., Thomas, W.L. and Darnell, J.E. (1972) Proc. Natl.Acad.Sci.U.S. 69, 3684-3688
32 Firtel, R.A. and Bonner, J. (1972) J.Mol.Biol. 66, 339-361
33 Kurnit, D.M., Schildkraut, C.L. and Maio, J.J. (1972) Biochim.Biophys.Acta 259, 297-312
34 Southern, E.M. (1970) Nature 227, 794-798
35 Wilson, D.A. and Thomas, Jr., C.A. (1974) Biochim.Biophys. Acta 349, 461 [Note added in proof to (1973) Biochim. Biophys.Acta 331, 333-340]
36 Tinoco, Jr., I., Borer, Ph.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. and Cralla, J. (1973) Nature New Biol. 246, 40-41
37 Mol, J.N.M. (1976) Ph.D.Thesis, The analysis of (dA.dT) clusters in DNA, University of Amsterdam
38 Tinoco, Jr., I., Uhlenbeck, O.C. and Levine, M.D. (1971) Nature 230, 362-367
39 Bautz, E.K.F. and Bautz, F.A. (1964) Proc.Natl.Acad.Sci. U.S. 52, 1476-1481