The binding of poly(rA) and poly(rU) to denatured DNA. I. Model studies with homopolymers.

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ABSTRACT

We have compared the properties of the poly(rA).oligo(dT)_n complex with those of the poly(rU).oligo(dA)_n complex. Three main differences were found. First, poly(rA) and oligo(dT)_n do not form a complex in concentrations of CsCl exceeding 2 M because the poly(rA) is insoluble in high salt. If the complex is made in low salt, it is destabilized if the CsCl concentration is raised. Complexes between poly(rU) and oligo(dA)_n, on the other hand, can be formed in CsCl concentrations up to 6.6 M. Second, complexes between poly(rA) and oligo(dT)_n are more rapidly destabilized with decreasing chain length than complexes between poly(rU) and oligo(dA)_n. Third, the density of the complex between poly(rA) and poly(dT) in CsCl is slightly lower than that of poly(dT), whereas the density of the complex between poly(rU) and poly(dA) in CsCl is at least 300 g/cm³ higher than that of poly(dA). These results explain why denatured natural DNAs that bind poly(rU) in a CsCl gradient usually do not bind poly(rA).

INTRODUCTION

Binding of polyribonucleotides to single-stranded DNA in concentrated CsCl provides a convenient method to separate the complementary strands of many DNAs [1-10], because more of the polyribonucleotide is bound to one strand than to the other. Interest in the mechanism of polyribonucleotide binding was raised by the finding that binding often correlates with transcriptional specificity [11-14]. With many DNAs the coding DNA strand preferentially binds rG-rich polymers; its complement, rU-rich polymers. It could be concluded that the binding sites for rG-rich polymers or rU-rich polymers have a specific role in transcription or translation. Their nucleotide sequences are, therefore, of interest.

On the basis of indirect evidence [1,5,13], Szybalski and

co-workers concluded that rG-rich polymers bind to dC-rich clusters in DNA by Watson-Crick base pairing. Previous experiments from this laboratory on the binding of poly(rI,G), poly(rC) and poly(rI) to the separated strands of animal mtDNA are compatible with this interpretation [14,15]. By extrapolation, poly(rU) is considered to bind to dA-rich clusters in DNA, but a major objection to this interpretation is the absence of poly(rA) binding to the complementary dT-rich clusters that should be present on the other strand. To get more information on the poly(rU) binding sites we have studied this discrepancy in detail, using homopolymer model systems (this paper) and natural DNAs (presented in the accompanying paper).

MATERIALS AND METHODS

Source of materials

Poly(dT) (2.3 S), poly(dA) (3 S) and poly(rA) (6.7 S) were purchased from Miles; poly(rU) (molecular weight $>10^5$ daltons) from Sigma. Molar extinction coefficients per nucleotide of all polymers were taken from ref. [16]; oligomers of (dT)_n were purchased from P.L.Biochemicals (n = 3-17); their molar extinction coefficients were taken from ref. [17].

Oligomers of (dA)_n were prepared by limited pancreatic deoxyribonuclease digestion of poly(dA) and separation of the products on a DEAE-cellulose column in 7 M urea, according to Bollum [18]; molar extinction coefficients were taken from ref. [17].

Melting profiles and mixing curves

Melting profiles were recorded on a Gilford spectrophotometer, model 2400 as described [19]. The temperature was measured with a type-402 thermistor of Yellow Springs Instruments. Polymer-polymer interactions were measured by mixing of the constituents at 22°C or 0°C (as indicated in the legends) in stoichiometric amounts. The total nucleotide concentration was 66 μM if double-stranded helices were studied and 99 μM for triple-stranded helices. The same holds for oligomer-polymer interactions, but mixing was always at 0°C. Whenever preformed complexes had to be melted at elevated CsCl concentrations, the salt con-

centration was raised by addition of the appropriate amounts of a 7 M CsCl solution.

Density determinations

CsCl and Cs_2SO_4 gradients were generated as described by Szybalski [20], using a Beckman Spinco SW-50.1 rotor. The amount of homopolymer in the gradient varied from 40-200 μg . ^3H -labelled bacteriophage T_7 DNA (ρ in CsCl = 1.710 g/cm^3 and ρ in Cs_2SO_4 = 1.426 g/cm^3) was used as internal marker. Gradients were dripped out through a hole in the bottom, followed by immediate measurement of the refractive index. The absorbance of the fractions was measured after suitable dilution and the position of the marker T_7 DNA was determined by trichloroacetic acid precipitation and counting of fractions collected on Whatman GF glass fibre filters.

Since μg amounts of poly(rA) form a visible precipitate band in CsCl at 50°C , which cannot be dripped out, we used ^3H -labelled poly(rA) of high specific activity (2 ng of $1.5 \times 10^6 \text{ cpm}/\mu\text{g}$); MS_2 RNA ($\rho = 1.893 \text{ g/cm}^3$ at 50°C) [21] was used as marker. After the run, the tube was placed on a holder coupled to a thermostat keeping the whole system at 50°C . The position of the marker RNA and the middle of the gradient were used to calculate the density of the ^3H -labelled poly(rA) assuming a linear gradient.

RESULTS

The solubility of poly(rA) in concentrated CsCl solutions

Attempts to form a complex between poly(rA) and poly(dT) in 6.6 M CsCl were initially unsuccessful, because the poly(rA) precipitated. From Fig. 1, which shows the relationship between the CsCl concentration and the turbidity of poly(rA)-containing solutions, we infer that at 18°C precipitation starts at salt concentrations slightly above 1 M. In 6.6 M CsCl, poly(rA) is insoluble even at 50°C ; at this temperature it yields a visible hyper-sharp precipitate band in a CsCl gradient at $\rho = 1.83 \text{ g/cm}^3$ (see below).

Stability and nature of the hybrid between poly(rA) and oligo(dT)_n

In 0.1 M CsCl poly(rA) and poly(dT) form a double-stranded helix which melts uneventfully (Fig. 2a), confirming the results

FIG. 1 - The turbidity of CsCl solutions containing poly(rA). To 0.1 ml 10 mM Tris-HCl (pH 7.4), containing 40 μ g poly(rA) the appropriate amounts of 7 M CsCl and 10 mM Tris-HCl (pH 7.4) were added under continuous stirring to give a final volume of 1.0 ml. The absorption was read at 18°C against a solvent blank. ●—●, A_{260}/A_{230} ; +—+, A_{400} .

FIG. 2 - Thermal melting curves of the complex between poly(rA) and poly(dT). The complex was preformed in 0.1 M CsCl and melted in 0.1 M CsCl (a) or 6.6 M CsCl (b). The constituents were mixed at 22°C. The insert in (a) shows the mixing curve of poly(rA) and poly(dT).

FIG. 3 - T_m values of complexes of poly(rA) and poly(dT) as a function of CsCl concentration. Whether a $3 \rightarrow 1$ or a $2 \rightarrow 1$ transition took place was determined from absorbance changes at 282 nm as described in the text.

of Riley *et al.* [16]. In 6.6 M CsCl this helix is not formed because the poly(rA) precipitates. The poly(rA).poly(dT) helix was, therefore, first made in 0.1 M CsCl and then the CsCl concentration was raised to 6.6 M. In this way, a sharp thermal transition could be observed, followed by a rapid decrease in $A_{260 \text{ nm}}$ caused by the precipitation of aggregated poly(rA) in the cuvette (Fig. 2b). Results at intermediate CsCl concentrations are presented in Fig. 3. It is obvious that very high CsCl concentrations destabilize the helix.

Since mixing curves of poly(rA) and poly(dT), of the type shown in Fig. 2a, cannot be made in 6.6 M CsCl, the nature of the complex must be inferred from the absorbance changes at 282 nm. At this wavelength the poly(rA).poly(dT) helix shows no hyperchromic effect on melting, whereas the triple-stranded helix poly(rA).2poly(dT) does [16]. From Fig. 4 we infer that in 6.6 M CsCl the triple-stranded helix is present; results at intermediate CsCl concentrations based on similar melting curves at 282 nm are presented in Fig. 3. None of these melting curves show evidence for the disproportionation reaction:

which should lead to a hypochromic effect at 282 nm [22]. Apparently, this reaction occurs whenever the double-stranded helix is placed in CsCl solutions greater than 3 M. The data further suggest that above 3 M CsCl the triple-stranded helix is more stable than the double-stranded helix over the whole temperature range from 0°C to the T_m .

FIG. 4 - Thermal melting curves of the complex between poly(rA) and poly(dT) preformed in 0.1 M CsCl. The constituents were mixed at 0°C. ●—●, Melting in 1.0 M CsCl; ○—○, melting in 6.6 M CsCl.

The effect of chain length and salt on the stability of the poly(rA).oligo(dT)_n helix is presented in Figs 5 and 6. Again there is a strong destabilizing effect of high CsCl concentrations and disproportionation occurs in high salt (checked for $n \gg 9$). These graphs further show that only triple-stranded poly(rA).2oligo(dT)_n is present in 6.6 M CsCl at 22°C for $n \gg 13$. In addition we observe the decrease of T_m with decreasing chain length already documented for other double-stranded and triple-stranded helices [17,23-27].

FIG. 5 - T_m values of complexes of poly(rA) and oligo(dT)_n as a function of CsCl concentration. Whether a $3 \rightarrow 1$ or a $2 \rightarrow 1$ transition took place was determined from absorbance changes at 282 nm as described in the text.

FIG. 6 - T_m versus reciprocal chain length graphs for complexes between poly(rA) and oligo(dT)_n at three different CsCl concentrations. + — +, 0.1 M; ●—●, 1.0 M; ○—○, 6.6 M.

Stability and nature of the hybrid between poly(rU) and oligo(dA)_n

The results are summarized in Figs 7 and 8. The mixing curve in Fig. 7 suggests that the complex between poly(rU) and oligo(dA)₁₀ is triple stranded, as also observed for poly(rU) and poly(dA) at all NaCl concentrations by Riley *et al.* [16]. This is further confirmed by the melting profile at λ_{282} nm (not shown). In this case, the stability of the complex increases up to 4 M CsCl and does not decrease when the salt concentration is raised to 6.6 M. From Fig. 8 we conclude that triple-stranded 2poly(rU).oligo(dA)_n complexes are stable in 6.6 M CsCl at 22°C for $n \gg 4$.

In 0.1 M CsCl, the 2poly(rU).oligo(dA)_n and poly(rA).oligo(dT)_n helices have equal T_m 's for $n = 23$. Since the T_m versus $1/n$ graph is much steeper for the latter than for the former, the 2poly(rU).oligo(dA)_n helix is less stable than its poly(rA).oligo(dT)_n counterpart for infinite n ; the situation is reversed for $n < 23$.

The density of homopolymers in cesium salts

To be able to quantitatively interpret density shifts in cesium salts induced by the binding of polyribonucleotides to DNA, one must know the densities of the polymers and the densities of the hybrid complexes. Some of the requisite data are presented in Table I.

Interesting points are that poly(rA) has a relatively low density in CsCl for a ribopolymer and that the density of triple-stranded poly(rA).2poly(dT) in CsCl is even below that of poly(dT) alone. Another unexpected finding is that double-stranded poly(rA).poly(dT) is undetectable (even at 0°C) irrespective of whether 1:1 or 1:2 mixtures of poly(rA) and poly(dT) are banded (after mixing in 0.1 M CsCl). According to Riley *et al.* [16] both the double-stranded and the triple-stranded complex between poly(rA) and poly(dT) are stable in a Cs₂SO₄ gradient at 20°C which is approximately 3.2 M in Cs⁺. Since our results in CsCl indicate disproportionation of the duplex complex at Cs⁺ concentrations exceeding 3 M, we repeated the analysis of 1:1 and 1:2 mixtures of poly(rA) and poly(dT) in Cs₂SO₄ and checked the

FIG. 7 - Thermal melting curves and T_m values for 2poly(rU).-oligo(dA)_n complexes. (a) Thermal melting curve of a 1:2 mixture of poly(dA) and poly(rU) (O—O) and of oligo(dA)₁₀ and poly(rU) (●—●) in 6.6 M CsCl. The insert shows the mixing curve of poly(dA) and poly(rU) in 6.6 M CsCl. (b) Melting temperature of the 2poly(rU).oligo(dA)₁₀ helix as a function of the CsCl concentration.

FIG. 8 - T_m versus reciprocal chain length graphs for complexes between poly(rU) and oligo(dA)_n at three different CsCl concentrations. + — +, 0.1 M; ●—●, 1.0 M; O—O, 6.6 M.

nature of the complexes by optical melting at 282 nm in 3.2 M Cs^+ . The analysis of the 1:1 mixture of poly(rA) and poly(dT) is presented in Fig. 9. Two bands are seen; one at $\rho = 1.440 \text{ g/cm}^3$ and the other at $\rho = 1.600 \text{ g/cm}^3$. Material at $\rho = 1.440 \text{ g/cm}^3$ is clearly poly(rA).2poly(dT) and not the double-stranded complex as stated by Riley *et al.* [16]. At $\rho = 1.519 \text{ g/cm}^3$, where Riley *et al.* locate the triple-stranded helix, no material is present in our gradients. The material at $\rho = 1.600 \text{ g/cm}^3$ forms a visible hyper-sharp band in the gradient and shows typical poly(rA) melting in 1 M Cs^+ (Fig. 9). Analysis of 1:2 mixtures of poly(rA) and poly(dT) in a Cs_2SO_4 gradient yields the same two bands with the same melting characteristics (not shown).

In conclusion, our experiments show that the sole soluble complex between poly(rA) and poly(dT) in CsCl and Cs_2SO_4 gradients is triple-stranded poly(rA).2poly(dT) and that the binding of the poly(rA) strand to the poly(dT) strands decreases rather than increases their density. Binding of poly(rU) to poly(dA) on the other hand, leads to a large increase in the density of the poly(dA) in these gradients.

DISCUSSION

The goal of these model experiments was to find out why denatured DNA that interacts with poly(rU) in 6.6 M CsCl does not do so with poly(rA). We have found three factors that may contribute to this difference. First, under the conditions used for density gradient centrifugation, poly(rU) is soluble whereas poly(rA) forms insoluble aggregates. Hence, complexes between poly(rA) and oligo(dT)_n cannot be made in 6.6 M CsCl and, if they are first made in low salt, they are destabilized when the CsCl concentration is raised to 6.6 M. Although the low solubility of poly(rA) in concentrated salt solutions had been reported before [28,29], this phenomenon had never been linked to the absence of poly(rA) binding to denatured DNA in CsCl gradients. Second, the T_m of the poly(rA).oligo(dT)_n helix decreases more strongly with decreasing n than the T_m of the 2poly(rU).oligo(dA)_n helix. Hence, at low n the relative stability of the former helix is much lower than one would anticipate from the data obtained at $n = \infty$. If these data hold for base clusters in natural DNAs, we

FIG. 9 - Equilibrium centrifugation of poly(rA).poly(dT) in a Cs_2SO_4 gradient and analysis of the banded material. 20 μg poly(rA) and 20 μg poly(dT) were mixed in 0.05 M Cs_2SO_4 at 22°C in a volume of 3 ml. The Cs_2SO_4 concentration was raised to 1.6 M by addition of 2.1 g of solid Cs_2SO_4 . The solution was centrifuged in a Beckman SW-50.1 rotor for 42 h at 44 000 rev./min at 20°C . Band positions were determined as described in *Methods*. The material banding at $\rho = 1.44 \text{ g/cm}^3$ was melted in 1.6 M Cs_2SO_4 ; the material at $\rho = 1.60 \text{ g/cm}^3$ was diluted to 0.5 M Cs_2SO_4 and melting was monitored at 282 nm.

TABLE I - HOMOPOLYMER DENSITIES IN CsCl AND Cs_2SO_4

Polymer	$\rho \text{ (g/cm}^3\text{)}$	
	CsCl	Cs_2SO_4
Poly(rA)	1.830 ^{a)}	1.570 [16]
Poly(dT)	1.740 ^{b)}	1.424 [16]
Poly(rA).poly(dT)	unstable	unstable 1.433 ^{c)} [16]
Poly(rA).2poly(dT)	1.725	1.440 1.519 ^{c)} [16]
Poly(rU)	>1.92 ^{d)}	1.650 [16]
Poly(dA)	1.620 ^{b)}	1.379 [16]
Poly(rU).poly(dA)	unstable	unstable
2Poly(rU).poly(dA)	>1.92 ^{d)}	1.620 [16]

a) At 50°C ; MS_2 RNA as marker ($\rho = 1.893 \text{ g/cm}^3$ at 50°C).

b) In agreement with ref. [33].

c) Possibly incorrect, see text.

d) Too dense to band in CsCl .

infer from the graphs presented in Figs 6 and 8 that the complex between poly(rA) and (dT)_n tracts is only stable for $n \gg 13$, whereas the complex between poly(rU) and (dA)_n tracts is already stable for $n \gg 4$ in 6.6 M CsCl at 22°C. Since (dT)_n tracts larger than 13 residues are not likely to occur on a large scale in the DNAs tested for poly(rU) binding [1-10], a poly(rA)-induced density shift after a suitable pre-annealing step might be below the resolution of the gradient. Third, the density of poly(rA) in CsCl is much lower than that of poly(rU) and the density of the poly(rA).2poly(dT) complex is much lower than that of the 2poly(rU).poly(dA) complex (Table I). Hence, binding of short poly(rA) to long DNA may not be detectable in a density gradient, because the density increase induced is too small if no aggregation of poly(rA) tails with free poly(rA) takes place.

Since the standard ribopolymer binding technique does not include an extended pre-annealing step, the first of these three factors already explains the lack of poly(rA) binding to a variety of natural DNAs in 6.6 M CsCl [1-10], whereas the last two factors may account for (unpublished) experiments where a pre-annealing step was included but no density shift was observed. Explanations that invoke binding of poly(rU) to DNA by forces other than standard Watson-Crick base pairs or to (dA)_n tracts interspersed with dG residues (*cf.* [15]) have, therefore, become unnecessary.

The data presented in this paper could in principle be used to estimate the size of (dA.dT)_n tracts in natural DNAs by comparing the T_m of the homopolymer.DNA hybrid with the T_m of the appropriate homopolymer.oligodeoxynucleotide system. In extrapolating from model systems to DNA, three complicating factors should be considered, however:

1. The T_m of a helix depends on its strand composition - Double-stranded and triple-stranded helices show a linear dependence of T_m on $\log[\text{salt}]$ [16,22,30]. Since these graphs are steeper for triple-stranded than for double-stranded helices, there is a critical salt concentration where inversion of relative stabilities occurs. For the poly(rA)+oligo(dT)_n system this critical salt concentration is about 2-3 M CsCl and does not depend on n ($n \gg 12$). For the poly(rU) + oligo(dA)_n system it cannot be

measured because the double-stranded helix is too unstable. To obtain meaningful data on the occurrence of homopolymer tracts in DNA from the T_m of the DNA-ribopolymer hybrid, this hybrid must have the same strand composition as the model system it is compared with. There exists suggestive evidence that poly(rU) forms a triple-stranded helix with (dA)_n tracts in natural DNAs [31]. If this is indeed true, the model system and the DNA-poly(rU) helix have the same strand composition and their T_m 's may be expected to exhibit the same dependence on the salt concentration. Poly(rA) is expected to form a double helix with (dT)_n tracts in denatured natural DNAs. Therefore, cluster length determinations in this system should be carried out in CsCl concentrations ≤ 2 M. Moreover, it is possible that longer tracts than the (dT)₁₃ inferred from the poly(rA).oligo(dT)_n model system are required to bind poly(rA) to DNA in 6.6 M CsCl at 22°C, if the resulting complex is double- rather than triple-helical. Since the stability of both complexes is mainly determined by the solubility of the poly(rA), we do not expect the T_m of double-stranded and triple-stranded complexes to differ very much, however, under these conditions. This expectation is borne out by the results presented in the accompanying paper.

2. The T_m of polymer.(oligomer)_n complexes depends on the oligomer concentration - In the poly(dA).oligo(dT)₈ system studied by Cassani and Bollum [17], a 5-fold rise in the oligo-(dT)₈ concentration raised the T_m of this double-stranded helix by 3°C. In the 2poly(rU).oligo(rA)₅ system [32] a 5-fold rise in the oligomer concentration stabilized this triple-stranded helix by 4.5°C. Since this is a mass-action effect, it should be most significant for small oligomers. In DNA the concentrations of homopolymer tracts are several orders of magnitude lower than in the model systems and, therefore, the effect on T_m could be large.

3. In polymer.(oligomer)_n complexes the oligomers interact - In poly(pyrimidine).oligo(purine)_n complexes the terminal bases of the oligomers strongly stack, thereby stabilizing the helix [25,26]. Such stacking interactions are presumably absent when the oligomer is part of a DNA chain and flanked by bases that are not complementary to the ribopolymer used.

To verify the magnitude of these complicating factors, we have studied the stability of hybrids of poly(rA) and poly(rU) with natural DNAs having well-known (dA.dT)_n tract compositions and compared them with the corresponding model systems. The results of this study are presented in the accompanying paper.

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