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Specificity in Formation of Triple-Stranded Nucleic Acid Helical Complexes: Studies with Agarose-Linked Polyribonucleotide Affinity Columns†

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ABSTRACT: The binding of a variety of deoxyribo and ribo homo- and copolynucleotide complementary duplexes to agarose-linked homopolynucleotide affinity columns has been studied. The results provide information concerning the specificity of recognition of complementary base pairs of nucleic acids through a mechanism that involves triple-helix formation under physiological conditions of ionic strength, pH, and temperature. The method employed made it possible, for the first time, to survey the full range of base triplets conceivable from the canonical nucleic acid bases and, in addition, hypoxanthine and thereby to differentiate between those triplets which can and cannot form. Certain previously observed features of the stereochemistry of double-helical targets for third-strand binding are confirmed, and some unrecognized features are elaborated. These include a general requirement for clusters of purine residues in one strand, protonation of third-strand C residues, the ability of natural third-strand residues to distinguish between A·T/U and G·C base pairs, and a capacity of third-strand (unnatural) I residues to recognize all base pairs within such clusters. Thus, the basis for a third-strand binding code is demonstrated.

The possibility that RNA–DNA interaction might occur through a general mechanism involving triple-stranded helix formation (Broitman et al., 1987; Fresco et al., 1989) has led us to examine the specificity and extent of base triplet formation in nucleic acid structures by several different methods. Here we report a study of the binding of nucleic acid duplexes by agarose-linked third-strand homopolyribonucleotide affinity columns under conditions that closely approximate the biological milieu. While some triple-stranded polynucleotide structures have been recognized for a long time (Felsenfeld et al., 1957; Fresco, 1963; Lipsett, 1964), generally they have been viewed as peculiarities of homopolynucleotide interaction. Moreover, they have not been widely studied under comparable conditions, and the full range of potential base triplets that can be formed starting with the Watson–Crick pairs has not been generally recognized [e.g., Broitman et al. (1987)]. Earlier, however, columns of poly(U)[1] and of poly(C) linked to Sephadex had been employed to screen for A·T and G·C clusters, respectively, in some eukaryotic and viral DNAs (Flavell & Van den Berg, 1975; Zuidema et al., 1978).

In the present work, a high level of specificity of recognition of A·U/T and G·C base pairs in homo- or co-purine·homo- or co-pyrimidine residue clusters by specific third-strand residues is demonstrated. This specificity suggests the plausibility of such a mechanism of recognition of complex target sequences by third-strand intermolecular RNA binding or intramolecular DNA binding that could conceivably serve some important biological functions.

Experimental Procedures

Materials

Affinity Columns. Short columns (1.4–2.5 × 0.5 cm) of agarose (Sepharose 4B) covalently linked polyribonucleotides, several hundred residues long, [Pharmacia Ag–poly(A), Ag–poly(G), Ag–poly(I), Ag–poly(C), and Ag–poly(U)] were prepared containing 0.3–0.7 mL of matrix (0.4–1.4 mg of polynucleotide).

Potential Polynucleotide Ligands. All single-stranded polyribonucleotides and polydeoxyribonucleotide duplexes were obtained from Pharmacia. Poly(A·U) and poly(I·C) were prepared by mixing equimolar stock solutions ($\sim 10^{-4}$ M) of their homopolymer constituents in the standard buffer (see

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[1] Abbreviations: Polynucleotide single strands, duplexes, and triplexes are designated by the standard symbols for the residues of homopolymer strands in parentheses, preceded by the word poly; a dot is inserted between the symbols for each strand of a duplex or triplex and a dash between the symbols for the residues of an alternating repeating sequence. Whereas a d preceding the letter symbol of a residue indicates that the backbone is deoxyribo, the absence of any symbol indicates that it is ribo. Base pairs and triplets are indicated by the letter symbols for their residues, with a dot between them. Column matrices are indicated by the abbrevation for agarose, Ag, followed by a dash and the symbols for the covalently linked homopolynucleotide. When a residue is protonated, its letter symbol carrier a (+) as a superscript.

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below) and allowing 1 week for annealing at room temperature.

In the case of poly(dG·dC), the sample was of such high molecular weight (10 000 base pairs) that homogeneous stock solutions were only obtained after suitable sonication (Broitman et al., 1989) to a mean size of 300 base pairs.

Samples of poly(dA·dT) and poly(dG·dC) were rendered blunt ended by degradation with the single-strand specific enzyme S1 nuclease. Conditions were as follows: 60 min, 37 °C, 137.5 units of enzyme, 0.5 mg of polynucleotide in a total volume of 8 mL of 3 mM $ZnCl_2$/0.05 M NaCl/0.01 M cacodylate, pH 6.0. (The activity of the enzyme under these conditions was verified by electrophoretic analysis of the digest.) The reactions were halted by chelation of Zn^{2+} with EDTA at pH 8.25. The polynucleotides were then purified and isolated by standard methods and dialyzed against the standard buffer.

For some experiments, polynucleotide samples were concentrated, prior to application to a column, in Centricon-10 microconcentrator tubes (Amicon) centrifuged for 60 min at 6000 rpm in a Sorvall centrifuge. As much as 10-fold isotonic concentration of solutions was so achieved.

Methods

Binding Experiments. These were generally performed with the sample of potential polynucleotide ligand (25–200 μg in 0.3 mL) and the column matrix equilibrated with the standard buffer, 0.01 M cacodylate/0.1 mM EDTA/0.15 M NaCl/5 mM $MgCl_2$, pH 7.0. For certain experiments, the samples were dialyzed exhaustively against a buffer in which the pH was reduced by changing the acid/salt ratio of the cacodylate or, for more acid pH, by using acetate of the same concentration to give the desired pH. In other experiments, the samples were dialyzed exhaustively against a buffer containing no Mg^{2+} and reduced levels of Na^+. Comparably equilibrated columns were always employed in these cases, but such equilibration was only attained after prolonged exposure of the columns to the modified buffer (several equilibrations of 6–12 h at room temperature). Because of this, the fraction of polynucleotide ligand bound had to be determined by calculating the difference between the amount applied to the column and the amount that did not bind (see below). Direct measurement of the amount of ligand bound by eluting it off the column under ionic conditions unfavorable for its retention proved tedius and unwieldy due to the time required. However, the material so eluted was intact, as judged from its spectral and melting properties.

The polynucleotide sample was allowed to percolate into an affinity column and incubate for 1 h at room temperature (unless otherwise noted). The column was then eluted with three successive 1-mL aliquots of the buffer (7–10 times the column volume), which was sufficient to remove any unbound polynucleotide. The amount of polynucleotide so eluted was determined spectrophotometrically on a Zeiss PMQII instrument. Binding experiments were performed at least twice and the values averaged. The values for the percent bound differed by no more than 10%. Control experiments were performed on a column of underivatized agarose.

RESULTS

Specificity in Column Binding of Single Nucleic Acid Strands. To test the capacity of the agarose-linked polynucleotide affinity columns to bind nucleic acids with specificity by base-dependent hydrogen-bond interaction, the binding specificity of single nucleic acid strands was examined. Such binding can be thought to occur by three different mechanisms. In one, the agarose-linked strands should bind their free Watson–Crick complementary strands. In another, the column-linked strands should bind free strands with which they can form a three-stranded complex as a result of the column-linked or the free strands folding back. Lastly, certain column-linked strands should bind free strands with which they can form a four-stranded rope-type helix. In fact, these three mechanisms are sufficient to explain all the cases of single-strand nucleic acid ligand binding in Table I.

Table I: Binding of Single-Stranded Polynucleotide Ligands by Agarose-Linked Polynucleotides

potential ligand	agarose-linked strand[a]					
	poly(A)	poly(U)	poly(G)	poly(C)	poly(I)	agarose
poly(A)	–	98	–	–	100	–
poly(U)	98	–	–	–	–	–
poly(G)	–	–	70	86	73	–[c]
poly(C)	–	–	96	–	85	–
poly(I)	81	–	32[b]	97	14[b]	–[c]

[a] Minus signs indicate an absence of binding; i.e., the percent of the potential ligand bound ≤ 7% of the amount applied to the column; numerical values indicate the percent of a ligand that is truly bound. [b] The values for the poly(I) ligand vary with the amount of secondary structure in the sample. When poly(I) was freshly denatured and fast cooled, or its development of secondary structure inhibited by the absence of Mg^{2+} in the sample solvent, much higher fractions of the ligand were bound. [c] The absence of binding was observed when poly(G) or poly(I) was freshly denatured and fast cooled; when these polynucleotides were allowed to form multistranded cross-linked networks, they were retained by the column, presumably by occlusion.

Thus, Ag–poly(U·A), Ag–poly(A·U), Ag–poly(C·G), Ag–poly(G·C), Ag–poly(I·C), and Ag–poly(C·I) all must form by complementary base pairing; Ag–poly(I) binding of poly(A) must occur by poly(I·A·I) formation, as must Ag–poly(A) binding of poly(I) (Rich, 1958a; Arnott & Bond, 1973) since the duplex poly(A·I) is not observed under the experimental conditions employed (Howard et al., 1977; Howard & Miles, 1977); and Ag–poly(I) binding of poly(I) and poly(G), as well as Ag–poly(G) binding of poly(G) and poly(I), must occur by formation of the appropriate four-stranded parallel helix (Rich, 1958b; Fresco & Massoulie, 1963; Zimmerman et al., 1975). The specificity of binding of single-stranded nucleic acids by these columns is further indicated by the absence of binding in all other (13) cases, as well as the absence of binding by the agarose column without linked nucleic acid strands.

Defining the System for Column Binding of Nucleic Acid Duplexes. In order to establish experimental conditions for measuring the binding of double helices to columns of agarose-linked third strands, the binding of poly(A·U) and of poly(I·C) by appropriate columns was examined as a function of time and temperature of incubation, and of the concentration of potential ligand.

It can be seen from Table II that binding of both these double helices is essentially complete after 5 min and that the level of binding is not markedly different between 4 and 37 °C. Also, the fraction of these helices bound is invariant when their concentration in the applied charge is varied between A_{260} of 0.4 and A_{260} of 4.0 (10^{-4}–10^{-3} M) (not shown), indicating that equilibrium is attained under the conditions of incubation. These findings indicate that incubation within this concentration range of ligand at 22 °C for 60 min is adequate to obtain equilibrium binding data; such conditions were used for the experiments leading to Tables I and III and Figure 2.

Since some of the experiments examine the potential binding of a double helix to a column whose nucleic acid strand is capable of base pairing to one of the strands of the double-helix, it was necessary to assure that binding does not occur by double-helix formation. One approach taken was to observe the extent of binding as the ionic strength of the medium was

Table II: Binding of Nucleic Acid Duplex Ligands by Agarose-Linked Polynucleotides as a Function of Time and Temperature of Equilibration[a]

	% ligand bound with time of incubation		
	1 min	5 min	60 min
poly(A·U) on Ag–poly(U)			
4 °C	52	71	81
22 °C	74	89	89
37 °C	69	74	72
poly(I·C) on Ag–poly(G)			
4 °C	11	42	47
22 °C	24	37	52
37 °C	45	54	45

[a] Solvent: 0.15 M NaCl/0.005 M $MgCl_2$/0.0001 M EDTA/0.01 M cacodylate, pH 7.0.

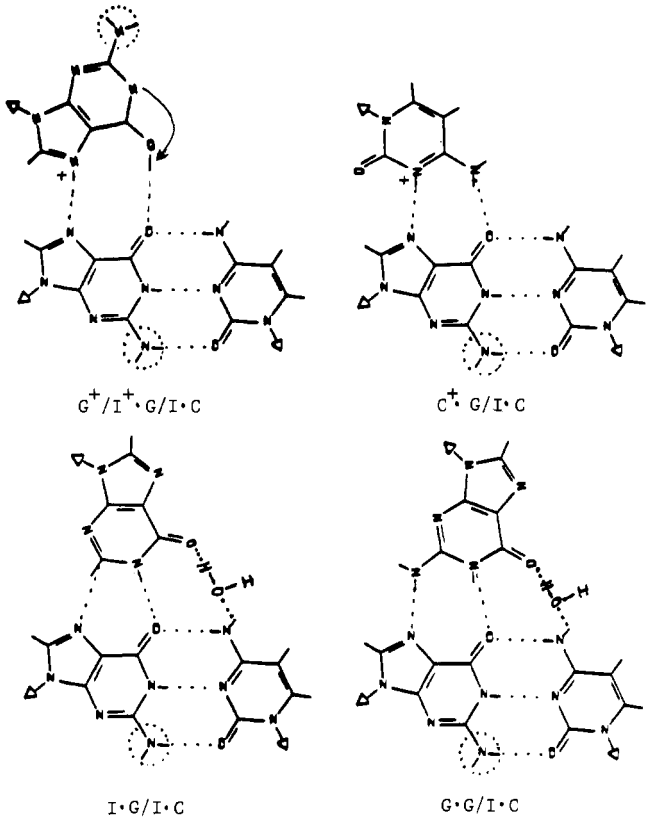

FIGURE 1: Hydrogen-bonding schemes for conceivable base triplets formed from the complementary base pairs I·C and G·C and the third-strand residues I, G, and C. (Top row) The third-strand residues are protonated. While all these triplets are isosteric, those with purine residues are in the enol form and in the syn configuration about the glycosyl bond. (Bottom row) The third-strand I and G residues are neutral and in the anti configuration about the glycosyl bond. As drawn, these triplets differ from the usual Hoogsteen pairing arrangement envisioned between third-strand residues and the purine component of the Watson–Crick pair in that they include a third water-bridged hydrogen-bond interaction between the C(6) carbonyl of the third-strand purine and the exocyclic amino of the pyrimidine (C) of the Watson–Crick pair. Note also that in the triplets at the left of this row the third-strand purine is linked by a weak CH···N interaction instead of a strong NH···N interaction, as in the triplets at the right.

reduced to a level where triple-helix formation is no longer supported, whereas double helices remain stable. For example, when the Na^+ concentration was reduced to 0.01 M in the absence of Mg^{2+}, conditions under which poly(A·U), poly(I·C), and nucleic acid duplexes in general are stable in solution at room temperature (Blake et al., 1967; Chamberlin & Patterson, 1965; Schildkraut & Lifson, 1965), the level of binding

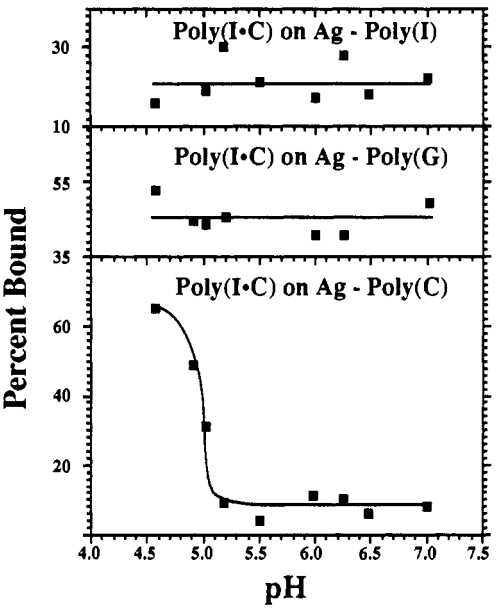

FIGURE 2: Percent of poly(I·C) bound to Ag–poly(C) (bottom), Ag–poly(G) (middle), and Ag–poly(I) (top) as a function of pH.

to Ag–poly(U) was reduced from 89% observed in the standard solvent to 0%. In the same way, poly(I·C) binding by Ag–poly(G) was reduced from 57% to 8% in 0.01 M Na^+ and to 0% in 0.006 M Na^+. This ionic strength dependence of stability is characteristic not of nucleic acid duplexes but of triplexes. Moreover, similar reduction of the ionic strength of the columns to which single strands had been bound to complementary strands covalently linked to the column matrix had no effect on the binding. Hence, it may be concluded that the observed column binding of duplexes occur by the third-strand binding mechanism.

Consistent with the foregoing conclusion, the absorption spectra of the fractions of poly(A·U) and poly(I·C) that were not bound to the columns were identical with those of the samples applied to the columns. Were binding to a column the result of displacement of a strand of double helix, the absorption spectrum of the unbound material would be expected to be altered. Another source of artifactual column binding could arise from dangling ends on the duplexes applied to the columns. However, when poly(dA·dT) and poly(dG·dC) were subjected to a blunting protocol with S1 nuclease, there was no effect on their fraction bound, providing further evidence that the binding of these duplexes does not arise by duplex formation but instead must be due to third-strand binding.

Specificity in Column Binding of Double Helices. The capacity of agarose-linked "third strands" to bind a variety of double helices that differ in composition and sequence (Table III) provides considerable insight into the factors that specify such interaction.

It is apparent that binding occurs only when the complementary strands are homo-polypurine·homo-polypyrimidine. Thus, poly(A·U) and poly(dA·dT) show the same specificity of binding, whereas poly(A-U·A-U) and poly(dA-dT·dA-dT) are not bound by any column matrix. Analogous behavior is observed for poly(I·C), poly(G·C), and poly(dG·dC) relative to poly(I-C·I-C) and poly(dG-dC·dG-dC). This requirement for asymmetric distribution of purines between the two strands of a duplex is most striking when comparing the binding of poly(dA-dG·dC-dT) to that of poly(dA-dC·dG-dT); while the former helix is bound (only) to Ag–poly(I), the latter is not bound by any column matrix.

Table III: Binding of Nucleic Acid Duplex Ligands by Agarose-Linked Polynucleotides

potential ligand	agarose-linked strand[a] poly-(A)	poly-(U)	poly-(G)	poly-(C)	poly-(I)	agarose
poly(A·U)	20[b]	89	–	–	72	–
poly(A-U·A-U)	–	–	–	–	–	–
poly(dA·dT)	85[c]	88[c]	–	–	85[c]	–
poly(dA-dT·dA-dT)	–	–	–	–	–	–
poly(I·C)	–	–	57	–[d]	19	–
poly(I-C·I-C)	–	–	–	–	–	–
poly(G·C)	–	–	43	29	32	–
poly(dG·dC)	–	–	80[c]	23[c]	35[c]	–
poly(dG-dC·dG-dC)	–	–	–	–	–	–
poly(dA-dG·dC-dT)	–	–	–	–	25	–
poly(dA-dC·dG-dT)	–	–	–	–	–	–

[a] Minus signs indicate an absence of binding; i.e., the percent of the potential ligand bound ≤7% of the amount applied to the column; numerical values indicate the percent of a ligand that is truly bound. [b] Consistent with the recently discovered intermediate poly(A) size requirement for formation of poly(A·A·U) (Broitman et al., 1987), this value was observed when the ligand contained poly(A) of dp ~ 105 but not when it contained poly(A) of dp > 220. [c] Duplex treated with S1 nuclease (blunt ends). The percent bound was within experimental error the same before and after the treatment. [d] Significant binding was observed in this case when the pH was dropped to 5 or below (see Figure 2).

Table IV: Third-Strand Binding Code

		third-strand residues A	U/T	I	G	C
Watson–Crick core polypurine strand residues	A	+	+	+	–	–
	G	–	–	+	+	+

Another generalization to be drawn from the data in Table III is that recognition of double helices is not qualitatively affected by the nature of the helix backbone and depends instead on the purine residues of the helix base pairs. Thus, poly(A·U) with its ribose phosphate backbone and poly(dA·dT) with its deoxyribose phosphate backbone are recognized by the same three third-strand residues, A, U/T, and I, whereas poly(I·C) and poly(dG·dC) are recognized by another set of three third-strand residues, in this case G, C, and I. At once, several features of third-strand recognition become apparent: there is degeneracy in the recognition of both A·T/U and G/I·C base pairs; yet, they can be distinguished by third-strand residues; and I in the third-strand can recognize any all purine·all pyrimidine double-helical segment.

The third-strand binding code indicated by these qualitative features is summarized in Table IV (Fresco et al., 1989). The specificity of this code is worthy of illustration in terms of the data presented in Table II. For example, both poly(A·U) and poly(dA·dT) are bound by Ag–poly(A), Ag–poly(U), and Ag–poly(I) but not by Ag–poly(G) and Ag–poly(C), whereas poly(A-U·A-U) and poly(dA-dT·dA-dT) are bound by none of the third-strand-linked column matrices. Poly(I·C), poly(G·C), and poly(dG·dC) show the reverse binding pattern, except with Ag–poly(I), and in this case, as well, the alternating-sequence double helices poly(I-C·I-C) and poly(dG-dC·dG-dC) are not bound by any of the column matrices. Lastly, the homo-purine·homo-pyrimidine alternating sequence poly(dA-dG·dC-dT) is bound only by Ag–poly(I), which recognizes both A·T and G·C pairs, but not by any column that recognizes only one of these pairs. There is, then, considerable potential for achieving great specificity in recognizing complex homo-purine·homo-pyrimidine sequences by third-strand binding.

Effect of pH on Binding. The effect of pH on the fraction of helix bound to a column is of interest because such data can indicate whether protonation of bases is involved in base triplet formation between a third-strand residue and the purine residue of a core helix base pair. As noted from the data in Figure 2 (see below), the binding of poly(I·C) by Ag–poly(C) is significantly enhanced on the acid side of neutrality. In fact, Job titration experiments in the same Mg^{2+}-containing solvent used in the present work have shown that the triplexes poly(C·G·C) and poly(C·I·C) do form at neutrality at 4 °C, suggesting the C residues in such triplets are protonated at neutrality (Fresco et al., 1989; Perlmutter & Fresco, 1989). Those results are consistent with the observation in the present work that Ag–poly(C) does bind poly(dG·dC) significantly at pH 7.

Besides C·G·C, several other triplets were modeled for potential stabilization by protonation (Figure 1). These include I·G·C, I·I·C, and G·G·C, for which hydrogen bonding with the third-strand purine residue in the syn orientation would require protonation at N(7) of I or G, whereas in the anti orientation it would not (Figure 1). Figure 2 shows the percent of poly(I·C) bound on Ag–poly(C), Ag–poly(I), and Ag–poly(G) between pH 7.0 and pH 4.6. It can be seen that the binding to Ag–poly(G) and Ag–poly(I) is invariant over this broad pH range, while that bound to Ag–poly(C) is distinctly enhanced as the pH drops below 5.2 (and the intrinsic pK of 4.3 for the C residue is approached). Thus, it would appear that protonation is required for base triplet formation only in the case of C^+·I/G·C. The hydrogen-bonding schemes for the other triplets in the top row of Figure 1 are therefore eliminated.

Quantitative Aspects of the Binding. It is evident from the data in Table III that there are distinct differences in the extent of binding among duplex ligands under the standard experimental conditions employed. Whereas values ≤ 7% are considered to indicate no binding (within experimental error), the positive values vary between 11% and 100%. Hence, the binding turns out not to be all or none. In addition, as expected (Thiele et al., 1971; Arnott et al., 1976), the pH variable was seen to affect the formation of Ag–poly(C·I·C), but neither Ag–poly(I·I·C) nor Ag–poly(G·I·C) (Figure 2); and the Na^+ concentration variable in the absence of Mg^{2+} was seen to affect the fraction of poly(A·U) and poly(I·C) bound to appropriate columns by specific third-strand binding. It is also noted that there is a positive correlation between the fraction bound and T_m for the dissociation of third strands from triplexes in those cases where this parameter has been measured. For example, T_m for the dissociation of the third strand is highest for poly(G·G·C), lower for poly(G·I·C), and lowest for poly(I·I·C) (Perlmutter & Fresco, 1989).

Nevertheless, it must be recognized that by utilizing third strands covalently linked to an insoluble matrix the possibility of observing third-strand binding is substantially enhanced. This is probably because the binding of a duplex ligand leads to its being transferred from the "free solvent" phase to the insoluble matrix phase. Therefore, the fraction bound must reflect the probability of being bound by a third strand once an encounter occurs, i.e., the binding affinity.

Discussion

It is likely that the present results demonstrate the full spectrum of base triplets possible from the interaction of the residues ubiquitous in RNA and the base pairs in DNA and RNA duplexes, i.e., A·A·T/U, U·A·T/U, G·G·C, and C^+·G·C. In addition, they reveal two triplets, I·A·U/T and I·G·C, with a third-strand residue not generally present in RNA. Hence, while it is unlikely that those triplets have any biological

relevance, they could have practical utility.

The observation of the latter two triplets and also G·G·C illustrates a significant advantage of the experimental system employed, which is that it allows formation of certain homopolymer triplexes that would not normally form in free solution at neutrality as the complexes of lowest energy because poly(I) and poly(G) form highly competitive multistranded helices.[2] Such self-structures cannot form when poly(G) and poly(I) strands of modest length are bound to agarose because they are then too far apart to interact with one another. As a consequence, the method also allows the determination of those of the conceivable triplets that cannot form; such data were not previously available.

The notion of a requirement for a purine residue cluster within a strand of a duplex target for third-strand binding is strongly supported by the current findings. A high degree of specificity of third-strand binding is also apparent; i.e., the binding code is well delineated. However, because of the relative simplicity of the sequences of the duplex ligands studied, the present experiments do not indicate how complex or unique the polypurine strand of the duplex can be and what frequency of occurrence and distance between pyrimidines in that sequence can be tolerated before third-strand binding is inhibited. Similarly, because the affinity strands on the agarose are homopolymers, it is not apparent whether both purines and pyrimidines can be tolerated within the same third strand and what sequences, if any, may reduce the specificity of the residues in a third strand. Some of these issues are addressed experimentally elsewhere (Fresco et al., 1989). On the other hand, the present results emphasize the potential value of oligo(I) as a nonspecific third strand for any target sequence. The results also clearly show that all combinations of third-strand backbone and target duplex backbone are compatible for all triplets that can mediate third-strand binding.

In sum, the potential for specific binding of third strands to segments of nucleic acid duplexes indicated by the present work suggests the possibility that such interaction could be exploited to modulate the expression of various genetic functions.

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[2] The occurrence of the triplets I·G·C and I·A·U has also been demonstrated by Job titrations of homopolymers with certain copolymers (Lomant & Fresco, 1975; Perlmutter & Fresco, 1989).

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