The Influence of Chain Length and Base Composition on the Specific Association of Oligoribonucleotides with Denatured Deoxyribonucleic Acid*

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SUMMARY

RNA made in vitro on a DNA (from T2, T3, T4, or T7 phage) template was subjected to a limited digestion with ribonuclease T1 and fractionated according to chain length. The oligonucleotides of various chain lengths were annealed at various temperatures to denatured DNAs immobilized on membrane filters. In the absence of Mg++, a chain length of 10 or more ribonucleotides is required to form a ribonuclease-resistant complex with denatured DNA. In the presence of Mg++, oligonucleotides as small as octanucleotides can form a detectable complex with denatured DNA. The shortest oligonucleotides capable of forming a complex with denatured DNA do so with a high degree of specificity for species; oligonucleotides produced from T-even phage DNAs will form a complex only with T-even DNAs, not with T-odd DNAs, and vice versa. The expression of heterology between the T-even and T-odd phage DNAs seems to occur on passing from a chain length of 9 to 10. As expected, the temperatures for optimum complex formation and the melting temperatures of the oligoribonucleotide-denatured DNA complexes increase with the chain length of the oligonucleotides. When chain length is constant, these temperatures are considerably higher for the oligonucleotides produced from T-odd phage DNAs than for those produced from T-even ones, suggesting that the former oligonucleotides, having higher guanine + cytosine contents, form complexes that are considerably more stable than those formed by the latter.

Several methods are available for the detection of polynucleotide complexes, e.g., changes in optical properties or density in CsCl gradients (1), association of labeled polynucleotides with complementary polynucleotides retained on agar columns (2) or nitrocellulose filters (3, 4), and fractionation in CsCl density gradients (5). There are two convenient ways to test the stability and, presumably, the specificity of such complexes: (a) stability to ribonuclease (4), a method suitable only for RNA-RNA and RNA-DNA complexes; and (b) stability to increased temperature (6, 7), a method suitable for RNA-RNA, RNA-DNA, and DNA-DNA complexes.

Some of the controlling factors believed to be important in polynucleotide interactions are: (a) the number of the interacting nucleotides (7-11); (b) the base composition of the interacting nucleotides (6, 9, 12); (c) the presence or absence of adjacent occupancy, that is, the degree of proximity of the oligonucleotides interacting with the complementary polynucleotide (13); and (d) the number, type, and location of mismatched (noncomplementary) bases (14, 15).

A knowledge of the number of interacting nucleotides is among the first requirements for an understanding of the question of homology or degree of complementarity between two polynucleotide chains. This paper describes experiments designed to determine the minimum number of nucleotides necessary to form a specific, stable complex between the interacting polynucleotides. These experiments also show an effect of base composition on the stability of such complexes.

RNA made in vitro on a DNA (T2, T3, T4, or T7) template was subjected to a limited digestion with ribonuclease T1 and fractionated according to chain length. The oligonucleotides of various chain lengths were then annealed at various temperatures to denatured DNAs immobilized on membrane filters. The results indicate that a chain length of 10 or more ribonucleotides is required to form a RNase-resistant complex with denatured DNA, even under optimum conditions. The shortest oligoribonucleotides capable of forming complexes with denatured DNA do so with a high degree of specificity for species; oligoribonucleotides produced with T-even phage DNAs will form complexes only with T-even DNAs, not with T-odd DNAs.

* This research was sponsored by the United States Atomic Energy Commission under contract with the Union Carbide Corporation.
Fig. 1. The separation according to chain length of oligonucleotides obtained from a partial ribonuclease T1 digestion of 3H-T2 RNA on a DEAE-Sephadex (A-25) column. The flow rate was 15 ml per hour. Fractions of 2 ml each were collected and 0.20-ml aliquots taken for counting. See "Experimental Procedure" for details.

### EXPERIMENTAL PROCEDURE

**Materials**—Unlabeled and 3H-labeled ATP, UTP, CTP, and GTP were purchased from Schwarz BioResearch. The four ribonucleoside triphosphates were adjusted to the same specific activity (1.8 x 10^7 cpm per pmole of nucleotide) for the synthesis in vitro of 3H-RNA.

*Escherichia coli* alkaline phosphatase was obtained from Worthington Biochemical Corporation (Freehold, New Jersey). Pancreatic RNase was also obtained from Worthington and freed of traces of DNase activity by heating a solution in 0.2 M NaCl at pH 5.0 to 90° for 10 min (4). RNase T1 was obtained from Calbiochem. Hydroxylapatite was prepared according to a modification (12) of the method of Tiselius, Hjertén, and Levin (17).

**Preparation of 3H-Oligonucleotides**—The growth and isolation of T2, T3, T4, and T7 phages and the isolation of their DNAs by phenol extraction were performed according to the method of Thomas and Abelson (18). The residual phenol was removed by repeated dialysis in the cold against 0.2 M NaCl at pH 5.0 to 90° for 10 min (4). RNase T1 was obtained from Calbiochem. Hydroxylapatite was prepared according to a modification (12) of the method of Tiselius, Hjertén, and Levin (17).

The oligonucleotides obtained from the DEAE-Sephadex (A-25) column can be desalted on small DEAE-Sephadex A-25 columns. Oligonucleotides longer than tetramers can be satisfactorily desalted on small hydroxylapatite columns. After adsorption, the column was washed with 0.15 M NaCl-0.01 M Tris, pH 7.5, to remove the urea. The oligonucleotide was then quantitatively eluted in a small volume of either 0.10 M sodium phosphate buffer, pH 6.8, or 5-fold concentrated SSC.2

2 The abbreviation used is: SSC, 0.15 M NaCl-0.015 M sodium citrate, pH 7.1.
Characterization of Oligonucleotides—The oligonucleotide chain length was identified by its elution position and confirmed by analysis (after phosphatase treatment) of the terminal nucleoside (guanosine) and the nucleotides released by alkaline hydrolysis. The average base compositions of the oligonucleotides were obtained by alkaline hydrolysis (after phosphatase treatment) and analysis of the nucleotides and nucleoside (guanosine) by paper chromatography. An aliquot of the sample (after desalting) was treated with E. coli alkaline phosphatase to remove the terminal 5'-phosphate. The solution was incubated in 0.3 M KOH for 18 hours at 37°. After neutralization with 1 N HClO4, the precipitated KClO4 was removed by centrifugation. The nucleotides and nucleoside (guanosine) in the supernatant fraction were separated by a two-step paper chromatographic procedure using first n-propyl alcohol-concentrated NH4OH-H2O (60:30:10, v/v/v) and then isobutyric acid-concentrated NH4 OH-H2O (66:1:33, v/v/v) in the same direction. Each separated spot was cut into small pieces, eluted by shaking with small volumes of water in scintillation vials which were filled with dioxane scintillator fluid (containing 5 g of 2,5-diphenyl oxazole, 50 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 130 g of naphthalene dissolved in a liter of dioxane), and counted in a Packard Tri-Carb liquid scintillation spectrometer.

**Annexing** The annexing of 3H-labeled oligoribonucleotides or RNA to denatured DNA fixed on membrane filters (Schleicher and Schuell, B 6) and the removal of background “RNA noise” by ribonuclease treatment were performed according to the method of Gillespie and Spiegelman (4), with some modifications. The blank filters and the DNA filters were subjected to the same washing procedure, drying at room temperature for 4 hours, and baking at 80° in a vacuum oven for 2 hours. This procedure consistently led to a reduction of the nonspecific association of the oligonucleotides or RNA to within 0.01% of the input. Similar observations have been made by Rigsby and Merrim (22). The filters were incubated (accompanied by stirring to attain equilibrium faster) in the oligonucleotide or RNA solution in 5-fold concentrated SSC (at indicated temperatures) for a period of at least 20 hours, then chilled in ice for at least 2 hours. The filters were then washed (22) by swishing them in large volumes of 5-fold concentrated SSC in successive beakers, thus omitting the filtration washing procedure of Gillespie and Spiegelman (4). The washed filters were treated with 20 μg per ml of RNase in 2-fold concentrated SSC at room temperature for 1 hour, chilled in ice, again washed in 5-fold concentrated SSC, dried, and counted.

**Thermal Chromatography**—The stability of the oligonucleotide-DNA complexes was determined by measuring the temperature of melting of the oligonucleotide from the DNA filters. Membrane filters containing each oligoribonucleotide-DNA complex were dried and inserted into a small jacketed column and washed with 2-fold concentrated SSC at increasing temperature. As the temperature is gradually raised, each oligoribonucleotide-DNA complex melts, thus releasing from the column the labeled oligonucleotide which is then assayed. The DNA is retained on the filter at all temperatures of the melting experiment. When the amount of oligonucleotide eluted per degree rise in temperature is plotted against temperature, it results in a thermal elution profile of the oligonucleotide. Such plots, called thermal chromatograms, have been described in detail previously (7, 12).

**RESULTS**

A typical separation according to chain length of oligonucleotides obtained from a partial T1 RNase digestion of 3H-T2 RNA is shown in Fig. 1. The oligonucleotides from the other RNAs gave similar results. The actual chain lengths determined for octamers to hexadecamers from T2 RNA were 8.1, 9.0, 10.2, 11.0, 11.9, 13.1, 14.3, 15.2, and 16.3, respectively. The oligonucleotides from the other RNAs gave similar satisfactory results. All the oligonucleotides had a guanine residue at the 3'-end. Oligonucleotides longer than undecamers usually contained on the average at least one internal guanine residue.

The ratio of (A + U):(G + C) of octamers to hexadecamers from T2- and T4-produced RNAs was in the range of 1.80 to 1.94, whereas that from T3- and T7-produced RNAs ranged from 0.89 to 1.13. The above oligonucleotides were obtained by partial T1 RNase digestion. Rushizky et al. (23) have also noted that the larger (n = 10 and longer) oligonucleotides obtained from a partial T1 RNase digestion of phage MS2 RNA have base ratios identical with those of the parent molecule.

Fig. 2 shows the results of annealing of oligonucleotides of various chain length (octamers to hexadecamers) from 3H-T2 RNA to 14 μg of denatured T2 DNA after a 20-hour incubation period at various temperatures. The amounts of input oligonucleotide (octamers to hexadecamers) were 5.6, 5.0, 8.0, 4.0, 5.0, 5.6, 3.1, 4.2, and 2.1 μg, respectively, each in 4 ml of 5-fold concentrated SSC. Separate experiments indicated that the amount

![Fig. 2](http://www.jbc.org/)
of oligonucleotide complexed is strictly proportional to the concentration of input oligonucleotide at the low oligonucleotide to DNA ratios used. The results are plotted as counts per minute fixed per constant amount (10 μg) of oligonucleotide input to indicate the efficiency of complex formation as a function of chain length. As can be seen (Fig. 2, inset), decamers form a detectable complex, whereas undecamers bind 3.5-fold better. The efficiency of complex formation and the optimum annealing temperature increase with the chain length. The optimum temperature of annealing for deca- and undecamers is 25° and shifts to 30° for dodeca- and tridecamers, 35° for tetradecamers, and about 40° for pentadeca- and hexadecamers.

Oligonucleotides from T4 RNA gave results very similar to those described above for oligonucleotides from T2 RNA. Here also a substantial enhancement of complex formation is observed on passing from decamers to trideca- and tetradecamers.

A previous publication (9) described the results of annealing of T7 RNA oligonucleotides to denatured T7 DNA. A detectable complex is formed by decamers with a 4-fold improvement upon passing to undecamers; the optimum temperature in both cases is 35°. Upon passing to dodecamers, an almost 60-fold enhancement (over undecamers) in complex formation is observed. The efficiency of complex formation increases with increasing chain length. The temperature of optimum complex formation also shifts from 35° for decamers and undecamers to a broad optimum at 45 to 55° for dodecamers and tridecamers and 50 and 55° for tetradeca- to hexadecamers. The temperature of optimum complex formation for the T-odd RNAs is about 70°, whereas that for the T-even RNAs is about 65° in 5-fold concentrated SSC.

Oligonucleotides from T3 RNA gave results very similar to those described above for oligonucleotides from T7 RNA. In this case again, a dramatic shift in the extent and optimum temperature of complex formation is observed on passing from undecamers to tetradecamers. The optimum annealing temperatures for T-odd oligonucleotides are substantially higher than those for T-even oligonucleotides of the same chain length.

Oligonucleotides smaller than decamers obtained from T2, T3, T4, or T7 RNA failed to anneal to any DNA at any temperature (0 65°), amount of oligonucleotide (1 to 20 μg), DNA loading (up to 100 μg), incubation time (up to 120 hours), or ionic strength (up to 20-fold concentrated SSC) that was tested. In other experiments, the incubation and washings were done at 4° and the RNase step was omitted. This resulted in an increase in 3H on both blank and DNA-loaded filters, but no net increase in the DNA-loaded filters could be detected. However, oligonucleotides smaller than decamers could be made to anneal to DNA in the presence of MgCl₂. The solvent used was 0.3 M NaCl, 0.01 M Tris- (pH 7.2), 0.01 M MgCl₂, DNA loading, incubation of the filters, RNase treatment, and washings were all done in this solvent for optimum binding of the oligonucleotides. Unfortunately, high backgrounds are obtained because the RNase step seems to be inhibited by Mg²⁺ ion. Omitting Mg²⁺ or lowering its concentration to 0.005 M during the RNase step led to either complete or partial dissociation of the oligonucleotides from the DNA filters. The results of annealing of hepta-, octa-, nona-, and decamers from 3H-T7 RNA to T7 and T2 DNAs are shown in Table I. Incubation was done at 30° for 20 hours. A detectable complex is formed by octamers with

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**Table I**

<table>
<thead>
<tr>
<th>T7-directed oligonucleotide</th>
<th>Blank</th>
<th>T7 DNA</th>
<th>T7 DNA blank</th>
<th>T2 DNA blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heptamers</td>
<td>134</td>
<td>136</td>
<td>139</td>
<td>2</td>
</tr>
<tr>
<td>Octamers</td>
<td>177</td>
<td>222</td>
<td>198</td>
<td>45</td>
</tr>
<tr>
<td>Nonamers</td>
<td>127</td>
<td>317</td>
<td>151</td>
<td>100</td>
</tr>
<tr>
<td>Decamers</td>
<td>159</td>
<td>482</td>
<td>166</td>
<td>323</td>
</tr>
</tbody>
</table>

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* S. K. Niyogi, unpublished experiments.
Oligonucleotides with chain lengths of 10 and longer will combine only with the template DNA or with the DNA of a related species. This is shown in Figs. 3 and 4. The shortest T2 oligonucleotide that is capable of forming a complex does so specifically with T2 or T4 DNA, not with T3 or T7 DNA (Fig. 3). Similarly, T7 oligonucleotides will form complexes with T7 or T3 DNA, not with T2 or T4 DNA (Fig. 4). Homology at the polynucleotide level is known (24) to be considerably greater between

The results of the thermal chromatograms of T7 and T2 oligonucleotides annealed to denatured T7 DNA on membrane filters are shown in Fig. 5. T2 oligonucleotides gave thermal chromatograms with similar elution profiles. The melting temperatures increase with increasing chain length of the oligonucleotides. However, T7 oligonucleotides melt at substantially higher temperatures than T2 oligonucleotides of the same chain length.

Table II

<table>
<thead>
<tr>
<th>Oligonucleotide chain length</th>
<th>Melting temperature</th>
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<tbody>
<tr>
<td></td>
<td>T2</td>
</tr>
<tr>
<td>12</td>
<td></td>
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<tr>
<td>13</td>
<td>40°</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
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<tr>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
</tr>
<tr>
<td>17</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>81</td>
</tr>
</tbody>
</table>

The solvent used was 2-fold concentrated SSC. The thermal chromatograms were performed as described in the text and in previous publications (7, 12). The ratio (A + U)/(G + C) of the oligonucleotides ranged from 1.80 to 1.94 for T2 and 0.89 to 1.13 for T7. The T2 oligonucleotides were bound to denatured T2 DNA, whereas the T7 oligonucleotides were bound to denatured T7 DNA.
T3 and T4 oligonucleotides gave results very similar to those for T7 and T2 oligonucleotides, respectively.

DISCUSSION

The present studies indicate that under conditions used by investigators in testing interspecies homology by RNA-DNA hybridization (2, 4, 25), a minimum of about 12 perfectly complementary nucleotides must be involved in the complex. This number is apt to vary somewhat with the G + C content of the interacting nucleotides; longer chain lengths should be required for oligonucleotides of lower G + C content.

The minimum number of nucleotides necessary to form a detectable complex with denatured DNA in the absence of Mg++ ion appears to be 10. In the presence of 0.01 M MgCl₂, oligonucleotides as small as octamers can form a detectable complex with denatured DNA. Gillespie and Spiegelman have done similar experiments, but their estimate of the minimum length required for stability is substantially larger. This apparent discrepancy may be partly due to the lower ionic strength of the solvent (2-fold concentrated SSC) in which they carried out their hybridizations as compared to 5-fold concentrated SSC used in the present investigations. The minimum length required should be greater at lower ionic strengths. However, the difference in ionic strengths does not seem sufficient to explain the difference in the results. In testing the binding of oligonucleotides from T4 RNA to denatured T4 DNA, Rüger and Bautz (11) reported detectable complexes starting from about dodecamers. Their inability to detect complexes with shorter chain lengths may be due to the very low inputs used, since we find that at the lower chain lengths the efficiency of complex formation is critically dependent on the concentration of oligonucleotide input. Testing oligodeoxyribonucleotides for their ability to bind to denatured DNA, McCouaughy and McCarthy (10) have also reported detectable complexes with minimum chain lengths of 10 and 14. However, in view of the absence of resolution of their oligonucleotides longer than heptamers, the above numbers may be subject to some degree of uncertainty. Moreover, considerable mismatching of the bases is possible because of the nonapplicability of the tool of RNase H to DNA-DNA complexes.

The shortest oligonucleotides capable of forming a detectable complex with denatured DNA do so with a high degree of species specificity (Figs. 3 and 4). The results presented in this paper indicate that the T-even oligonucleotides show a greater degree of homology than the T-odd oligonucleotides. As shown in Table 1, the expression of heterology between the T-even and T-odd DNAs seems to arise on passing from a chain length of 9 to 10.

As expected, the temperatures for optimum complex formation and the melting temperatures increase with the chain length of the oligonucleotide. However, these temperatures for the T-odd oligonucleotides are considerably higher than those for the T-even ones of the same size. This is apparently a reflection of the higher G + C contents of the T-odd oligonucleotides and accounts for the discrepancy observed by Rüger and Bautz (11) when comparing the optimum incubation temperatures for their T4 oligonucleotides with those for T7 oligonucleotides reported by Niyogi and Thomas (9).

In the case of the T-odd oligonucleotides, upon passing from undecamers to dodecamers there is a great enhancement of complex formation (9). A similar shift in the extent of complex formation is observed with the T-even oligonucleotides (Fig. 2); however, this shift occurs on passing from dodecamers to tridecamers and tetradecamers. This shift in stability at a longer chain length for the T-even oligonucleotides is probably due to the lower G + C content of these oligonucleotides as compared to the T-odd ones.

It is rather curious that oligonucleotides shorter than octamers fail to anneal to denatured DNA even under optimum conditions. It should be recalled that the situation is considerably improved in homopolymer interactions, for example, oligonucleotides as short as pApA can form complexes with polyuridylic acid (7, 8). It is likely that the higher stability of oligoadenylate acid-polyuridylic acid complexes is partly due to "stacking" or the presence of neighboring oligoadenylate acids in adjacent positions, since the thermal stability of RNA-RNA complexes is only slightly greater than RNA-DNA complexes (20). Such adjacent occupancy would not be expected in the RNA oligonucleotides bound to DNA, since each oligonucleotide would be combined with its own (unique) complementary sequence. The formation of three-stranded complexes [(A)n . (U), . (U),] also possibly stabilizes the complexes between oligoadenylate acids and polyuridylic acid. Such three-stranded complexes are not formed between denatured DNA and RNA oligonucleotides.

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