THE SPECIFIC ASSOCIATION OF RIBOOLIGONUCLEOTIDES OF KNOWN CHAIN LENGTH WITH DENATURED DNA *

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The interaction of polynucleotides is now understood to be one of the most fundamental in biology. It is through this interaction that genetic information flows to succeeding generations by replication and recombination, and from DNA to protein by the steps of transcription and translation. Therefore, this subject has been studied by many workers.

In general, the specific union of polynucleotides can be detected by a change in optical properties or density in CsCl gradients (Marmur and Doty, 1961) or by the association of labeled polynucleotides to complementary polynucleotide chains that are retained on agar columns (Bolton and McCarthy, 1962), nitrocellulose filters (Gillespie and Spiegelman, 1965) or fractionated in CsCl density gradients (Hall and Spiegelman, 1961). The character of the polynucleotide complex has been probed by testing its stability to increased temperature (Bolton and McCarthy, 1964) or by testing its stability to nucleases (Gillespie and Spiegelman, 1965). While the available assays for complex formation and complex quality are not ideal in all respects, some controlling factors have been interpreted directly or indirectly. These include:

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1. The number of interacting nucleotides (Lipsett et al., 1961).

2. The composition of the interacting nucleotides (Bolton and McCarthy, 1964; Miyazawa and Thomas, 1965).

3. The presence or absence of adjacent occupancy (Naylor and Gilham, 1966).

4. The number, type and location of mismatched pairs (Bautz and Bautz, 1964).

In any theoretical or experimental approach to the question of homology or degree of complementarity between polynucleotide chains, the number of interacting nucleotides is among the first variables that must be defined. In the present communication we describe experiments bearing on the question of the minimum number of nucleotides required to form a specific, stable complex. RNA made in vitro, on T4 or T7 DNA template was degraded with T1 ribonuclease and fractionated with respect to chain length. The oligomers of various lengths were annealed to denatured T4 and T7 DNA that was immobilized on membrane filters. It was found that only oligonucleotides containing 10 or more nucleotides would form a RNAase resistant complex, even under optimum conditions. The shortest oligomers that were capable of complexing with denatured DNA, did so with a high degree of species specificity.

EXPERIMENTAL

Materials:

Unlabeled and ^{3}H-labeled ATP, UTP, CTP and GTP were purchased from Schwarz BioResearch, Inc. The four ribonucleoside triphosphates were adjusted to the same specific activity for making ^{3}H - RNA in vitro.

Ribonuclease T1 was obtained from California Biochemical Corporation. Pancreatic ribonuclease (Worthington) was freed from traces of deoxyribonuclease activity by heating a solution in 0.2 M NaCl at pH 5.0 to 90°C for 10 minutes (Gillespie and Spiegelman, 1965). Hydroxyapatite was prepared according to a modification by Miyazawa and Thomas (1965) of the method of Tiselius et al. (1956).

Methods:

The growth and isolation of T_4 and T7 phages and the extraction of their DNA's by mild phenol extraction were done according to Thomas and Abelson (1966). The excess phenol was removed by repeated dialysis in the cold against 0.02 M Tris, pH 7.8, containing 0.01 M NaCl. The DNA was denatured by adjusting the solution to pH 13.0, incubating at room temperature for 10 minutes, then adding a small volume of appropriately acidified buffer. RNA polymerase was prepared from E. coli B according to the procedure of Stevens and Henry (1964).

^3H - RNA was made in vitro using E. coli RNA polymerase and T_4 or T7 DNA as a template, according to Stevens and Henry (1964). After a 15-minute incubation period, the reaction mixture was deproteinized by phenol extraction. The ^3H - RNA was freed from unlabeled DNA on a hydroxyapatite column. After adsorption, the column was first washed with 0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 6.8, to remove the excess phenol. The ^3H - RNA was quantitatively eluted with 0.1 M NaCl, 0.20 M sodium phosphate buffer, pH 6.8. Under these conditions, native DNA is not released from the column.

The ^3H - RNA was dialyzed in the cold against 0.05 M Tris, pH 7.5. The solution was then made up to 0.20 M Tris, pH 7.5, 0.005 M EDTA[1] and incubated with ribonuclease T1 (250 units/mg. RNA) for two hours at 0°C, then deproteinized with phenol and extracted several times with ether. The last traces of ether were removed by blowing a gentle stream of air through the solution at room temperature.

Oligonucleotides thus obtained were separated according to chain length on a DEAE-Sephadex (A25) column using a linear NaCl gradient in 0.02 M Tris, pH 7.4, and 7 M urea (Rushizky et al., 1964; Rushizky et al., 1965). Chain

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[1] The abbreviations used in this paper are EDTA, ethylene diaminotetraacetic acid; SSC, 0.15 M NaCl - 0.015 M Na-citrate, pH 7.1. Oligonucleotides of different chain lengths, for example, decanucleotides, undecanucleotides, and dodecanucleotides are denoted by 10-mers, 11-mers, and 12-mers, respectively.

lengths from 1 to 11 could be separated quite well using a linear gradient between 0.15 M NaCl and 0.45 M NaCl. Higher members were eluted from the column with 1 M NaCl and rechromatographed on another column using a linear gradient between 0.35 M NaCl and 0.60 M NaCl; chain lengths from 12 to 16 could be resolved by this procedure. The oligomer chain length was identified by elution position and confirmed by analysis of the terminal nucleoside after phosphatase treatment.

The oligonucleotides thus obtained can be desalted on small DEAE-Sephadex columns. Oligonucleotides longer than 4-mers were satisfactorily desalted on hydroxyapatite columns. After adsorption, the hydroxyapatite column was washed with 0.15 M NaCl, 0.01 M Tris, pH 7.4, to remove the urea. Quantitative elution was then done using a small volume of 5 x SSC.

The hybridization of ^{3}H-labeled oligonucleotides 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 done according to the procedure of Gillespie and Spiegelman (1965). The non-specific association of oligomers could be reduced to within 0.01% of the input. The incubation of the filter in the oligonucleotide or RNA solution in 5 x SSC was done (at indicated temperatures) for a period of 6 hours, then chilled in ice for 15-20 hours. The filters were washed with 5 x SSC and treated with 20 µg/ml RNAase in 2 x SSC at room temperature (Gillespie and Spiegelman, 1965).

RESULTS AND DISCUSSION

Figure 1 shows the net RNAase resistant radioactivity fixed to membrane filters bearing 10 µg of denatured T7 DNA after a 6 hour exposure at various temperatures to ^{3}H - labeled T7 ribooligomers of various chain lengths. The input ^{3}H - labeled oligomer was present at relatively low concentrations (10, 8, 14, 11 µg in 4 ml 5 x SSC for the 8, 9, 10 and 11-mers respectively and 4.1, 4.0, 4.4, 5.8 and 4.5 µg in 3 ml 5 x SSC for the 12, 13, 14, 15 and 16-mers). Less than 9% of the input is complexed even in the most favorable case shown

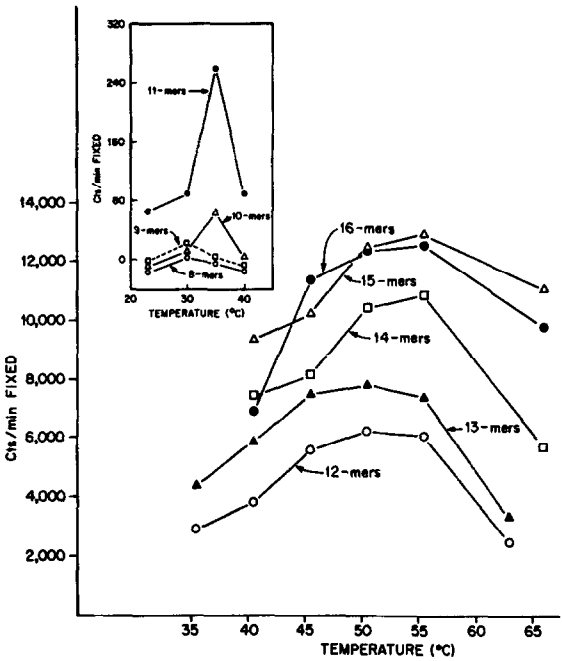

Figure 1. Annealing of oligonucleotides (8-mers to 16-mers) from ^{3}H - T7 RNA to denatured T7 DNA at different temperatures. Each point represents the RNAase-resistant counts per minute fixed to one filter containing 10 µg of denatured T7 DNA minus the counts per minute fixed to one filter containing no DNA. The amounts of 8-mers to 11-mers were 10, 8, 14 and 11 µg, respectively, in a volume of 4 ml each of 5 x SSC. The amounts of 12-mers to 16-mers were 4.1, 4.0, 4.4, 5.8 and 4.5 µg, respectively, in a volume of 3 ml each of 5 x SCC. The specific activity of the oligonucleotides was 4.0×10^{4} counts per minute/µg. The ratio $\frac{A + U}{G + C}$ of these oligomers was in the range of 0.89 to 1.13.

here, and less that 3.3% of the membrane-bound DNA would be complexed under these conditions. Separate experiments indicate that the amount of oligomer complexed is strictly proportional to the concentration of input oligomer.

The temperature of optimum complex formation for oligomers of length 8 to 11 can be seen in Figure 1 (inset). A detectable complex can be seen with 10-mers and a 3-fold improvement with 11-mers. Upon passing to 12-mers a 25-fold enhancement is observed. When this is normalized for the 2-fold difference in the concentration of the oligomer input, the difference is nearly 50-fold. The temperature for optimum complex formation now shifts from 35°C for 10 and 11-mers to a broad maximum at 45 to 55°C for 12 and 13-mers and 50 to 55°C for

14 to 16-mers. This shift in the extent and optimum temperature of complex formation has been observed repeatedly on passing from 11-mers to 12-mers. Results similar to those shown in Figure 1 have been obtained for T4 RNA and DNA.

Oligonucleotides smaller than 9-mers obtained from T4 or T7 RNA failed to anneal to either DNA at any temperature (0° to 65°C), oligonucleotide concentration (1 - 20 µg), DNA loading (up to 100 µg) or incubation time (up to 120 hours) that was tested. In other experiments, incubation and washing were performed at 4°C and the RNAase treatment omitted. This resulted in an increase in ^3H on both experimental and control filters, but no net increase on those filters bearing DNA could be detected. However, oligomers of length 10 will complex, but only with the homologous DNA. This is shown in Figure 2. Here it can be seen that the shortest T7 ribo-oligomer that is able to form a complex does so specifically to T7 DNA, not to T4 DNA. Analogous results were obtained with T4 ribo-oligomers.

These results mean that in experiments testing interspecies homology by

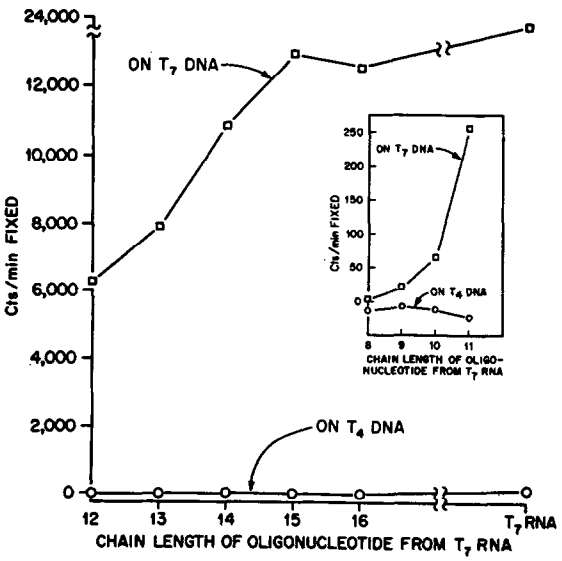

Figure 2. Annealing of ^3H - T7 RNA and oligonucleotides (8-mers to 16-mers) from ^3H - T7 RNA to denatured T7 DNA as compared to denatured T4 DNA. The incubations were done at the optimal temperature for each oligonucleotide (see Figure 1). The amounts of denatured DNA were 10 and 25 µg for T7 and T4, respectively. Other conditions were similar to those described under Figure 1.

RNA-DNA hybridization (Bolton and McCarthy, 1962; Green, 1963; Gillespie and Spiegelman, 1965), about 12 perfectly complementary nucleotides must be involved in the complex. Gillespie and Spiegelman (1966) have done similar experiments leading to similar conclusions although their estimate of the minimum length required for stability is substantially larger. This apparent discrepancy may possibly be due to an ionic strength effect. Gillespie and Spiegelman (personal communication) carried out their hybridizations in 2 x SSC, whereas we have done ours in 5 x SSC. The minimum length required should be greater at lower ionic strengths.

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