Ligation with T4 RNA ligase of an oligodeoxyribonucleotide to covalently-linked cross-sectional base-pair analogues of short, normal, and long dimensions

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ABSTRACT

Compounds that are covalent analogues of nucleic acid base pairs of normal, long, and short C1' to C1' dimensions [B. Devadas and N. J. Leonard (1990) *J. Am. Chem. Soc.*, 112, 3125–3135.] have been added to the oligodeoxyribonucleotide d(A)₆ with bacteriophage T4 RNA ligase as a prelude to placing them at defined loci within nucleic acid duplexes. Analogue cross sections that represent a normal Watson-Crick base pair as well as a pyrimidine-pyrimidine and a purine-purine apposition were ligated in modest yields (~ 20%) to the oligonucleotide. Ligation conditions were optimized for each analogue, and the cross section was joined to only a single oligonucleotide in each case. The structures of the ligated products were proved by HPLC, enzymatic degradation, and spectroscopic analyses.

INTRODUCTION

We have described recently the synthesis of 'cross-sectional' covalent compounds related to nucleic acid double helix components (1–3). These compounds comprise deoxyribonucleoside and ribonucleoside pairs that are held together by covalent bonds rather than by the hydrogen-bonding interactions that pair single strands of DNA, RNA, or DNA and RNA with one another in nature. The concept of covalently-linked cross sections involves a formal pinching (or cinching) of the normal eight-membered, doubly-hydrogen-bonded ring of a Watson-Crick pair to form two five-membered, coplanar rings. Compound 1 is an example that mimics the cross section of a duplex at a purine-pyrimidine base pair. The cogent comparison is that the calculated N----N interatomic distances indicated in 1 are within 0.2–0.3 Å of those (N—H---O and N---H—N) in the natural pair, dA·dT. Accordingly, compound 1, 3,9-bis(2'-deoxy-β-D-ribofuranosyl)-3H-pyrimido[1'',6'':1',

2']imidazo[4',5':4,5]imidazo[2,1-i]purin-8(9H)-one, is a covalently-linked cross section of 'normal' (or near-normal) dimensions. The C1'–C1' distance is approximately 10.6 Å, a value close to that (10.85 Å) found in B-form DNA. Compound 2, 2,9-bis(2'-deoxy-β-D-ribofuranosyl)pyrimido[1'',6'':1',2']imidazo[4',5':4,5]imidazo[1,2-c] pyrimidine-1,10(2H,9H)-dione, is a covalently-linked pyrimidine-pyrimidine cross section with

1 Y = Y' = H

2 Y = H

3 Y = H

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'short' dimensions such as might be produced in the hypothetical apposition of dC and dU or dT. The C1'-C1' distance of **2** is approximately 8.1 Å. Compound **3**, 3,10-bis-(2'-deoxy-β-D-ribofuranosyl)-3H,10H-purino[1'',6'':1',2']imidazo[4',5':4,5]imidazo[2,1-i]purine, is a covalently-linked purine-purine cross section with 'long' dimensions such as would be produced in the Watson-Crick apposition of dA and dI or dG, generating a bulge in a double-helical DNA. The C1'-C1' distance of **3** is approximately 13.0 Å. There is the potential with these molecules **2,3**, when phosphorylated and incorporated in a double-helical oligodeoxynucleotide sequence, of constricting or bulging the helix. Such analogues could thereby be used to contribute to our full appreciation of the effect of DNA distortion and stiffening on enzyme recognition and reactivity.

We wished to investigate the incorporation of covalent cross sections into DNA by means of the enzyme T4 RNA ligase (4). Both ribo- and deoxyribonucleoside 3',5'-bisphosphates serve as donor substrates while oligoadenylates act as acceptors for linkage by means of RNA ligase (4,5). Since conditions have been optimized for the ligation of deoxynucleotide substrates (6–12), we tested phosphorylated derivatives of the covalent cross sections **1–3** as substrates, with the aim of subsequent construction of a double helix (Scheme 1). The steps involved include the phosphorylation of the covalently-linked cross section, represented as **4**, ligation of the resulting tetrakisphosphate **5** with d(A)₆ (**6**) to form **7**, and removal of the phosphate groups **8**, in anticipation of further ligations. These reactions have been performed with modest yields and portend the construction of intact duplexes containing cross sections at defined loci.

EXPERIMENTAL

Materials

The oligodeoxyribonucleotide d(A)₆ was purchased from Sigma and d(A)₁₂, from Pharmacia. Diethylaminoethyl (DEAE) cellulose was from Pierce Chemical Company. Bond Elut C 18 columns for desalting were purchased from Analytichem Int., Inc. Analytical anion-exchange high-performance liquid chromatography (HPLC) was carried out on a Whatman Partisil 5 RAC II SAX column (4.5×100 mm). Preparative anion-exchange HPLC separations were carried out on a Whatman Magnum M9 SAX column (4.9×250 mm). Reversed phase HPLC was performed on an Ultrasphere Octyl column from Altex. The following solvent systems were used for HPLC: I, A: 10% methanol; B: 1 M KH₂PO₄ in 10% methanol, pH 4.2; II, A: 40 mM sodium phosphate, pH 5.9; B: 50 mM sodium phosphate, pH 5.9, 25% methanol. Potassium phosphate buffer was filtered through Chelating Resin (Sigma) to remove

UV absorbing impurities (13). Thin-layer chromatography was performed on cellulose plates purchased from Eastman Kodak using saturated ammonium sulfate : 1.3 M sodium acetate : 1-propanol = 80 : 18 : 2 as the solvent system. Homochromatography was performed on Polygram CEL 300 DEAE/HR plates purchased from Macherey-Nagel using Homomix 5 (14) at 65°C as the solvent system. Triethylamine for the preparation of triethylammonium bicarbonate (TEAB) buffer was purified by fractional distillation of the commercial compound after the addition of 0.1% of benzenesulfonic acid. The pH value of the TEAB buffer was adjusted to 7.5 with carbon dioxide. Stock solutions of reagents were prepared and stored as described (12); T4 RNA ligase was 97.7 μM in 50% glycerol, stored at –20°C (12).

Phosphorylation

The tetrakisphosphates **5** of compounds **1, 2**, and **3** were all obtained in a similar manner. For example, a suspension of **1** (30 mg, 0.06 mmol) and pyrophosphoryl chloride (15) (0.53 g, 2.10 mmol) was stirred at –15°C for 4 h in an argon atmosphere. When the solution became clear, it was stirred for an additional hour and then added slowly to a precooled (–15°C) mixture of 1.0 M triethylammonium bicarbonate (TEAB) (20 mL) and triethylamine (2.1 mL). The solution was allowed to warm to 0°C, stirred for 10–15 min at this temperature, and then concentrated under reduced pressure with the bath temperature maintained below 30°C. The residue was triturated with chloroform (3×20 mL) to remove excess triethylammonium chloride. Addition of 50% aqueous methanol (10 mL) to the residue was followed by evaporation. This process was repeated three times, and the residue was finally dried under vacuum for one hour to remove traces of organic solvent. The residue was dissolved in 0.1 M TEAB and the pH adjusted to 7.4, if necessary, by adding TEA. The solution was loaded onto a DEAE-cellulose column (125 mL), and the column was eluted with eight volumes of 0.1 M TEAB followed by a linear gradient of 0.1 M – 1 M TEAB. Appropriate fractions were combined and concentrated. Excess TEAB was removed by co-evaporation with 50% aq. MeOH. The product **5₁** was purified on a Partisil M-9 preparative anion-exchange column using solvent system I and a gradient of 40–100% B during 30 min, followed by 100% B for 15 min with a flow rate of 4 mL/min. The fractions containing a product with retention time centered at 14.7 min were combined, diluted fifteen-fold with distilled H₂O, and loaded on a DEAE column (10 mL) which had been preequilibrated with 0.1 M TEAB. The column was washed with 10 volumes of 0.1 M TEAB, followed by elution with a linear gradient of 0.1 M to 1 M TEAB. The fractions containing the tetrakisphosphate **5₁** were combined and concentrated. Excess TEAB was removed by repeated co-distillation with 50% aqueous MeOH. The product was dried in vacuum for 4 h to give **5₁** as an off-white powder: yield, 15 mg (27%); UV λ_{max} (H₂O) 324, 280, 249, 229 nm. Low resolution FAB mass spectrum m/z 818.7 (MH⁺), 919.8 (MEt₃NH⁺). High resolution FAB mass spectrum m/z 819.0334 (C₂₁H₂₇N₈O₁₉P₄ requires 819.0345 amu).

The tetrakisphosphate (**5₂**) of compound **2** was unstable below pH 5.5 and above pH 9.0, so that conditions of phosphorylation and isolation were carefully selected. The phosphorylation procedure was similar to that used for **5₁**, but the quenching of the reaction mixture of **2** and pyrophosphoryl chloride was effected with a 1:1 solution of 1 M KH₂PO₄ and K₂HPO₄

(pH 6.4–6.5) cooled at -20°C . The reaction mixture was allowed to warm slowly to room temperature, and solid K_2HPO_4 was added slowly until the pH value stabilized. After a ten-fold dilution with water, chromatography was carried out on a DEAE cellulose column as described for **5**₁. Combination of the fractions with UV spectra corresponding to **2** and lyophilization were followed by dissolution in water and purification on an HPLC anion-exchange column using the solvent system 40–100% B (A = 1% aqueous MeOH, B = 1 M K_2HPO_4 : 1 M KH_2PO_4 (1:1), pH 6.4–6.5 in 1% MeOH). The major peak with a retention time of 10.4 min was collected, diluted ten-fold with water, and applied to a DEAE-cellulose column for desalting. The final work-up of **5**₂ was similar to that for **5**₁: yield, 31%; $\text{UV}\lambda_{\text{max}}$ (H_2O) 350, 333, 256, 222 nm. Low resolution FAB mass spectrum m/z 795.1 (MH^+); 896.2 (MET_3NH^+). High resolution mass spectrum m/z 896.1429 ($\text{C}_{26}\text{H}_{42}\text{N}_7\text{O}_{20}\text{P}_4$ requires 896.1435 amu).

The tetrakisphosphate **5**₃ of compound **3** was synthesized and isolated in much the same manner as compound **5**₁: yield, 23%; $\text{UV}\lambda_{\text{max}}$ (H_2O) 322, 278, 238 nm. High resolution FAB mass spectrum m/z 843.0458 ($\text{C}_{22}\text{H}_{27}\text{N}_{10}\text{O}_{18}\text{P}_4$ requires 843.0455).

T4 RNA Ligase Coupling

The ligations of **5**₁, **5**₂, and **5**₃ with **6** were carried out at least three times each under the most efficient conditions. The major difference among the three cases was in the optimum molar ratios of d(A)₆ acceptor **6** to donor **5**, which were: 5:1, 1:8, and 3.5:1, respectively. The general procedure can be illustrated by the coupling of **5**₃ with **6** or with the hexamer lacking the 5'-monophosphate of **6**. Solutions containing the following components were mixed to give the indicated final concentrations in a 10 μL final volume and were dried in vacuum in siliconized Eppendorf tubes: **5**₃, 1 mM; **6** (or without the 5'-monophosphate), 3.5 mM; ATP, 0.4 mM; spermine, 8 mM; creatine phosphate, 1 mM. The residue was dissolved by addition, in the order and to the final concentrations indicated: HEPES, pH 7.9, 50 mM; bovine serum albumin (BSA), 0.01 mg/mL; dithiothreitol (DTT), 20 mM; MgCl_2 , 5 mM; hexamine cobalt chloride (HCC), 1 mM. The components were mixed and reaction was initiated by the addition of the indicated final concentrations of the enzymes: myokinase, 169 units/mL; phosphocreatine kinase, 175 units/mL; T4 RNA ligase, 58.5 μM . The components were mixed and incubated at 25°C . The progress of the reaction was followed by dilution of 1 μL aliquots to 11 μL with 10 mM EDTA and subsequent separation of the components by anion-exchange HPLC. Solvent system I was used with a gradient of 40–100% B in 30 min followed by 100% B in 15 min with a flow rate of 1 mL/min. The compounds were detected by their absorbance at 260 nm. Extension of the reaction time beyond 117 h did not improve the yield of **7**₃.

On a preparative scale, the reaction was scaled up 10-fold with pd(A)₆ or d(A)₆ as the acceptor. After 117 h at 25°C , water (0.1 mL) was added to the reaction mixture and the resulting suspension was filtered through a Millipore HV 0.45 μm filter. The filter was washed twice with water (0.1 mL), and one-third of the combined filtrates was injected onto an analytical anion-exchange column. The elution conditions were as described above. In addition to the fractions containing pd(A)₆ (elution time 8.6 min) (Fig. 1A), two other fractions containing products were collected: pd(A)₁₂ (elution time 24.6 min) and **7**₃ 5'-phosphate (elution time 28.5 min) (Fig. 1B). The products

Figure 1. Anion exchange HPLC of T4 RNA ligase-catalyzed reactions. Reactions and chromatography were performed as described in the Experimental section. A: Reaction mixture for the addition of **5**₃ to pd(A)₆ before incubation. B: As in A after incubation for 120 h. C: Reaction mixture for the addition of **5**₃ to d(A)₆ before incubation. D: As in C after incubation for 120 h. The ligation product in D lacks a 5'-phosphate on the oligonucleotide terminus.

were desalted by application of a ten-fold-diluted fraction to a DEAE cellulose column (2 mL) pre-equilibrated with 0.1 M TEAB, washing with 10 column volumes of 0.1 M TEAB, and elution of the compounds with 1 M TEAB. Analogous reactions with **6** lacking a 5'-phosphate similarly produced **7**₃ lacking a 5'-phosphate (compare Fig. 1C and D). The base compositions of pd(A)₁₂ and **7**₃ (with 5'-phosphate) were determined by degradation to deoxyribonucleosides with venom phosphodiesterase (VPD) and bacterial alkaline phosphatase (BAP), and separation of the products by HPLC. Approximately 0.1 A₂₆₀ unit of the compound to be analyzed was incubated at 37°C for 20 h in 0.05 mL containing 0.1 M sodium glycinate, pH 9.20; MgCl_2 , 5 mM; VPD, 0.4 mg/mL; BAP, 0.08 mg/mL. The reaction mixture was analyzed by HPLC on a C8 column (solvent system II), with a 40–100% gradient over 20 min and 100% B for 30 min at a flow rate of 1 mL/min. The UV was monitored at 270 nm to maximize detection of adduct containing **3**. The compound with the elution time of 24.6 min (Fig. 1B, see above) contained only 2'-deoxyadenosine as indicated by its spectrum. Incubation of this compound with BAP at 65°C gave a product with the same retention time as an authentic sample of d(A)₁₂. No adenylated donor was observed in any of these reactions. The compound with the elution time of 28.5 min furnished 2'-deoxyadenosine and **3** after

Figure 2. Reverse phase HPLC of nucleoside components of ligation products. Analysis was performed as described in the Experimental section. A: 7_3 before treatment with VPD and BAP. B: 7_3 after treatment with VPD and BAP. C: $p(dA)_{12}$ before treatment with the enzymes. D: As in C after treatment with VPD and BAP. The products deoxyadenosine and 4_3 were identified by co-injection with standards followed by HPLC analysis.

incubation with VPD and BAP (compare Fig. 2A/B with Fig. 2C/D). The ratio of the integrated UV absorption peak areas at 270 nm divided by the respective molar extinction coefficients at 270 nm was 1.19 to 1 for 2'-deoxyadenosine versus 3_3 , compared with the normalized ratio of 1.29 to 1 found for $6 \times \epsilon_{\max}$ for 2'-deoxyadenosine to the $\epsilon_{\max}$ for 3_3 . This corresponds to a single ligation, i.e., 7_3 (yield, 20% with respect to 5_3). Double ligation would have produced an absorbance ratio closer to a calculated ratio of normalized molar extinction coefficients of 2.58 to 1. The structure of the product of the ligation of 6 lacking a 5'-phosphate (Fig. 1D) was similarly proved.

The enzymatic ligation of 6 to 5_2 required a molar ratio of 1:8 (a reversal of the effective ratio for $6 + 5_3$), and incubation proceeded for 130 h at 17°C. The rest of the general methodology applicable to the formation of 7_3 was followed in the isolation of 7_2 . On an analytical ion-exchange column monitored at 260 nm, the relative elution times of 11.0, 18.5, and 31.5 corresponded, respectively, to $d(A)_6$, 5_2 , and 7_2 (20% yield with respect to 6). The first two of these were readily checked by means of authentic samples. The ligated product 7_2 , $\lambda_{\max}$ (H_2O)

336 and 258 nm, was characterized by treatment with VPD to cleave the phosphodiester bonds and BAP to hydrolyze the terminal phosphates. The retention times of the components, 2'-deoxyadenosine and 2 , on a C8 reversed phase HPLC column were compared with those of standard samples. The ratio of the peak areas at 260 nm adjusted for the molar extinction coefficients of 2'-deoxyadenosine and 2 at that wavelength was 4.37:1. Since the value calculated for the components of 7_2 is 4.43:1, a single ligation had again occurred. The result is not surprising in this case because the covalent cross section was present in a large molar excess.

A molar ratio of 5:1 was optimal for the enzymatic ligation of 6 to 5_1 . The T4 RNA ligase incubation took place at 25°C for 130 h. The relative retention times on an analytical anion-exchange column monitored at 260 nm were 6.4, 12.1, 22.9, and 27.1 min, corresponding, respectively, to $d(A)_6$, 5_1 , $d(A)_{12}$, and 7_1 (20% yield with respect to 5_1). The ligated product 7_1 , UV $\lambda_{\max}$ (H_2O) 330 and 258 nm, was characterized by enzymatic degradation to 2'-deoxyadenosine and 1 . The ratio of the peak areas at 260 nm adjusted for the molar extinction coefficients of 2'-deoxyadenosine and 1 was 6.01. Since the value calculated for the components of 7_1 is 5.97, a single ligation had occurred in this case as well.

Hydrolysis of the Phosphomonoester Bonds by BAP

The hydrolysis of the phosphomonoester bonds of 7_1 , 7_2 , and 7_3 was effected as exemplified for the conversion of 7_1 to 8_1 . A solution containing 1.8 O.D. units of 7_1 was dried under reduced pressure. The residue was dissolved in 320 μ L of H_2O and adjusted to 0.1 M Na glycinate (pH 9.2), 5 mM $MgCl_2$, and 80 μ g/mL BAP in a total of 500 μ L.

The solution was incubated at 37°C for 6 h, and the product was isolated and purified on a reversed phase C8 HPLC column, using gradient elution with solvent system II: 0–100% B during 20 min, 100% B for 15 min. The fraction with the elution time of 22.5 min contained the product. It was evaporated, and the residue was dissolved in 0.5 mL of water. A Bond Elut C18 column was washed with 2 mL of methanol, 10 mL of water, and then the solution of 8_1 was applied. The remaining salts were washed out with 2 mL of water, and the product was eluted with 0.5 mL of 30% methanol. Vacuum evaporation gave 8_1 in 85% yield with respect to the phosphorylated precursor. Identification was implicit in the structure of the precursor, the known activity of the enzyme BAP, and the similarity in the UV spectra of starting material and product. The >98% purity made 8_1 a candidate for further ligation experiments. A similar procedure was employed for obtaining 8_2 and 8_3 in their pure forms.

RESULTS

The covalently-linked cross sections 1 – 3 , represented in Scheme 1 by the abbreviated formula 4 , i.e., 4_1 , 4_2 , 4_3 , were phosphorylated to the corresponding tetrakisphosphates 5 with pure pyrophosphoryl chloride at low temperature in the absence of solvent (16,17). This is a new application of the 3',5'-bisphosphorylation method (15). Each of these tetraphosphates was used as donor in the RNA ligase reaction, for which the minimal sufficient donor is pNp (4,5). Trinucleoside diphosphates are the minimal effective acceptors, and oligodeoxyribonucleotides are much less effective substrates than oligoribonucleotides (12). Since we had prepared a

deoxyribonucleotide cross section, we were committed to the deoxy series for initial experiments. As the first example, we used d(A)₆ as the acceptor in the reaction sequence (Scheme 1), with the 5'-hydroxyl either phosphorylated or free. Both were equally effective. Low temperature incubation (4–17°C) was employed (7,9), RNA ligase concentration was maintained (18), and ATP concentration was kept at the optimum level through the use of an ATP regeneration system (8,9), all in accord with the recommended conditions for ligation of oligodeoxynucleotides (6–12).

Under optimized conditions, which included a lengthy reaction time, compounds with elution times greater than those of the starting materials (**5** and **6**) were formed and were isolated by anion-exchange HPLC. The base compositions of the products were determined by degradation to deoxyribonucleosides with VPD and BAP, followed by separation of the resulting compounds by HPLC. When pd(A)₆ was used as the acceptor (or was present as a contaminant in the d(A)₆ used as the acceptor), pd(A)₁₂ was identified as one of the slowly-eluting products of the ligation reaction. The other product of ligation, for example in the case of **5**₃ + **6**, was the result of a single donor addition, **7**₃. (Double addition was recognized as a possibility since **5**₃ contains two 5'-phosphate groups.) Analysis of the desired ligation product was effected by the degradation and separation methodology described above. The UV peak areas of 2'-deoxyadenosine and **7**₃ were integrated and normalized by the respective extinction coefficients at 270 nm. The quantitative spectroscopy showed that only one oligonucleotide had been added to one covalent cross section. The product of self ligation was confirmed as pd(A)₁₂ by VPD and BAP degradation to 2'-deoxyadenosine as the single product and by BAP degradation alone to yield authentic d(A)₁₂.

The phosphate groups were removed from **7**₃ by BAP, followed by separation by HPLC and desalting, to yield **8**₃, which was further characterized by its ultraviolet absorption spectrum. The preparations of **8**₂ and **8**₁ followed the same reaction sequence shown in Scheme 1. Some variation was necessary in the ratio of d(A)₆ to tetraphosphate **5** for each case to obtain optimal yields, but the salient feature was that the RNA ligase reaction joined each of the 'normal,' 'short,' and 'long' cross sections to the acceptor oligonucleotide.

DISCUSSION

The enzyme T4 RNA ligase was prepared, purified, and assayed as described (12). Although it was recognized that the enzyme shows little specificity at its donor pNp site (4,5,17,19), the tetraphosphorylated, covalently-linked cross sections **5** offered a rather extreme test for binding, adenylation, and subsequent displacement of AMP from the activated donor by the 3'-hydroxyl of the acceptor to form the phosphodiester bond. All three donors of varying cross-sectional dimensions, **5**₁, **5**₂, and **5**₃, were acceptable substrates. No adenylated cross sections were observed suggesting that they do not accumulate in the reaction mixtures. It is also worthy of note that a single rather than double ligation occurred in each case. Since **5**₂ and **5**₃ are symmetrical, ligation on either side would produce identical products, **7**₂ and **7**₃, respectively. In the case of **5**₁, ligation on the pyrimidine side would produce a different product from that arising from a ligation on the purine side. We hoped to differentiate these possibilities because of the relative donor efficiencies previously established (4), i.e., pyrimidine pNps were more efficiently

joined than purine pNps, but the relative efficiencies of **5**₁, **5**₂, and **5**₃ were not definitively different. The fact that the optimum ratio of d(A)₆ to cross section required for **2** was different (by forty-fold) from those for **1** and **3** suggests that ligation favors the adenosine side. It will be necessary to block one side of compound **1** so that 3',5'-bisphosphorylation will occur regioselectively to yield a ligated product of unequivocal structure. The best yields attainable were approximately 20% of any cross section converted to product. Low yields of deoxyribonucleotide acceptors are common with T4 RNA ligase. The lack of accumulation of adenylated cross sections suggests that the addition of a second acceptor may be prevented by the inability of the primary product **7** to be efficiently adenylated. The dephosphorylation of **7** to **8** by means of BAP was effective and unexceptional in all three cases. The retention times of the components were identical with those of authentic 2'-deoxyadenosine and **1**, **2**, or **3**.

In initial attempts to add a second strand, thus making a short segment of double-helical DNA with a blunt-end, covalently-linked cross section, compound **8**₃ was incubated with [5'-³²P]pd(T)₆ and T4 DNA ligase at 4°C. Variations of temperature, ratios of **8**₃ to pd(T)₆, and concentrations of NaCl and of polyethylene glycol failed to yield any detectable product as judged by homochromatography of the reaction mixtures (14,20–22). A control reaction containing pd(T)₆ was dimerized successfully using d(A)₁₂ as a template under the same reaction conditions. Because of the general substrate requirements for T4 DNA ligase (23–25), it may be necessary to join the strand complementary to the d(A)₆ in **8** by chemical means or by a combination of chemical and enzymatic means (26,27). This course would be adaptable to the incorporation of other specific sequences, first introduced on one side by RNA ligase and then matched on the other side in synthetic succession.

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