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# Sequence Complementarity-Driven Nonenzymatic Ligation of RNA

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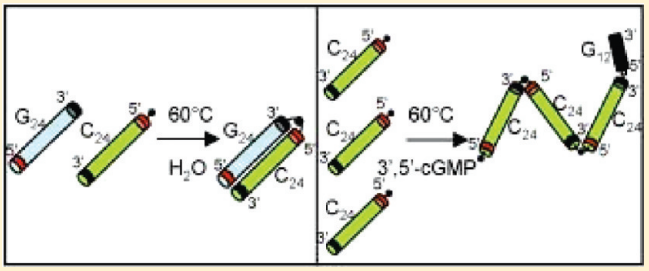
Supporting Information

**ABSTRACT:** We report two reactions of RNA G:C sequences occurring nonenzymatically in water in the absence of any added cofactor or metal ion: (a) sequence complementarity-driven terminal ligation and (b) complementary sequence adaptor-driven multiple tandemization. The two abiotic reactions increase the chemical complexity of the resulting pool of RNA molecules and change the Shannon information of the initial population of sequences.




The abiotic generation of (pre)-genetic polymers is best conceived if at least the following conditions are satisfied.

The first is the (i) availability of precursors that are sufficiently activated to spontaneously polymerize. Previous studies showed that nucleic bases and acyclonucleosides can be synthesized from formamide $H_2NCOH$ by being heated in the presence of prebiotically available mineral catalysts (last reviewed in ref 1).

Formamide-based syntheses add to a large body of findings that, starting from the pioneering observation of the formation of adenine in the frame of HCN chemistry,[2] explored the possibilities of the abiotic origin of nucleic bases.[3,4] Nucleosides, whose prebiotic synthesis is possible in principle,[5,6] can be abiotically phosphorylated in every possible position (2′; 3′; 5′; cyclic 2′,3′; and cyclic 3′,5′).[7−9] The higher stability of the cyclic forms in water at high temperatures relative to the open forms allows their selective accumulation over time.[1]

The second condition is a (ii) polymerization mechanism. The abiotic origin of the first RNA polymers has been the focus of several proposals[10−16] entailing different physicochemical scenarios, often suggesting distinct mechanisms for solving the problem of the presumably highly dilute concentration of the initial reactants. The simple nonequilibrium environment of a temperature gradient, compatible with conditions present in pores in hydrothermal rocks, suggests the physical setup for efficient and prebiotically plausible concentration and replication mechanisms.[17,18]

The role of mineral surfaces in concentrating and promoting the polymerization of activated or preligated mononucleotides was successfully shown.[19,20] The potential relevance of intercalation as a means of suppressing oligomer cyclization and promoting polymerization of base-pairing oligonucleotides in abiotic conditions was recently reported.[21] One limitation to the actual prebiotic relevance of the solutions proposed for polymerization, as originally mentioned by Orgel,[22] is their requirement for highly activated precursors whose formation entails complex chemistry. Sophisticated chemistry is difficult to reconcile with harsh prebiotic conditions. As a possible solution to the problems highlighted by the ensemble of these studies, a reaction showing the nonenzymatic generation of RNA polymers in water starting from prebiotically plausible precursors (3′,5′-cyclic GMP and 3′,5′-cyclic AMP) was reported.[23] However, polymerization under these conditions rapidly yields a steady state because of synthesis–degradation reactions, limiting the length of the polymer produced to a maximum of ∼40 nucleotides for polyG and ≥100 nucleotides for polyA.

The third condition is (iii) ligation of the synthesized oligomers. If ligation could occur on the basis of a terminus-joining mechanism through standard 3′,5′-phosphodiester bonds, rapid growth of the neo-synthesized oligomers would be realized, overcoming the steady state determined by synthesis–degradation equilibrium. This type of reaction was reported,[24] limited to homogeneous polyA sequences and leading to the di- and tetramerization of polyA oligomers.

What is missing in the line of reactions affording polymers potentially able to kick-start evolution are mechanisms providing the proof of principle for (iv) multiple tandemizations. These reactions would result in generation of sequence complexity.

We present evidence of the existence in simple RNA sequences of two mechanisms based on sequence complementarity, compatible

**Received:** December 13, 2010
**Revised:** February 28, 2011
**Published:** March 02, 2011

**Table 1. Specificities of the RNA-Degrading Enzymes Used in This Work**

| | yes | no | | |
|---|---|---|---|---|
| ribonuclease | | | | |
| A | 3′−5′ | 2′−5′ | C↓, U↓ | endo |
| T1 | 3′−5′ | 2′−5′ | G↓ | endo |
| SVPD | 3′−5′<br>2′−5′ | – | A↓, G↓, C↓, U↓ | exo 3′ → 5′ |
| phosphatase<br>shrimp<br>alkaline | | | 5′-P↓ | exo |

with such proof of principle, namely, nonenzymatic sequence complementarity-driven terminal ligation and formation of long mixed-sequence polymers by multiple tandemizations. This latter reaction is promoted by the presence of complementary adaptors generated nonenzymatically under the same conditions.

## MATERIALS AND METHODS

**Materials.** *Oligonucleotides.* Oligonucleotides $5'G_{24}3'$, $5'C_{24}3'$, $5'A_{24}3'$, $5'A_{18}C_63'$, $5'A_{12}C_{12}3'$, $5'A_6C_{18}3'$, $5'G_{23}A3'$, $5'G_{23}C3'$, $5'G_{23}U3'$, $5'C_{23}A3'$, $5'C_{23}U3'$, and $5'C_{23}G3'$ were purchased from Dharmacon and were provided unphosphorylated, at both the 5′- and 3′-extremities. Guanosine 3′,5′-cyclic monophosphate (3′,5′-cGMP) was from Sigma Aldrich (analytical grade).

*Enzymes.* T4 polynucleotide kinase (EC 2.7.1.78) from England BioLabs (catalog no. M0201L) catalyzes the transfer and exchange of $P_i$ from the $\gamma$-position of ATP to the 5′-hydroxyl terminus of polynucleotides, and the removal of 3′-phosphoryl group from 3′-phosphoryl polynucleotides. One unit is defined as the amount of enzyme catalyzing the production of 1 nmol of phosphate to the 5′-OH end of an oligonucleotide from [$\gamma$-$^{32}$P]ATP in 30 min at 37 °C.

*Ribonucleases.* Phosphodiesterase I from *Crotalus adamanteus* venom (snake venom phosphodiesterase I SVPD I) (EC 3.1.4.1) from Sigma (catalog no. P3243) is a 5′-exonuclease that hydrolyzes 5′-mononucleotides from 3′-hydroxy-terminated ribo-oligonucleotides. It cleaves both 2′,5′- and 3′,5′-phosphodiester linkages, and it was here typically used at a level of 1 milliunit/assay in 40 mM Tris-HCl (pH 8.4) and 10 mM $MgCl_2$ in 20 $\mu$L assays. One unit hydrolyzes 1.0 $\mu$mol of bis(*p*-nitrophenyl) phosphate/min at pH 8.8 and 37 °C.

Ribonuclease T1 from *Aspergillus oryzae* (EC 3.1.27.3), from Sigma (catalog no. R1003-100KU), is a 3′−5′ specific ribonuclease. It cleaves with a strong preference at the 3′-end of G residues but at high concentrations or at longer times will cleave also at other residues.[25] One unit produces acid soluble oligonucleotides equivalent to a $\Delta A_{260}$ of 1.0 in 15 min at pH 7.5 and 37 °C in a reaction volume of 1 mL.

Ribonuclease A (EC 3.1.27.5) from bovine pancreas catalyzes the cleavage of the P−$O^{5'}$ bond of RNA at the 3′-side of a pyrimidine nucleotide leaving a phosphate group connected at the 3′-end, similar to what occurs in the water-based hydrolytic reaction. RNase A is specific for 3′−5′ bonds yielding a 2′,3′-cyclic phosphodiester[26,27] from Sigma (catalog no. ENO531-10MG), via an assay in $H_2O$ at 98 °C and pH 6.2. One unit of the enzyme causes an increase in absorbance of 1.0 at 260 nm when yeast RNA is hydrolyzed at 37 °C and pH 5.0.

Shrimp alkaline phosphatase (orthophosphoric-monoester phosphohydrolase) (EC 232-631-4) from Promega (catalog no. M8201) removes phosphate groups from the 5′-position of RNA. One unit will hydrolyze 1 $\mu$mol of 4-nitrophenyl phosphate/min at pH 9.8 and 37 °C.

Table 1 summarizes the specificities of these enzymes.

**Methods.** *Terminal Labeling of RNA Oligonucleotides and of the Material Polymerized from Unlabeled Cyclic Nucleotides.* The oligo RNA (300 pmol) was labeled with [$\gamma$-$^{32}$P]ATP using polynucleotide kinase. The oligo was then purified on a 16% denaturing acrylamide (19:1 acrylamide/bisacrylamide, 8 M urea) gel. After elution, the residual polyacrylamide was removed with a NuncTrap Probe purification column (Stratagene). Subsequently, the RNA, suspended in STE buffer [100 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 10 mM EDTA], was precipitated by addition of 5 $\mu$L of glycogen (20 $\mu$g/$\mu$L bidistilled sterile water) and 3 volumes of ethanol, kept overnight at −20 °C, centrifuged, washed once with a 70% ethanol/water mixture, and dehydrated (Savant, 13000 rpm, 10 min, room temperature, environmental atmospheric pressure). The pellet was suspended in $H_2O$, distributed in aliquots, immediately frozen, and conserved at −20 °C. Typically, one aliquot was used for each experiment. Each experimental point consisted of 2−4 pmol of RNA (typically 15000 cpm/pmol).

The products of the polymerization reactions from cyclic nucleotides were precipitated with ethanol and dissolved in 44 $\mu$L of water. For dephosphorylation, 1 $\mu$L of shrimp alkaline phosphatase (1 unit/$\mu$L) was added along with 5 $\mu$L of 10× buffer [1× buffer being 0.05 M Tris-HCl (pH 9.0) and 10 mM $MgCl_2$], and the reaction mixture was incubated at 37 °C for 30 min, followed by phenol extraction and ethanol precipitation. Glycogen (1 $\mu$L of a 20 mg/mL stock) was added to facilitate precipitation. RNA was pelleted by centrifugation, then dissolved in 16 $\mu$L of water, and labeled at the 5′-termini with $^{32}$P. Phosphorylation was conducted via addition of 1 $\mu$L of T4 polynucleotide kinase (T4 PNK, 10 units/$\mu$L, New England Biolabs), 2 $\mu$L of 10× PNK buffer, and 0.5 $\mu$L of [$\gamma$-$^{32}$P]ATP, followed by incubation at 37 °C for 30 min. For gel electrophoresis, 10 $\mu$L aliquots of the RNA samples were added to 30% formamide buffer and separated by electrophoresis on 12 or 16% polyacrylamide gels containing 7 M urea, along with the indicated markers. In the dephosphorylation−rephosphorylation assay (Figure 4), this latter protocol (e.g, dephosphorylation with SAP and rephosphorylation with PNK) was used.

*Polymerization from 3′,5′-cGMP.* Polymerization from 3′,5′-cGMP was performed in water as reported previously,[23] at the concentration and temperature detailed where appropriate.

## RESULTS

**Sequence Complementarity-Driven Ligation.** PolyG and PolyC were reacted in two similar complementary assays. (a) A 24mer polyC bearing a phosphate group at the 5′-end and a nonphosphorylated hydroxyl terminus at the 3′-end (in short $5'$-$^{32}$P-$C_{24}$) was reacted with a 24mer polyG that was not phosphorylated at the 5′- or 3′-end ($G_{24}$). (b) For the complementary reaction, 5′-phosphorylated polyG 24mer ($5'$-$^{32}$P-$G_{24}$) was reacted with nonphosphorylated 24mer polyC ($C_{24}$). Although not strictly necessary, the presence or absence of the phosphate groups is indicated throughout

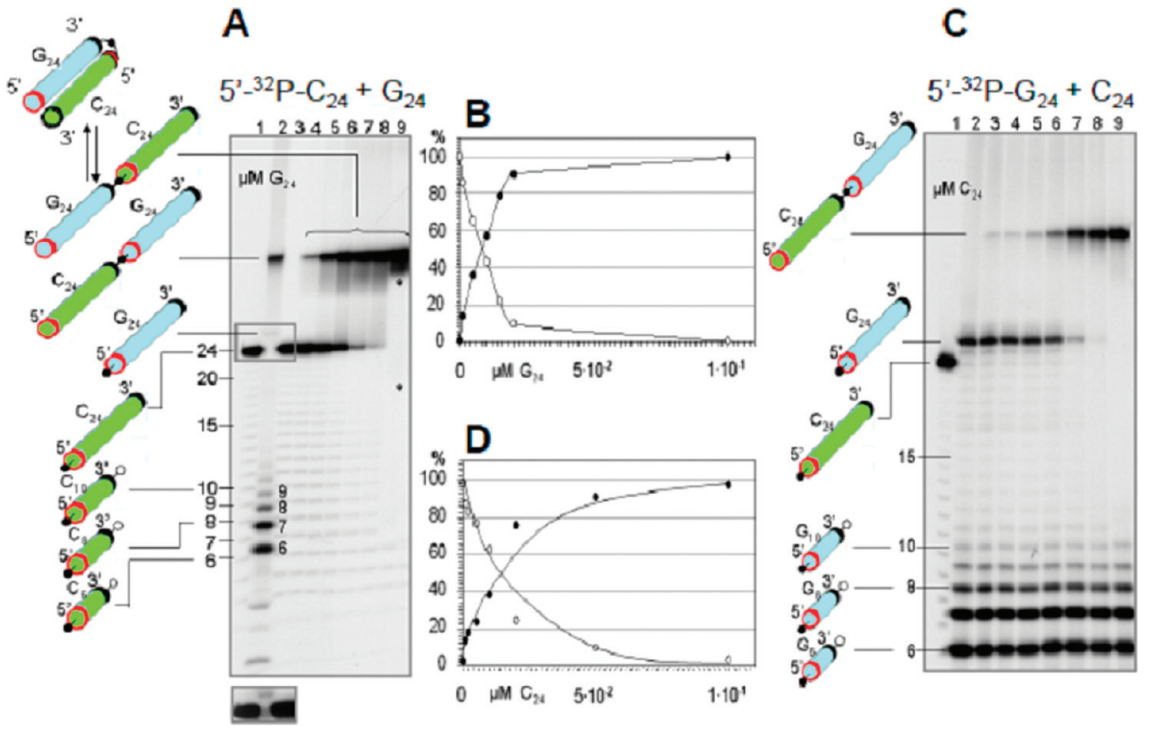
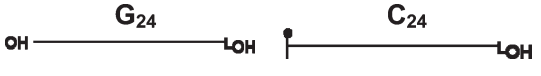



**Figure 1.** Terminal ligations of oligo C and oligo G. (A) Terminal ligation of polyC$_{24}$ to polyG$_{24}$. PolyC$_{24}$ $\gamma$-$^{32}$P-phosphorylated at the 5′-end was reacted with increasing amounts of unphosphorylated polyG$_{24}$. The following cartoons are shown at the left: G sequences (blue), C sequences (green), 3′-extremity (black), 5′-extremity (red), empty dots for phosphate groups, and filled dots for [$^{32}$P]phosphate groups. The numbering at the left side indicates the size in nucleotides of 5′-$^{32}$P-C$_{24}$ and the numbering at the right side of lane 2 that of 5′-$^{32}$P-G$_{24}$. The G$_{24}$—C$_{24}$ dimer (A) is shown both as a hairpin and as an open form, the first being the likely form present in water and the second that under denaturing gel conditions. The same is expected for the oppositely oriented C$_{24}$—G$_{24}$ dimer (not shown for the sake of simplicity): lane 1, unreacted 5′-$^{32}$P-C$_{24}$ (2.25 pmol in 15 $\mu$L = 0.15 $\mu$M); lane 2, 5′-$^{32}$P-G$_{24}$ (0.13 $\mu$M) reacted with C$_{24}$ ($2 \times 10^{-2}$ $\mu$M); lanes 3—9, 5′-$^{32}$P-C$_{24}$ (0.15 $\mu$M) reacted with increasing amounts of G$_{24}$ (0, $1 \times 10^{-3}$, $2 \times 10^{-3}$, $5 \times 10^{-3}$, $1 \times 10^{-2}$, $2 \times 10^{-2}$, and $1 \times 10^{-1}$ $\mu$M, respectively). The reaction was conducted for 30 min at 60 °C in water (pH 5.3). The specific activity of the full-length polyG 24mers, as measured from the amount of starting oligo compared with the amount of labeled 24mers, is 10-fold lower than that of polyC. The values given in the plot were corrected accordingly. The bottom inset shows a higher exposure of the 5′-$^{32}$P-C$_{24}$- and 5′-$^{32}$P-G$_{24}$-containing gel area, reported to allow identification of the 5′-$^{32}$P-G$_{24}$ molecule. (B) Quantitative evaluation of the formation of the dimeric 48mer (●) relative to the disappearance of the 5′-$^{32}$P-C$_{24}$ monomer (○). Data are from panel A. The line connecting experimental points has no mathematical meaning and is given to facilitate identification, here and in the rest of the figures. (C) Terminal ligation of 5′-$^{32}$P-G$_{24}$ to polyC$_{24}$. As in panel A, with a fixed amount of 5′-$^{32}$P-G$_{24}$ (0.13 $\mu$M) and increasing amounts of C$_{24}$ in lanes 2—9 (0, $1 \times 10^{-3}$, $2 \times 10^{-3}$, $3 \times 10^{-3}$, $6 \times 10^{-3}$, $1 \times 10^{-2}$, $5 \times 10^{-2}$, and $1 \times 10^{-1}$ $\mu$M, respectively). Lane 1 contained unreacted 5′-$^{32}$P-C$_{24}$, reported here as a size control. (D) Quantitative evaluation of the reactions shown in panel C.

the text to facilitate understanding of the relative position of the reactants and the direction of the reactions.

Phosphorylation was performed with T4 polynucleotide kinase by transferring a nonisotopically labeled or $^{32}$P-labeled phosphate on the 5′-end of polyG or polyC. The enzyme from New England Biolabs was routinely used. Thus, in instance b, the ends of the reacting species were not phosphorylated at 5′-G, not phosphorylated at 3′-G, phosphorylated at 5′-C, and not phosphorylated at 3′-C, schematically indicated as follows:




The "dot" in the scheme indicates a $^{32}$P phosphate group. In complementary reaction b, phosphorylation is on the 5′-G end. The following ends were present: not phosphorylated at 5′-C, not phosphorylated at 3′-C, phosphorylated at 5′-G, and not phosphorylated at 3′-G:


In addition to these full-length molecules, shorter molecules were present:


"P" indicates a nonlabeled phosphate group. The relative abundance of these shorter fragments is visible in the electrophoretic images (Figure 1, and later, as indicated); their average chain length (taking into account the most abundant population,

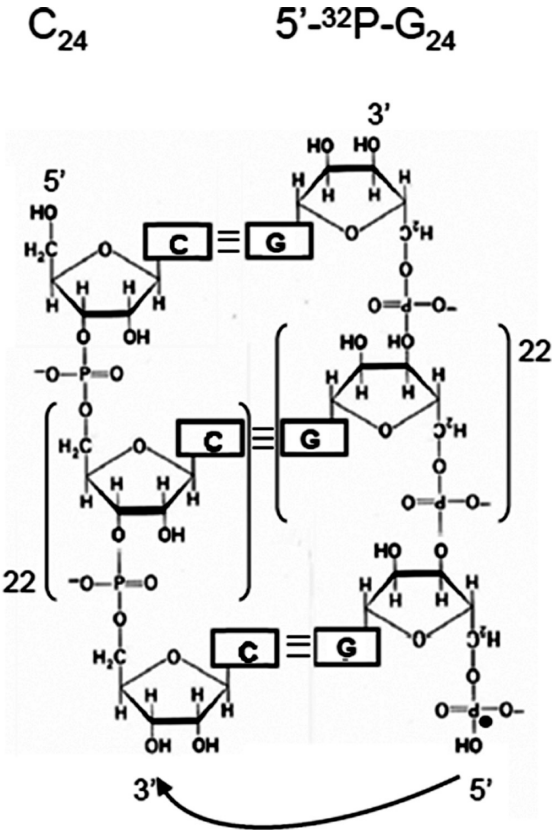



**Figure 2.** Sequence complementarity-driven terminal ligation. The 5′-$^{32}$P-$G_{24}$ oligonucleotide schematically shown in its 5′-extremity (right) ligates to the 3′-extremity of a $C_{24}$ oligonucleotide (left) through a standard 3′−5′ phosphodiester bond.

encompassing fragments 6−11 nucleotides in length) is 8.5 nucleotides. These molecules are produced by hydrolytic attack of the RNA molecules, thus affording 3′-phosphorylated ends, according to a well-characterized reaction.[28]

The molecules produced by hydrolysis, being shorter than the starting ones, are easily identified (visible in Figure 1A, lane 2) and quantified. The treatment of the 24mer with T4 polynucleotide kinase at the same time introduces a phosphate group at the 5′-extremity and removes the 3′-phosphate by its 3′-phosphatase activity.[29] The procedure followed (consisting of labeling the 24mer by polynucleotide kinase, purification via gel electrophoresis, and elution of the 24mer) implies that hydrolysis to shorter fragments occurs after the treatment with polynucleotide kinase and that the fragments with fewer than 24 nucleotides are phosphorylated at their 3′-ends.

Figure 1 shows the terminal ligation of 5′-$^{32}$P-$C_{24}$ with $G_{24}$ (panel A) and of 5′-$^{32}$P-$G_{24}$ with $C_{24}$ (panel C). Panel A shows the reaction of 5′-$^{32}$P-$C_{24}$ with $G_{24}$ affording the 48mer dimer molecule 5′-$G_{24}$-$^{32}$P-$C_{24}$-3′, the labeled phosphate group having provided the bridging phosphoester bond, as shown below. Lane 1 shows the 5′-$^{32}$P-labeled $C_{24}$ (marker, not reacted). Lanes 3−9 show the products of the reaction of 5′-$^{32}$P-$C_{24}$ with increasing amounts of unlabeled, non-5′-phosphorylated $G_{24}$. All the 5′-$^{32}$P-$C_{24}$ molecules react and yield a 48-nucleotide molecule, the bona fide dimer. Lane 2 shows a control size marker of the sequence inversely oriented reaction: 5′-phosphate-labeled $G_{24}$ (5′-$^{32}$P-$G_{24}$) reacted with non-labeled, non-5′-phosphorylated $C_{24}$. This reaction is detailed in panel C and is reported in panel A as a marker. The results show that

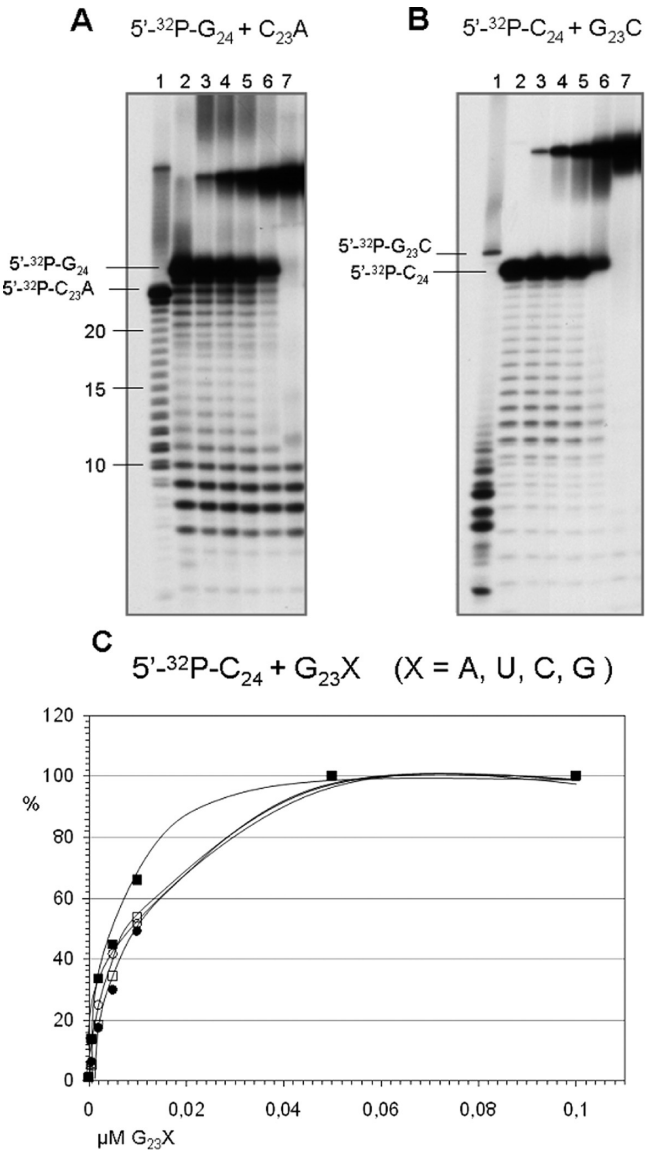



**Figure 3.** Terminal ligation of oligos with varying ends. (A) 5′-$^{32}$P-$G_{24}$ (2 pmol in 15 $\mu$L = 0.13 $\mu$M) was reacted (as detailed in Figure 1) with increasing concentrations of $C_{23}$A: lane 1, 5′-$^{32}$P-$C_{23}$A run as a size position control, present in the rest of the assay in the 5′-nonphosphorylated, nonlabeled form; 5′-$^{32}$P-$G_{24}$ in the absence of acceptor $C_{23}$A (lane 2) or in the presence of $5 \times 10^{-4}$, $2 \times 10^{-3}$, $5 \times 10^{-3}$, $1 \times 10^{-2}$, and $5 \times 10^{-2}$ $\mu$M $C_{23}$A (lanes 3−7, respectively). (B) 5′-$^{32}$P-$C_{24}$ reacted with increasing amounts of $G_{23}$C: lane 1, 5′-$^{32}$P-$G_{23}$C, run as a size position control, but present in the assay in the 5′-nonphosphorylated, nonlabeled form; lanes 2−7, 5′-$^{32}$P-$C_{24}$ in the presence of 0, $5 \times 10^{-4}$, $2 \times 10^{-3}$, $5 \times 10^{-3}$, $1 \times 10^{-2}$, and $5 \times 10^{-2}$ $\mu$M $G_{23}$C, respectively. (C) Formation of dimer 48mers via the 5′-$^{32}$P-$C_{24}$ + $G_{23}$X reaction (X = A, G, C, or U) as a function of the 3′-terminal base in the acceptor moiety. Plot of the data from panel B for $G_{23}$C (■) and from similar titrations (not shown) for $G_{23}$A (●), $G_{23}$U (□), and $G_{24}$ (○).

5′-$^{32}$P-$G_{24}$ (which migrates slower because of its higher molecular weight) yields, upon reaction with a $C_{24}$, a dimer whose size and migration are identical to those of the product of the specular reaction of 5′-$^{32}$P-$C_{24}$ with $G_{24}$.

The fact that the two dimer bands [the one obtained from the reaction of 5′-$^{32}$P-$C_{24}$ with $G_{24}$ (as indicated in lanes 3−9) and the one obtained from the reaction of 5′-$^{32}$P-$G_{24}$ with $C_{24}$ (as indicated

**Table 2. Quantitative Analysis of Ligation Test System Results**

| ligating polymers | | ligation rate[a] | half-max[b] ($\mu$M) |
|---|---|---|---|
| 5′•-P-$G_{24}$ | $C_{24}$ | 10000 | $2.1 \times 10^{-2}$ |
| | $C_{23}U$ | 1000 | $2.4 \times 10^{-1}$ |
| | $C_{23}A$ | 7000 | $4 \times 10^{-2}$ |
| | $C_{23}G$ | 800 | $2.9 \times 10^{-1}$ |
| 5′•-P-$C_{24}$ | $G_{24}$ | 5800 | $4 \times 10^{-2}$ |
| | $G_{23}U$ | 2960 | $8.2 \times 10^{-2}$ |
| | $G_{23}A$ | 3350 | $7 \times 10^{-2}$ |
| | $G_{23}C$ | 9760 | $2.4 \times 10^{-2}$ |

[a] Rates of dimer formation for the 5′-$^{32}$P-$G_{24}$ + $C_{23}X$ and 5′-$^{32}$P-$C_{24}$ + $G_{23}X$ reactions, as indicated. Only the 48mer band was considered as the ligation product, disregarding the minor bands with no more than 47 nucleotides. The fixed concentrations of the P-donor (see the text) were 0.13 and 0.15 $\mu$M, respectively. Complete reaction was reached at $5 \times 10^{-2}$ $\mu$M for the best case ($C_{24}$), which has been scaled to 10000. The efficiencies of the other reactions are relative to this value. [b] Half-max is the concentration of the acceptor oligo at which the rate of product yield is one-half of the maximal ligation rate.

in lane 2)] migrate identically shows their oppositely oriented base complementary $G_{24}$-$^{32}$P-$C_{24}$ and $C_{24}$-$^{32}$P-$G_{24}$ composition. $G_{48}$ or $C_{48}$ would in fact migrate quite differently. This interpretation is supported by the findings that (1) neither 5′-$^{32}$P-$C_{24}$ nor 5′-$^{32}$P-$G_{24}$ taken alone dimerizes (independent of the concentration, tested up to 0.2 mM) (data not shown), (2) 5′-$^{32}$P-$G_{24}$ treated alone in water under the same conditions (as tested at 60 and 90 °C) cyclizes but does not dimerize (data not shown), and (3) formation of heterogeneous dimers is exactly what is expected by the sequence complementarity-driven interaction of $G_{24}$ with $C_{24}$, followed by terminal ligation. The reaction is schematically presented in Figure 2.

The bottom asterisk (Figure 1A, right side) points to the region of less-than-unit sized 5′-$^{32}$P-$C_{24}$ molecules that, reacting with $G_{24}$, yield the less-than-dimer-sized molecules resulting in the compressed blurred population indicated by the top asterisk. This reaction occurs for molecules between the 23mer and ≥13mers.

Figure 1B shows the quantitative evaluation of the dimerization reaction, as a function of the increase in the amount of the nonphosphorylated reactant ($G_{24}$) over a fixed amount of 5′-$^{32}$P-$C_{24}$. Panel D shows the same for the complementary reaction. The fact that the migration of the 5′-$C_{24}$-$^{32}$P-$G_{24}$-3′ 48mer is identical to that of the 5′-$G_{24}$-$^{32}$P-$C_{24}$-3′ 48mer (as reported in the direct comparison in panel A, lane 2) shows that the terminal ligation reaction can occur in both orientations and that the phosphate donor can be a pyrimidine nucleotide (as in 5′-$G_{24}$-$^{32}$P-$C_{24}$-3′, panel A) or a purine nucleotide (as in 5′-$C_{24}$-$^{32}$P-$G_{24}$-3′, panel C). The acceptor molecule is in both instances topologically located upstream of the donor molecule, as shown by the set of analyses that follows. The oligo providing the phosphate group leading to dimer formation and located downstream is the "P-donor", and its partner located upstream is the "P-acceptor".

The reaction approximates a plateau at $2 \times 10^{-2}$ $\mu$M P-acceptor over $1.5 \times 10^{-1}$ $\mu$M P-donor for the 5′-$^{32}$P-$C_{24}$/$G_{24}$ reaction, and at similar values ($2 \times 10^{-2}$ over $1.3 \times 10^{-1}$) for the complementary reaction. This similarity shows the nonfastidious nature of these transphosphorylations.

**Base Requirements for Ligation.** The P-donor 5′-$^{32}$P-$C_{24}$ was also reacted with P-acceptors whose 3′-terminal residues involved in the reaction were A, U, or C, namely, $G_{23}A$, $G_{23}U$, or $G_{23}C$, respectively. The complementary reactions were also performed: 5′-$^{32}$P-$G_{24}$ reacted with $C_{23}A$, $C_{23}U$, or $C_{23}G$. Two representative examples of different P-donor−P-acceptor combinations are reported in Figure 3A (5′-$^{32}$P-$G_{24}$ + $C_{23}A$) and Figure 3B (5′-$^{32}$P-$C_{24}$ + $G_{23}C$). Figure 3C shows the quantitative evaluation of the 5′-$^{32}$P-$C_{24}$ + $G_{23}X$ dimer formation reactions. Reaction rate differences are induced by the different bases present at the acceptor extremity (Table 2). The same occurred for the complementary reaction, confirming both a specific base-related preference and the robustness of the transphosphorylation leading to terminal ligation.

The ligation of two oligomers to form a dimer can occur by formation of a phosphoester bridge between the 5′-phosphate of one oligonucleotide and the 2′- or 3′-OH extremity of another oligonucleotide or, in the other direction, between a phosphate at the 2′- or 3′-position and an OH at the 5′-position. The resulting phosphodiester bond may be a 2′−5′ or 3′−5′ bond. The enzymatic analyses described below show that in this system the ligation involves a 5′-phosphate and a 3′-OH, via 3′−5′ phosphodiester bonds.

**Ligation Rates and $K_{cat}$.** The ligation rates of the formation of dimers by the eight different sequence combinations tested are listed in Table 2.

$K_{cat}$. The amount of reactant converted to ligated products per unit time ($K_{cat}$) could not be determined because the sequence complementarity-driven ligation reaction is faster that the handling time of the samples, which is different from the cases for stacking-driven ligation[24] and the simultaneous polymerization−ligation reactions.[23] In the experimental system described here, the $K_{obs}$ of the reactions is as elusive as the $K_{cat}$. Base pairing of sequence homogeneous polyC and polyG is faster than measured times. Lower concentrations would prevent detection.

**Ligation Mechanism.** The ligation requires the presence of a phosphate group at the 5′-end of the oligonucleotide that ligates at the 3′-extremity of the acceptor oligo. This directionality is shown by results of the two oppositely oriented reactions (Figure 1). The efficiency of dimer formation is highly dependent on the dose of the acceptor oligo (Figure 3C). Similar but not identical values are observed for the concentration required to complete the reaction when the P-donor is a purine as opposed to a pyrimidine (Figure 3), showing the relevance of the chemical nature of the donor nucleotide. The thermodynamics involved is that of a simple transphosphorylation, activated by the moderate temperature of 60 °C.

The ligation reactions were routinely conducted at pH 5.6. Reactions occurring at this pH value are consistent with a simple general acid catalysis mechanism. The reactions were also conducted at higher pH values (6.7 and 8.3) (data not shown). The results showed that ligation also occurs under these conditions. However, given that the general acid catalysis mechanism is only proven by a kinetic dependence of the reaction on pH and given the nonmeasurable kinetics of this reaction, the general acid catalysis mechanism involved cannot be formally proven.

The RNase analyses reported below showed that the resulting phosphodiester bond is a standard 3′−5′ bond. In conclusion, the catalytic strategy in action here is that typical of ribozymes: positioning the reaction groups in an optimal alignment,[27] resulting in a transphosphorylation reaction that in principle does not differ from that reported previously[24] for terminal ligation of polyA.

**Analysis of the Products.** We characterized the products as follows. (1) Shrimp alkaline phosphatase (SAP) was used to treat the 5′-$C_{24}$-$^{32}$P-$G_{24}$-3′ dimer. SAP removes a phosphate group from the 5′-extremity of RNA. SAP treatment of the reactants

and of the products of the dimerization reaction promptly removed the 5′-labeled phosphate from the starting 5′-$^{32}$P-$G_{24}$, but not from the product of dimerization. This shows (as detailed in Figure S1 of the Supporting Information) that the addition of the $C_{24}$ oligo occurs at the 5′-end, with internalization of the 5′-terminal label. (2) Snake venom phosphodiesterase (SVPD RNase) cleaves RNA at the 3′-side at P-O$^{5'}$, with no sequence bias and in a manner independent of the 2′−5′ or 3′−5′ nature of the phosphodiester bond. SVPD being an exonuclease, it completely digested the RNA forms studied here (data not shown), showing that they were linear and not circular. (3) Ribonuclease A (RNase A) was used at a high temperature (98 °C). RNase A catalyzes the cleavage of the P−O$^{5'}$ bond of RNA at the 3′-side of a pyrimidine nucleotide leaving a phosphate group at the 3′-end. RNase A from bovine pancreas is resistant to high temperatures,[25] allowing digestion assays close to its boiling point. The RNase A reaction is better controlled by varying the amount of enzyme rather than the length of time of the treatment. Complete digestion of the 5′-$C_{24}$-$^{32}$P-$G_{24}$-3′ dimer was conducted ($2 \times 10^2$ units for 2 h at 98 °C), showing the 3′−5′ nature of the phosphodiester bond bridging the $C_{24}$ and $G_{24}$ moieties. Full details of RNase A digestion assays are given in Figure S2 of the Supporting Information. The properties of the ribonucleases used in this study are detailed in Methods. (4) The kinetic stability toward hydrolysis of the presumptive dimer at different temperatures in water was analyzed. This analysis (shown in Figure S3 of the Supporting Information) provides information based on the fact that double-stranded RNA is more resistant to hydrolysis than its single-stranded form. The product of dimerization (self-complementary 48mer) had at 90 °C a half-life of $3 \times 10^3$ min, while the half-lives of the single-stranded RNAs under these conditions were shorter: $6.3 \times 10^2$ min for poly$C_{24}$ and $2.4 \times 10^2$ min for poly$G_{24}$ (data not shown).

Given that these reactions were conducted with molecules carrying the $^{32}$P isotope, the remote possibility that ligation could be somehow activated by radiation damage or by an activated species generated by radiation exists. This possibility was discarded by performing the reactions on molecules phosphorylated with non-isotopically labeled phosphates. The labeling was performed *a posteriori* at the final analytical step. No difference was found between the two procedures (data not shown).

The products of the reaction were also analyzed on a more extremely denaturing electophoretic system: 10% acrylamide, 8 M urea, and 30% formamide. The results confirmed the interpretation given above (data not shown).

**Formation of Long Mixed-Sequence Heteropolymers by Terminal Polymerization and Tandemization.** Under the conditions in which terminal ligation takes place (water, 60 °C, absence of enzyme activities), 3′,5′-cGMP rapidly polymerizes.[23] We have thus explored the possibility that this polymerization could lead, when occurring in the presence of complementary oligo sequences, to the generation of new molecules by combinatorial processes. We observed that treatment of C oligomers (and/or of mixed-sequence oligomers containing sufficiently long C stretches) with 3′,5′-cGMP induces the formation of long complex polymers by a molecular glueing process.

A group of five different 24mers ($A_{24}$, $A_{18}C_6$, $A_{12}C_{12}$, $A_6C_{18}$, and $C_{24}$) was selected, and each oligo was treated in water at 60 °C with increasing amounts of 3′,5′-cGMP. Figure 4A shows that the polymerization of 3′,5′-cGMP induced the ligation of the poly$C_{24}$ oligos and of the oligos containing 18 or 12 C residues. For the sake of clarity, the five different oligos are schematically shown and numbered on the left side of Figure 4. The extent of gel migration of the untreated oligos increases as a function of the increasing C content (lanes 1−5) because of the known phenomenon of C compression. When reacted (lanes 6−10) with 1 mM 3′,5′-cGMP, oligos $A_{12}C_{12}$, $A_6C_{18}$, and $C_{24}$ (lanes 8−10) formed higher-molecular weight molecules that are interpreted (and as shown by RNases analyses) as follows: molecules 6−8 are the starting 24mer oligos on whose 3′-extremity neo-synthesized oligo G has polymerized (or has ligated after untemplated polymerization in solution) (lanes 8−10, respectively). This reaction was reported.[23] The analysis presented here shows that the complementary C sequence is necessary for this 3′-terminal reaction to occur and that it must be more than six residues in length, presumably to allow stable hybridization of neo-synthesized G oligomers on the C acceptor sequence. No terminal polymerization occurs on the $A_{24}$ or $A_{18}C_6$ molecules (lanes 6 and 7). Longer molecules also form (numbered 9−12), consisting of an increasing number of 24mer units, as depicted on the right side. This process is descriptively dubbed "tandemization".

These long molecules are formed by 24mers ligated at the 5′-extremity through 3′−5′ bonds, the phosphate being provided at the 5′-end by the labeled oligomer. This interpretation is supported by RNase analyses (see below). Also in this case, a C stretch of more six residues is required. At a higher 3′,5′-cGMP concentration (10 mM, lanes 11−15), the reaction pattern is conserved, and in the case of $A_6C_{18}$ and $C_{24}$, all the initial 24mer molecules are involved in multimeric products (asterisk on the right of lane 15).

These reactions were explored in detail by titrating their dependence on the 3′,5′-cGMP concentration and by determining the amount of oligo G polymerized from 3′,5′-cGMP during the reaction. Figure 4B shows the effect of increasing amounts of 3′,5′-cGMP on 5′-$^{32}$P-$C_{24}$ polymerization, focusing on the transition concentrations (between 0.1 and 1 mM 3′,5′-cGMP). The C 24mer is the most efficient substrate for both G tail polymerization and tandemization. The detailed analysis of the concentration dependence for the least efficient $A_{12}C_{12}$ (panel C) shows that this substrate supports G tail polymerization but almost no tandemization.

What is the correlation between the generation of G oligos from 3′,5′-cGMP and tandemization?

3′,5′-cGMP polymerizes in solution in the absence of any template starting at the low concentration of 0.1 mM,[23] and as seen in lane 2 of Figure 4B, tandemization becomes appreciable already at this low concentration. To better define the role of the neo-polymerized G oligos, the following two-step experiment was performed.

5′-$^{32}$P-$C_{24}$ was reacted with increasing amounts of 3′,5′-cGMP, and part of the sample was analyzed directly (Figure 4D). The remaining part was dephosphorylated and rephosphorylated by two successive treatments, first with SAP and then with T4 polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]ATP. The first step removes the 5′-labeled phosphates, and the second step phosphorylates the 5′-extremity of the initial 24mer, of the multimers formed by tandemization, and of the neo-synthesized oligo G forms. The results (Figure 4E) show that the formation of G polymers becomes appreciable at a concentration of 1 mM at which the tandemization process is already complete (Figure 4E, lane 3). At higher 3′,5′-cGMP concentrations, polymerization continues but tandemization does not proceed further because of a lack of the 24mer substrates feeding the reaction. Polymerization of Gs continues up to the highest concentration tested, showing that G polymerization in solution occurs only when the complementary oligo C is no longer available for hybridization. No G polymerization was observed (data

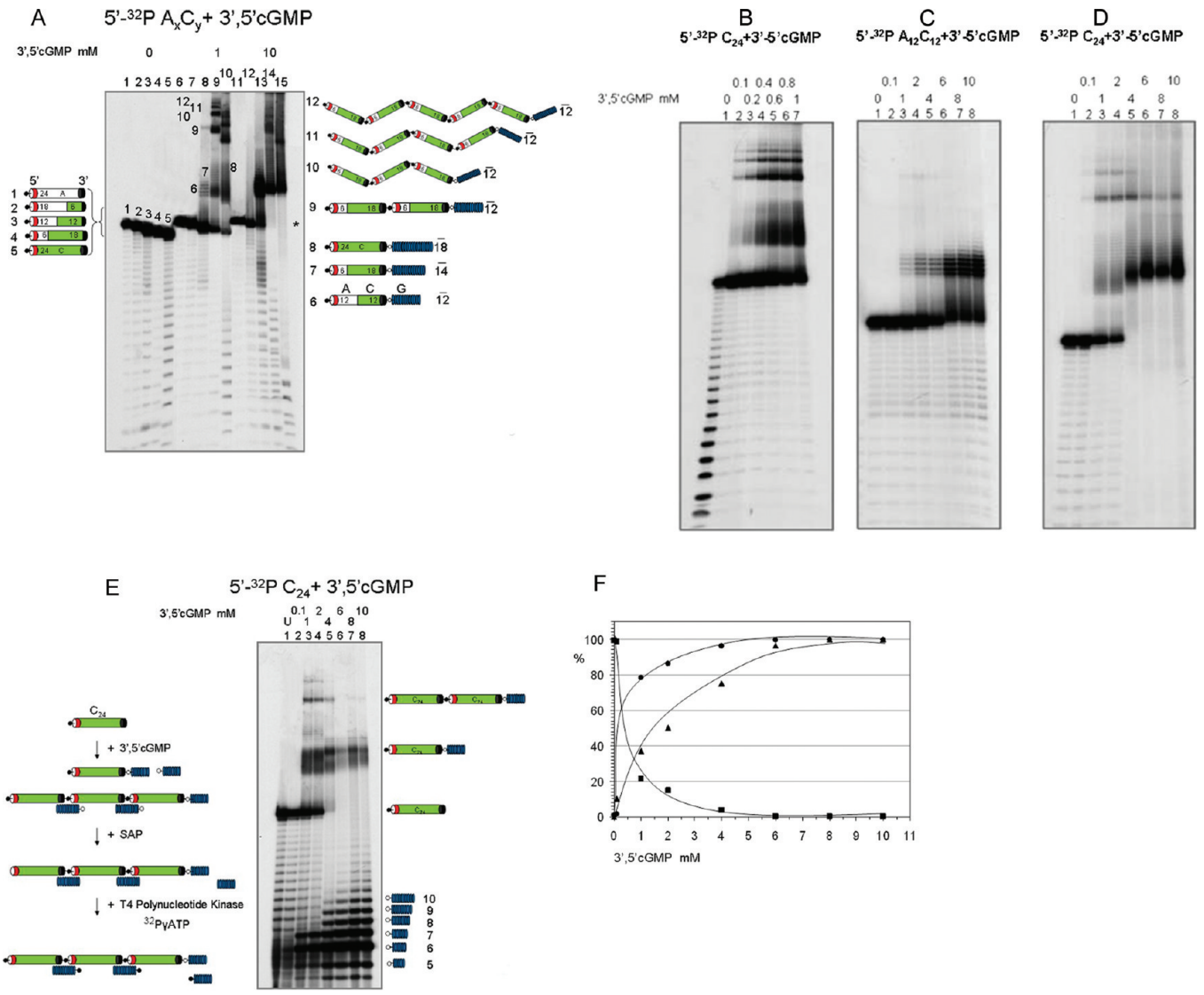



**Figure 4.** Generation of long complex sequences by 3′-terminal polymerization and adaptor-mediated tandemization. (A) The 5′-terminally labeled $A_{24}$, $A_{18}C_6$, $A_{12}C_{12}$, $A_6C_{18}$, and $C_{24}$ species at 0.13 $\mu$M (lanes 1−5, respectively) each reacted with 1 (lanes 6−10) or 10 mM 3′,5′-cGMP (lanes 11−15) for 30 min at 60 °C, analyzed as described. Numbers 1−5 refer to the different oligos, as indicated in the cartoon on the left and on top of the full-size band in the first five lanes. Numbers 6−8 refer to the 3′-terminally grown molecules (G tails on $A_{12}C_{12}$, $A_6C_{18}$, and $C_{24}$ oligos, respectively) as indicated in the gel and in the cartoon on the right. Numbers 9−12 refer to the tandemized polymers (in-gel and right side cartoon numbering). The asterisk points to the run out 24mer. Values 12, 14, and 18 indicate the average lengths of the polymerized G tails. (B) 5′-$^{32}$P-$C_{24}$ reacted with increasing amounts of 3′,5′-cGMP (0.1−1 mM), as indicated. (C) 5′-$^{32}$P-$A_{12}C_{12}$ reacted with increasing amounts of 3′,5′-cGMP (0.1−10 mM), as indicated. (D) 5′-$^{32}$P-$C_{24}$ reacted with increasing amounts of 3′,5′-cGMP (0.1−10 mM), as indicated. (E) Samples identical to those shown in panel D were dephosphorylated by SAP and rephosphorylated by T4 polynucleotide kinase as detailed in the text and indicated in the cartoon on the left. The products of rephosphorylation are shown. The bands on the lower right side are the neo-synthesized G polymers not bound to the 24mer or to the multimers. Panel F shows the quantitative evaluation of the transformation of the starting 5′-$^{32}$P-$C_{24}$ (■) into more complex forms (●), each calculated as a percentage (ordinate) relative to the total amount of signal present in the top part of the gel from the 24mer up, as a function of the increasing 3′,5′-cGMP concentration (abscissa). The amount of increasing G polymer is also indicated (▲), as a percentage of the maximum obtained (lane 8 = 100%).

not shown) at 3′,5′-cGMP concentrations between 0.1 and 1 at which (see panel B) efficient multimerization was observed. Figure 4F shows a quantitative description of this experiment.

In conclusion, 3′,5′-cGMP polymerizes free in solution and/or on a complementary C template and then terminally ligates to its 3′-extremity. In addition, the neo-synthesized G polymers also bind by sequence complementarity to oligo C stretches and, in the appropriate sequence constructs (see above), may protrude and provide a sequence complementarity adaptor structure. The tandem multimerization observed simply occurs by terminal ligation of the 3′-OH extremity of one polyC oligo to the 5′-phosphate extremity of another oligo, held in the correct position by the neo-synthesized oligo G adaptor (Figures 4E and 5).

Stacking-mediated terminal ligation was described for oligoA stretches,[24] and sequence complementarity-mediated terminal ligation is reported in the first section of this study. The tandemization described above concerns homogeneous pyrimidine stretches and is favored by an external adaptor synthesized in loco and in real time, not by a sequence provided by an external source involved in the final product of the reaction.

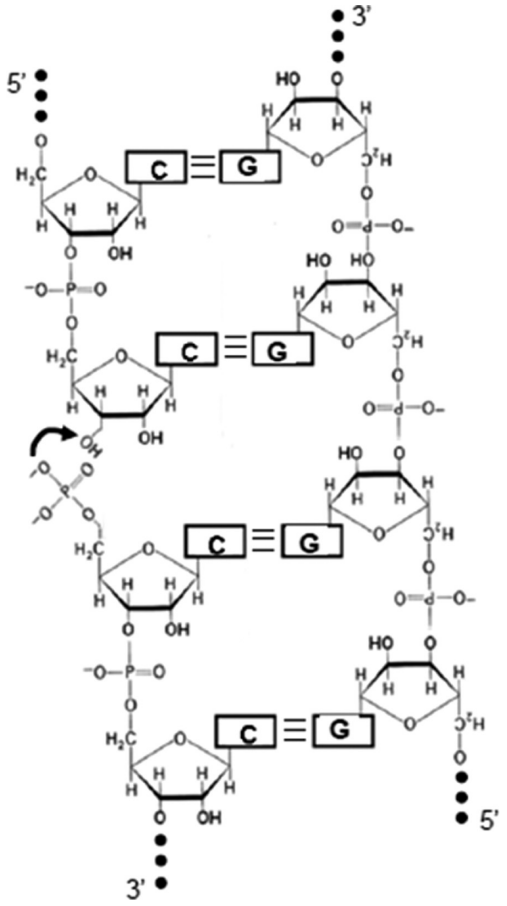



**Figure 5.** Tandemization of C or C-containing polymers via terminal ligation (arrow, center left) of oligos held in position by complementary neo-synthesized G oligomers.

**Characterization by RNases.** RNase A treatment (Figure S2 of the Supporting Information) shows the digestion of a 5′-$A_{12}C_{12}$ oligomer (lane 1) on which 3′,5′-cGMP has tail-grown a 12mer (lane 2). Increasingly intense treatment with RNase A (lanes 3−7) shows the validity of the models shown alongside. RNase A destroys the central C stretch, thus leading to the disappearance of the 3′-terminal G tail and leaving only the 5′-proximal A 12mer (cartoon on the right side). Similar assays were conducted at higher 3′,5′-cGMP concentrations and on $A_6C_{18}$, affording more complex molecular combinations. The results were always consistent with the models depicted as cartoons in Figure 4.

The models were also confirmed by shrimp alkaline phosphatase used with the same analytical approach detailed in Figure S1 of the Supporting Information (data not shown) and by RNase T1. RNase T1 cleaves highly preferentially at the 3′-ends of G residues.[25] RNase T1 treatment of the molecular species interpreted as 5′-$A_{12}C_{12}$-$G_{12}$-3′ selectively removed the 3′-G tails, confirming the molecular structure given above (data not shown).

**MALDI-ToF MS Analysis.** This structural analysis was performed for (1) the ligation of the 5′-phosphorylated polyC 24mer to the polyG 24mer leading to the formation of the $G_{24}$-$C_{24}$ 48mer and (2) the polymerization of 3′,5′-cGMP on the 3′-end of the polyC 24mer. The results are consistent with the evidence provided by the electrophoretic and enzymatic analyses and are detailed in the Supporting Information.

## DISCUSSION

We describe reactions occurring upon interaction of G and C oligos in water at 60−80 °C: terminal ligation of polyG on polyC and, complementarily, of polyC on polyG. This reaction occurs nonenzymatically in water in the absence of any added organic or inorganic cofactor.

A second reaction consists of the ligation of cytosine-containing mixed sequences with no internal sequence complementarity in the presence of a cyclic guanosine nucleotide. These ligation events afford long polymers that may, according to the composition of the initial species, abiotically become long sequence-heterogeneous molecules. Sequence complementarity-driven terminal ligation and terminal growth concur, resulting in the generation of complex and controlled sequence combinations.

Ribopolymers in water are bound to rapidly disappear. At 60 °C, polyG in water rapidly undergoes degradation, and what is not hydrolyzed undergoes cyclization (to be detailed elsewhere). As for polyC, no circularization is observed, and hydrolysis ($t_{1/2} = 3.5 \times 10^3$ min at 60 °C) will slowly lead to its dissolution. The reactions reported provide the initial homogeneous molecules with the potential to acquire new properties and to improve their environmental fitness.

The numerous mechanisms proposed to solve the origin of the first RNA polymers[10−16] are characterized by the plausibility of the mechanistic aspects of polymerization involved. However, the lack of a strong prebiotic relevance due to the need for highly activated precursors works against them. The reported generation of long RNA chains in water from 3′,5′-cyclic AMP and 3′,5′-cyclic GMP[23] and the production of polyA from AMP by repeated hydration−dehydration cycles in a lipid environment[15] potentially point to a possible solution. The reported abiotic synthesis of acyclonucleosides[30] and nucleosides[5,6] and the possibility of obtaining cyclic nucleotides by abiotic phosphorylation of nucleosides[7−9] provide a potential solution to the problem of precursor supply. The onset of evolution of sequence information, potentially leading to complexity, or the generation of homogeneous RNA oligomers (as in the case of polyG and polyA)[23] or their terminal ligation (as in the case of PolyA)[24] indicates the path to genetic information.

A system potentially capable of entering this path requires a proof of principle for the possibility of combining the sequences existing in the pool. In partial fulfilment of this necessity, the instances of covalent combination reported here result in the generation of long covalently bound molecules from simpler shorter sequences. The reactions described here involve robust properties of simple RNA sequences and point to the intrinsic ability of RNA to afford sequence complexity.

Complexity in this case is not an answer to external stimuli. Rather, it derives from rules dictated by the sequences themselves: sequence complementarity or stacking (as in the case of the polyA ligation previously reported)[24] and the ability of the chain backbone to ligate by simple essentially iso-energetic transphosphorylation.

Should this behavior be considered as the emergence of new properties? In the largely accepted definition of this term, emergence is a key concept in complexity theory in which certain features of a complex system occur as a result of the collective behavior of the system. Accordingly, the collective behavior of a given number of RNA sequences could result, if the system emerges, in different properties, as a function of the generated complexity. Given the formal definition (Oxford Dictionary of Sciences) of complexity as "the levels of self-organization of the system" and its accepted attribute (ibidem) that "it is not necessary for a system to have a

large number of degrees of freedom in order for complexity to occur", a population of RNA molecules generated from monomers and able to undergo ligation spontaneously acquires new properties (such as increased resistance to temperature, largely different average sizes, different quantitative relationships between linearity and circularity, etc.), thus approaching the definition of emergence of complexity. The new properties emerging in the system described here are as follows. The first is formation of double-stranded hairpins, introducing properties as increased resistance to the temperature and ribonuclease attack (considering RNases as evolutionary mimics of hydrolysis). The second is largely differing average sizes. Terminal ligation reactions have the potential of unlimited logarithmic size growth. Sequence complementarity-driven terminal growth is conceptually superimposed on replication. The third is increased heterogeneity, resulting in new sequence combinations. The fourth is resistance to circularization. It was pointed out that one specific obstacle to abiotic nucleic acid polymerization is strand cyclization. Chemically activated short oligomers efficiently cyclize, impairing polymer growth.[21,31] Intercalation alleviates this problem.[21] Other possible solutions are provided by duplex formation and the exponential size increase by tandemization. All these reactions occur without external added cofactors or metal ions, thus highlighting the very basic intrinsic catalytic properties of the RNA structure in water.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Enzymatic, kinetic, and chemical characterization of the ligation products is described. Shrimp alkaline phosphatase analysis (Figure S1) determines the position of the labeled phosphate group. RNase A analysis (Figure S2) defines the relative position of the different sequence moieties. The relatively high stability of the hairpinlike 48mer is detailed in Figure S3. MALDI-ToF MS analyses refer to the identification of the $C_{24}$, $G_{24}$, and $G_{24}C_{24}$ oligomers (Figure S4A) and to the 3′-end growth of a $C_{24}$ oligomer in the presence of 3′,5′-cGMP (Figure S4B). These analyses confirm the chemical interpretation reported in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Funding Sources

This work was supported by Italian Space Agency "MoMa project" and by Italian Space Agency National Institute for Astrophysics Grant I/015/07/0 "Esplorazione del Sistema Solare".

## ■ ACKNOWLEDGMENT

We thank Silvia Lopizzo for helpful contributions.

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## Author Contributions

S.P. and G.C. planned and conducted the experiments and the overall project. A.G. performed the MALDI-ToF analyses. E.D. M. planned, interpreted, and wrote.