Review Article

Self-Replicating Systems†

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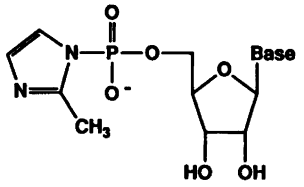
Som considerable time ago, chemistry and physics spawned biology and it is likely that a single molecule existed at that interface. While the attributes of that molecule have stimulated much debate and speculation, one property is agreed upon: its ability to self-replicate. Many researches believe that by looking at today's molecules, RNA and DNA, it is possible to trace back to the structures of the earliest replicators. Others believe that the road is 'washed out', while still others place their faith in synthetic systems as models. In this article, we review some of the work with nuclei acid components as replicating systems and then describe in some detail our own studies.

Self-replicating systems based on oligonucleotides

The past five years have seen increasing efforts to devise nucleotides that replicate without the aid of enzymes. The finding that RNA has the capability to function both as a carrier of genetic information and as a catalyst has inspired much of this research. This discovery has also made RNA the most likely candidate for the first prebiotic self-replicating molecule.[1]

Early model studies by Orgel and coworkers support this theory.[2] When guanosine 5′-phosphorimidazolide **1**, a polycytidylic acid template, and Zn^{2+} catalyst are allowed to react, 3′-5′-linked oligonucleotides longer than $(polyG)_{30}$ form.[3] Under the same conditions, Pb^{2+} efficiently catalyzes the formation of exclusively 2′-5′-linked oligomers.[2e] When poly(C) was incubated with a mixture of equimolar amounts of the activated guanosine G, adenosine A, uracil U and cytosine C monomers, G was incorporated 200 times more efficiently than the 'incorrect' nucleotides. The pentahomopolymer $poly(C)_5$ facilitated the synthesis of mainly G-containing oligonucleotides from a mixture of activated C and G whereby the complementary polyguanylic acid $[poly(G)_5]$ was formed in 17% yield.[2c] When random copolymer templates with C as the major component were used, nucleotides were incorporated into the product when its complement was present. With poly(CU), for example, only **1**-A and **1**-G oligomerized. The products formed had a mean chain length of six nucleotides and the incorporation of the nucleotides occurred with very high fidelity.[2d] Limited regions of each template were found to be copied more accurately than others, leading to the conclusion that only a restricted range of oligomers were efficient templates. In these examples, the newly synthesized strand does not separate from the template strand, so template molecules are not used more than once.



1 Base = U, C, A or G

An example of self-replicating oligonucleotides was given by von Kiedrowski, who showed the self-replicating capability of a protected hexadeoxynucleotide,[4] the palindromic template, 5′-H_3CO-d(CpCpGp-CpGpGp)-O-(2-ClC_6H_4) **2**, was formed in presence of the carbodiimide ethyl(dimethylaminopropyl)carbodiimide (EDC) **3** [CH_3CH_2-N=C=N-$(CH_2)_3$-$N(CH_3)_2$] from the complementary trideoxy-nucleotides 5′-H_3CO-d(CpCpGp) **4** and 5′-HO-d(CpGpGp)-O-(2-Cl-C_6H_4) **5** to a small extent via an autocatalytic pathway. The reaction order for **2** in the kinetics of its own formation was 1/2, and the amount of product synthesized increases with the square root of the template concentration. The major by-product was the pyrophosphate ^{3}p-GGC-$^{5'}$pp-$^{5'}$CGGp$^{3'}$, resulting from the self-condensation of **5**. When the more nucleophilic 5′-H_2N-d(CpGpGp)-O-(2-Cl-C_6H_4) **6** was used instead of **5**, together with the template **2**, the related aminotemplate was found to be the only product. Kinetic studies of this system revealed that the time course of the formation of **2** follows a sigmoidal curve.[3c]

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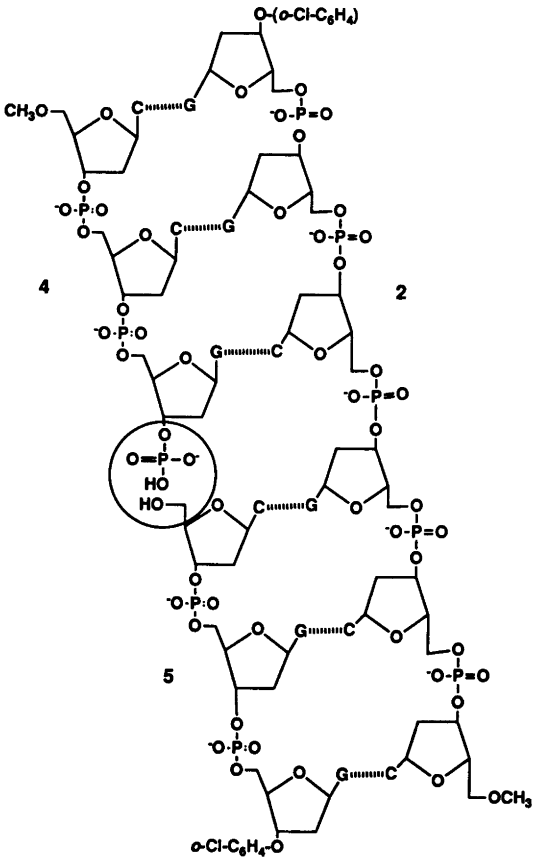
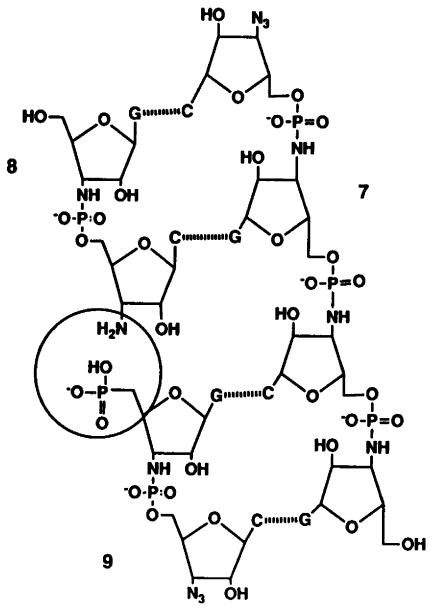
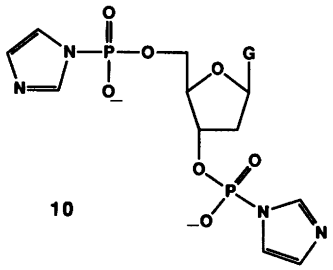


Similarly, Zielinski and Orgel[5] showed the autocatalytic synthesis of the tetranucleotide triphosphoramidate GNHpCNHpGNHpCN$_3$ **7** from the dinucleotide analogs GNHpCNH$_2$ **8** and pGNHpCN$_3$ **9** using the same carbodiimide activator **3**. The presence of **7** significantly increases the rate of its own formation. This system exhibits essentially the same autocatalytic properties as the systems studied by von Kiedrowski.[4]



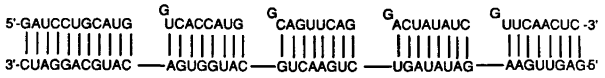
By using an oligonucleotide template based on pyrophosphate linkages, the synthesis of the corresponding pyrophosphate product strand was achieved by Visscher *et al.*[6] The oligomerization of the monomer **10** was studied in the absence and in the presence of the template poly-d $(CppC)_{1-4}$ **11**. The cyclization of the monomer was the primary reaction in the absence of the template. When **11** was present, the total yield of oligomers doubled, including the formation of pyrophosphate-linked oligomers of at least nine nucleotides in length. Nucleic acid analogs with a pyrophosphate backbone were proposed as the possible precursors of the first RNA molecules,[7] which makes this result especially relevant for theories on the origins of life.



An approach towards a self-replicating RNA was described by Doudna and Szostak, who demonstrated the synthesis of a complementary strand RNA on an external template, catalyzed by the *Tetrahymena* ribozyme.[8] The key step was the separation of the template region of the ribozyme (the stem loop of the isolated internal guide sequence) from the catalytic center (the part of the RNA that catalyzes the ligation reaction). The reactants were short oligonucleotides, nine to ten bases in length. These oligonucleotides line up on the external template, and are ligated by the catalytic part. The consequence of the separation of the template region from the catalytic center is a weakened interaction of the substrates with the catalytic region. After ligation, the complementary strand pairs with the template, and the guanosine formed during the ligation is rapidly released from the enzyme. This enables another catalytic cycle to begin. The full-length 40 nucleotide product strand, however, was obtained in only 1 % yield. Such low efficiency makes it impossible to realise self-replication of a replicase as large as the *Tetrahymena* ribozyme (413 bases in length).

A remarkable improvement of this reaction was achieved with a modified version of the self-splicing *sunY* intron from bacteriophage T4.[9] For this study, the intron was divided into three short RNA fragments, 59, 75 and 43 bases in length. When five oligonucleotide substrates complementary to one of the mutant sun*Y* subunits were used, the multisubunit ribozyme catalyzed the synthesis of the subunit's own antisense strand. Ligation of the substrates even occurred when no additional template beyond that in the enzyme complex itself was added. Thus, the enzyme complex was sufficiently stable to function as a ligase, in

the presence of the synthesized antisense strand, while some fraction was unfolded enough to allow the annealing of the substrates. These experiments suggest that it might be possible to assemble RNA oligomers of modest length, which can assemble to give a RNA replicase.



Replication in synthetic model systems

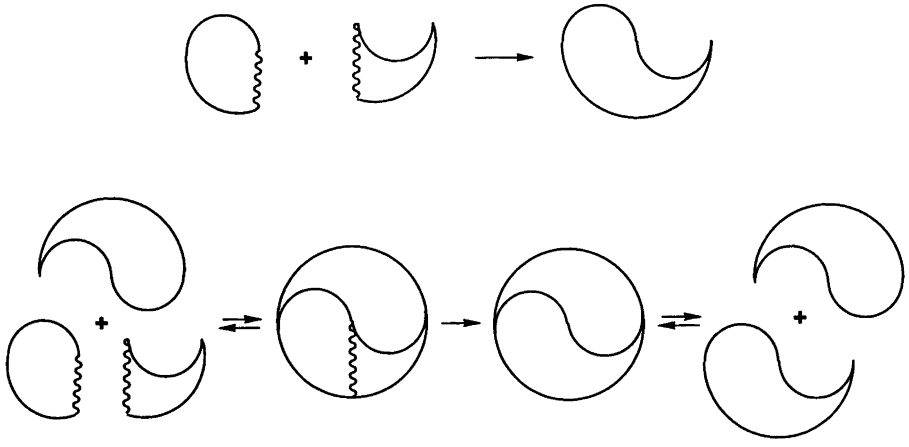
In our laboratory, we have chosen to forsake oligonucleotides in favor of simpler synthetic molecules of our own design. We use Nature as an example of what is possible, rather than as a blueprint. Part of the challenge is taking words such as regulation, catalysis, recognition and replication and working out what structural features might result in these behaviors. These words have no *structural* content, and its up to the imagination of the researcher to create systems or molecular devices that express such phenomena. Our approach is minimalist, one that defines the simplest requirements. We have identified that replication invariably involves molecules that are complementary. One could even say that a principle of self-complementarity exists. We take comfort in the possibility that this idea, like so many others of bio-organic chemistry, may be traced to Linus Pauling.[10]

In Scheme 1, we show a simple cartoon, a two-dimensional example of how self-complementarity operates. Within the circular figure, the sigmoid line represents a weak intermolecular contact between the two self-complementary (and here, identical) components. If these units are broken along the jagged line, the fusion of any of the new pieces leads to a shape that can act as a template for the assembly of an identical subunit. At first glance, then, such simple notions should be easily reduced to real structures and we are somewhat embarrassed by the amount of time that it took us to do so. Let us begin with the intermolecular forces and the general types of molecules involved.

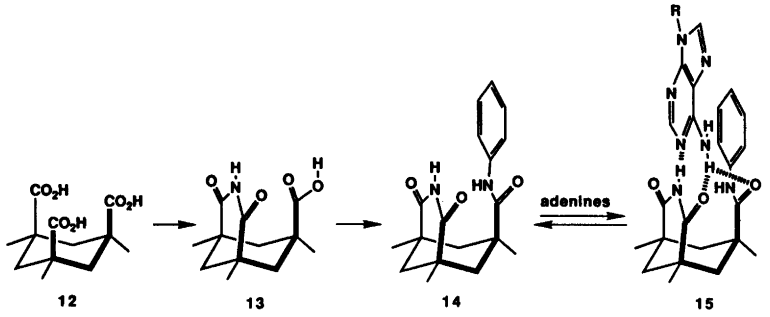
Like others, we have used base pairing to provide the recognition and the organization of the two components with respect to one another. The binding event involves both hydrogen bonding and aryl stacking interactions in organic solvents.[11] We make no apologies for working in non-aqueous media. Indeed, it has been quite useful in exposing the subtle and intrinsic, such as secondary, effects[12] in hydrogen bonding patterns that are so difficult to observe in aqueous solution. Moreover, the magnification of association constants available in non-competing solvents such as chloroform ensure high levels of association. As a result, it contracts the timescale of our experiments to manageable lengths. To be sure, chloroform is not likely to be the prebiotic soup, but so what? We attribute the difficulties encountered by previous workers to their insistence on the use of aqueous media. There, association effects are generally so small that only very long reaction times and large structures could reveal their expressions in self-replication.

The specific structures involve derivatives of Kemp's triacid **12**[13] (Scheme 2) and feature both hydrogen bonding edges and aryl stacking interactions. These converge from perpendicular directions in receptor **14** and provide a complementary microenvironment to adenine derivatives. We have spent considerable effort in mapping how the two components fit together and these are described in experimental detail elsewhere.[14] For the present purposes, structure **15** represents these systems adequately. Base pairing is shown in the Watson–Crick sense, and a bifurcated hydrogen bond is involved in binding the amino function of adenine. These complexations are very rapid on the NMR timescale at room temperature. Association constants are on the order of 100 M^{-1} and involve contributions from not only Watson–Crick, but also Hoogsteen base-pairing modes.

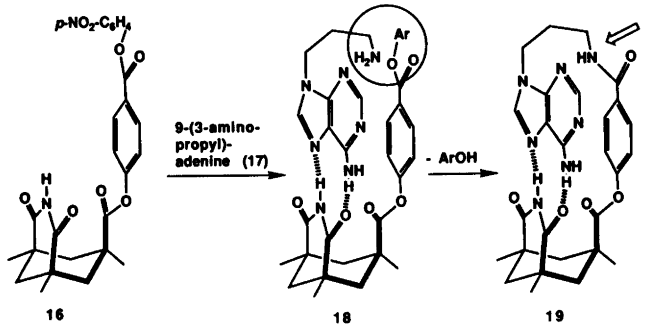
The complementary binding surfaces provide the free energy for association and it should have been an easy



Scheme 1.



Scheme 2.

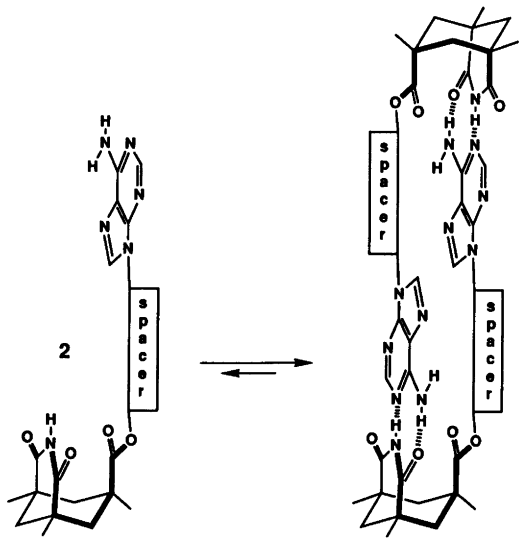


Scheme 3.

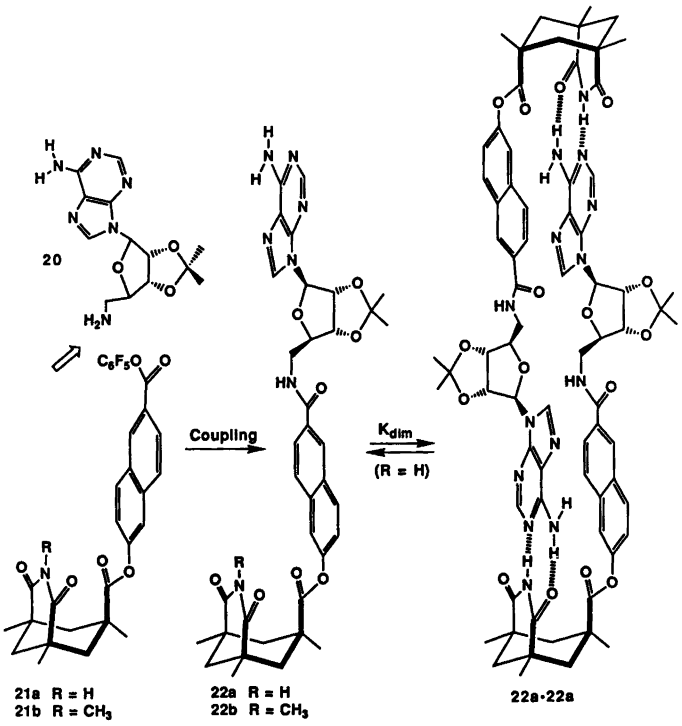
matter to position complementary *reactive* functionality in both components that could lead to a covalent bond to be formed within the complex. Nonetheless, our early attempts were dismal failures. For example, base pairing does take place between the 9-(aminopropyl)adenine **17** and receptor **16**, and intramolecular aminolysis does occur. However, the resulting product **19** remains folded shut (Scheme 3). The short aryl spacer can accommodate a perfectly stable *trans* amide bond while maintaining base pairing, specifically as shown in the Hoogsteen sense. These intramolecular contacts were established by NOE experiments.

Our second system also failed, and it seemed like the whole project would face early extinction. Here, the product molecules remained stuck together in a bimolecular fashion as the dimer. Though the spacer was longer, the self-affinity of the product was so high that its ability to act as a template was severely limited. Nonetheless, the general shape of structures (shown in Scheme 4) suggested that building a large bulge in the center of the molecules might diminish their self-affinity. Specifically, the 5′-aminoadenosine[15] derivative **20** was prepared and coupled to an active ester bearing a naphthalene surface **21a**.[16] The expectation was that the ribose acetonide subunits of the resulting adduct **22a** would provide spherical, lumpy bulges, and destabilize the dimer **22a** · **22a**.

This indeed proved to be the case. For example, the dimerization constant of **22a** was a manageable 630 M^{-1}. Even simpler systems established that base pairing for individual components was on the order of 60 M^{-1}. Since the two ends of the structure were not expected to communicate, one might have anticipated an association constant for the dimer of greater than $(60)^2$. That is, the chelate effect would enhance the association, since translational entropy involved in each base pairing would not have to be paid for twice. Apparently, the bulge was doing its job. As for geometry, NOE experiments showed no particular bias in the arrangement of the dimer; both Watson–Crick (Scheme 5, **22a** · **22a**) and Hoogsteen (not shown) senses were represented among the populations of dimeric molecules. For



Scheme 4.



Scheme 5.

the active ester component, the pentafluorophenyl derivative gave coupling rates that were on a compassionate and humane timescale. HPLC provided reasonable accuracy to determine the rate of product formation.

Preliminary studies established that added product template **22a** indeed speeded up the reaction of **20** and **21a**, whereas addition of 2,6-(diacetylamino)pyridine slowed the coupling rate down. Moreover, the corresponding *N*-methyl imide derivative **21b**, incapable of base pairing, showed greatly reduced coupling rates. The initial rates of product formation were linear. The results are plotted in Fig. 1.

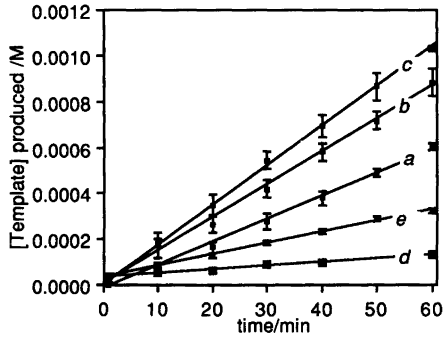


Fig. 1. Generation of the template **22a** or **22b** as a function of time. All reactions were performed with initial concentrations: [**20**] = [**21a** or **b**] = 0.0082 M in $CHCl_3$. Lines are linear least-squares fit of data, and correspond to the initial rates of reaction. Error bars represent standard deviations of multiple, independent runs. (*a*) Reaction of **20** and **21a**; (*b*) reaction of **20** and **21a** with 0.20 equiv. of **22a** added as an autocatalyst; (*c*) reaction fo **20** and **21a** with 0.50 equiv. of **22a** added as an autocatalyst; (*d*) reaction of **20** and the *N*-methylated **21b** (single run); (*e*) reaction of **20** and **21a** with 1 equiv. of 2,6-di(acetylamino)pyridine added as an inhibitor.

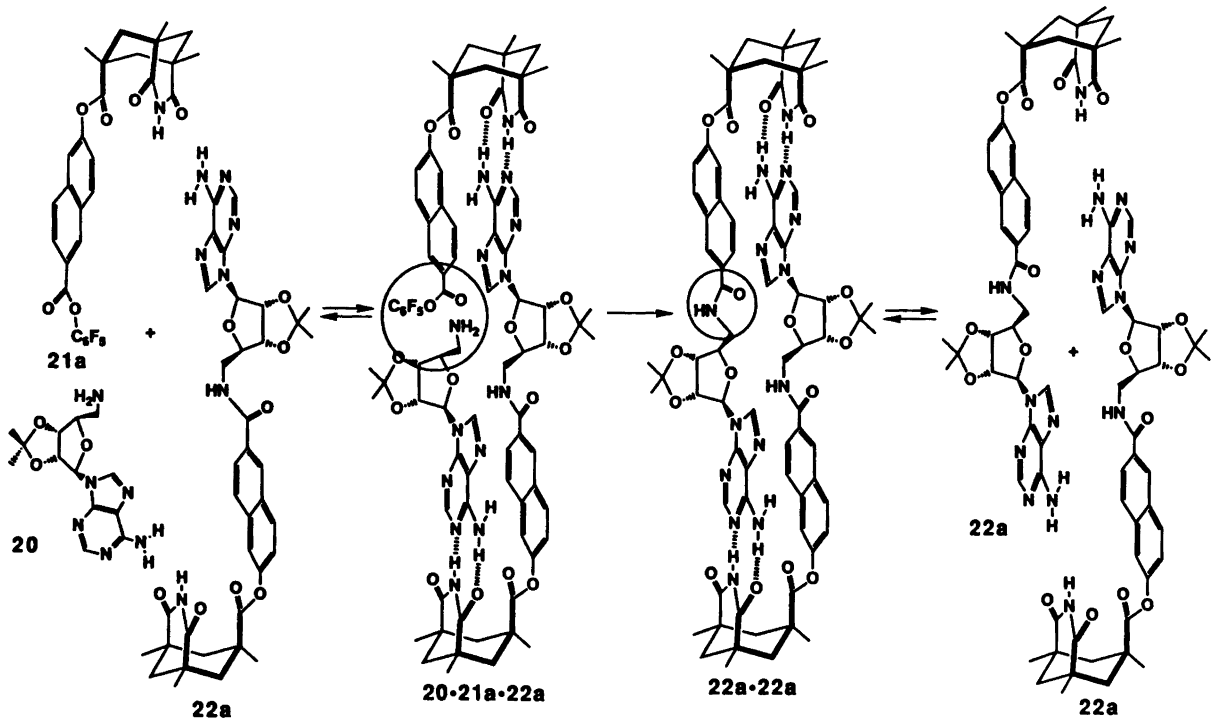
These results indicate that self-replication is occurring. Preassociation of the reactants and template to form the complex **20** · **21a** · **22a** leads to intramolecular acyl transfer in the coupling event (shown in Scheme 6). *This is the act of replication,* since the new structure is an identical copy of the template.

In actuality, three distinct mechanisms generate template **22a**: (1) a background bimolecular reaction, in which aminolysis takes place without base pairing; (2) a base-paired bimolecular reaction, in which the amine **20** and ester **21a** form a hydrogen-bonded dimer **20** · **21a** prior to reaction; (3) the termolecular or template-catalyzed process (shown in Scheme 6), in which the amine **20**, ester **21a** and template **22a** form a trimeric complex **20** · **21a** · **22a** prior to reaction. The following equations represent these pathways. Only the latter pathway is responsible for self-replication and sigmoidal product growth. The observed kinetic curves are a result of the interplay of all three reactions.

$$\mathbf{20} + \mathbf{21a} \xrightarrow{k_1} \mathbf{22a}$$

$$\mathbf{20} + \mathbf{21a} \rightleftarrows \mathbf{20 \cdot 21a} \xrightarrow{k_2} \mathbf{22a}$$

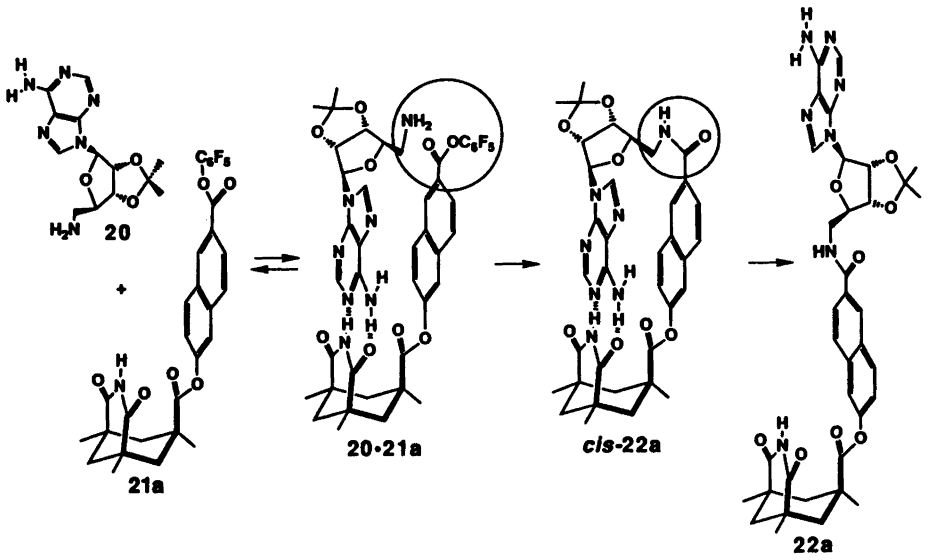
$$\mathbf{20} + \mathbf{21a} + \mathbf{22a} \rightleftarrows \mathbf{20 \cdot 21a \cdot 22a} \xrightarrow{k_3} \mathbf{22a \cdot 22a} \rightleftarrows \mathbf{22a} + \mathbf{22a}$$



Scheme 6.

The base-paired mechanism involves complex **20** · **21a**, in which the amino and pentafluorophenyl groups are brought into proximity and react readily to generate the template. Because of the length of the aromatic spacer (the napthalene), the product is initially formed in the less stable *cis*-amide conformation. Isomerization to the *trans* conformation follows. Scheme 7 summarizes these processes. In contrast, the termolecular process *initially* generates the template *trans*-amide conformation.

From the experiments carried out with varying amounts of added template, it is possible to extract kinetic parameters for all three reactions.[17] Analysis of the data presented in Fig. 1 afforded rate constants that fit the data quite well. The background bimolecular reaction occurs with $k_1 = 0.023\ M^{-1}\ min^{-1}$, the bimolecular base-paired process with $k_2 = 0.0036\ min^{-1}$, and the template-catalyzed process with $k_3 = 0.070\ min^{-1}$. This permits a calculation of the effective molarity of the reacting groups in the various complexes. For example, the effective molarity in the termolecular complex is actually higher (3.0 M) than in the



Scheme 7.

bimolecular complex (0.16 M). This 19-fold difference probably reflects the higher activation energy involved in the formation of the *cis*-amide bond.

Although the effective molarity in the template termolecular process is considerably higher than in the base-paired bimolecular process, a very large fraction of the product is formed by the bimolecular pathway. This occurs because of the difference in molecularity of the two pathways. For example, at 10 millimolar total concentration of **20** and **21a**, and 3 millimolar total concentration of template, 67% of the amine and ester are free, 27% are associated, and only 1.5% are present as a termolecular complex. Under these conditions, the relative contributions of the three reactions are 1.0, 4.3, and 4.6, for the background, base-paired and termolecular components, respectively.

Although the reaction of **20** and **21a** is autocatalytic, it does not exhibit a sigmoidal product-growth curve. Longer reaction times and higher concentrations of the reactants failed to show the expected sigmoidal behavior. For example, Figs 2 and 3 show the results of increased time and concentration. Perhaps this is not surprising; von Kiedrowski[3] and Orgel[4] have also found it quite difficult to observe sigmoidal growth in related systems. Nonetheless, we were convinced that it was possible to devise a system that exhibited sigmoidal growth.

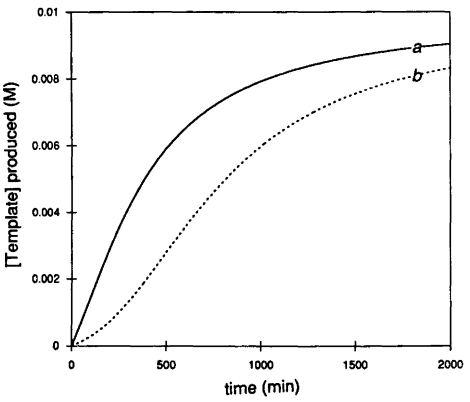


Fig. 4. Calculated effect of the removal of the base-paired bimolecular pathway upon the growth curve. All calculations were performed for initial concentrations [**20**] = [**21a**] = 0.01 M. (*a*) Present system in which background bimilecular, base-paired bimolecular, and template termolecular pathways operate (k_1 = 0.023 M^{-1} min^{-1}, k_2 = 0.0036 min^{-1}, k_3 = 0.070 min^{-1}); (*b*) system in which only background bimolecular and template termolecular pathways operate (k_1 = 0.023 M^{-1} min^{-1}, k_2 = 0, k_3 = 0.070 min^{-1}).

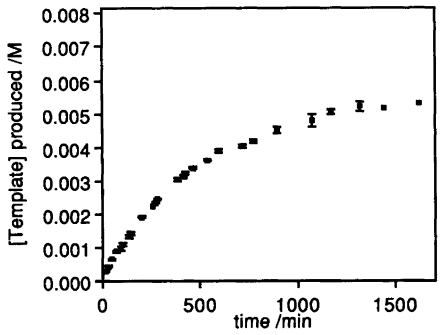


Fig. 2. Generation of the template **22a** as a function of time. Initial concentrations: [**20**] = [**21a**] = 0.0082 M in $CHCl_3$. Error bars represent standard deviations of multiple, independent runs.

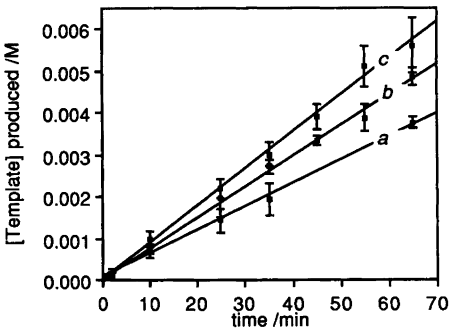
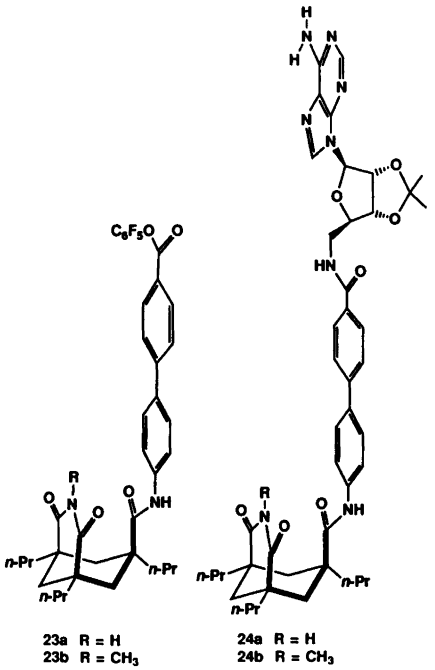


Fig. 3. Generation of the template **22a** as a function of time. All reactions were performed with initial concentrations [**20**] = [**21a**] = 0.0165 M in $CHCl_3$. Lines are linear least-squares fits of data, and correspond to the initial rates of reaction. Error bars represent standard deviations of multiple independent runs. (*a*) Reaction of **20** and **21a**; (*b*) reaction of **20** and **21a** with 0.20 equiv. of **22a** added as an autocatalyst; (*c*) reaction of **20** and **21a** with 0.55 equiv. of **22a** added as an autocatalyst.

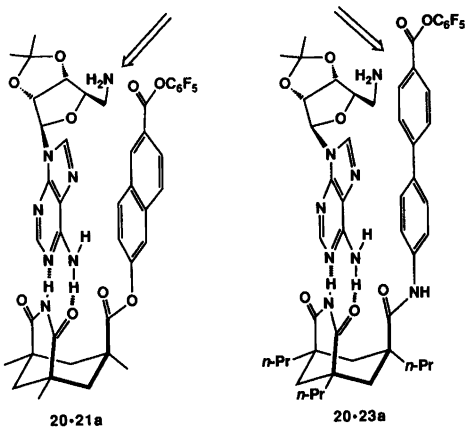
The sigmoid behavior characteristic of an autocatalytic pathway is expected only in systems where *most of the product* is formed by way of autocatalysis. Calculations suggested that increasing the concentration of reactants would increase the contribution of autocatalysis. However, the limiting solubility of these systems prevented us from reaching the appropriate concentrations. If the base-paired bimolecular pathway were not operative a noticeably sigmoidal growth curve would be predicted. Fig. 4 shows the calculated effect of shutting down this reaction pathway. Curve *a* corresponds to the the present system, whereas curve *b* corresponds to a system in which the base-paired pathway is lacking.

Accordingly, we have restructured the system to reduce the bimolecular base-paired pathway. We used the simple artifice of replacing the hydroxynaphthyl spacer with an even longer aminobiphenyl group. Structures **23** and **24** represent the pentafluorophenyl ester and template components in this system. Although the ester can still base-pair with aminoadenosine **20**, the reactive amino and pentafluorophenyl ester functions reach each other only with difficulty (structure **20** · **23a** vs. structure **20** · **21a**). The tripropyl analog[18] of Kemp's triacid was used as the structural scaffold, to enhance further the solubility of these systems.

This system exhibits modest sigmoidal growth curves.[19] Fig. 5 illustrates the formation of template upon reaction of aminoadenosine **20** with the biphenyl imide **23a** or the *N*-methyl imide **23b**. Curve *a* corresponds to the formation



Scheme 8A.



Scheme 8B.

of template **24a**, and is distinctly sigmoidal. Curve *b* represents the formation of **24b**, and is typical of a simple bimolecular reaction. Only the NH derivatives, which are able to participate in base-pairing, exhibits sigmoidal behavior. Furthermore, addition of 0.20 equivalents of the template enhances the coupling rate by more than a factor of nearly two (Fig. 6). The system exhibits efficient autocatalysis!

It is interesting to note that the structural modifications on going from the naphthyl to the biphenyl spacer do not substantially affect the rates of the background bimolecular and template termolecular processes. The biphenyl spacer does change the position of the ribose bulges, however, and the dimerization constant ot the biphenyl template **24a** is considerably larger than that of the naphthyl template **22a** (80000 m^{-1} vs. 630 M^{-1}). This observation has led us to reconsider the effect of a large dimerization constant upon the efficacy of replication. The dimerization constants of the replicating oligonucleotides of von Kiedrowski and Orgel are obviously quite large. The same forces that result in better binding of the template will also lead to better binding of the transition state. We are currently developing self-replicating systems that employ Hoogsteen and Watson–Crick binding simultaneously, in order to develop more efficient replicators.

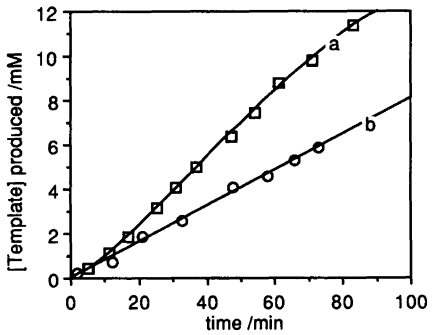


Fig. 5. Plots of appearance of **24a** and **24b** vs. time as determined by HPLC. Initial concentrations of **20**, **23a** and **23b** were 50 mM in $CHCl_3$ with 9 equiv. Et_3N added. (*a*) Reaction of **20** and **23a**; (*b*) reaction of **20** and **23b**. Boxes represent approximate uncertainty (± 0.1 mmol).

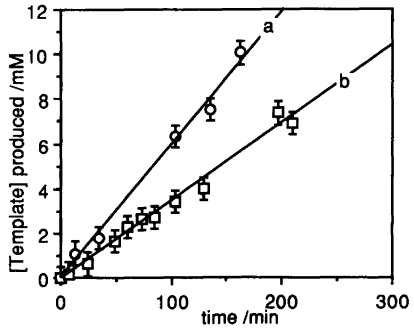
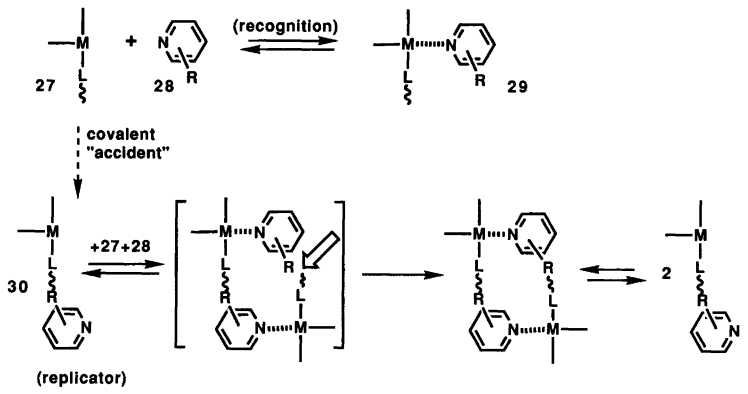


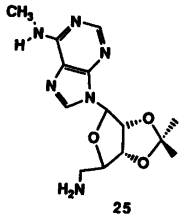
Fig. 6. Plots of initial product formation of **24a** vs. time as determined by HPLC. Initial concentrations of **20** and **23a** were 25 mM in $CHCl_3$ with 9 equiv. Et_3N added. (*a*) Reaction of **20** and **23a** with 0.2 equiv. of template **24a** added. (*b*) Reaction of **20** and **23a** without added template.



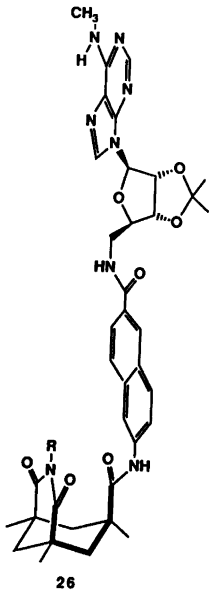
Scheme 9.

Prospects for the future

Can such systems show evolution? The ability to make mistakes and mutate into more or less effective replicators appears to be one of the requirements for evolution, and we have made some preliminary progress on this goal. For example, using the *N*-methyladenine derivative **25**, a second replicator **26** has been developed.[20] In this compound, the *N*-alkyl group serves to limit the number and quality of base-pairs to largely Hoogesteen senses. The unsubstituted adenine derivative **22a** can also catalyze the formation of the other replicator **26**. However, in experiments in which both **25** and **20** compete in the same flask for the active ester **21a**, the unsubstituted adenine **20** is the more effective replicator; its population grows at the expense of the *N*-methylated product. It can catalyze its own formation in both Watson–Crick and Hoogsteen senses, whereas the *N*-methyladenine **25** is restricted.



25



26

Our current plans involve the introduction of mutational-type events that alter the efficiency of replicating systems. In principle, changes in temperature, pH, salt concentration, etc., could be used to trigger such an event. Our plan is to use radiation. Specifically, a photochemically removable blocking group has been incorporated into an adenine nucleophile, and the competition of the two replicators is quite evenly matched. However, shining light on the system and cleaving the photo group introduces a new, *unsubstituted* adenine which can rapidly take over because of its superior base-pairing abilities.

We conclude that designing replicators is easier than once believed and we point out a simple principle that can be used to generate replicators: covalent attachment of a host to its guest is all that is required. Tethering a crown ether to an ammonium ion, a cyclophane to an aromatic, or a cyclodextrin to an aryl all give self-complementary systems that could act as templates for their own assembly. The situation holds even for metals **27** and their ligands **28**; the facile rise of a replicator **30** is depicted in Scheme 9.

Other research in the group has as its goal the use of the informational content of base pairing to help drive acyl-transfer reactions. The intent is to catalyze the formation of peptides with more recognizable shape and functions, i.e. to develop a simple genetic code. We will report on these in due course.

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3. *Abbreviations:* In the oligonucleotides discussed here the bases are abbreviated as follows: G = guanine, A = adenine, T = thymine, C = cytosine, U = uracil; polyhomonucleotides = $(polyN)_x$ [N = A, G, C or T; x = number of bases]; polyheteronucleotides = $(NpN)_x$, deoxyheterooligonucleotides = $d(NpN)_x$, polypyrophosphate linked deoxyoligomers = poly-$d(NppN)_x$.
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