Dynamic assembly of primers on nucleic acid templates

Nicole A. Leal¹,*, Makoto Sukeda² and Steven A. Benner¹,3,*

¹Foundation for Applied Molecular Evolution, Ayers Medical Plaza, Suite 208, 720 SW 2nd Avenue, Gainesville FL 32601, USA, ²Firebird Biomolecular Sciences, Inc., Gainesville Technology Enterprise Center, 2153 Hawthorne Road, Gainesville, FL 32641-7553, USA and ³The Westheimer Institute for Science and Technology, Ayers Medical Plaza, Suite 200, 720 SW 2nd Avenue, Gainesville FL 32601, USA

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ABSTRACT

A strategy is presented that uses dynamic equilibria to assemble in situ composite DNA polymerase primers, having lengths of 14 or 16 nt, from DNA fragments that are 6 or 8 nt in length. In this implementation, the fragments are transiently joined under conditions of dynamic equilibrium by an imine linker, which has a dissociation constant of $\sim$1 μM. If a polymerase is able to extend the composite, but not the fragments, it is possible to prime the synthesis of a target DNA molecule under conditions where two useful specificities are combined: (i) single nucleotide discrimination that is characteristic of short oligonucleotide duplexes (four to six nucleobase pairs in length), which effectively excludes single mismatches, and (ii) an overall specificity of priming that is characteristic of long (14 to 16mers) oligonucleotides, potentially unique within a genome. We report here the screening of a series of polymerases that combine an ability not to accept short primer fragments with an ability to accept the long composite primer held together by an unnatural imine linkage. Several polymerases were found that achieve this combination, permitting the implementation of the dynamic combinatorial chemical strategy.

INTRODUCTION

A general problem is associated with architectures to detect specific nucleic acids in complex genomic environments. First, as there are 4ⁿ different sequences of length n within a target genome G nucleotides in length, a probe that is specific for a single sequence must have (on average) a length L (nucleotides) given by the formula $L \approx (\log G)/0.6$. Thus, as the human genome has $\sim 5 \times 10^{9}$ nt sequences, a probe that is 16 nt long will bind, on average, to just one sequence within that genome.

Such calculations suggest that a probe must be 16 nt in length to seek a specific DNA segment in a human genome. Unfortunately, for duplexes of this length under standard hybridization conditions, single mismatches depress the melting temperatures only slightly. Further, the AT and GC nucleobase pairs have different intrinsic affinities, and contribute to duplex stability differently depending on the local ‘sequence context’. Together, this means that a duplex built from a pair of two 16mers having two, three or occasionally more mismatches can easily be more stable than another duplex built from a pair of two perfectly matched 16mers. This creates difficulties throughout the analytical chemistry of nucleic acids, especially in complex biological mixtures.

Of course, if the duplex is shorter, then any pair of perfectly matched sequences will form a more stable duplex than any pair of duplexes that fail in complementarity by a single mismatch. For DNA–DNA duplexes under standard hybridization conditions, this is met by duplexes as short as six nucleobase pairs. These, however, lack specificity in the human genome (a 6mer is found on average a million times in the human genome).

Two recent developments suggest a possible way to obtain primers that bind with the specificity of a 16mer, but display the discriminatory power of a 8mer (e.g.). The first is dynamic combinatorial chemistry. This technology, invented independently by Benner (1), Sanders (2) and Lehn (3), presents a library of n ligand fragments to a target. Each of these fragments can reversibly form a covalent bond with another fragment in the library to form a composite, all in the presence of the receptor under conditions of dynamic equilibrium.

Although not originally conceived to apply to nucleic acids, dynamic combinatorial chemistry can potentially be used in DNA-targeted analytical chemistry. This differs from the irreversible template ligation suggested by Kool (4), von Kiedrowski (5,6), Ellington (7) and others, in that the reversible ligation permits the system to achieve the thermodynamically preferred combination without kinetic traps.

*To whom correspondence should be addressed. Tel: +1 352 271 7005; Fax: +1 352 271 7076; Email: nleal@ffame.org
*Correspondence may also be addressed to Steven A. Benner. Tel: +1 352 271 7005; Fax: +1 352 271 7076; Email: sabenner@ffame.org
Present address:
Makoto Sukeda, Japan Association for International Chemical Information (JAICI), 6-25-4 Honkomagome Bunkyo-ku Tokyo 113-0021, Japan

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Therefore, dynamic combinatorial assembly offers the opportunity to assemble a long composite oligonucleotide fully complementary to a short template from, in principle, an unlimited number of DNA fragments under reversible conditions. Schematically, one possible implementation of DNA-targeted dynamic combinatorial chemistry that joins two fragments is shown in Figure 1a, and with greater chemical detail in Figure 1b.

The chemical details are adapted from work by Lynn and coworkers (8–10). These scientists were interested in

Figure 1. (a) Architecture of an assay for a DNA template that dynamically assembles its own composite primer from two fragments. Two DNA fragments, each 8 nt in length, terminated with 3'-CH2CHO (on the 5'-DNA fragment) and 5'-NH2 (on the 3'-DNA fragment) should reversibly form a composite, joined via an imine linker under conditions of dynamic equilibrium. Imine formation is easily reversible in aqueous conditions, and this reversibility ensures that the tightest binding complement perfectly matched to the template is formed. Should this composite be able to prime the synthesis of DNA using a DNA polymerase, the specificity of priming should be characteristic of a 16mer (and therefore unique in the human genome), as both sequences must be adjacent on the template for priming to occur. The discrimination against mismatches, however, should be that characteristic of an 8mer, and therefore be very high. (b) Details of the chemical implementation of this architecture.
exploring alternative ways to create replicating systems capable of Darwinian evolution, in the hope of gaining a better understanding of how life might have emerged on early Earth. They showed that it was possible to assemble, under conditions of dynamic equilibrium, nucleotide trimers using imine chemistry (11). In a broad sense, this is a dynamic combinatorial chemistry experiment where DNA is the target. The assembled trinucleotide fragments and the hexanucleotide composites were too short to bind stably, however. Therefore, Lynn and his coworkers detected the assembly of Darwinian evolution, in the hope of gaining a better understanding of how life might have emerged on early Earth.

Given that the disassociation constant of the imine intermediate is ~1 μM, if a polymerase could be found to accept primers that have an unnatural imine linkage, an architecture for selective priming of DNA might be constructed by Lynn and his coworkers (10), adapted as described below. To a mixture of 3'-deoxy-3'-C-allyl-5'-O-[cyanoethyl]-N,N-diisopropylamino)phosphinyl)thymidine (5), the building block for the synthesis of the 3'-terminal aldehydes unit.

This compound was prepared using the procedure described by Lynn and coworkers (10), adapted as described below. To a mixture of 3'-deoxy-3'-C-allyl-5'-O-[cyanoethyl]-N,N-diisopropylamino)phosphinyl)thymidine (5), the building block for the syntheses of the 3'-terminal aldehydes unit.

**MATERIALS AND METHODS**

**Synthesis of 3’-terminal fragments having a 5'-deoxy-5'-aminonucleoside**

The 3'-terminal fragments, each carrying a 5'-deoxy-5'-amino nucleoside, were prepared by standard solid phase synthesis using an Expedite 8900 DNA synthesizer according to standard protocols. Phosphoramidites, including the 5'-amino nucleoside analog where the 5'-amino group was protected as a 5'-monomethoxytrityl ether, were purchased from Glen Research. The final detritylation of the protected amine was done with 2% dichloroacetic acid for 15 min. The product was purified by high-performance liquid chromatography (HPLC) after the 5'-monomethoxytrityl group was removed.

**Synthesis of 5’-terminal fragments having a 3'-deoxy-2'-carbonylmethylene unit**

The 5'-terminal fragments (8, 10, 12) were obtained using standard DNA synthesis done in the unconventional 5’→3’ direction (13) on an Expedite 8900 DNA synthesizer. Figure 2 shows the scheme for their preparation.

**Polymerases**

Bst, Deep Vent®, Deep Vent® (exo−), Taq DNA polymerase 1 Large (Klenow) Fragment, Klenow Fragment (exo−), 9°N™ and Therminator™ polymerases were purchased from New England Biolabs (Beverly, MA). M-MuLVRT, AMVRT and Tth polymerases were purchased from Promega (Madison, WI). Pfu and Pfu (exo−) polymerases were purchased from Stratagene (La Jolla, CA). Buffers used in these experiments were supplied by the manufacturer. Bst, Taq, Vent, Deep Vent, 9°N and Therminator use Thermopol Buffer (1X): 20 mM Tris–HCl (pH 8.8, 25°C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, Klenow and Klenow exo− use NEB 2 (1X): 50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl₂, 1 mM DTT (pH 7.9, 25°C). AMVRT uses buffer (1X): 50 mM Tris–HCl (pH 8.3, 25°C), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine and 10 mM DTT. M-MuLVRT uses buffer (1X): 50 mM Tris–HCl (pH 8.3, 25°C), 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT.

**Primer extension assays**

For primer extension assays (10 μl reaction volume) formyl 8mer (20 pmol) and/or amine 8 or 6mer (20 pmol) and DNA Template (30 pmol) were annealed by incubation at 96°C for 5 min and allowed to cool to room temperature over 1 h. Polymerases were used at 2 U per reaction with the exception of AMVRT (10 U/reaction) and M-MuLVRT (200 U/reaction). Manufacturer’s supplied buffer and
Polymerase were added to primer template complex and incubated at appropriate temperature for 30 s. Each reaction was initiated by adding dNTP solution (1 μl, 100 μM each; dATP, dTTP, dGTP, and dCTP) and 33 nM [α-32P]dCTP to the reaction mixture (9 μl) and were incubated at appropriate temperatures for 2 min. Reactions were quenched by the addition of 5 μl PAGE loading/quench buffer (98% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF and 1 mg/ml bromophenol blue). Samples (1 μl) were resolved on denaturing polyacrylamide gels (7 M Urea and 20% 40:1 acrylamide:bisacrylamide) and analyzed with a Molecular Imager FX system (BioRad, Hercules, CA).

**Primer extension using alternative templates**

Oligonucleotides (Table 1) were synthesized and PAGE purified from Integrated DNA Technologies (Coralville, IA). These various oligonucleotides have 1 nt mismatch at different sites where the formyl and amine primers anneal. Modified nucleotides are numbered based on their location in the template. The assays were run as described above however the standard template in the reaction mixture was substituted with an alternative template. These primer extensions were run with the formyl 8mer, amine 8mer and M-MuLV RT at 35°C. Additional primer extensions were run with the formyl 8mer, amine 6mer and 9°C 70°C. Samples (1 μl) were resolved on a 20% denaturing PAGE and analyzed with a Molecular Imager FX system.

**Methoxylamine treatment**

After completion of the primer extensions assays an aliquot was treated with methoxylamine hydrochloride (1 M, Sigma) and incubated at 94°C for 1 h. The sample was then resolved on a 20% denaturing PAGE and analyzed with a Molecular Imager FX system.

**RESULTS**

The basic architecture in Figure 1 assembles two fragments: (i) a 3'-fragment, which has an elongatable 3'-OH group and a

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**Figure 2.** Schematic showing the synthesis of oligonucleotides having a CH2CHO unit at their 3' ends. This synthesis follows closely that reported by Lynn and his coworkers (10). Numbers assigned to the oligonucleotides refer to the melting curves in Supplementary Figure A.
Table 1. Oligonucleotides used in this study

| Name | Sequence (5'-3') | Modification |
|------|----------------|--------------|
| T1   | CGTTTATGAGAGTGGG | None         |
| T2   | CGTTTATGAGAGTGGG | 12G to A     |
| T3   | CGTTTATGAGAGTGGG | 12G to T     |
| T4   | CGTTTATGAGAGTGGG | 12G to C     |
| T5   | CGTTTATGAGAGTGGG | 15T to C     |
| T6   | CGTTTATGAGAGTGGG | 15T to G     |
| T7   | CGTTTATGAGAGTGGG | 15T to A     |
| T8   | CGTTTATGAGAGTGGG | 19A to G     |
| T9   | CGTTTATGAGAGTGGG | 19A to C     |
| T10  | CGTTTATGAGAGTGGG | 19A to T     |
| T11  | CGTTTATGAGAGTGGG | 20A to G     |
| T12  | CGTTTATGAGAGTGGG | 20A to C     |
| T13  | CGTTTATGAGAGTGGG | 20A to T     |
| T14  | CGTTTATGAGAGTGGG | 23G to A     |
| T15  | CGTTTATGAGAGTGGG | 23G to T     |
| T16  | CGTTTATGAGAGTGGG | 23G to C     |
| T17  | CGTTTATGAGAGTGGG | 27A to G     |
| T18  | CGTTTATGAGAGTGGG | 27A to C     |
| T19  | CGTTTATGAGAGTGGG | 27A to T     |
| T20  | CGTTTATGAGAGTGGG | 17G to A     |
| T21  | CGTTTATGAGAGTGGG | 17G to T     |
| T22  | CGTTTATGAGAGTGGG | 17G to C     |
| Region copied 3'-terminal | 5'-amino fragment (3'-formyl) Binding site | Binding site |

*Boldface indicates the portions of the template that are matched by the fragments. The vertical line | indicates the junction between the fragment binding sites. The 5'-amino sequence of oligonucleotides is written in lowercase. Each table indicates whether the 3'-terminal fragment binds to the left of the vertical line.*

Table 2. List of oligonucleotide analogs used in this work

| Name | Sequence (3'–5') |
|------|----------------|---------------|
| 5'-Fragment precursor (allyl) | 3'-H2C(:CH2:C:TTTCACT-5') |
| 5'-Fragment precursor (dial) | 3'-HOH2C(:HOH:C:TTTCACT-5') |
| 5'-Fragment (aldehyde) | 3'-OHCH2:C:TTTCACT-5' |
| 3'-Fragment(NH2,5mer) | 3'-AAACCT-NH2-5' |
| 3'-Fragment(NH2,8mer) | 3'-AAAACCT-NH2-5' |

5'-amino group able to form an imine, and (2) a 5'-fragment, where CH2=CHO unit replaces its 3'-OH, permitting it to form an imine with the 3'-fragment. The first is readily available from commercial supply houses. The second, having a CH2=CHO unit at its 3' end, was synthesized from the appropriate precursor, which in turn was prepared by the scheme shown in Figure 2.

For the dynamic combinatorial architecture outlined in Figure 1 to be implemented, polymerases must be found that do not prime from a 3'-terminal fragment by itself, but do prime from it in the presence of the 5'-terminal fragment. Naively, we expected that short DNA molecules would generally not prime if the temperature of the polymerase incubation was sufficiently higher than the melting temperature for the short primer–template duplex. Examination of a variety of polymerases showed that this was not the case with all polymerases (Figure 3). Thus, many polymerases were able to use short 8mer DNA molecules as

**Figure 4.** Primer extension assays using a complementary 3'-terminal 5'-amino-8mer and 5'-terminal 3'-aldehyde- 8mer with M-MuLVRT, Pfu, and Pfu exo polymerases, selected based on the data in Figure 3. Final concentrations: dNTPs (100 µM each, for A, T, G, C, with 33 nM [α-32P]dCTP) template (30 pmol, 3 µM), and 3'-terminal 8mer fragment carrying a 5'-amino group (20 µmol, 20 pmol). The mixtures containing the indicated polymerase were brought to the indicated temperature for 30 s and dNTPs were added. The mixtures were incubated at the indicated temperatures for 2 min. Reactions were terminated with quench buffer (5 µl, formamide EDTA, dyes, dilution factor of 33%). An aliquot (1 µl) was loaded on a polyacrylamide gel (20%, 7 M urea) and resolved. Surprisingly, a large number of polymerases extended the 8mer amine at temperatures well above the melting temperature of the duplex (Supplementary Figure A).
primers at temperatures above the nominal melting temperature of the DNA duplexes. Only M-MuLV reverse transcriptase rejected the 8mer primer entirely, although Pfu exo\(^+\) generated only traces of full-length product at 55°C, and no detectable product at higher temperatures.

For the remaining polymerases, significant amounts of priming were observed at temperatures above the nominal melting temperatures. In most cases, the amount of priming decreased as the temperature was raised. With various Klenow fragments, whose thermal instability prevented

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**Figure 5.** Mismatch discrimination using M-MuLVRT. PAGE gel (20%) of primer extension assays with mismatched DNA 38mer templates using M-MuLVRT. (A and B) contains the mismatches in the site of binding of the 3'-fragment 8mer and 5'-fragment 8mer, respectively (see Table 1 for details of mismatches). Final concentrations: dNTPs (100 \(\mu\)M each, for A, T, G, C, with 33 nM \(\alpha\)-\(\text{\textsuperscript{32}}\)PdCTP) template (30 pmol, 3 \(\mu\)M), 3'-terminal 8mer fragment carrying a 5'-amino group (20 pmol, 2 \(\mu\)M), and 5'-terminal 3'-aldehyde-8mer (20 pmol, 2 \(\mu\)M). The reaction mixtures were brought to 35°C (30 s) and dNTPs were added. The mixtures were incubated for 2 min at the appropriate temperature. Reactions were terminated with quench buffer (5 \(\mu\)l, formamide EDTA, dyes, dilution factor of 33%). An aliquot (1 \(\mu\)l) was loaded on a polyacrylamide gel (20%, 7 M urea) and resolved. The 27mer band comes from residual imine. The lanes are paired, with, following the amine fragment lacking the formyl, fully matched, one nucleotide mismatched.

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**Figure 6.** Seeking a 3'-terminal fragment that does not prime by itself (A and B). Screening polymerases for 8 + 6 extension with just the 3'-terminal fragment carrying a 5'-amino group. Final concentrations: dNTPs (100 \(\mu\)M each, for A, T, G, C, with 33 nM \(\alpha\)-\(\text{\textsuperscript{32}}\)PdCTP), template (30 pmol, 3 \(\mu\)M), and 3'-terminal 6mer fragment carrying a 5'-amino group (20 pmol, 2 \(\mu\)M). The mixtures containing the indicated polymerase were brought to the indicated temperature for 30 s and dNTPs were added. The mixtures were incubated at the indicated temperatures for 2 min. Reactions were terminated with quench buffer (5 \(\mu\)l, formamide EDTA, dyes, dilution factor of 33%). An aliquot (1 \(\mu\)l) was loaded on a polyacrylamide gel (20%, 7 M urea) and resolved. The full-length product is now N + 13 (19mer), because the primer is a 6mer. Note that the synthesis of full-length product with Klenow is almost certainly due to the fact the temperature is lower. Only the therminator polymerase effectively extends the shorter primer.
incubation at high temperature, the extent of full-length product produced tracked the activity of the polymerase itself.

This screen allowed M-MuLVRT and Pfu polymerase to be viewed as lead polymerases to implement the desired architecture. These did not efficiently use the 8mer 3'-fragment by itself as a primer. For them to be used, however, the 8mer 3'-fragment bearing a 5'-amino group must be accepted by the polymerases as a primer when it forms transiently an imine complex with an 8mer that bound immediately upstream.

The data collected in Figure 4 shows that both Pfu and M-MuLVRT were able to extend an 8mer fragment bearing a 5'-amino group, but only in the presence of a second fragment carrying a 3'-CH$_2$CHO unit complementary to the sequence immediately adjacent in the DNA template. Two types of evidence were collected that suggested that the imine was actually being formed. First, it was possible to detect the imine intermediate by the presence of a band at the position on the gel where a 19mer would be expected to run (see higher bands in Figure 5 and following). The fact that this band was the imine was demonstrated by showing that its intensity was reduced when the mixture was incubated with methoxylamine before gel analysis; the methoxylamine should disrupt the imine complex by itself forming an imine with the aldehyde group.

Further, in several test cases, the imine presumed to be formed transiently was reduced in situ with sodium cyanoborohydride, a procedure that was expected to join the two fragments via a stable secondary amine linker. Mass spectrometry identified the expected amine product (C$_{156}$H$_{202}$N$_{56}$O$_{90}$P$_{14}$, calculated mass: 4704.9216, observed: 4707.87, 2355.51 and 1571.24 assigned as the monoanion, dianion and trianion, respectively).

With polymerases in hand that extended only composite primers formed in situ under conditions of dynamic equilibrium, we asked whether the dynamically assembled primer could be used to discriminate against single nucleotide mismatches. Figure 5 collects data obtained with M-MuLVRT that shows it does. Here, the primer extension assays examined with DNA 38mer templates containing single mismatches at various positions throughout the binding region (see Table 1 for details of mismatches).

Perhaps not surprisingly, a mismatch at the 3' end of the composite primer resulted in no detectable elongation. Mismatches at every other site resulted in decreased elongation, with the overall pattern roughly consistent with expectations based on general concepts of the impact of mismatches on duplex stability. Thus, mismatches at the 5' end of the 5'-fragment had the smallest (but still detectable) impact on composite primer extension, while mismatches in the middle of the fragments had the largest impact. Purine–purine and pyrimidine–pyrimidine mismatches generally diminish elongation more than purine–pyrimidine mismatches. The only clear exception to this generalization is at Site 20, on the 5' end of the imine junction. Nevertheless, mismatches at the imine junction uniformly diminished the efficiency of priming by the composite; a priori, this outcome was not obvious, as mismatches could conceivably have been favored given the distortion of the geometry of the backbone caused by the imine.

These results demonstrate the use of M-MuLVRT as a part of an 8 + 8 architecture where one could discriminate between single mismatches. With this proof of concept in hand, we asked whether shorter primer fragments might allow discrimination to be more complete, or allow other polymerases to be used, especially thermostable polymerases that allowed a wider range of temperatures to be exploited to obtain higher levels of mismatch discrimination. Figure 6 shows the results when the same DNA 38mer template was presented with a 6mer (instead of an 8mer) as the 3'-fragment. Among the thermostable polymerases at high temperature, only Therminator and Bst generated detectable amounts of full-length product using the 3'-terminal 6mer fragment alone, and these only at the lower temperatures examined. Klenow fragment again primed efficiently from the 6mer by itself, again presumably because the lower incubation temperature required by this polymerase permitted less stable duplexes to form.

The identification of conditions where many thermostable polymerases did not elongate a 6mer primer terminated with a 5'-amino group allowed us to use several of these to selectively elongate a composite primer. Figure 7 shows a collection of data. Virtually all of the polymerases primed
from the 8 + 6 composite to yield full-length product. As before, some of the imine composite could be recovered on the gel if electrophoresis was done without substantial dilution of the mixture.

From this set of data, the 9°N polymerase was selected as a candidate for more detailed study to determine whether assay conditions could be obtained with a thermostable polymerase that would allow for complete mismatch discrimination. The results in Figure 8 show that at 70°C, the 9°N DNA polymerase efficiently elongated the 8 + 6 composite, but did not elongate any composite primers containing the mismatches examined in the 6mer fragment to any detectable extent. Further, the polymerase did not elongate composite primers containing most of the selected mismatches in the 8mer 5'-fragment. The notable exceptions were mismatches at the 5' end of the 5'-fragment (where a TT mismatch still pre-vented composite primer elongation) and at the junction at the 3' end of the 5'-fragment (but even here, TT and TC mismatches disrupted elongation).

**DISCUSSION**

These results provide the framework for implementing a new type of dynamic combinatorial chemistry that permits the template-directed assembly of primers that have the specificity of long oligonucleotides, but the discriminatory power of short oligonucleotides. In just one implementation, employing a 8mer as the 5'-fragment carrying a 3'-aldehyde, a 6mer as the 3'-fragment carrying a 5'-amino group, the 9°N DNA polymerase (from New England Biolabs), and a temperature of 70°C, high discriminatory power could be achieved across the entire length of the composite primer. While further development is needed to optimize the strategy, this particular implementation is certainly useful.

Thus, the DNA dynamic combinatorial experiment differs from the irreversible template-directed ligation demonstrated by von Kiedrowski, Kool and Ellington, for example (5–7). In the latter case, kinetics determines the outcome of the ligation, and partial mismatches are frequently ligated with nearly the same frequency as full matches (14,15). This difference underscores the principle of having the primer assemble reversibly under conditions of dynamic equilibrium.

Further, this result is reminiscent of a pre-genome strategy proposed by Studier for the sequencing of DNA using random primers (16) as modified by Szybalski (17), who proposed to assemble primers *in situ* by enzymatic ligation. It is different from the work reported here, of course, in various aspects, including the dynamic equilibrium feature of our approach. These differences notwithstanding, our approach is clearly applicable in multiplexed PCR, sequencing and other technologies.

From a scientific perspective, an additional comment is warranted. The results in Figure 3 suggest that polymerases stabilize short primer–template complexes, allowing them to serve as substrates at temperatures considerably higher than those that would allow them to exist over long periods in the absence of the polymerase. This feature of polymerase behavior has not been extensively studied. Its functional significance is uncertain; it is not clear that such short primers are ever present physiologically.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR online.

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**REFERENCES**

1. Benner, S.A. (1995) Receptor assisted combinatorial chemistry. U.S. Patent 5 958 702. Filed: February 6.
2. Otto, S., Furlan, R.L.E. and Sanders, J.K.M. (2002) Recent developments in dynamic combinatorial chemistry. Curr. Opin. Chem. Biol., 6, 321–327.

3. Huc, I. and Lehn, J.M. (1997) Virtual combinatorial libraries: dynamic generation of molecular and supramolecular diversity by self-assembly. Proc. Natl Acad. Sci. USA, 94, 2106–2110.

4. Sando, S., Abe, H. and Kool, E.T. (2004) Quenched auto-ligating DNAs: multicolor identification of nucleic acids at single nucleotide resolution. J. Am. Chem. Soc., 126, 1081–1087.

5. Sievers, D. and von Kiedrowski, G. (1994) Self replication of complementary nucleotide-based oligomers. Nature, 369, 221–224.

6. Bag, B.G. and von Kiedrowski, G. (1996) Templates, autocatalysis and molecular replication. Pure Appl. Chem., 68, 2145–2152.

7. James, K.D. and Ellington, A.D. (1999) The fidelity of template-directed oligonucleotide ligation and the inevitability of polymerase function. Origins Life Evol. Biosphere, 29, 375–390.

8. Goodwin, J.T. and Lynn, D.G. (1992) Template-directed synthesis. Use of a reversible-reaction. J. Am. Chem. Soc., 114, 9197–9198.

9. Zhang, M. (1997) Identification of protein coding regions in the human genome by quadratic discriminant analysis. Proc. Natl Acad. Sci. USA, 94, 565–568.

10. Zhan, Z.Y.J., Ye, J.D., Li, X.Y. and Lynn, D.G. (2001) Replicating DNA differently. Curr. Org. Chem., 5, 885–902.

11. Zhan, Z.Y.J. and Lynn, D.G. (1997) Chemical amplification through template-directed synthesis. J. Am. Chem. Soc., 119, 12420–12421.

12. Chu, C.K., Doboszewski, B., Schmidt, W. and Ullas, G.V. (1989) Synthesis of pyrimidine 3′ allyl 2′,3′-dideoxyribonucleosides by free-radical coupling. J. Org. Chem., 54, 2767–2769.

13. Bogan, J., Ignatovich, L. and Stankevich, E. (1999) Reversed (5′–3′) oligonucleotide synthesis on oxalyl-CPG support. Nucleosides Nucleotides Nucleic Acids, 18, 1183–1185.

14. James, K.D., Boles, A.R., Henckel, D. and Ellington, A.D. (1998) The fidelity of template directed oligonucleotide ligation and its relevance to DNA computation. Nucleic Acids Res., 26, 5203–5211.

15. James, K.D. and Ellington, A.D. (1997) Surprising fidelity of template-directed chemical ligation of oligonucleotides. Chem. Biol., 4, 595–605.

16. Studier, F.H. (1989) Strategy for high-volume sequencing of cosmid DNAs. Random and directed priming with a library of oligonucleotides. Proc. Natl Acad. Sci. USA, 86, 6917–6921.

17. Szybalski, W. (1990) Proposal for sequencing DNA using ligation of hexamers to generate sequential elongation primers (SPEL-6). Gene, 90, 177–178.