An Artificial Primosome:
Design, Function, and Applications

Vadim V. Demidov,* Natalia E. Broude, Irina V. Lavrentieva-Smolina, Heiko Kuhn, and Maxim D. Frank-Kamenetskii*[a]

Double-stranded (ds) DNA is capable of the sequence-specific accommodation of an additional oligodeoxyribonucleotide strand by the peptide nucleic acid(PNA)-assisted formation of a so-called PD-loop. We demonstrate here that the PD-loop may function as an artificial primosome within linear, nonsupercoiled DNA duplexes. DNA polymerase with its strand displacement activity uses this construct to initiate the primer extension reaction at a designated dsDNA site. The primer is extended by several hundred nucleotides. The efficiency of dsDNA priming by the artificial primosome assembly is comparable to the single-stranded DNA priming used in various assays. The ability of the PD-loop structure to perform like an artificial primosome on linear dsDNA may find applications in biochemistry, molecular biology, and molecular biotechnology, as well as for DNA diagnostics. In particular, multiple labels can be incorporated into a chosen dsDNA site resulting in ultrasensitive direct quantification of specific sequences. Furthermore, nondenaturing dsDNA sequencing proceeds from the PD-loop. This approach opens the way to direct isothermal reading of the DNA sequence against a background of unrelated DNA, thereby eliminating the need for purification of the target DNA.

KEYWORDS:

DNA structures · oligonucleotides · peptide nucleic acids · primosome · sequence determination

Introduction

Local opening of an extended region within the DNA double helix provides access for DNA and RNA polymerases to specific DNA sequences. This can be achieved with the use of reagents that are capable of sequence-specific formation of single-stranded (ss) loops within double-stranded (ds) DNA. As a result, initiation of transcription and replication can take place at designated sites. Peptide nucleic acids (PNAs), which are able to invade complementary targets within dsDNA yielding P-loops,[1, 2] exemplify this ability. It has been found that the P-loop complexes formed by homopyrimidine PNAs[1] act as artificial transcription promoters in linear dsDNA.[3]

Single and merged P-loops are known to be able to accommodate an oligodeoxyribonucleotide (ODN) by forming so-called PD-loops and similar structures.[4, 5a] When assembled at inverted repeats within negatively supercoiled DNA, the PD-loop-like complexes were shown to be capable of initiating the primer extension reactions through strand displacement.[4] These reactions are facilitated by the excessive energy of supercoiling and may be used for monitoring probe hybridization to supercoiled DNA by its direct sequencing.[4] Still, the design of an artificial primosome capable of initiating the primer extension reactions within nonsupercoiled dsDNA is of considerable interest from both the viewpoint of basic research and the perspective of practical applications.

Recently, we have demonstrated that the PD-loop formed by a pair of homopyrimidine PNA openers has such a potential and used it as a tool for the direct quantification of specific targets in linear, nonsupercoiled dsDNA.[5b] Here we demonstrate that the primer extension reaction carried out through the PD-loop by DNA polymerases with their strand displacement activity allows to selectively incorporate multiple labels into a dsDNA target, thereby significantly amplifying the hybridization signal. We also verify the feasibility of nondenaturing sequencing linear dsDNA starting from the PD-loop against a background of unrelated duplex DNA.

We show that the PD-loop structure, when formed by homopyrimidine PNAs, performs like an artificial primosome within nonsupercoiled dsDNA and also demonstrate the utility of this construct for some applications. Because of the high sequence specificity of PD-loop formation,[5a–d] the primer extension reaction initiated on linear duplex DNA by the artificial primosome is remarkably selective. Together with the simplicity of assembly/operating and the feasibility of evading tedious procedures of target purification, this feature provides a significant advantage for possible applications of artificial primosomes, as compared to traditionally used ssDNA templates.

[a] Dr. V. V. Demidov,[+] Prof. M. D. Frank-Kamenetskii, Dr. N. E. Broude,[+]
Dr. I. V. Lavrentieva-Smolina, Dr. H. Kuhn
Center for Advanced Biotechnology
and
Department of Biomedical Engineering
Boston University
36 Cummington Street, Boston, MA 02215 (USA)
Fax: (+ 1) 617-353-8501
E-mail: vvd@bu.edu, mfk@bu.edu

[+] These authors contributed equally to this work.

Results

The PD-loop performs like an artificial primosome within a linear DNA duplex

Figure 1 shows our design of the artificial primosome and schematizes its function. In our first experiments we targeted pHIV, the plasmid carrying a short PD-loop-forming insert of HIV-1 DNA, by homopyrimidine bis-PNAs 1 and 2 together with the 5′-biotinylated ODN 1 (see Experimental Section for details). To analyze the potential of the PD-loop to initiate the primer extension reaction, we used modified T7 DNA polymerase as a highly processive enzyme[6] that is capable of strand displacement replication.[7] The incorporation of radiochemically pure [125I]dCTP with a specific activity of 5×10^{15} dpm mmol^{-1} (2200 Ci mmol^{-1}) into the extending primer allowed us to quantitatively monitor the primer extension reaction by radioactivity counting.

The kinetics of the increase in radioactivity due to the incorporation of [125I]dCTP into target dsDNA is shown in Figure 2. These data clearly indicate that the incorporation of radioactivity proceeds exclusively through the extension of the primer associated with the PD-loop. Indeed, when the PD-loop is assembled, a significant amount of radioactivity, up to a 30- to 40-fold excess over the background, is rapidly incorporated into the DNA duplex. In contrast, without the PNA openers or without the primer only background radioactivity can be detected. It is important to emphasize that only purines can be incorporated by DNA polymerase into the primer that is being extended within the PD-loop. This is because homopyrimidine PNA openers, while forming the PD-loop, displace the pyrimidine-rich strand of the dsDNA target. Since we observe the incorporation of [125I]dCTP, the data in Figure 2 can be explained
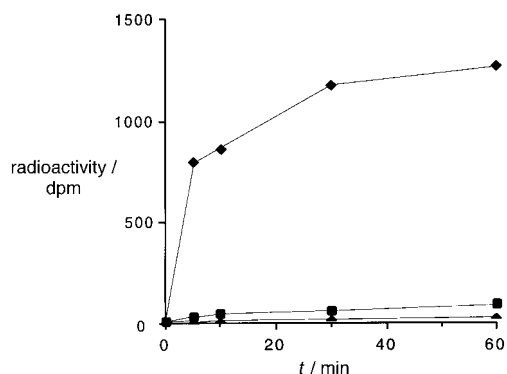

Figure 2. The time course for the increase in radioactivity incorporated into a primer that was bound to pHIV through the PD-loop (diamonds). The PD-loop was formed by bis-PNAs 1 and 2 together with the ODN 1 primer. Incorporation of [125I]dCTP was performed by T7 DNA polymerase. In control experiments, the reaction mixture contained all components except PNA openers (squares) or the ODN primer (triangles). Instrumental background and counting efficiency of the radioactivity counter were ca. 30 dpm and 99.9%, respectively.

only in terms of expansion of the primer beyond the PD-loop by strand displacement.

Hence, in the presence of DNA polymerase, the PD-loop complex acts as a primosome that selectively initiates replicative synthesis at a specific site on linear dsDNA. To check the sequence specificity of artificial primosome formation and functioning, we assembled the PD-loop within pHIV when this plasmid DNA was mixed with either pBR322 or phage λ DNA. These two DNAs do not contain sites that can form PD-loops with ODN 1 plus PNA openers 1 and 2. When the PD-loop-initiated primer extension reaction was performed with the mixtures of pHIV and unrelated DNA, virtually the same incorporation of radioactivity was observed as compared to
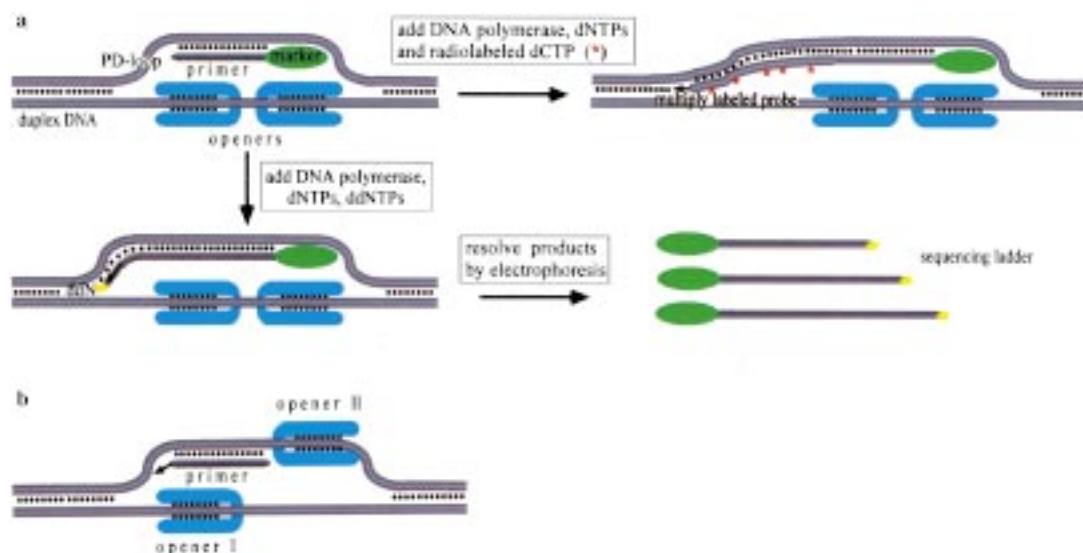

Figure 1. Design and function of the artificial primosome. a: The PD-loop forms sequence-specifically inside dsDNA as a hybrid complex of an oligonucleotide primer with the DNA target locally exposed by homopyrimidine bis-PNA openers. Addition of DNA polymerase performing the strand displacement replication results in the primer extension reaction. When labeled dNTPs are used, a multiply labeled probe associated with the dsDNA target emerges (top right). The sequencing ladder (bottom right) can be isothermally generated directly on dsDNA by DNA polymerase, which extends the primer in the presence of dNTPs and ddNTPs (bottom left). The primer may carry a special marker, for example biotin for probe affinity capture or fluorescein for optical detection of the sequencing ladder. b: An alternative arrangement of the primosome: bis-PNA openers can be bound in trans within the PD-loop as compared to binding in cis, which is shown in part a of this figure.

PD-loop assembly in the presence of pHIV alone (data not shown).

Table 1 contains the quantitative estimations of primer elongation, which were obtained from the data presented in Figure 2. The values given for the fraction of radiolabeled cytosines incorporated into the primer at a given time were estimated by taking into consideration the specific activity of [125I]dCTP. It is reasonable to assume that DNA polymerase incorporates both [125I]dCTP and dCTP with comparable rates. Under this assumption, and taking into account the molar ratio of these dNTPs in the reaction (1:800), the total number of incorporated cytosines can be calculated (Table 1). Furthermore, cytosine occurs in plasmid DNA as every fourth nucleobase, on average. Hence, multiplying the total number of cytosines by four gives an estimate of the entire length of primer accretion (Table 1).

Table 1. *Elongation of a primer through strand displacement polymerization.[a]*

Reaction time [min]	Fraction of radiolabeled cytosines per PD-loop[b]	Total number of cytosines in the probe	Overall length of the probe [nt][c]
5	0.056	45	180
10	0.061	49	197
30	0.079	63	252
60	0.082	66	265

[a] ODN 1, PNAs 1 and 2, pHIV, and T7 DNA polymerase were used. [b] 1.25 nM [125I]dCTP together with 1 μM dCTP were used for labeling. [c] Estimations obtained from the data given in the second and third column (see text for details).

The data show that after 5 min the primer is extended by 180 nucleotides (nt), which corresponds to the rate of replicative synthesis (ca. 1 nt s^{-1}). Although the rate is sufficiently high to provide the primer elongation by several hundred nucleotides within a few minutes, it is significantly lower than the rate of the primer extension reaction (ca. 300 nt s^{-1}) estimated for the action of this DNA polymerase on a ssDNA template.[6] This result could be expected because DNA polymerase should somehow disrupt the stable DNA double helix to perform the strand displacement replication. Otherwise, this enzyme has to wait for infrequent base pair opening caused by thermal fluctuations at the duplex edge bordering the primer elongation point. This consideration is supported by the fact that, when the ssDNA-binding (SSB) protein is added to the reaction mixture, DNA polymerase performs the primer extension reaction through the PD-loop approximately two times faster (data not shown).

When 5 nM [125I]dCTP was used as the only source of cytosine, up to ten radiolabels were incorporated into the extended primer, thus allowing to monitor sub-attomolar amounts of dsDNA target. In this experiment, the extent of primer elongation after 1 h was not greater than 50 nt even in the presence of the SSB protein. Here, a lesser extent of primer elongation as compared to previous experiments (see Table 1), in which 1.25 nM [125I]dCTP was used together with 1 μM dCTP, can be attributed to the lower concentration of dCTP, causing a decrease in the polymerization rate. Further studies are required

to find the optimal quantity and proportion of (radio)labeled and nonlabeled nucleotides for multiply labeling DNA through the PD-loop.

PD-loop-primed direct isothermal sequencing of linear dsDNA

Since the PD-loop works like an artificial primosome, it may be used for the nondenaturing sequencing of duplex DNA by a standard dideoxy (dd) sequencing protocol.[8] Figures 3a and b show a part of the sequence data obtained for the linearized dsDNA plasmid pPL3 by priming with modified T7 DNA polymerase through the PD-loop and by a common denaturing procedure (see Experimental Section for details). Clear Sanger dideoxy sequencing ladders yielding the correct read were generated with the PNA-assisted priming method, by using the 15-mer ODN 2 as a primer. The sequence data in Figures 3a and b confirm that dsDNA priming occurred at the expected PD-loop site. Taking into consideration the similar intensity of the corresponding bands in these figures and equal DNA amounts used in both experiments, we conclude that the efficiency of priming dsDNA through the artificial primosome is comparable to that of priming ssDNA. No ladders were generated from the dsDNA template primed by ODN 2 without PNA openers (Figure 3c). The sequence data obtained by using another PD-loop, specifically, with pHIV primed by the 16-mer ODN 3, demonstrate that about 300 bp can be read within the DNA duplex (Figures 4a, b). Hence, these data show that the primer extension reaction proceeds quite far in agreement with the kinetic data presented above.

Well-readable data with virtually the same signal-to-background ratio, although with a shorter unambiguous sequence, have been obtained with pHIV when the PD-loop complex was assembled and subsequently used for priming in the presence of an equimolar amount of 48.5-kb-long dsDNA from *Escherichia coli* bacteriophage λ (compare Figures 4c and a). Note that we failed to read the pHIV sequence with the same primer against the background of λ DNA when an alkaline denaturation procedure was used (data not shown).

Notwithstanding some cases when cosmid DNA as well as wild-type or recombinant λ DNA can be directly sequenced by using a conventional denaturing protocol,[9] the direct sequencing of inserts cloned into cosmids and λ vectors by alkaline denaturation is still a technically challenging task. Typically, if vector DNA is not removed prior to sequencing, substantial background and abundant ambiguities hamper successful sequencing. By contrast, our sequencing strategy, which is able to resolve the nucleotide sequences of double-stranded templates against the background of long unrelated DNA, has a promising potential for isothermal sequencing of cloned DNA inserts.

To demonstrate this potential, we have cloned the entire plasmid pHIV (2.7 kb) into the *Eco*RI cloning site of λgt11 vector (43.7 kb) thus obtaining the 46.4-kb-long λ-HIV recombinant DNA (Figure 5a). Next, we accomplished the isothermal non-denaturing sequencing of this DNA by using the artificial primosome strategy (Figure 5b). Again, we failed to read the
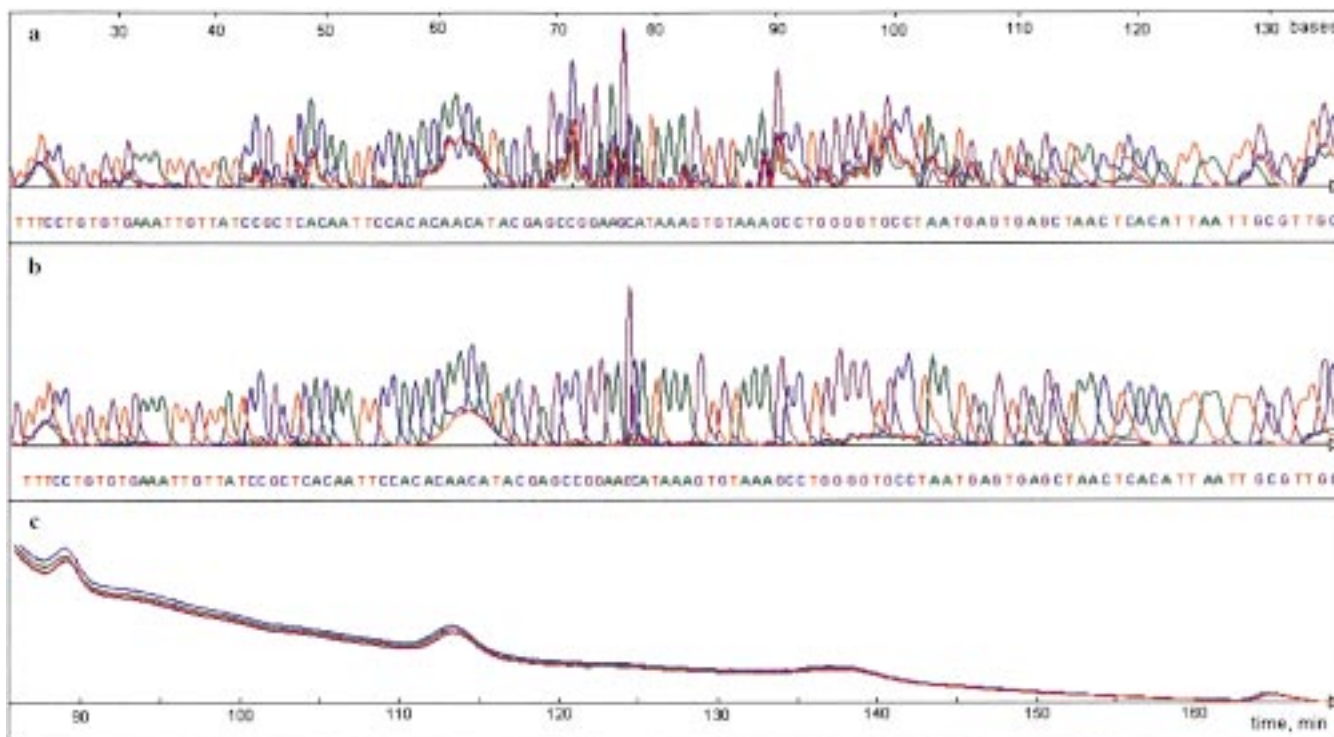
Figure 3. *a: Result of sequencing the double-stranded plasmid pPL3 by PNA-assisted priming with T7 DNA polymerase. Here and in Figures 3b and c, only part of the sequences is shown. The PD-loop was formed by bis-PNAs 3 and 4 together with the ODN 2 primer. b: As a positive control, the same sequence read was obtained with prior alkaline denaturation. c: As a negative control, the sequencing reactions were performed with the same primer for the duplex DNA template but without PNA openers. Note that the sequence read can be obtained quite far, typically several dozen base pairs ahead, from the priming point. That is why the purine-rich stretch, which should be the first part of the extended sequence, cannot be observed by sequencing.*
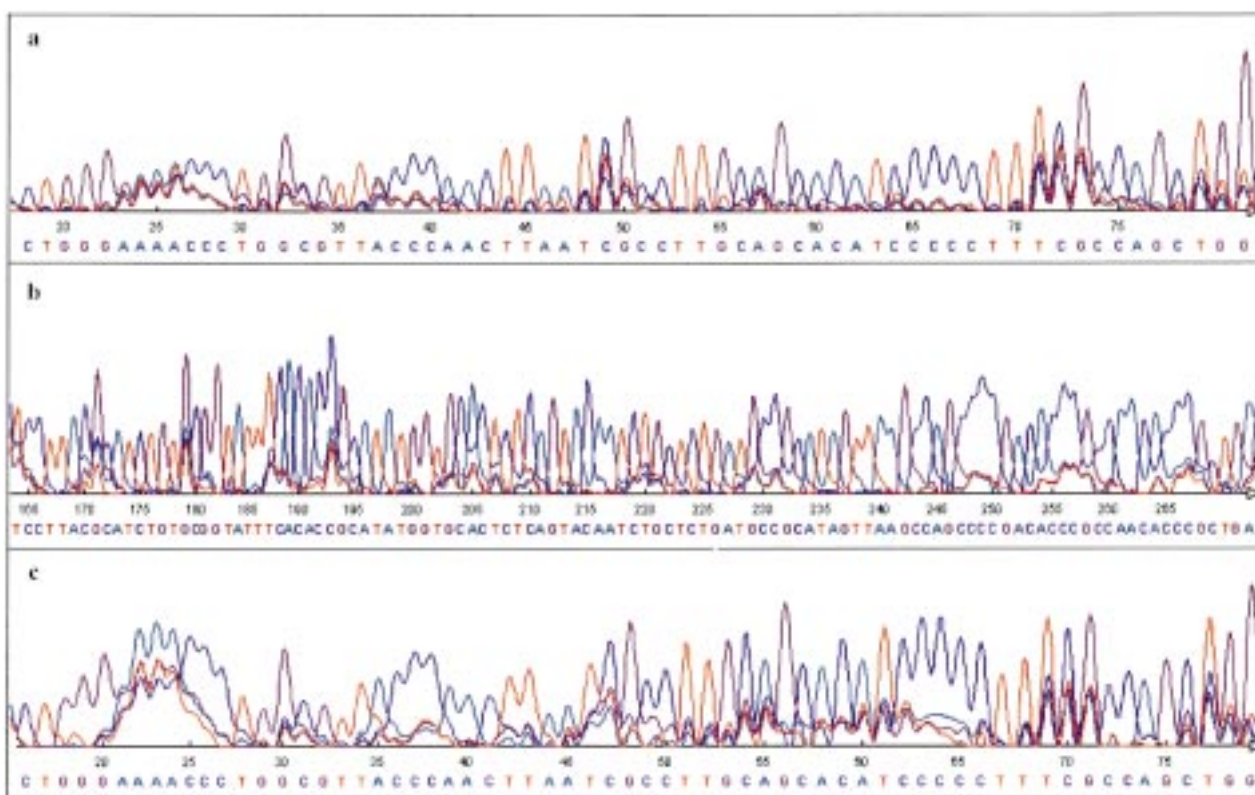

Figure 4. *The sequence reads obtained by PNA-assisted priming of pHIV alone (a, b) or in the presence of the equimolar quantity of λ DNA (c). The PD-loop was formed by bis-PNAs 1 and 2 together with the ODN 3 primer. The sequence reads are given in nucleobases.*
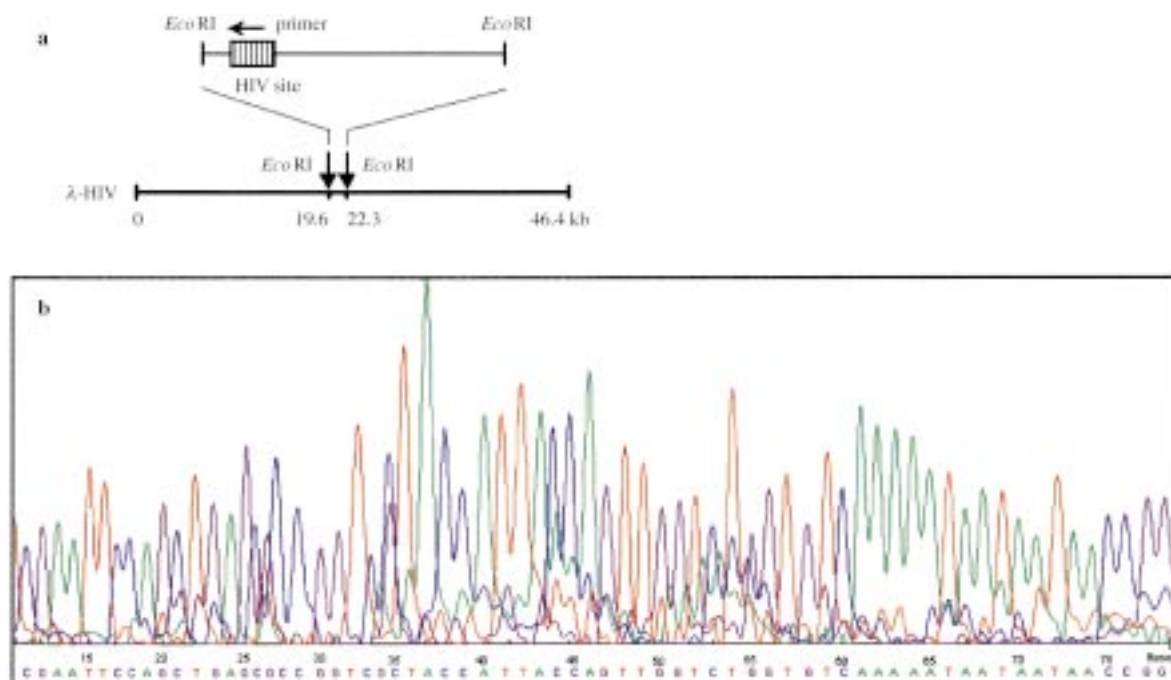
Figure 5. *Direct nondenaturing sequencing of the recombinant λ-HIV clone. a: Schematic representation of the arrangement of the whole-length plasmid insert (pHIV) within the λgt11 vector and the direction of the PNA-assisted priming. b: A part of the sequence read obtained for λ-HIV dsDNA digested by* Aat II *and sequenced in the presence of fluorescein-labeled dATP by using an artificial primosome (nearly 200 bp of λgt11 vector DNA was sequenced in total; see legend of Figure 4 and Experimental Section for details).*

same λ-HIV sequence with the alkaline denaturation procedure (data not shown).

Discussion

Primer extension assays employing ordinary oligonucleotide primers are widely used for DNA analysis.[10] PNA oligomers conjugated with either deoxynucleosides or dinucleotides are also able to initiate the primer extension reaction,[11] thus overcoming an evident inability of PNAs themselves to act as primers. With few exceptions,[4, 7] these assays are performed on ssDNA templates. This is due to the lack of a general approach, which would allow DNA polymerases to initiate the primer extension reaction at a designated site within linear dsDNA.

The design of an artificial primosome reported here has the capacity to sequence-specifically direct the primer extension reaction within nonsupercoiled DNA duplexes. In the course of this reaction, the primer is extended by several hundred nucleotides. High selectivity of the formation of the artificial primosome has been demonstrated when target plasmids were mixed with unrelated DNA. Such selectivity is the result of the exceptional sequence specificity of PD-loop formation.[5a–d] Note that the entire PD-loop requires 20 – 25 bases for its formation, thus ensuring a single priming site even in large genomes. This means that only the preselected site should be opened within dsDNA by a pair of PNAs, thereby becoming accessible for the binding of complementary oligonucleotides. The rest of the DNA should retain its duplex structure and thus remain inaccessible for mispriming.

Note that the PNA-based design of an artificial primosome is radically different from the naturally occurring primosome. The cellular primosome is a very complex multicomponent assembly containing numerous proteins and short RNA primers. It forms an open structure at the origin of replication, a unique site within the entire genome that initiates replicative DNA synthesis.[12] By contrast, the artificial primosome described here is quite a simple construct consisting of a pair of PNA openers and a primer.

In principle, a sufficiently long single PNA opener is able—through duplex or triplex invasion—to locally expose dsDNA for binding of an ODN primer. Nevertheless, we consider our design of an artificial primosome, which is based on two PNA openers, as more robust for several reasons. First, due to sequence limitations on the strand invasion mode of PNA binding, long openers would provide access to rather special DNA sequences such as stretches of about a dozen purines and pyrimidines[1c, 2b–d] or extended trinucleotide and inverted repeats,[2a, 4] some of which may additionally require DNA supercoiling for PNA binding.[4] Second, a pair of openers pries apart the DNA duplex in between thus exposing an extra DNA sequence for recognition by a primer. Third, in this case three independent site-specific ligands are involved in the recognition of a designated target. As a result, the specificity of dsDNA priming is exceptionally high.

The ability of the PD-loop structure to behave as an artificial primosome in linear dsDNA offers new practical benefits in biochemistry, molecular biology, and molecular biotechnology, as well as for DNA diagnostics, thus expanding the range of PNA applications.[13] In particular, numerous labels can be incorpo-

rated into a chosen site of the DNA duplex, in contrast to nonspecific multiple labeling by nick translation. This allows the direct, ultrasensitive quantification of specific DNA sequences and avoids tedious procedures of target purification. We also anticipate that artificially driven primer extension can be used for intracellularly manipulating gene activity, similar to the recent in vivo use of artificial P-loop transcription promoters for inducing gene-specific expression.[3b]

Furthermore, nondenaturing isothermal sequencing of designated regions within linear double-stranded DNA, which is preferable for many applications, becomes possible. Typically, high-quality sequence data are obtained when sequencing reactions proceed on purified ssDNA templates.[9f, 14] This type of sequencing requires tiresome steps of ssDNA isolation. To evade this, methods for sequencing dsDNA templates that have been denatured by alkaline treatment or heating have been developed.[9f, 15] Direct, nondenaturing sequencing of dsDNA avoids complications caused by folded loops that may form in denatured or ssDNA templates,[8b, 16] but not in linear dsDNA templates.

When coupled with a proper detection method, our sequencing approach should enable reading DNA sequences directly against the background of unrelated DNA or within large templates (see Figure 5 b) without the need for a thermal cycler, which is used for these purposes in cycle sequencing.[17] Therefore, our sequencing strategy could be miniaturized for DNA nanoanalysis.[18] It could also be adopted by other DNA-polymerase-based methods of sequencing DNA, for example in real-time DNA pyrosequencing,[19] to make them more robust for the direct sequencing of dsDNA.

Applications of the artificial primosome technique to manipulate linear dsDNA are currently limited by the fact that bis-PNAs open only homopurine/homopyrimidine targets. However, these limitations are rather mild. Indeed, the data presented here, along with the data obtained earlier, demonstrate the formation of stable PD-loops with short, 7 – 8-bp-long homopurine sites separated by an arbitrary sequence of up to 10 bp.[5] Moreover, design of homopyrimidine bis-PNA openers terminating in a duplex-forming PNA extension with all four nucleobases is feasible. Such openers should be capable of effectively prying apart dsDNA sites consisting of short purine tracts separated by more than 10 bp. In addition, our data prove that the PD-loop forms also when PNA-binding sites are located on the opposite DNA strands,[20] as shown schematically in Figure 1 b.

Statistically, different PD-loop-forming sites that meet these conditions should occur quite frequently, on average every 400 to 500 base pairs of a random DNA sequence.[5a,e,f] According to the data presented above, the length of an extended primer lies in the same range. This means that a copy and, as a result, the sequence of many dsDNA sites of interest (e.g. for single-nucleotide polymorphism (SNP) analysis) could be obtained isothermally through strand displacement extension of a primer starting from a PD-loop located nearby. Significantly, DNA copies can be made starting from many different artificial primosomes assembled on genomic DNA at multiple PD-loop-forming sites.

The sequence limitations on the design of the artificial primosome can be additionally softened by the use of recently developed pseudocomplementary PNAs (pcPNAs).[21] pcPNAs carry specially chosen modified nucleobases that hinder an association of two PNA oligomers with seemingly complementary sequences into a corresponding PNA – PNA homoduplex. Still, such modifications allow pcPNA to form a stable PNA – DNA heteroduplex with the complementary DNA strands. As a result, appropriate pairs of pcPNAs are able to invade mixed purine/pyrimidine sequences of linear DNA duplex by a double-duplex invasion mode.[21] We expect that one of two homopyrimidine bis-PNA openers within the artificial primosome can be replaced by a pair of pcPNAs with mixed purine/pyrimidine composition that recognizes all four nucleobases within a DNA duplex. Then, only one short homopurine site, which should occur ca. every hundred base pairs, will be required to assemble an artificial primosome within nonsupercoiled dsDNA. Our preliminary data indicate that a PD-loop of this kind can be formed with the aid of pcPNAs.

Experimental Section

Molecular biology reagents: DNA from phage λ and pBR322 were purchased from New England Biolabs. Recombinant plasmids pPL3 and pHIV containing the PD-loop-forming sequences were obtained by cloning the appropriate oligodeoxynucleotides into the *Bam*HI site of the pUC19 polylinker. The first plasmid, pPL3,[5a] contains the artificially designed purine-rich insert 5′-**AG$_4$A$_2$G**CT$_2$**G$_2$A$_2$GA$_3$**-3′ (here and below, PNA target sequences are printed in boldface). The second plasmid, pHIV,[5b] carries the natural purine-rich insert 5′-**AGAG$_2$A$_2$G**GCTACT**G$_2$AG$_2$AGA**-3′, which is a part of a coding region of the *nef* gene from human immunodeficiency virus (HIV-1 subtype A, strain 92ug037). All plasmids were linearized with the restriction enzyme *Aat*II (New England Biolabs).

Recombinant λ-HIV DNA was obtained by insertion of pHIV into the *Eco*RI site of the λgt11 vector (see Figure 5 a) using λ DNA cloning and packaging kits (Promega) with subsequent purification by the lambda maxi system (Qiagen). Before sequencing, recombinant λ-HIV DNA was digested with *Aat*II.

With the exception of PNA 4 (a gift from Dr. P. E. Nielsen, Copenhagen), cationic bis-PNAs were obtained from PE Biosystems. All PNA oligomers were HPLC-purified and characterized by MALDI-TOF mass spectrometry. PNA 1, HLys$_2$-TJTJ$_2$T$_2$J-eg1$_3$-CT$_2$C$_2$TCT-LysNH$_2$ (eg1, Lys, and J denote bis-PNA linker segment, the amino acid lysine, and the nucleobase pseudoisocytosine, respectively), and PNA 2, HLys$_2$-TCTC$_2$TC$_2$-eg1$_3$-J$_2$TJ$_2$TJT-LysNH$_2$, were used as openers for pHIV. PNA 3, H-TC$_4$T$_2$C-eg1-Lys$_2$-eg1-JT$_2$J$_4$T-LysNH$_2$, and PNA 4, HLys$_2$-T$_3$JT$_2$J$_2$-eg1$_3$-C$_2$T$_2$CT$_3$-LysNH$_2$, were used as openers for pPL3.

HPLC-purified ODNs were from Operon. Biotin-5′-GAG$_2$A$_2$GCTACT-G$_2$AG-3′ (ODN 1), was used in the primer extension experiments with pHIV. Fluorescein-5′-A$_2$GA$_2$G$_2$T$_2$CGA$_2$G$_2$-3′ (ODN 2) and fluorescein-5′-GAG$_2$A$_2$GCTACTG$_2$AG-3′ (ODN 3) were used for sequencing pPL3 and pHIV, respectively.

Assembly of PD-loops: Target DNA (typically ca. 10 μg) was incubated overnight at 37 °C in a siliconized micro test tube with 200 pmol of PNA openers in 100 μL of 10 mM Na-phosphate buffer (pH 6.8). Excess of PNA was then removed by gel filtration through a Sephadex-G50 spin column (Pharmacia Biotech), equilibrated with TEP buffer (10 mM Tris, 0.1 mM EDTA, 10 mM Na-phosphate, pH 6.8). The resulting DNA – PNA complexes were precipitated with ethanol and redissolved in 10 μL of TEP buffer. To form the final PD-loop,

these complexes were incubated in 5 µL of the annealing buffer (200 mM Tris-HCl, pH 7.6, and 20 mM MgCl₂) for 15 min at 37 °C with the specific ODN used subsequently as a primer.

Primer extension reaction: This reaction was performed with 0.1 µg of linearized pHIV and 1 pmol of biotinylated ODN 1 at 37 °C by 4 U of modified T7 DNA polymerase (Pharmacia Biotech) in the extension buffer (15 mM citric acid, 16 mM DTT, and 2 mM MnCl₂, pH 7.5) containing 10 µM dATP, dTTP, and dGTP, 1 µM dCTP, and 1.25 nM (25 nCi µL⁻¹) [¹²⁵I]dCTP (radiochemically pure; New England Nuclear). The reaction was terminated by addition of EDTA to give a final concentration of 50 mM. For multiple labeling (1 h incubation), the conditions of the primer extension reaction were the same, except that nonlabeled dCTP was omitted and the concentration of [¹²⁵I]dCTP was increased up to 5 nM. In some experiments, 1 µg of *E. coli* SSB protein (Promega) was added to the reaction mixture to facilitate the primer extension by strand displacement.[7]

The reaction products were incubated at 65 °C for 5 min to dissociate extended primers from the target dsDNA. After cooling on ice, samples were incubated at room temperature for 1 h with 15 µL of a suspension containing streptavidin-coated iron microbeads (PerSeptive Biosystems). This procedure was done to selectively capture the products on the microbeads through the 5′-biotin label originally present in ODN 1. The microbeads were washed three times with 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8,0) containing 0.2 M NaCl and twice with 100 µL of TE buffer, and were collected with a magnetic separator (Sigma – Aldrich). Finally, the radioactivity of the microbead suspension was counted on a Beckman LS 6500 multipurpose counter by using the ReadySafe liquid scintillation cocktail (Beckman).

DNA sequencing: The Sanger dideoxy sequencing reactions were performed with 0.5 µg of the PD-loop-containing linearized plasmids or 9 µg of the λ-HIV recombinant digested with *Aat*II at 37 °C by using the AutoRead sequencing kit (Pharmacia Biotech). For sequencing the plasmids and λ clones, 20 – 40 pmol of fluorescein-labeled primers was used. In case of λ-HIV sequencing, fluorescein-labeled dATP was added to the same amount of nonlabeled dATP. In some experiments, an equimolar mixture of pHIV (0.5 µg) and wild-type λ DNA (9 µg) was involved in the PNA-directed sequencing. A standard procedure with alkaline denaturation was used to sequence the control samples. For PNA-directed sequencing, denaturation and neutralization steps were omitted. DNA sequencing was recorded automatically on the A.L.F. DNA Sequencer (Pharmacia Biotech). To facilitate the strand displacement sequencing through dideoxy termination, 1 µg of SSB protein was added to all reactions.[7]

[1] a) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, *Science* **1991**, *254*, 1497 – 1500; b) D. Y. Cherny, B. P. Belotserkovskii, M. D. Frank-Kamenetskii, M. Egholm, O. Buchardt, R. H. Berg, P. E. Nielsen, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 1667 – 1670; c) V. V. Demidov, M. V. Yavnilovich, B. P. Belotserkovskii, M. D. Frank-Kamenetskii, P. E. Nielsen, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2637 – 2641.

[2] a) L. C. Boffa, E. M. Carpaneto, V. G. Allfrey, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 1901 – 1905; b) P. E. Nielsen, L. Christensen, *J. Am. Chem. Soc.* **1996**, *118*, 2287 – 22888; c) G. Haaima, H. F. Hansen, L. Christensen, O. Dahl, P. E. Nielsen, *Nucleic Acids Res.* **1997**, *25*, 4639 – 4643; d) P. Wittung, P. E. Nielsen, B. Nordén, *Biochemistry* **1997**, *36*, 7973 – 7979.

[3] a) N. E. Møllegaard, O. Buchardt, M. Egholm, P. E. Nielsen, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3892 – 3895; b) G. Wang, X. Xu, B. Pace, D. A. Dean, P. M. Glazer, P. Chan, S. R. Goodman, I. Shokolenko, *Nucleic Acids Res.* **1999**, *27*, 2806 – 2813.

[4] S. V. Smulevitch, C. G. Simmons, J. C. Norton, T. W. Wise, D. R. Corey, *Nat. Biotechnol.* **1996**, *14*, 1700 – 1704, and references therein.

[5] a) N. O. Bukanov, V. V. Demidov, P. E. Nielsen, M. D. Frank-Kamenetskii, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5516 – 5520; b) N. E. Broude, V. V. Demidov, H. Kuhn, J. Gorenstein, H. Pulyaeva, P. Volkovitsky, A. K. Drukier, M. D. Frank-Kamenetskii, *J. Biomol. Struct. Dyn.* **1999**, *17*, 237 – 244; c) H. Kuhn, V. V. Demidov, M. D. Frank-Kamenetskii, *Angew. Chem.* **1999**, *111*, 1544 – 1547; *Angew. Chem. Int. Ed.* **1999**, *38*, 1446 – 1449; d) H. Kuhn, V. V. Demidov, M. D. Frank-Kamenetskii, *J. Biomol. Struct. Dyn*. **2000**, *18*, 221 – 225; e) V. V. Demidov, N. O. Bukanov, M. D. Frank-Kamenetskii in *Peptide Nucleic Acids: Protocols and Applications* (Eds.: P. E. Nielsen, M. Egholm), Horizon Scientific Press, Wymondham, UK, **1999**, pp. 187 – 196; f) V. V. Demidov, N. O. Bukanov, M. D. Frank-Kamenetskii, *Curr. Issues Mol. Biol.* **2000**, *2*, 31 – 35.

[6] S. Tabor, C. C. Richardson, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 4767 – 4771.

[7] D.-J. Fu, H. Köster, C. L. Smith, C. R. Cantor, *Nucleic Acids Res.* **1997**, *25*, 677 – 679.

[8] a) F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463 – 5467; b) T. A. Brown, *DNA Sequencing: The Basics*, IRL Press, Oxford, UK, **1994**, p. 101.

[9] a) G. Manfioletti, V. Schneider, *Nucleic Acids Res.* **1988**, *16*, 2873 – 2884; b) H. Voss, J. Zimmermann, C. Schwager, H. Erfle, J. Stegemann, K. Stucky, W. Ansorge, *Nucleic Acids Res.* **1990**, *18*, 1066; c) H. Voss, J. Zimmermann, C. Schwager, H. Erfle, J. Stegemann, K. Stucky, W. Ansorge, *Nucleic Acids Res.* **1990**, *18*, 5314; d) B. S. Kim, C. Jue, *BioTechniques* **1990**, *8*, 156 – 159; e) D. L. Steffens, R. W. Gross, *Methods Enzymol.* **1993**, *218*, 233 – 240; f) A. N. Malik in *DNA Sequencing Protocols* (Eds.: H. G. Griffin, A. M. Griffin), Humana Press, Totowa, USA, **1993**, pp. 141 – 148.

[10] a) H. Htun, B. H. Johnston, *Methods Enzymol.* **1992**, *212*, 272 – 294; b) R. Wu, *Trends Biochem. Sci.* **1994**, *19*, 429 – 433.

[11] a) M. J. Lutz, S. A. Benner, S. Hein, G. Breipohl, E. Uhlmann, *J. Am. Chem. Soc.* **1997**, *119*, 3177 – 3178; b) H. S. Misra, P. K. Pandey, M. J. Modak, R. Vinayak, V. N. Pandey, *Biochemistry* **1998**, *37*, 1917 – 1925.

[12] a) R. L. P. Adams, J. T. Knowler, D. P. Leader, *The Biochemistry of the Nucleic Acids*, Chapman and Hall, New York, USA, **1986**; b) B. M. Alberts in *Structure and Function of Nucleic Acids and Proteins* (Eds.: F. Y.-H. Wu, C.-W. Wu), Raven Press, New York, USA, **1990**, pp. 223 – 237.

[13] a) D. R. Corey, *Trends Biotechnol.* **1997**, *15*, 224 – 229; b) E. Uhlmann, A. Peyman, G. Breipohl, D. W. Will, *Angew. Chem.* **1998**, *110*, 2954 – 2983; *Angew. Chem. Int. Ed.* **1998**, *37*, 2796 – 2823; c) P. E. Nielsen, *Curr. Opin. Biotechnol.* **1999**, *10*, 71 – 75; d) M. D. Frank-Kamenetskii in *Triple Helix Forming Oligonucleotides* (Eds.: C. Malvy, A. Harel-Bellan, L. L. Pritchard), Kluwer Academic Publishers, Norwell, USA, **1999**, pp. 285 – 294.

[14] a) S. Gal in *DNA Sequencing Protocols* (Eds.: H. G. Griffin, A. M. Griffin), Humana Press, Totowa, USA, **1993**, pp. 183 – 190; b) *DNA Sequencing Strategies: Automated and Advanced Approaches* (Eds.: W. Ansorge, H. Voss, J. Zimmermann), Wiley, New York, USA, **1997**, p. 202.

[15] H. G. Griffin, A. M. Griffin in *DNA Sequencing Protocols* (Eds.: H. G. Griffin, A. M. Griffin), Humana Press, Totowa, USA, **1993**, pp. 131 – 135.

[16] a) A. Dayn, G. M. Samadashwily, S. M. Mirkin, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 11 406 – 11 410; b) A. S. Krasilnikov, I. G. Panyutin, G. M. Samadashwily, R. Cox, Y. S. Lazurkin, S. M. Mirkin, *Nucleic Acids Res.* **1997**, *25*, 1339 – 1346; c) V. N. Potaman, J. J. Bissler, *Nucleic Acids Res.* **1999**, *27*, e5.

[17] C. R. Heiner, K. L. Hunkapiller, S.-M. Chen, J. I. Glass, E. Y. Chen, *Genome Res.* **1998**, *8*, 557 – 561.

[18] a) M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man, D. Jones, D. Heldsinger, C. H. Mastrangelo, D. T. Burke, *Science* **1998**, *282*, 484 – 487; b) D. Schmalzing, N. Tsao, L. Koutny, D. Chisholm, A. Srivastava, A. Adourian, L. Linton, P. McEwan, P. Matsudaira, D. Ehrlich, *Genome Res.* **1999**, *9*, 853 – 858.

[19] M. Ronaghi, M. Uhlén, P. Nyrén, *Science* **1998**, *281*, 363 – 365.

[20] V. V. Demidov, M. D. Frank-Kamenetskii, unpublished results.

[21] a) J. Lohse, O. Dahl, P. E. Nielsen, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11 804 – 11 808; b) K. I. Izvolsky, V. V. Demidov, P. E. Nielsen, M. D. Frank-Kamenetskii, *Biochemistry* **2000**, *39*, 10 908 – 10 913.