Cell. Mol. Life Sci. (2009) 66:3325–3336
DOI 10.1007/s00018-009-0094-3

Cellular and Molecular Life Sciences

REVIEW

Isothermal DNA amplification in vitro: the helicase-dependent amplification system

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Received: 22 May 2009 / Revised: 26 June 2009 / Accepted: 1 July 2009 / Published online: 24 July 2009

Abstract Since the development of polymerase chain reaction, amplification of nucleic acids has emerged as an elemental tool for molecular biology, genomics, and biotechnology. Amplification methods often use temperature cycling to exponentially amplify nucleic acids; however, isothermal amplification methods have also been developed, which do not require heating the double-stranded nucleic acid to dissociate the synthesized products from templates. Among the several methods used for isothermal DNA amplification, the helicase-dependent amplification (HDA) is discussed in this review with an emphasis on the reconstituted DNA replication system. Since DNA helicase can unwind the double-stranded DNA without the need for heating, the HDA system provides a very useful tool to amplify DNA in vitro under isothermal conditions with a simplified reaction scheme. This review describes components and detailed aspects of current HDA systems using *Escherichia coli* UvrD helicase and T7 bacteriophage gp4 helicase with consideration of the processivity and efficiency of DNA amplification.

Keywords DNA helicase · DNA replication · Helicase-dependent amplification · Nucleic acid amplification · Processivity

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Introduction

Amplification of nucleic acids in a living system is a pivotal process that is required to copy the progenitor. In addition to its biological significance, nucleic acid amplification is an elemental method in conventional molecular biology and biotechnology. Nucleic acids have been manipulated in many diverse ways for in vitro amplification. The method of DNA amplification using temperature cycling was invented and widely used in the early 1980s, and was termed polymerase chain reaction (PCR) [1–5]. This wonderful technology has revolutionized traditional research in molecular biology, detection methods, and diagnostic and forensic fields [6]. Enzymatic amplification of scarce amounts of nucleic acid in vitro has replaced the old and slow biological amplification for sequence-dependent and fast amplification. Researchers working on nucleic acids in the laboratory all over the world exploit nucleic acid amplification techniques for their own research purposes. Especially, the field of clinical microbiology as well as basic science have benefited from the development of PCR technology for detection and identification of many pathogens [7–14]. The demand to improve the sensitivity and easy use of diagnostics has driven many companies to invent diagnostic devices for simple and prompt tests [15–20].

Despite the widespread use of PCR-based amplification, a drawback of this technology is its need for temperature cycling, which is inappropriate for preserving the morphology of chromosomal structure as well as for device portability, e.g., point-of-care diagnosis [21, 22]. Another limitation of PCR is that it is reliably used as a qualitative method rather than a quantitative method due to the exponential nature of the amplification, which skews the initial amount of the target. Therefore, there have been many

attempts to develop isothermal amplification methods that do not require heating the double-stranded nucleic acid for the separation of templates [21]. Strand-displacement amplification (SDA) is an isothermal DNA amplification method that is based on nicking an unmodified strand of DNA using restriction enzymes and extending the 3′ end at the nick through the action of an exonuclease-deficient DNA polymerase to displace the downstream DNA strand [23]. These processes, nicking/polymerization/displacement steps, cycle continuously and produce single-stranded DNA copies of the target sequence at a linear amplification rate. In rolling circle amplification (RCA), the reaction is started by annealing a linear ssDNA primer to a specific circular ssDNA template that is generated by joining two ends of the DNA using a DNA ligase [24, 25]. A suitable DNA polymerase (e.g., Φ29 DNA polymerase) extends the annealed primer, generating tandemly linked copies of the complementary sequence of the template. Displacement of the synthesized DNA strand is caused by the intrinsic property of the Φ29 DNA polymerase during replication. However, these isothermal methods are difficult to execute successfully; they require complicated reaction protocols and are incapable of amplifying a sufficient length of nucleic acids. In addition, both SDA and RCA methods require an initial heat denaturation step even though the rest of the amplification reaction is isothermal.

To accomplish an isothermal amplification of DNA strands of sufficient length, researchers have attempted to develop new technologies that mimic DNA replication in vivo [22]. As a result of this work, recent progress has been made on isothermal nucleic acid amplification using the nucleic acid helicase, the so-called helicase-dependent amplification (HDA) system. In this system, DNA helicase is used to separate two complementary DNA strands into each single-stranded DNA during DNA replication. Initially, the HDA system was developed using *Escherichia coli* UvrD helicase, which can unwind blunt-end substrates (fully duplex DNA) as well as nicked circular DNA [26]. Since the DNA helicase enzymatically unwinds duplex DNA that is synthesized by DNA polymerase, the initial heat denaturation and subsequent thermocycling are not necessary. Since DNA helicase can unwind the double-stranded target DNA without the need of heating, the entire HDA process can be performed at one temperature. Therefore, the HDA system provides a very useful tool to amplify DNA in vitro under isothermal conditions with a simplified reaction scheme. In this review, we describe the components and detailed aspects of the HDA system and discuss the potential for using other helicases and their effects on the processivity factor.

Helicase-dependent DNA amplification

Although the PCR method is widely used for amplification of dsDNA, it requires a temperature cycling apparatus and thermostable polymerase. Therefore, amplification of dsDNA at a defined temperature is an attractive alternative method to avoid these cumbersome requirements. Due to advances in our understanding of the molecular components and mechanisms of nucleic acid replication in living cells, new technologies have been established to overcome these problems by mimicking DNA replication in vivo [22]. Inspired by the replication fork mechanism, isothermal nucleic acid amplification using a dsDNA unwinding helicase has been developed and is referred to as the helicase-dependent amplification (HDA) system. The reaction scheme of the HDA system is shown in Fig. 1. In this

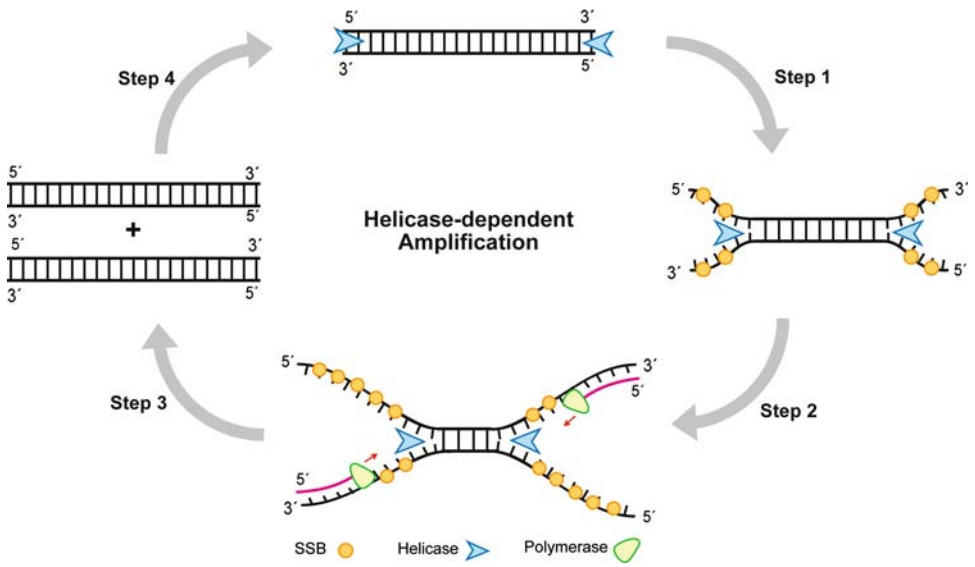

Fig. 1 Schematic diagram of the Helicase-dependent DNA amplification. Helicases unwind dsDNA and SSB proteins bind to exposed ssDNA. Subsequently, DNA polymerases start synthesizing the complementary strand from the bound primers, and the cycles repeat continuously

system, duplex DNAs are unwound by a DNA helicase in the presence of ATP, and the displaced DNA strands are coated by single-stranded binding proteins (SSBs; step 1 in Fig. 1). Two-sequence specific primers anneal to the 3′-end of each ssDNA template, and exonuclease-deficient DNA polymerases produce dsDNA by extending the primers annealed to the target DNA (step 2 in Fig. 1). Completion of DNA synthesis produces another copy of the dsDNA template (step 3 in Fig. 1). The two newly synthesized dsDNAs are used as substrates by the DNA helicase and enter the next round of the reaction (step 4 in Fig. 1). Thus, exponential amplification of the selected target DNA sequence is possible by simultaneous chain reactions.

Among the several elemental proteins in the HDA system, which mimics the replication fork, the DNA polymerase and helicase are the main components that enable DNA synthesis to occur by using chemical energy. Since the discovery of helicase in 1976 [27], biochemical studies have shown that helicases perform diverse functions such as nucleic acid metabolism, genome replication, repair, and recombination in all living organisms [28–31]. Approximately 2% of open reading frames in eukaryotes code for helicases or helicase-like proteins. Besides eukaryotes, many viruses also code for helicases [32] that have conserved motifs, and helicases are good targets for developing antiviral compounds [33–37]. Helicases are molecular motor proteins that translocate along nucleic acids and separate double-stranded nucleic acids using the energy generated by nucleoside 5′-triphosphate (NTP) hydrolysis. Helicases are classified into families and superfamilies depending on their directionality (5′–3′ or 3′–5′) and substrate specificity (RNA vs DNA) [38, 39]. Oligomerization is also an important standard for classifying helicases [30, 31]. Even though many helicases function as a monomer, a class of helicases assembles into oligomers (dimer or hexamer) to enhance their activity [40–45]. The most well-known oligomerization state is a ring-shaped hexamer and, interestingly, the monomers of hexameric helicases do not show NTP hydrolysis and nucleic acid unwinding activity. The presence of NTP, Mg^{2+}, or nucleic acids usually stabilizes the formation of the hexamer [30, 31, 46–48]. Recent progress in understanding the function of helicases has enabled researchers to apply helicases, in combination with polymerases and other accessory proteins, to nucleic acid amplification.

Nucleic acid polymerases catalyze the addition of nucleotides to the 3′-hydroxyl terminus of a nucleic acid chain (DNA or RNA). There are many types of polymerases in all living organisms and their activity depends on the template, synthetic product, or the absence and presence of a primer [49, 50]. Since the discovery of the *E. coli* polymerase by Dr. Arthur Kornberg, extensive studies have been performed to understand the mechanism and function of various types of polymerases. Due to the usefulness of polymerases, a few of them are used widely and extensively (e.g., *Taq* polymerase [51, 52], *Pfu* polymerase [53, 54], reverse transcriptase [55, 56], and Klenow fragment [57, 58], etc.) when in vitro nucleic acids synthesis is necessary. In addition, depending on the needs of researchers, purified DNA or RNA polymerases from various different microorganisms are also available from many manufacturers. The recent increase in demand for in vitro production of RNA drove manufacturers to develop in vitro RNA production kits for easy preparation of RNA. The common RNA polymerases used for this purpose are T7, T3, and SP6 polymerases, where each of them requires distinct and specific promoter sequences [59, 60].

Another protein to be considered is the single-strand binding (SSB) protein, which binds specifically to the single-stranded part of DNA in a sequence-independent manner and is found in the viruses from humans [61–64]. SSB proteins are essential in DNA replication, repair, and recombination [65, 66]. They prevent unwound ssDNAs, which are produced by helicase activity, from reannealing and protect them from degradation [67]. These proteins are also involved in destabilizing DNA secondary structure and enhancing polymerase activity. This allows the DNA replication machinery to proceed properly, and SSB proteins are required for in vitro transcription of unwound ssDNA templates. In the initial HDA system, which uses the UvrD DNA helicase, two SSBs, such as the T4 gene 32 or RB 49 gene 32 proteins, were suggested to be included in the reaction to stimulate in vitro DNA amplification [22]. Recent studies have shown that SSB proteins have far more complex roles in DNA metabolism, besides simple ssDNA coating and protection [68]. Here, a few model systems for in vitro nucleic acid synthesis are described, all of which use a combination of the aforementioned proteins.

HDA system using bacterial UvrD DNA helicase

The first HDA system developed used the *E. coli* UvrD helicase, in which several hundred base pairs of DNA were amplified in the presence of DNA polymerase and two accessory proteins in vitro [22]. *E. coli* UvrD helicase (~82 kDa) was chosen due to its ability to unwind blunt-end dsDNA. UvrD belongs to the superfamily 1 (SF1) DNA helicase and is involved in DNA metabolic processes such as methyl-directed mismatch repair, UvrABC-mediated nucleotide excision repair of DNA, replication restart, and rolling circle replication of plasmids [26, 69, 70]. The UvrD helicase shows unidirectionality in dsDNA unwinding (3′–5′), which is facilitated in the presence of a 3′ ssDNA overhang covalently attached to the dsDNA. Monomers of UvrD helicase are able to bind to ssDNA; however, they cannot unwind dsDNA. Instead, single

turnover pre-steady state kinetic analysis of DNA unwinding showed that the active form of UvrD helicase in vitro is a dimer [71]. Kong and his colleagues have developed an in vitro isothermal DNA amplification method using *E. coli* UvrD helicase as an alternative to DNA separation by the thermocycling apparatus [22, 72]. They also included MutL protein to stimulate helicase activity, because UvrD helicase plays its role as the master coordinator of mismatch repair through direct physical interaction between UvrD and MutL; MutL is responsible for loading the UvrD onto the DNA substrate [73, 74]. Based on the current model for loading of UvrD by MutL [75], a dimer of MutL in the ATP-bound form, which may be attached to the end of blunt-ended DNA, recruits and loads multiple molecules of UvrD onto the end of dsDNA substrates (Fig. 2a). In addition, phage T4 gene 32 proteins, which are SSB proteins, were also included in the HDA reaction, without which no amplification product was obtained [22]. Thus, SSB proteins are required in the reaction in order to prevent reannealing of the complementary ssDNA templates. In fact, DNA replication in vivo begins with dsDNA separation by helicase, and then SSB proteins associate with unwound ssDNA templates for primer hybridization. The DNA polymerase then extends the primers bound to the templates and one dsDNA is finally amplified to two dsDNAs. Thus, the initially developed HDA system, which contains a mixture of UvrD helicase with MutL, exo$^-$ Klenow fragment, and SSB protein in one tube, mimics the in vivo DNA replication system. In the HDA system, the two newly synthesized dsDNAs enter the next round of amplification and the chain reaction repeats itself, thus accomplishing an exponential enrichment of target DNA sequences [22].

However, this initial UvrD HDA system could not efficiently amplify long target sequences, probably due to the intrinsic properties of the UvrD helicase. It has been reported that the UvrD helicase has a limited speed (20 bp/s) and processivity (less than 100 bp/binding) [22]. Although MutL can stimulate the UvrD helicase DNA unwinding activity by loading onto the ssDNA, the processivity of the UvrD helicase was not enhanced in the presence of MutL [74]. Kong's group improved the efficiency of HDA by using a thermostable UvrD helicase (*Tte*-UvrD) purified from thermophilic bacteria (*Thermoanaerobacter tengcongensis*) and the *Bacillus stearothermophilus* polymerase I large fragment without that addition of the MutL and SSB protein, which can amplify the target sequences at 60–65°C [72]. Unlike the HDA system, which utilized the *E. coli* UvrD helicase, no significant effect on the thermostable UvrD helicase activity by the *mutL* homolog from *T. tengcongensis* was observed. The active dimeric form of the UvrD helicase has been known to require 3′-ssDNA tails several nucleotides in length [71]. The ends of duplex DNA

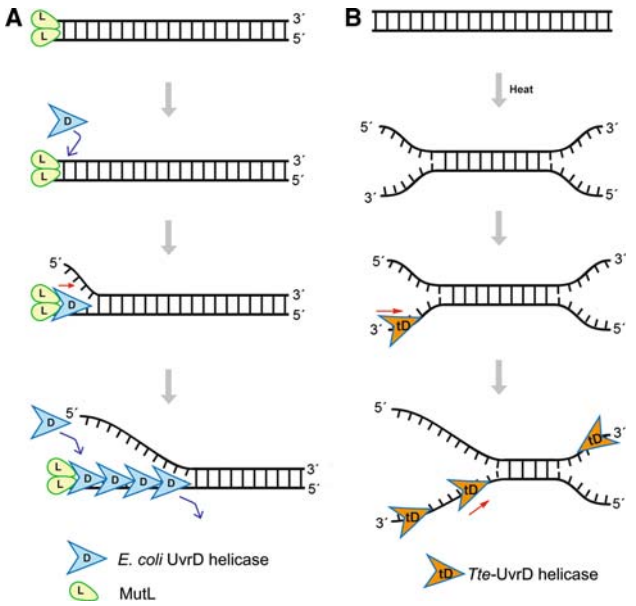

Fig. 2 Model for the mechanism of UvrD loading on ssDNA. A model for loading onto ssDNA and dsDNA unwinding by UvrD + MutL at 37°C (**a**) or by thermostable UvrD alone at high temperature (60–65°C) (**b**). See text for details

may become single-stranded because of thermal breathing at high temperatures. Thus, the thermostable UvrD helicase (*Tte*-UvrD) unwinds both blunt-ended and 5′-ssDNA-tailed duplexes without MutL by capturing transient 3′-ssDNA tails generated at high temperatures (Fig. 2b) [72]. In addition, the SSB protein was not required in the thermostable HDA system, which was probably due to the slower rate of reannealing of separated DNA strands at higher temperatures [72]. The fact that the SSB protein and MutL were not required to enhance the helicase activity led to a more simplified reagent composition.

Because the sensitivity and specificity of DNA amplification in the HDA reaction was improved at higher temperatures (60–65°C) with the thermostable UvrD helicase, adaptation of the HDA system for the detection of antibiotic-resistant pathogens *N. gonorrhoeae* and *S. aureus* on surface bound primers was performed [76]. This work, referred to as "OnChip HDA", provides a way for the HDA system to be developed into a miniaturized and multiplexed detection system for point-of-care diagnosis in conjunction with a commercially available thermostable HDA kit (IsoAmp II tHDA kit; BioHelix, Beverly, MA, USA). Thermophilic HDA was also used for the detection of *Helicobacter pylori* in combination with ELISA using a digoxigenin-labeled primer by Gill and colleagues. In addition, they also developed a colorimetric method to detect *H. pylori* using thermophilic HDA and gold nonoparticles [77, 78]. It has been reported that RNA targets as well as DNA were also amplified and detected by

thermophilic HDA followed by reverse transcription [79]. Recently, a new chimeric protein, named helimerase, was constructed by physically linking a thermostable helicase and thermostable DNA polymerse, *T. tengcongensis* UvrD helicase and *Bacillus stearothermophilus* polymerase I large fragment, using structural motif of coiled-coil [80]. The fusion protein showed both helicase and polymerase activity and was more efficient at DNA amplification than when the individual proteins were used. The helimerase system was able to amplify fragments that were up to 2.3 kb in length, whereas when the UvrD helicase and DNA polymerase were used separately only fragments less than 400 bp could be successfully amplified.

HDA system using the T7 bacteriophage replisome machinery

Since the initial HDA system using UvrD helicase was only able to amplify DNA fragments up to several hundred base pairs, which was due to the limited processivity and speed of the UvrD helicase, a new HDA system with high processivity and speed was developed using the T7 bacteriophage replication machinery, which can replicate its 40-kb genome in one initiation event [81]. The high processivity of the T7 bacteriophage replication system compared with the *E. coli* UvrD system makes this replication machinery attractive for long DNA amplification in vitro.

The T7 bacteriophage replisome consists of four proteins, phage-encoded gp4 (helicase and primase), gp5 (DNA polymerase), gp2.5 (ssDNA binding protein), and host-encoded thioredoxin [82, 83], and only these four proteins are needed to replicate its genome, as shown in Fig. 3. The 63-kDa gp4A has both helicase and primase activities, whereas the shorter 56-kDa gp4B (lacks 63 residues) has only helicase activity [82, 84–87]. Similar to the UvrD helicase, T7 gp4A (referred to as T7 helicase) is involved in various aspects of DNA metabolism and uses NTP hydrolysis as an energy source. As depicted in Fig. 3, in which the T7 replication system consists of the above mentioned proteins at a replication fork, the T7 helicase assembles into a ring-shaped hexamer in the presence of nucleotide ligands such as ATP, dTTP, dTDP, and dTMP-PCP [88, 89]. Although the T7 helicase hydrolyzes various NTPs, dTTP is preferred for efficient dsDNA unwinding. The dTTP hydrolysis activity is enhanced by ssDNA binding and is almost 100 times faster than in the absence of DNA [90]. The T7 helicase shows unidirectional movement (5′–3′), and dsDNA unwinding is facilitated in the presence of a 5′-ssDNA overhang covalently attached to the dsDNA [91, 92]. Unlike many other helicases, the T7 helicase does not need other proteins to bind to the

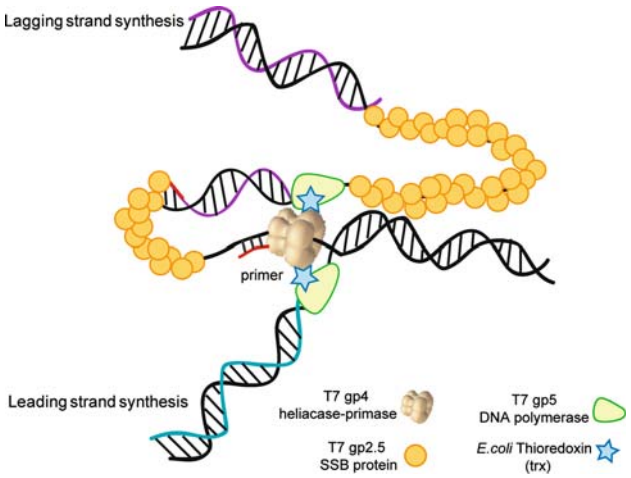

Fig. 3 Model of T7 bacteriophage replisome at the replication fork. The schematic shows the DNA replication system of T7 bacteriophage, which consist of the DNA polymerase (*gp5*), the helicase-primase (*gp4*), the ssDNA-binding protein (*gp2.5*), and the processivity factor *E. coli* thioredoxin (*trx*). Hexameric helicase T7 gp4 unwinds the dsDNA at the fork and the primase domain of T7 gp4 produces the RNA primer (*red segment*). Extruded ssDNA (lagging strand) is coated by gp2.5 and aligns its polarity with the leading strand through a loop formation. The leading strand is synthesized by the T7 gp5/trx complex tethered to gp4 as Okazaki fragments, whereas the leading strand is synthesized by T7 gp5/trx complex continuously

substrate DNA. The T7 helicase can bind equally well to both linear and circular ssDNA [93], suggesting that the hexameric ring must open or disassemble to encompass the ssDNA in the central channel of the helicase [94]. EM images show that the T7 helicase surrounds only one strand of the dsDNA, which goes through the central channel of the ring, and the complementary strand is excluded from the channel [95].

The T7 DNA polymerase (gp5) is encoded by gene 5 of the phage. The T7 gp5 protein was found to be complexed with the host *E. coli* thioredoxin (trx) in the replisome. The T7 DNA polymerase itself is not processive; but, when it forms a complex with *E. coli* thioredoxin, the speed and processivity are enhanced by up to >100 nt/s and >10 kb per binding, respectively [96]. Although the exact mechanism of the enhanced processivity by thioredoxin has not been well established, thioredoxin is believed to clamp the DNA polymerase to the DNA template for better processivity (Fig. 3). It was recently proposed that thioredoxin mediates the interaction between the T7 helicase and the DNA polymerase through electrostatic binding, which further increases the processivity of the T7 DNA polymerase [97]. The T7 gp2.5 protein (∼26 kDa) is a SSB protein that binds about seven nucleotides per monomer and exists as a dimer of two identical subunits [98, 99]. The gene 2.5 protein binds to the exposed ssDNA on the lagging strand in the replication fork. If gene 2.5 is deleted

in T7 bacteriophage, phage DNA synthesis is greatly decreased to a level of less than 1% relative to wild-type. The gp2.5 proteins have been known to interact with the T7 DNA polymerase and helicase, which is important for coordination of leading and lagging strand synthesis [100, 101].

It was reported that T7 helicases translocate along ssDNA at a speed of about 130 bases/s, which is considered to be the maximal speed of the T7 helicase by itself [102]. However, pre-steady state kinetic analysis of dsDNA unwinding shows that the T7 helicase separates dsDNA at a very slow speed of about 15 bps/s [92]. Stano et al. [103] showed that the T7 DNA polymerase can increase the dsDNA unwinding rate of the T7 helicase up to a similar speed when it translocates along ssDNA. In addition, the T7 DNA polymerase by itself is not able to catalyze strand displacement DNA synthesis; however, duplex DNA can be efficiently synthesized when it is combined with the T7 helicase. Studies using a T7 helicase mutant with the 17 C-terminal residues deleted showed that the interaction between the 17 C-terminal residues and T7 DNA polymerase does not increase helicase activity [104]. However, the interaction between the T7 helicase and T7 DNA polymerase increased the processivity of the complex to copy long DNA.

Using the T7 replication system, isothermal amplification of DNA strands that were as long as 10 kb was reported with circular DNA templates as well as for a defined length of target DNA by two primers [81]. The method allows for complicated rolling circle amplification to be performed without initial heat denaturation and DNA contamination (Fig. 4). In this system, referred to as the circular HDA system (cHDA), circular DNA template was used to amplify a very long DNA fragment by performing a helicase-dependent and strand-displacement reaction with the T7 gp4B helicase and $3' \rightarrow 5'$ exonuclease-deficient T7 DNA polymerase (T7 Sequenase; USB, Cleveland, OH, USA). Using circular DNA as a template, DNA amplification was accomplished through helicase-dependent rolling circle amplification (RCA), producing multiple copies of a specific product defined by two primers and concatemers of circular DNA. In this method, the T7 replisome extends a primer annealed to the complementary region in the single-stranded circular template DNA (step 1). After one round of rolling circle synthesis of DNA, the $5'$-end of the newly synthesized DNA strand is displaced by the T7 replisome (step 2). The rolling circle synthesis of DNA is continued and the displaced strand provides multiple sites for annealing of the reverse primer, which is extended by the T7 replisome (step 3). The synthesized ssDNAs that are extended from the reverse primers are released due to the activity of strand displacement by the T7 replisome, which provide annealing sites for the forward primers (step 4). The T7 replisome then extends the forward primers annealed to the released ssDNAs, generating dsDNA products defined by two primers and concatemers of the circular DNA template (step 5). The T7 replisome continues this process with the released ssDNA produced at step 5 for the next round of strand displacement synthesis.

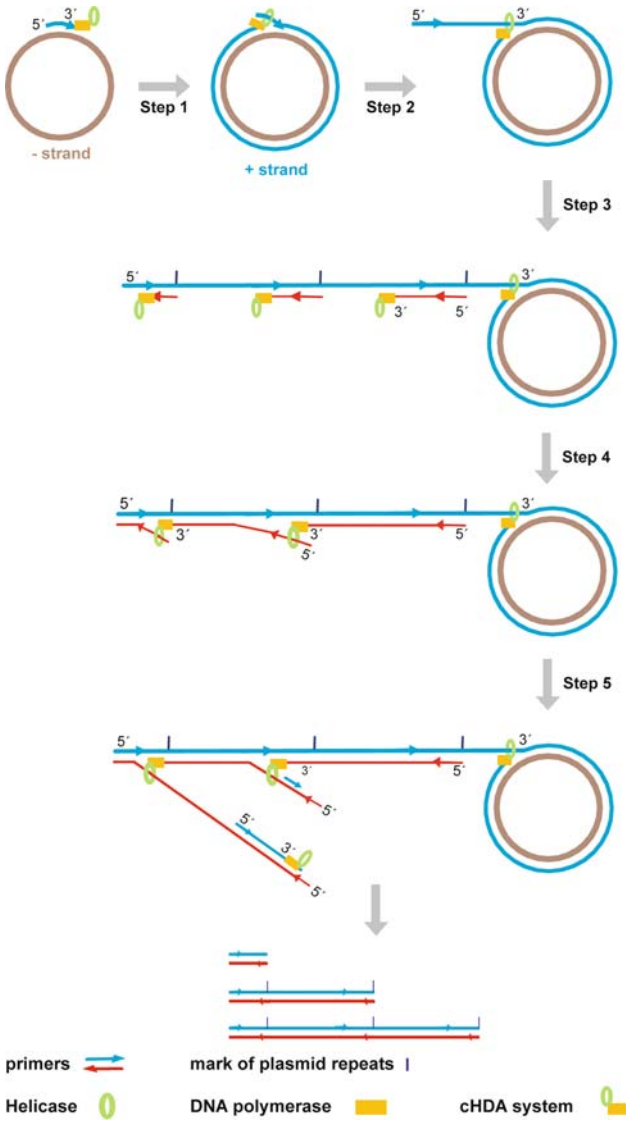

Fig. 4 Schematic of circular helicase-dependent DNA amplification. The T7 replisome machinery consists of a T7 gp4B helicase and T7 DNA polymerase gp5/trx complex. Primer extension and strand displacement produces a concatamer of the circular template DNA. Multiple reverse primers anneal to the concatamer and are extended by the T7 DNA polymerase. The helicase/DNA polymerase complex displaces the non-template strands, which provide complementary sites for forward primers to anneal. Duplex DNAs are produced by the T7 replisome after the release of ssDNAs for the next round of strand displacement synthesis. This figure is reproduced and modified from [81]

Recently, another type of DNA amplification using T7 gp4 primase was developed to remove the requirement of adding primers [105]. T7 gp4 contains the primase activity,

which resides on the N-terminus half of the protein, and predominantly recognizes only the following nucleotide sequences: 5′-(G/T)GGTC-3′, and 5′-GTGTC-3′. Thus, it generates short RNA primers for lagging strand synthesis [106, 107]. The T7 DNA polymerase can synthesize DNA by extending from the short RNA primers. In this method, referred to as primase-based whole genome amplification (pWGA) [105], which utilizes the dual activities of T7 gp4 as helicase and primase, dsDNA templates are denatured and primers are generated without addition of DNA primers and thermocycling. After T7 gp4 denatures the dsDNA template and synthesizes short RNA primers, the T7 DNA polymerase subsequently extends the strand with the aid of *E .coli* thioredoxin and T7 gp2.5. Overall, this technique is quite close to the cHDA method described above (Fig. 5). Using this method for DNA amplification, microgram quantities of DNA product could be obtained from nanogram quantities of input DNA within an hour.

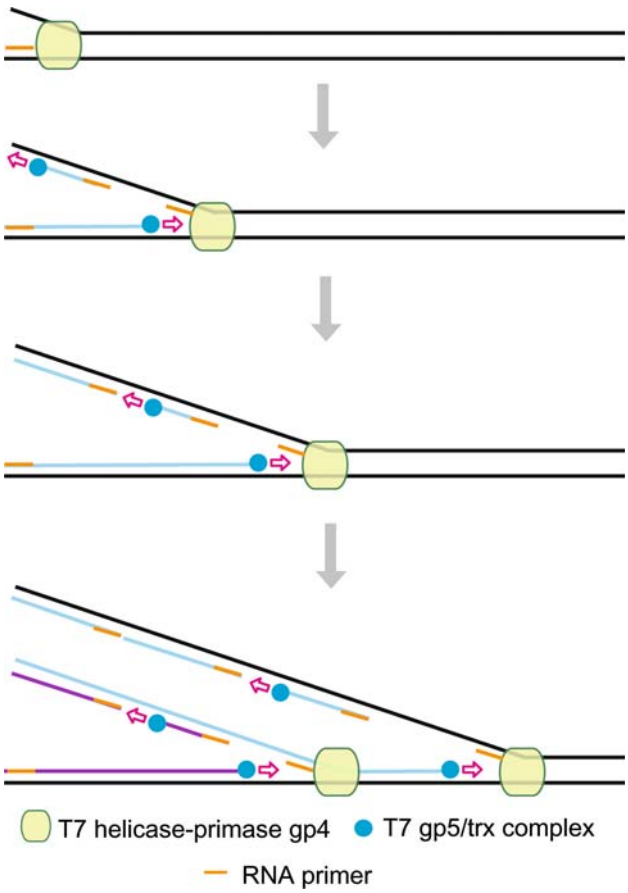

Fig. 5 Mechanism of primase-based whole genome amplification. The T7 helicase-primase gp4 denatures the dsDNA template and synthesizes primers. Primers are extended by the T7 DNA polymerase gp5/trx complex, resulting in DNA replication in both strands. Newly synthesized DNA is displaced and serves as a template for whole genome amplification. This figure is reproduced and modified from [105]

More importantly, elimination of primer annealing reduces the risk of amplification bias due to uneven annealing of the added primers.

Perspective use of other helicases in HDA system

Despite the fact that the process of genome replication in prokaryotes and eukaryotes has been investigated for decades, a lot of questions still remain unresolved. The replication of dsDNA requires the proper assembly of many proteins, and their coordinated functions are essential to maintain biological integrity. From viruses to humans, the fundamental components for genome replications are very similar; these components must be able to unwind nucleic acid strands, synthesize complementary DNA, and bind to exposed ssDNA with proteins, although the numbers and intrinsic properties of the individual proteins are different [108]. Of the many replication systems, only a few species, including *E. coli*, T7, and T4 bacteriophage, have been well studied, and their replication machineries have been shown to be applicable to nucleic acid amplification in vitro, as described in this review. For example, in the case of T4 bacteriophage, at least eight proteins are involved in the replication system: gp43 (DNA polymerase), gp41 (helicase), gp61 (primase), gp32 (SSB protein), gp44/62 (clamp loader), gp45 (sliding clamp), and gp59 (helicase loading protein) [109, 110]. According to the results of von Hippel et al. [111], a minimal replication complex, which consisted of T4 gp41 and gp43, can be formed in a DNA-mediated fashion and synthesize complementary DNA at a speed of ∼90 nts/s. Although T4 gp43 is nonprocessive by itself, strand-displacement DNA synthesis by the T4 DNA polymerase holoenzyme complex (gp43, gp44/62, and gp45) is processive and efficient in DNA synthesis at the replication fork with the aid of the T4 gp41 helicase, showing an enhanced rate of ∼250 nts/s [111]. This result demonstrates the importance of the processivity clamp and clamp-loader complex. The processivity clamp (gp45) is a trimeric protein, and the clamp-loader complex consists of four molecules of gp44 and one molecule of gp62 [108].

Similar to the T4 replication system, the ring-shaped processivity factor and its clamp-loader proteins are required in the *E. coli* replication machinery. Compared to T7 bacteriophage, the replication system of *E. coli* is more complex because many proteins are involved in the replisome machinery. The replication proteins of *E. coli* consist of polymerase III core (α, polymerase catalytic subunit; ε, 3′–5′ exonuclease; and θ, stimulates ε exonuclease), τ protein (mediates the connection of polymerase to DnaB and stimulates DnaB activity [112]), β protein (sliding clamp), γ complex ($\gamma\delta\delta'\chi\psi$, clamp loader), DnaA (replication

initiator protein), DnaB (helicase), DnaC (helicase loading partner), DnaG (primase), and single strand binding proteins, etc. [113]. Thus, proper organization of replication proteins is very important for strand unwinding and synthesis in *E. coli*. Among these proteins, the hexameric helicase (DnaB) is an essential component of the DNA replication machinery in *E. coli*, which requires Mg^{2+} for hexamer formation. The DnaB helicase shows poor processivity and unwinding efficiency by itself, but within the replisome DnaB supports highly processive DNA synthesis [114]. Single-turnover kinetic analysis of dsDNA unwinding studies show that *E. coli* DnaB alone unwinds DNA at a rate of ~291 bp/s at 25°C with an unwinding processivity of 0.89 [115]. The DnaB helicase shows a similar dsDNA unwinding mechanism to the T7 gp4 helicase; that is, the ssDNA passes through a central channel of the hexameric ring during dsDNA unwinding with a 5′–3′ directionality[116]. However, unlike the T7 gp4 helicase/primase, the DnaB helicase is separated from the primase polypeptide (DnaG) and needs a loader protein (DnaC) to bind to ssDNA[117]. In the T4 replication system, the helicase loader protein (gp59) is also required for the T4 helicase to bind to ssDNA [118]. Thus, in contrast to T7 replication, the T4 and *E. coli* replication system requires more complex components such as processivity clamp, clamp-loader, and helicase loader, which are not necessary in the T7 replisome system.

The non-hexameric helicases also hold promise for use in denaturing dsDNA molecules to facilitate isothermal amplification of target DNA. A lot of biochemical and structural studies of non-hexameric helicases, most of them belonging to superfamily-1 (SF1) or SF2 (for example, SF1; PcrA, Rep, UvrD, and SF2; NS3, etc.), have been conducted to understand the mechanism of ssDNA translocation and dsDNA unwinding. Pre-steady state kinetic studies have shown that monomeric forms of a few SF1 helicases (PcrA, Rep, and UvrD) are rapid and processive ssDNA translocases, but do not function as processive helicases [119]. To overcome this limited helicase activity, oligomerization or interactions with accessory proteins were employed to enhance the helicase activity in vitro [43, 45]. As in *E. coli* UvrD and accessory protein MutL, *Bacillus stearothermophilus* (*B. stear*) PcrA and hepatitis C virus (HCV) NS3 require *B. stear* RepD and HCV NS4A as accessory proteins, respectively, for better helicase function [120, 121].

Isothermal DNA amplification in a tube without thermocycling was attempted using *E. coli* UvrD or T7 bacteriophage helicase system, and isothermal nucleic acid amplification kits were developed commercially at BioHelix. However, nucleic acid amplification using the T4 replication system has not yet been reported. Whereas the processivity of T4 bacteriophage is attained by interaction of gp44/62 and gp45 with the polymerase (gp43), the processivity of T7 bacteriophage is assured only by a tight interaction between the host thioredoxin and polymerase, rendering the T7 system simpler than the T4 system in terms of on-spot amplification of nucleic acid. Nevertheless, it is expected that more useful and simpler devices will be invented by making use of the nucleic acid replication systems of diverse microorganisms such as other viruses or thermopile bacteria.

Conclusions

Amplification of nucleic acids has emerged as an elemental tool for molecular biology, genomics, and biotechnology. Since the development of PCR, there have been numerous attempts to amplify DNA and RNA in vitro. Similar to PCR, some of amplification methods utilize temperature cycling to exponentially amplify nucleic acids. In contrast, isothermal amplification methods have also been developed which do not require heating the double-stranded nucleic acid to dissociate the synthesized products from templates. DNA amplification under isothermal conditions was possible by using the reconstitution of protein components present in the DNA replication fork, which is referred to as the helicase-dependent amplification (HDA) system. In the HDA system, duplex DNA is denatured by DNA helicase binding to ssDNA. This also serves as a reservoir for the DNA polymerase, which can initiate DNA synthesis. The first HDA system for isothermal DNA amplification was developed by using *E. coli* UvrD helicase and the helicase-loader MutL protein. This method was further simplified with increased amplification specificity by using the thermostable UvrD helicase at an elevated temperature. In this modified method, the SSB protein and MutL were not needed to enhance the helicase activity. However, the amplification of a long target sequence was not possible due to the low processivity and limited speed of DNA synthesis by UvrD helicase. A new HDA system with high processivity and speed was developed using the T7 bacteriophage replication machinery, in which the ring-shaped hexameric T7 DNA helicase gp4 played a pivotal role in maintaining the processivity and fidelity of DNA synthesis. The T7 helicase-based HDA system has been applied to amplify circular ssDNA templates, and the primase activity of T7 gp4 allows for whole genomes to be amplified in the HDA system without the need for additional DNA primers. These current HDA techniques are summarized with their strengths and weaknesses in Table 1. Replicative helicases from other sources such as T4 bacteriophage or thermostable bacteria might be useful in HDA systems by optimizing and improving the processivity and fidelity of DNA synthesis in vitro. The simplicity and isothermal nature of the HDA technology based on DNA helicase

Table 1 Properties of various isothermal helicase-dependent amplification (HDA) methods

HDA technique (helicase used)	Overview of method	Strength	Weakness
Ambient temperature HDA (*E. coli* UvrD helicase)	UvrD helicase with MutL, *exo*$^-$ Klenow fragment, and SSB protein in one tube mimics the DNA replication system for DNA amplification at ambient temperature	The first developed HDA system without temperature cycling	Limited speed (20 bp/s) and processivity (less than 100 bp/binding)
Thermostable HDA (*Tte*-UvrD helicase)	Thermostable UvrD helicase purified from thermophilic bacteria (*Thermoanaerobacter tengcongensis*) and polymerase I large fragment from *Bacillus stearothermophilus* amplify the target DNA sequences without MutL and SSB protein at high temperature (60–65°C)	MutL and SSB are not required. Higher stringency than *E. coli* UvrD helicase system. Applicable to the OnChip HDA	Same as above
Circular HDA (cHDA) (T7 gp4B DNA helicase)	Circular DNA template is used to amplify a long DNA fragment by helicase-dependent and strand-displacement reaction with T7 gp4B helicase and 3′ → 5′ *exo*$^-$ T7 DNA polymerase, supplemented with *E. coli* thioredoxin (trx) and T7 gp2.5 SSBs. DNA amplification produces multiple copies of a specific product defined by two primers and concatemers of circular DNA (Fig. 4)	High speed (>100 bp/s) and processivity (>10 kb/binding)	Processivity factor (trx) and T7 gp2.5 SSB are required for high processivity and yield
Primase-based whole genome amplification (pWGA) (T7 gp4 DNA helicase-primase)	Dual activities of T7 gp4 as helicase and primase are utilized; dsDNA templates are denatured and primers are generated without addition of DNA primers and thermocycling. T7 DNA polymerase extends the strand subsequently with the aid of *E. coli* thioredoxin and T7 gp2.5 (Fig. 5)	High yield in a short time; microgram scale of DNA can be obtained from nanogramscale of input DNA within an hour. Primers are not required	Same as above

activity provides great potential for the development of portable diagnostic tools to detect pathogens in the field.

Acknowledgments This work was supported by a grant (20080401034026) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea, a grant from Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A080123), a grant (10032113) from Industrial Technology Development, Ministry of Knowledge Economy, and a grant from the Korea Science & Engineering Foundation (KOSEF) through a general research grant (R01-2008-000-20301-0). K. Park is supported by the second stage of Brain Korea 21. Y.-J. Jeong was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-313-C00531).

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