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# Seryl-Histidine as an Alternative DNA Nicking Agent in Nick Translation Yields Superior DNA Probes and Hybridizations

Yunsheng Li,[a] Scott Hatfield,[a] Jing Li,[a] Mark McMills,[b,c] Yufen Zhao[d] and Xiaozhuo Chen[a,b,c,e,*]

[a]*Edison Biotechnology Institute, Ohio University, Athens, OH 45701, USA*
[b]*Department of Chemistry and Biochemistry, Ohio University, Athens, OH 45701, USA*
[c]*The Molecular and Cellular Biology Program, Ohio University, Athens, OH 45701, USA*
[d]*Department of Chemistry, Tsinghua University, Beijing 100084, China*
[e]*Department of Biomedical Sciences of School of Osteopathic Medicine, Ohio University, Athens, OH 45701, USA*

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**Abstract**—Nick translation is a commonly used method for labeling DNA to make DNA hybridization probes. In this approach, the use of DNase I to generate nicks in double-stranded DNA presents an inherent drawback, because the enzyme's high rate of reaction causes significant fragmentation and shortening of the hybridization probes. Based on our recent findings regarding the nucleolytic activity of the dipeptide seryl-histidine (Ser-His) and generation of free 3′ hydroxyl and 5′ phosphate groups at the cleavage sites of the substrate DNA by Ser-His, it was hypothesized that this disadvantage may be overcome by using Ser-His in place of DNase I as an alternative DNA nicking agent. In this study we demonstrate that like DNase I, Ser-His randomly nicks DNA, but the dipeptide has a much lower rate of reaction that enables more complete labeling of the DNA probes with less fragmentation. DNA probes labeled through nick translation using Ser-His as the DNA nicking agent were consistently larger in size and exhibited significantly higher specific activities, and enhanced hybridization signals in Southern blot analyses compared to control DNA probes that were made using DNase I as the nicking agent. Furthermore, the degree of nicking and consequently the quality of the probes could be easily controlled by adjusting the temperature and time of the Ser-His nicking reaction. These results affirm our hypothesis that Ser-His can serve as an alternative DNA nicking agent in nick translation to yield superior DNA probes and hybridization results and suggest the possible general utility of Ser-His for wide range of biological and biomedical applications that require more moderated nicking of nucleic acids. Based upon these and computer modeling results of Ser-His, a mechanism of action is proposed to explain how Ser-His may nick DNA. 

## Introduction

Nucleic acid hybridization is an important method for analyzing specific DNA or RNA sequences, in which a targeted sequence is detected by binding a complementary labeled nucleic acid probe.[1–7] A common way of labeling nucleic acid probes, with either radioisotope labeled nucleotides or otherwise, is nick translation.[8–11] This method typically employs DNase I as the DNA nicking agent to generate single-stranded breaks in double-stranded DNA, exposing new 5′-phosphate groups and 3′-hydroxyl groups (Fig. 1). The nicks are subsequently translated along the DNA in the 5′-3′ direction as DNA Polymerase I uses the newly generated 3′-hydroxyl groups to introduce labeled deoxy-nucleotides into the nicked DNA while removing native nucleotides ahead of the nick.[8–11] Ideally, nicking of the DNA will be sufficiently extensive to allow maximal labeling without occurring so frequently that proximal nicks on opposite strands result in excessive fragmentation of the DNA. Because DNase I is an efficient enzyme that has an intrinsic tendency to nick both strands at the same base-pairs and severely fragment the substrate DNA, the nicking reaction is usually performed at a temperature significantly lower than room temperature and with very low DNase I concentrations in the presence of $Mg^{2+}$ to reduce DNA fragmentation.[10–12] Even with such precautions, DNase I frequently makes nicks at corresponding positions on both strands, fragmenting the DNA substrate and leaving a significant proportion of the DNA probes in

*Corresponding author at: Edison Biotechnology Institute, 109 Konneker Research Center, Ohio University, the Ridges, Athens, OH 45701, USA. Tel.: +1-740-593-4713; fax: +1-740-593-4795; e-mail: chenx@ohiou.edu

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various degrees of truncation. Since these shortened probes cannot bind the target sequence as well as full-length probes, the hybridization signal is diminished. To address this shortcoming of DNase I in nick translation, it was hypothesized that more full-length probes could be generated using the dipeptide Ser-His instead of DNase I to nick the DNA. Recent findings that Ser-His, a dipeptide with two amino acid residues also function as essential amino acids in the active sites of serine proteases,[13] lipases,[14,15] esterases,[16] and in intein–extein junctions of homing endonucleases,[17–19] is capable of cleaving an array of biological molecules, including DNA, protein, and ester substrates, with reaction rates easily moderated by controlling reaction concentrations, pH, or temperature.[20] These findings suggest the feasibility of using Ser-His in nick translation. In considering Ser-His as an alternative to DNase I as the nicking agent in nick translation, two questions were addressed. First, though the mechanism by which Ser-His cleaves DNA is not fully understood, the dipeptide would need to generate 5′-phosphate groups and 3′-hydroxyl groups on the nicked DNA to be suitable for nick translation. The generation and presence of 5′-phosphate groups and 3′-hydroxyl groups on the ends of the DNA fragmented with Ser-His was previously indirectly demonstrated by the capability of re-ligation of DNA fragments produced by Ser-His cleavage.[20] Second, Ser-His would have to generate sufficient nicking of the DNA to allow ample labeling of the probe by DNA Polymerase I. The effectiveness of the probes generated using Ser-His in nick translation were demonstrated in comparison to probes nicked with DNase I by comparing the size of the probes and the strength of the hybridization signals in Southern blot analyses. Optimal conditions for using the dipeptide as the DNA nicking agent were investigated by examining the effects of the duration of the nicking reaction and the reaction temperature on the quality of the resulting DNA probes.

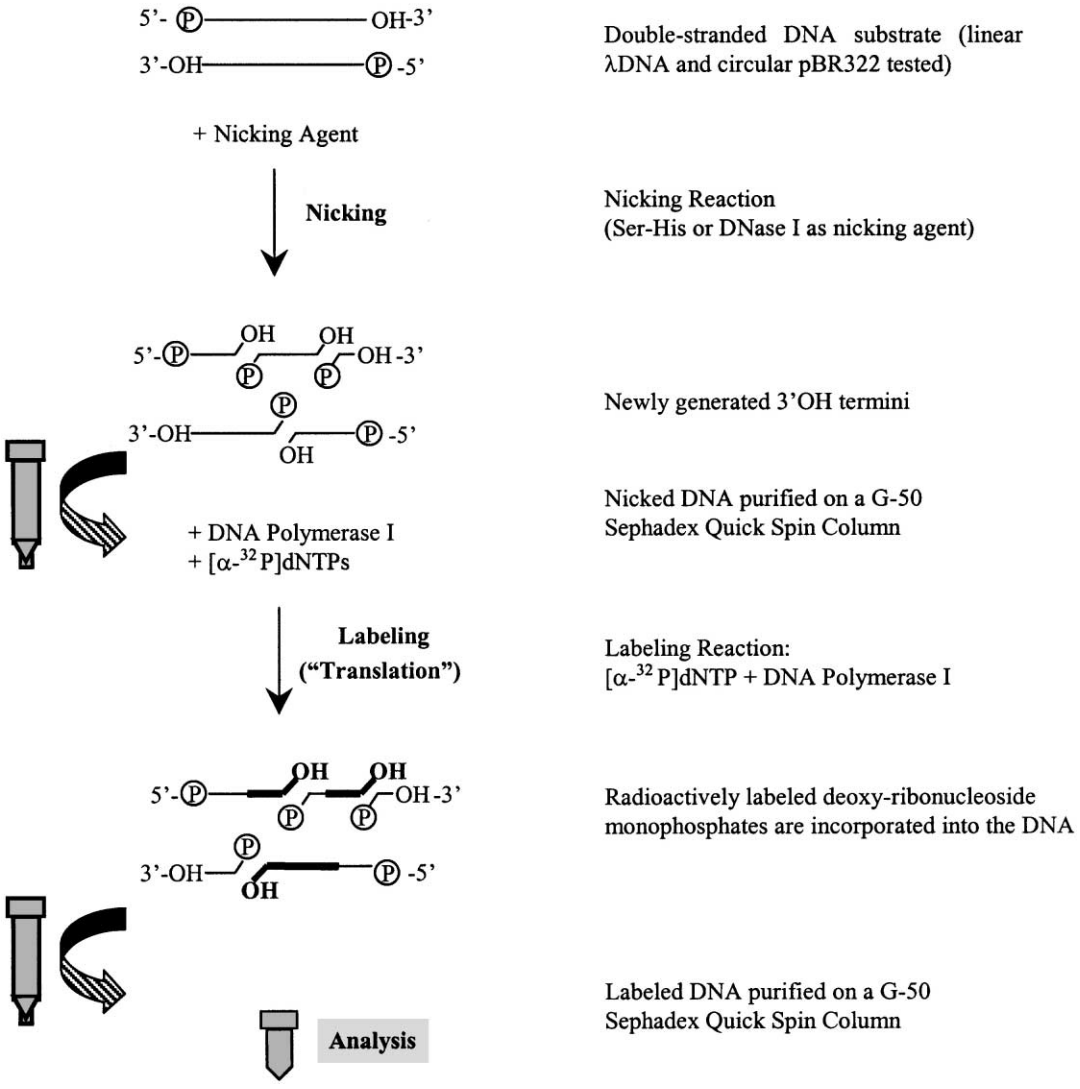


**Figure 1.** Flow-chart of the nick translation procedures using either Ser-His or DNase I. Double-stranded DNA was nicked with either Ser-His or DNase I as indicated. DNA that was nicked with DNase I and then labeled was processed according to the instruction of a nick translation kit (standardized nick translation). The procedure for making radioactive probe from DNA nicked with Ser-His is essentially the same as the procedure for DNase I nicked DNA except that Ser-His was removed from the nicked DNA by a G-50 Sephadex Quick Spin Column as an additional step.

## Results and Discussion

Dipeptide Ser-His has been found to be capable of cleaving DNA, protein, and carboxyl esters.[20] Furthermore, it has also been revealed that DNA fragments cleaved by Ser-His could be re-ligated by T4 DNA ligase.[20] This result suggests that the DNA cleavage carried out by Ser-His generates 3′-hydroxyl and 5′-phosphate groups in the newly generated ends of the cleaved DNA fragments. In addition, the DNA cleavage activity of Ser-His was shown to be greatly affected by reaction temperature.[20] All of these previous findings led us to speculate that Ser-His can be used to replace DNase I for DNA nicking in nick translation, which requires free 3′-hydroxyl and 5′ phosphate groups for the translation process to occur.[8–12]

The agarose gel analysis of the radio-labeled DNA fragments nicked by different agents revealed a upward shift of the smear generated by the radio-labeled DNA fragments nicked by Ser-His compared to that generated by DNase I under the optimal conditions specified by the nick translation kit (Fig. 2A and 2B). This result indicates the average size of the DNA nicked with Ser-His is noticeably larger than the same DNA nicked by DNase I. This observation may reflect a fact that Ser-His cleaves DNA at a slower rate than DNase I and the undesired DNA fragmentation can be more easily controlled and avoided with Ser-His than with DNase I. Furthermore, the DNA nicked with Ser-His was consistently labeled to higher specific activities than those nicked by DNase I (Fig. 2A and 2B), possibly also due to fewer and more random nicks and fewer DNA fragmentation generated by Ser-His compared to DNase I. It is known that the $k_{cat}$ and $K_m$ for DNase I are 15 per second and 34-50 mg/mL, respectively. Because of the technical difficulties involved in accurately measuring very slow cleavage reactions, the $k_{cat}$ and $K_m$ for DNA cleavage by Ser-His have not been determined. However, the difference in the DNA cleavage (nicking) rates between DNase I and Ser-His is estimated to be between 10,000-fold to 1,000,000-fold depending upon reaction conditions used.

Following the radio-labeling, the probes made from the DNA nicked either by Ser-His or DNase I (Fig. 2A and 2B) were used and compared for their relative performance in Southern blot analyses.[21] As shown in Figure 2C and 2D, the probes prepared from Ser-His nicked DNA consistently generated stronger hybridization signals to the complementary target DNAs than those generated by the probes prepared from the DNA nicked with DNase I on the same amount of target DNAs. Larger sizes and higher specific activities of the Ser-His nicked probes relative to the DNase I nicked probes appear to be the major factors contributing to the stronger hybridization signals. Thus, exposure time required by the Ser-His nicked probes can be proportionally shortened due to higher specific activity of the probes. Lower specific activity (Fig. 2B) and lower

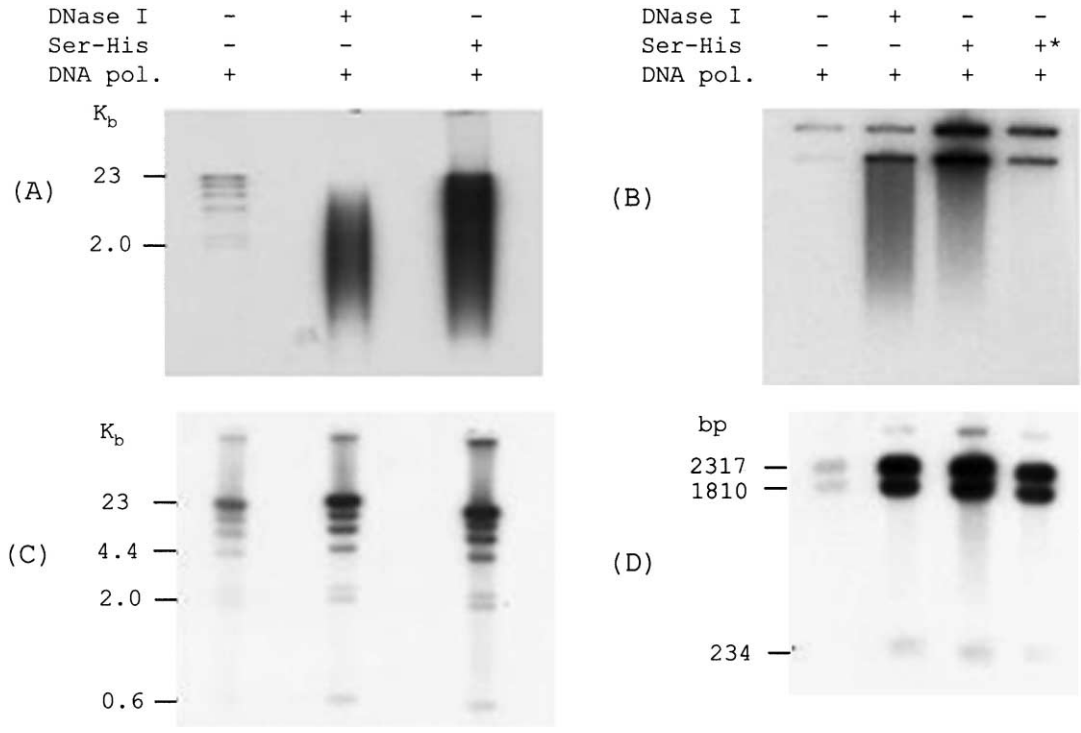


**Figure 2.** DNA probes prepared with Ser-His or DNase I as nicking agents and Southern blot analyses of target DNAs with these probes. Linear DNA of λ-DNA/Hind III fragments (A) or circular DNA of pBR322 (B) were used as DNA substrates for making probes. Probes were prepared with procedures described in Fig. 1 and in the Experimental. Following the nick translation procedures, the probes were subjected to 1% agarose gel electrophoresis, gel fixation, and autoradiography. DNAs of λ-DNA/Hind III fragments and pBR322 DNA without nicking (the lanes furthest to the left in both panels A and B) were used as negative controls. The same DNAs were nicked with DNase I under the optimal conditions specified by a nick translation kit. B. Following the nick by Ser-His, Ser-His was either removed (second lane from right) or not removed (first lane from right) prior to the labeling (translation) reaction. C. Southern blot analysis of λ-DNA/Hind III fragments using probes made from A. D. Southern blot analysis of pBR322/Bgl I cleaved DNA fragments using probes made from B.

hybridization signal from the probe translated with DNA polymerase I without prior Ser-His removal (Fig. 2D) suggests that the presence of Ser-His during the translation reaction interfered with the labeling reaction and thus Ser-His should be removed for preparation of probes with high specific activity.

It was previously shown that, with increasing incubation time, linear or circular plasmid DNA could be gradually cleaved by Ser-His into smear consisting of short heterogenous oligonucleotides.[20] It is conceivable that an optimal incubation time exists at which the specific activity of the probe is higher than that of traditional nick translation probe, but at the same time the average size of the probe is also larger than that of the traditional nick translation probe. The result of the nicking and labeling experiment with variable incubation time indicates that 4 h at 50 °C is the best incubation time tested, resulting in larger average size for the probe compared to the probe made from a standard nick translation kit under the optimal conditions as specified by the kit (Fig. 3A). More significantly, the specific activity of the probe prepared from DNA nicked with Ser-His for 4 h at 50 °C consistently doubles ($\geq$100%) that of the traditional DNase I nicked probe ($\geq 8\times10^8$ DPM/μg for Ser-His probe vs $\sim 4\times10^8$ DPM/μg for DNase I probe. (Fig. 3B). Shorter incubation time resulted in lower specific activity (under-nicking and under-labeling) and longer incubation resulted in no further increase in the specific activity, and smaller average size of the probe (over-nicking and over-cleavage; Fig. 3).

It can be time-consuming and inconvenient to make probes with 4 h additional nicking time compared to the standard nick translation procedure, and it is highly desirable to shorten the nicking reaction time. It was shown previously that Ser-His cleaves DNA with increasing rates when the incubation temperature is increased.[20] In this study, when the temperature of the Ser-His nicking reaction was increased from 50 °C to 60, 70, and 80 °C, and the reaction time was shortened to 2 h, 1 h, and 30 min, correspondingly (Fig. 4). It was found that probes made under these conditions are in general comparable in specific activity to that of the probe generated from the DNA nicked by Ser-His at 50 °C for 4 h (Fig. 4). This result is consistent with our hypothesis that the nicking reaction time can be shortened by increasing the nicking reaction temperature. On the other hand, nicking reaction performed at 70 °C and 80 °C resulted in relatively high specific activity but smaller average size of the probes (Fig. 4). This phenomenon can be explained by much higher non-catalyzed DNA strand breaks at higher incubation temperatures (open bars in Fig. 4B). These results indicate that nicking temperature and nicking time can be selected to achieve the best labeling and hybridization results suitable for different DNA templates and DNA targets.

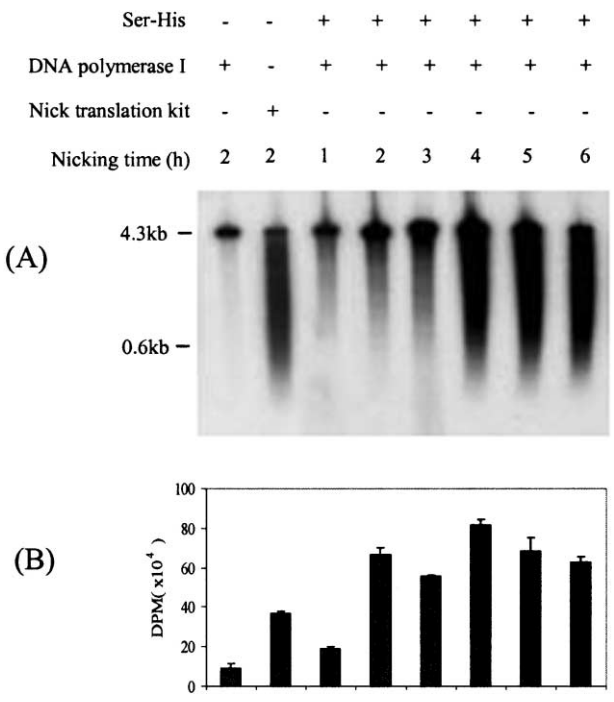


**Figure 3.** Probes prepared from DNA nicked with Ser-His for various nicking reaction times. Plasmid DNA pBR322 linearized with Bam H1 was used as DNA substrate. The DNA was nicked at 50 °C for various times, and then radio-labeled with DNA polymerase I. One ng of the labeled probe was subjected to 1% agarose gel electrophoresis, dried, and autoradiographed (A), or measured for its labeled radioactivity using a scintillation counter (B). Each bar corresponds to the probe shown in the gel on top, and represents mean ± SEM. Probes without nicking (lane 1) or prepared using a nick translation kit with kit's specified optimal conditions (lane 2) were used as controls.

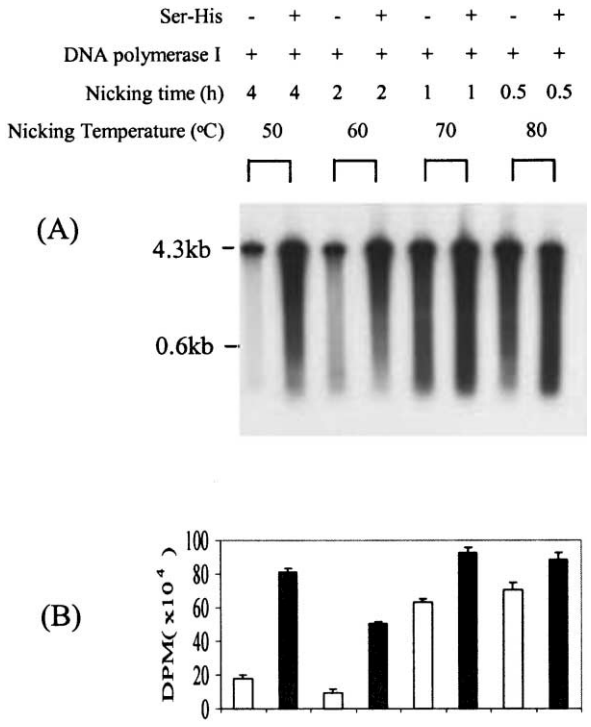


**Figure 4.** Probes prepared from DNA nicked with Ser-His for shortened incubation time and increased incubation temperature. Plasmid DNA pBR322 linearized with Bam H1 was used as DNA substrate. The DNA was nicked under increasing temperatures with decreasing nicking times, and then radio-labeled with DNA polymerase I. One ng of the labeled probe was subjected to 1% agarose gel electrophoresis, dried, and autoradiographed (A), or measured for its labeled radioactivity using a scintillation counter (B). Each bar corresponds to the probe shown in the gel on top, and represents mean ± SEM.

The successful labeling (polymerization) of the DNA nicked by Ser-His further supports the previous conclusion that the ends (gaps) of the DNA generated by the nicking of Ser-His contain 3′-hydroxyl and 5′-phosphate groups,[20] an activity that cannot be accomplished by transition metals or other nonpolypeptide nucleolytic agents.[22–25]

A possible structural explanation for the DNA cleavage activity of Ser-His is evidenced by computer modeling of the dipeptide. Using a Silicon Graphics System and a computer modeling software to predict a minimal energy conformation of Ser-His, it was found that the predicted conformation (Fig. 5A) is remarkably similar to the distances and relative orientations of the catalytically active Ser and His residues in the active site of chymotrypsin (Fig. 5B). The predicted distances between the oxygen in the hydroxyl group of Ser to the two nitrogen atoms in the imidazole group of His are 2.76 and 4.87 Å (Fig. 5A), compared to the corresponding distances 2.68 and 4.49 Å, respectively, in the chymotrypsin active site (Fig. 5B). Likewise, the distance between the amino nitrogen of Ser to its hydroxyl oxygen predicted in the minimized Ser-His conformation is also similar to the corresponding distance in chymotrysin: 2.83 Å compared to 3.11 Å (Fig. 5A and B), respectively. It is conceivable that unencumbered of the tertiary structure of a polypeptide enzyme like chymotrypsin, while retaining the critical aspects of the enzyme's catalytic center, Ser-His is able to extend beyond the range of chymotrypsin in cleaving peptide and ester bonds to include phosphodiester bonds among its substrates.

Based upon previous studies[20] and the computer modeling result (Fig. 5) as well as the mechanism by which chymotrypsin cleaves esters,[26] a mechanism of reaction is proposed to account for the DNA cleaving (nicking) activity of Ser-His (Fig. 6). The mechanism involves an $S_N2$ nucleophilic attack[27] by oxygen in the hydroxyl group of serine on a phosphorus of a phosphodiester bond in the DNA to form a pentacoordinate phosphorane transitional state, which is subsequently hydrolyzed, resulting in cleavage of the DNA. In this proposed mechanism, a positively charged nitrogen in the imidazole group of histidine facilitates the nucleophilic attack by interacting with one of the phosphate oxygen and functioning as a general base to stabilize the formation of the transitional state. The imidazole then assists the reaction by functioning as a general acid to protonate the leaving group. The low energy conformation of Ser-His predicted by computer modeling (Fig. 5A), which apparently enables to dipeptide to approach and interact with the phosphodiester, is likely the conformation required for this mechanism (Fig. 5C). The free 3-hydroxyl groups and 5-phosphate groups resulting from hydrolysis of phosphodiesters by Ser-His enable Ser-His to serve as a suitable substitute for DNase I in nick translation. Significant amount of future study in catalysis and kinetics, which are difficult

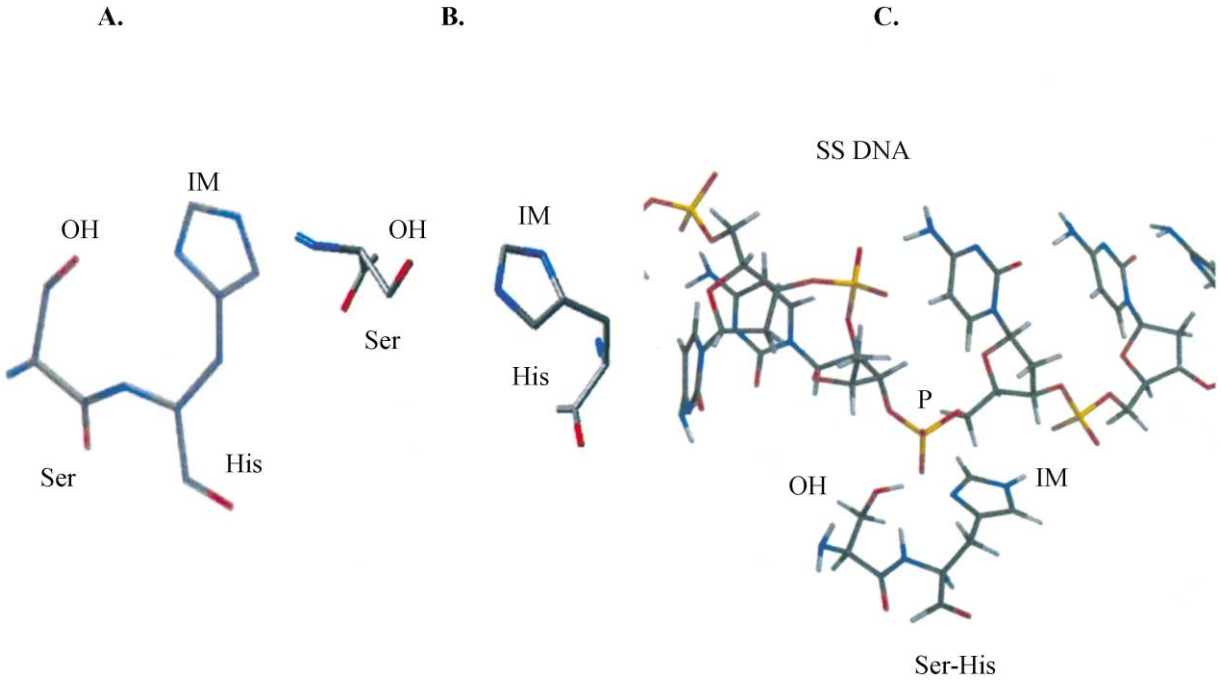


**Figure 5.** Computer modeling of Ser-His and Ser-His′ interaction with DNA substrate. Computer modeling was used to simulate the low energy conformation of Ser-His. The conformation was compared to that of the active site of a protease and esterase chymotrypsin. The molecular interaction between Ser-His and a single-stranded DNA substrate was also simulated. Atoms are denoted by colors as follows: oxygen (red), nitrogen (blue), carbon (black), and hydrogen (white). (A) A 3-D minimized energy conformation of Ser-His in vacuum environment. OH, hydroxyl group of Ser; IM, imidazole group of His. (B) Three-dimensional conformation of Ser and His residues in the active site of crystallized chymotrypsin. The conformation was constructed by downloading the crystal structure of chymotrypsin from Brookhaven National Protein Database, and removing all other amino acid residues of the protein from the structure. (C) Three-dimensional representation of the minimized energy conformation Ser-His

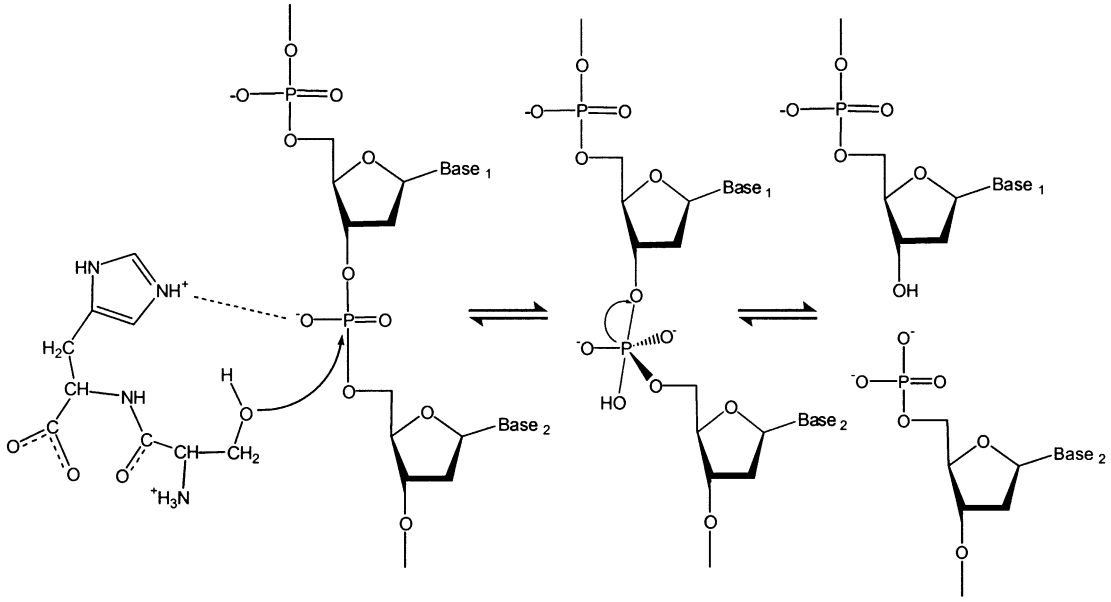


**Figure 6.** Proposed DNA nicking (cleavage) mechanism by Ser-His. As a first step of the reaction, nucleophilic attack on phosphorous ($S_N2$) by hydroxyl group of serine forms a pentacoordinated intermediate (or transition state). The subsequent cleavage (hydrolysis) produces a 3′-hydroxyl and a 5′-phosphate groups at the new ends of the cleaved substrate DNA. Dotted line indicates the interaction between the imidazole group in His and the oxygen atom linked to phosphorous, neutralizing the negative charge and facilitating the nucleophilic attack.

to perform due to the very slow and inefficient cleavage kinetics of Ser-His compared to those of polypeptide enzymes, needs to be done to substantiate and verify the hypothesis that Ser-His functions as a primitive enzyme and nicks DNA enzymatically.

## Conclusion

We have demonstrated that Ser-His can substitute for DNase I as a DNA nicking agent to produce superior DNA probes and hybridizations. This application can be easily extended to making non-radioactive nick translation probes.[5–7,28–31] Due to its simple composition, slow nicking rate, and flexibility in reaction temperature, particularly being active at temperatures significantly higher than physiological temperature, Ser-His can also be used in other experimental settings where random DNA nicking and high reaction temperature are required. Furthermore, when associated with a targeting molecule, Ser-His may be able to produce site-specific nucleic acid and/or protein cleavage.

## Experimental

### DNA substrates

Linear bacteriophage λ-DNA/Hind III fragments, a circular plasmid DNA pBR322 (Life Science Technologies, MD, USA), as well as a pBR322 fragment linearized with Bam H1 were used as substrates for DNA nicking reactions and for making probes.

### Nick translation

Four hundred ng of the DNA was incubated with 10 mM of Ser-His (BACHEM) in 40 mM of Britton-Robinson (B-R) buffer in a final volume of 20 μL at 50 °C for 4 h. After the incubation, the nicked DNA was isolated from Ser-His and B-R buffer with a G50 column by centrifugation. The subsequent nick translation reaction for Ser-His nicked DNA was performed exactly as instructed by a nick translation kit (Roche, Mannheim, Germany) using the materials provided by the kit except the DNase I-containing enzyme mixture. One hundred ng of the isolated nicked DNA was mixed with 1 unit of DNA polymerase I (Roche, Mannheim, Germany), [α-$^{32}$P]dCTP, and regular dNTPs (N = A, C, G, T) in standard nick translation buffer (Roche, Mannheim, Germany) at 15 °C for 60 min. As a negative control, the same amount of non-nicked DNA was subjected to the same procedure as Ser-His nicked DNA. The nick translation procedures for Ser-His and for DNase I are schematically described and compared in Figure 1. In order to compare with the probe made from the nick translation kit, the same amount of non-nicked DNA was incubated with reaction components provided by the nick translation kit (the enzyme mixture contains both DNase I and DNA polymerase I) plus [α-$^{32}$P]dCTP under the identical conditions, which were also consistent with the conditions recommended by the kit instruction. Following the nick translation reaction, labeled DNA from all three samples was separately isolated with G50 columns. The sizes and labeling intensities of the differentially labeled DNA were detected by subjecting 1 ng of each DNA sample (by serial dilution) to 1% agarose gel electrophoresis followed by autoradiography. The specific activities of the labeled DNA probes were measured with a liquid scintillation counter.

### Southern blot analysis

To determine the hybridization performance of the Ser-His nicked probe relative to the regular nick translation

probe, Southern blot analyses[21] were carried out. λ/Hind III cleaved DNA or pBR322/Bgl I cleaved DNA fragments were used as the targets for hybridization and were subjected to 1% agarose gel electrophoresis. The gel-separated DNA was then transferred to Nylon membranes. The membrane-bound DNA fragments were detected by hybridization to the differentially labeled probes followed by autoradiography.

### Computer modeling

A Silicon Graphic system O2 with an R5000 processor (Silicon Graphic Inc., Portland, OR, USA) and the molecular simulation software Spartan 5.0 from Wavefunction (Irvine, CA, USA) and a Macromodel 7.0 from Schrodinger Inc. were used to simulate the low energy conformations of Ser-His as well as the molecular interactions between Ser-His and DNA.

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