行政院國家科學委員會專題研究計畫 成果報告

單股未配對核酸環修復反應機制分析
研究成果報告(精簡版)

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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
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單股未配對核酸環修復反應機制分析

計畫類別：■ 個別型計畫　　□ 整合型計畫
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共同主持人：
計畫參與人員： 秦維燦、徐悅芳、林郁倫

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執行單位：國立台灣大學醫學院醫學檢驗暨生物技術學系

中　華　民　國　九十五　年　　十　月　　三十 日

中文摘要

在正常的核酸代謝過程可能會發生核酸環的異狀，特別是在重覆序列或是反向重覆序列中，這些核酸環結構如果不予修正則會產生突變。而且反向重覆序列的核酸會形成髮夾結構，對於核酸合成或表現會造成抑制，因此對基因體的穩定有負面影響。為了解原核細胞如何處理核酸環結構，我們使用一套環狀異雙股核酸，包含 20-, 50-, 或 70-核甘酸的反向重覆環，以評估它們在大腸菌萃取液中的處理機制，我們發現真核細胞會以股斷指引方式進行修復，與先前任意單股環的修復模式相似，股斷無論發生在錯誤的 5'-或是 3'端都可以有效的修復插入式或刪除式的髮夾環結構，在大腸菌中這種股斷指引活性，與目前的 DNA 修復系統皆不相同，像是核酸配對錯誤修復(MMR)，核酸切除修復(NER)，SbcDC 系統等基因產物皆無關，限制修復反應的核酸合成，以分析髮夾環與任意環修復的中間產物發現，在核酸環部位皆產生股斷的現象，因此推測這兩類錯誤皆經由同一個修復系統，且透過在錯誤部位切割以進行修復的機制。

關鍵詞：核酸環修復 / 髮夾結構 / 配對錯誤修復 / 股斷指引修復 / 核酸環切割

Abstract

DNA loop heterologies may be generated during regular DNA transactions, particularly within palindromic and/or repetitive sequences. These DNA loop structures are mutagenic if they are not corrected. Furthermore, palindromic sequences present in DNA may form hairpin loops that have the potential to block DNA synthesis and transcription also causing adverse effects on genome stability. To understand how prokaryotic cells process DNA hairpin loop structures, we used a set of circular heteroduplexes containing 20-, 50-, or 70-nucleotide palindromic loops to evaluate their processing in *Escherichia coli* cell-free extracts. We demonstrate here that prokaryotic cells can process hairpin structures in a nick-directed manner similar to the in vitro large loop repair reaction. A strand break, regardless of its orientation to the hairpin, is sufficient to activate repair on both insertions and deletions of the nicked strand. This nick-directed reaction in *E. coli* extracts is independent of many known DNA repair pathways, including mismatch repair, nucleotide excision repair, and SbcCD pathway. Analysis of repair intermediates generated by limiting DNA synthesis revealed that processing of palindromic loop heterologies in prokaryotic cells may proceed through the same mechanism as large loop repair reaction.

Keywords: DNA loop repair/ hairpin structure/ mismatch repair/ nick-directed repair/ loop nicking

二、緣由與目的

DNA loop heterologies are unpaired, single-stranded DNA structures that can be formed during DNA metabolism. These structures can lead to severe genetic instabilities if unrepaired. DNA loops can range in size from a single nucleotide to several thousand nucleotide (nt). Smaller loops (<20nt) are generally formed during replication of slipped DNA sequences (1), and larger loops can arise during recombination events between non-identical sequences (2). Loops containing secondary structures formed by palindromic sequences may further disrupt the integrity of DNA causing genetic instability (3). These secondary structures include hairpin, hairpin-loops, and heteroduplex (4) (5) structures. Such architectures may act as a block to replication fork progression and obstruct transcription (6).

In vitro experiments have been demonstrated that both prokaryotic and eukaryotic cells are capable of processing DNA insertion/deletion loops ranging from 1 up to several hundred-nt in length. The mismatch repair (MMR) pathway can correct single base mismatches and small loops in a strand-specific manner in both prokaryotes and eukaryotes (7,8). The MMR in *Escherichia coli* can correct loops up to 7 unpaired nucleotides (9-11), but repair efficiency decreases as the loop size increases. Similarly, the human mismatch repair system can correct loops up to about 16 unpaired nucleotides (12-14).

In addition to mismatch repair, an alternative pathway can also correct loops. Previously we demonstrated that 8- to 429-nt loops were efficiently processed by a nick-directed pathway in *E. coli* (15). Studies have reported that loops of 16, 27 and 216 bases can be repaired both *in vivo* and *in vitro* by mismatch repair-deficient yeast strains (16,17). Human mismatch repair-deficient cell extracts are known to repair 8- and 16-nucleotide loops in a nick-directed fashion (13). Larger heterologies were also processed by a strand-specific manner independent of the human mismatch repair system (18). A loop-directed repair activity that requires little DNA synthesis was also reported in human extracts (19). Recently, we demonstrated that human cell-free extracts were capable of processing palindromic loops (20). Judging from their in vitro repair requirements and nick-directed dependence, suggested palindromic loops and random large loops could be processed through a common large loop repair pathway in human (20).

Previous studies have indicated that a bacterial large loop repair pathway can process large loops containing a strand break 5’ or 3’ to the heterology (15). However, very little is known about the specificity of this pathway toward palindromic loops and its mechanism in large loop processing. To understand the nature of large DNA loop repair in prokaryotic cells, we constructed a series of DNA loop heteroduplexes containing a nick (either 3’ or 5’ to the heterology) and a palindromic loop of 20-, 50-, or 70-nucleotides to evaluate their processing in *Escherichia coli* cell-free extracts. We demonstrate here that prokaryotic cells can process hairpin structures in a nick-directed manner regardless of the nick orientations and that the repair of both palindromic loops and random loops appears to involve a nick-dependent site-specific excision mechanism(s).

三、研究方法

***Escherichia coli* strains and extracts preparation**

Strain NM522 was used to propagate fully methylated replicative form f1 phage DNA and was used to make mismatch repair proficient cell extracts. Strain RS5033 was used to propagate unmethylated viral strand f1 phage DNA (11) for heteroduplex preparation. Mismatch repair deficient strain RK1517 (*mut*S201::Tn5) was as described (11). Strains SMR690 (recJ284::Tn10), SMR839 (*xon*A300::cat), SMR3488 (recJ284::Tn10 xseA18::amp), SMR3481 (*xon*A300::cat, *recJ*284::Tn10, *xseA*18::amp) were kindly provided by Dr. S.M. Rosenberg (Baylor College of Medicine). Strain DL733 (kindly provided by Dr. D.R. Leach, University of Edinburgh) is an *sbcCD* mutant (21). NER pathway mutants AB1886 (uvrA), AB1883 (uvrB), AB1884 (uvrC), and NK7512 (uvrD) were from *E. coli* Genetic Stock Culture Center, Yale University. All the strains were grown at 37ºC in Luria-Bertani broth supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50μg/ml), or tetracycline (12 μg/ml). *E. coli* cell extracts were prepared as described for mismatch repair and loop repair (15,22).

Preparation of heteroduplex DNA

The random or hairpin looped substrates were as described previously (20) (Fig. 1). The nick generated by the unique restriction endonuclease was on complementary (C) strand, while viral (V) strand remain continuous. Mutant f1hp phages used in this study are summarized in Table I. Heteroduplex DNA substrates were prepared as described (20). For making covalently closed circular substrates, nicked substrates were sealed with *E. coli* DNA ligase in the presence of ethidium bromide then purified as described (20). The covalently closed circular DNA molecules that we prepared were in supercoiled form with only 3-12% nicked DNA as judged by agarose gel electrophoresis.

Heteroduplex Repair assays

In vitro heteroduplex repair is as described (15). Repair was scored by restriction endonuclease digestion and agarose gel electrophoresis. The 6.7-kilobase circular DNA was linearized by AlwNI and treated with one of the diagnostic restriction endonucleases. Bands of 3.6- and 3.1-kilobase fragments indicate corrected products. The DNA products were quantified after ethidium bromide staining using a gel documentation CCD camera (UVP Ltd.) (11).

Analysis of Repair Intermediates Produced under Conditions of Limited Repair DNA Synthesis

Trapping of intermediates resulting from excision or incision of loop heterologies was accomplished by omission of exogenous dNTPs or by addition of ddNTPs and thereby restricting repair DNA synthesis. The reactions were scaled up 6-fold and purified reaction products were digested with SspI and NheI. Incision sites or excision tract end points were mapped relative to an indicated restriction endonuclease cleavage site by indirect end labeling after electrophoresis through 5% denaturing polyacrylamide gels (50 mM Tris borate, pH 8.0, 1 mM EDTA, 8.3M urea). After transfer and UV cross-linking to a nylon membrane, that portion of a heteroduplex strand of interest was visualized by probing with a 5'^{32}P-labeled oligonucleotide that hybridized near the 5' terminus produced by SspI/NheI restriction cleavage. Oligonucleotide V6106

(5'-CCGATTTCGGCCTATTGGTT-3', corresponding to viral strand nucleotides 6106-6125 of f1P, but hybridizing to the C strand) were used as probes. Size standard were created by digestion of f1P double strand DNA with SspI and NheI (552 bases), SspI and EcoRV (506 bases), SspI and XbaI (352 bases), SspI and HindIII (321 bases). For nucleotide level resolution a G+A ladder was generated by Maxam-Gilbert chemical cleavage of SspI/NheI fragment. Labeled fragments were visualized by exposure of membranes to Kodak MXG film.

結果與討論

Palindromic loops are processed in a nick-directed fashion in E. coli extracts

Using circular heteroduplex substrates like that shown in Fig. 1, we have previously shown that human cell extracts repair loops of hairpin structures in a fashion similar to human large loop repair (20). We have extended this study to test palindromic loop repair in a bacterial system. By pairing palindromic linker containing f1hp mutants with f1P phage DNA, we have prepared a set of heteroduplexes with a mismatch-containing hairpin structure. Each of these heteroduplexes contains a 20, 50, or 70-nt hairpin structure at the HindIII site in either complementary or viral DNA strand. All the substrates contained a nick at the ClaI site (3293 bases 5' to the heterologies) unless otherwise stated.

All substrates constructed permitted independent evaluation of correction on either DNA strand. Digestion of the unprocessed heteroduplex DNA with AlwNI and the indicator restriction endonuclease will yield a 6.7-kb fragment only (Fig. 2, upper panel). A similar digestion of DNA after the repair reaction will yield 3.6- and 3.1-kb product fragments.

To determine whether hairpin structures were corrected via the nick-directed loop repair pathway *in vitro*, heteroduplexes containing hairpin structures and a site-specific nick on the complementary strand were tested for repair in *E. coli* (NM522) cell extracts. If repair occurred on the nicked complementary strand, then the heterology in the nicked C strand must first be excised, followed by repair resynthesis using the continuous strand (the V strand) as a template. The product can then be digested by the indicator restriction endonuclease present on the viral strand. This type of repair is designated as C in this study. When repair occurred on the viral strand, the complementary strand was used as the template and repaired DNA can be digested by the indicator restriction endonuclease site present in the complementary strand and is designated as V.

When subjected to bacterial cell-free extracts treatment, all hairpin substrates were significantly repaired with a bias (1.8-4.4, average 3.0) to the nicked C strand (*Nicked* entries), when heteroduplexes contained a pre-existing nick. In nick-directed reactions, repair efficiencies of hairpin structures containing mismatches (range from 3.8-6.3 fmol) are generally higher than those of perfectly matched hairpins (range from 2.4-3.1 fmol). The repair specificities toward substrates containing different mismatches in the hairpin were also shown to have a certain degree of variations (up to 1.7 fold difference). These observations are similar to our previous study of bacterial large loop repair (15).

In contrast to the nicked substrates, our results show that heteroduplexes in covalently closed

circular form can only undergo low level of hairpin loop repair with less strand bias (1-2.9, average 1.5). Base on our experience in the preparation of covalently closed circular heteroduplex; there is a small fraction (3-12%) of substrates which still contain a nick. Therefore, the higher repair in the C strand of ccc substrates is most likely derived from a background nick-directed repair of the unligated substrates.

The presence and location of the nick affects repair efficiency

In order to assess the dependence of the reaction on the location of the strand break, we further tested five other hp50TT heteroduplexes that differ only in location of the strand-break within the complementary strand. This set includes two substrates with breaks 3' to the hairpin, at EcoRV (154 bp) or NheI (200 bp) sites, as viewed along the shorter path joining the two sites in the circular molecule. Three substrates containing nicks 5' to the hairpin were also prepared, with strand breaks located at XbaI, AccI, or HincII sites: 31 bp, 517 bp, and 833 bp respectively from the hairpin along the shorter path.

A hairpin loop containing a T-T mismatch was subject to low level processing in *E. coli* extracts and displayed little strand bias when present in a covalently closed circular DNA. The presence of a strand break increases repair efficiency and directs repair to the nicked C strand. However, the efficiency of hairpin DNA processing is independent of the distance separating the heterologies and the strand break since the substrates with a nick at the AccI site (5'-517 nt) exhibited higher activity than substrates containing other nicks closer to the hairpin structures (5'-31 nt, 3'-154 nt, and 3'-200 nt). This observation is similar to our recently reported bacterial nick-directed loop repair pathway (15); both 3' and 5' nicks can activate repair to the nicked strand, and the repair efficiency is independent of the location of the nick.

Repair efficiency and hairpin size

To determine the repair efficiencies for hairpin structures of different sizes, we compared Chp50CG and Vhp50CG with additional two sets of substrates, one with a 20-nt (Chp20 and Vhp20) and the other a 70-nt (Chp70 and Vhp70) perfect-matched hairpins at a HindIII site. A strand break at the ClaI site was also employed in both substrates. This allows all hairpin structures to reside in a similar DNA context that minimizes the effects of peripheral sequence on repair efficiency. As shown in Fig. 4, there is a significant difference in repair efficiency between different hairpin lengths. The substrate with the shorter hairpin structure (hp20; 20-nt) of both configurations exhibited the highest repair efficiency when compared to Chp50, Vhp50, Chp70, and Vhp70.

Requirements for hairpin repair are the same as nick-directed loop repair in E. coli

In vitro loop repair by *E. coli* cell extracts requires the addition of $MgCl_2$, the four dNTPs, and low amount of ATP as described previously (15). In order to find out whether any of these components are essential for hairpin repair, we omitted the cofactors in separate reactions. The repair efficiency of the reaction with all the components present was normalized to 100%. The exogenous cofactors required for *in vitro* hairpin repair by *E. coli* extracts are Mg^{2+} and the four dNTPs; unexpectedly ATP was not required. We observed a considerable decrease in repair of the hairpin substrates tested when any of these components were omitted.

Although omission of exogenous ATP only causes a minor decrease in loop repair, both loop repair and hairpin repair levels were significantly decreased in the presence of a non-hydrolysable ATP analog. In addition, the hairpin reaction was partially inhibited by chain terminator dideoxyTTP (ddTTP). In contrast, eukaryotic DNA polymerase inhibitor aphidicolin had no effect, similar to previous findings of loop repair system in *E. coli* (15).

Palindromic and random loop substrates are processed by a common mechanism

Aside from restriction endonuclease scoring described above, using Southern blot analysis the repair of large DNA loops can also be visualized by taking advantage of the 32-50-nt difference in size between two stands of looped substrates. Fig 5 shows examples of five looped substrates (lanes 3, 6, 9, 15, and 18, standard reaction of 3'-Chp50TT, Chp50GG, Vhp50GG, 3'-xC32, and 3'-xV32). In this assay, a DNA fragment encompassing the loop region was separated by denaturing polyacrylamide gel electrophoresis, transferred to a nylon membrane, and probed for the 5' end of the nicked complementary strand. A conversion of strand size from a shorter strand to a longer strand or vice versa reflects the repair of the loop by removing insertions or filling in deletions during the repair. Consistent with the restriction endonuclease assay described above, significant repair of the nicked strand was observed for substrates containing a nicked C strands regardless of the orientation of nicks. For 3'-Chp50TT and Chp50GG, a new species with a size of 552-nt in length was detected in our standard reaction compared to an unprocessed 602-nt fragments (Fig. 5, lanes 6and 9). In the case of Vhp50GG, a new species of 602-nt in size was observed after exposing the substrate to an active *E. coli* extract (Fig.5, lane 3). These conversions indicate that repair excision/incision occurred in the nicked strand, followed by repair synthesis using the continuous strand (the V strand) as a template.

Restriction of repair DNA synthesis by omission of dNTPs or inclusion of ddNTPs has permitted visualization of excision intermediates that are produced during mismatch repair and human loop repair (18). We have used a similar approach in an attempt to trap repair intermediates that are produced during nick-directed repair of large heterologies. By blocking repair DNA synthesis, repair events prior to DNA synthesis are trapped, and the reaction intermediates can by visualized by Southern blot analysis. Blocking DNA synthesis resulted in a substantial reduction in production of repair products, an effect easily seen with Chp50GG and 3'-Chp50TT where the 552-nt repair fragment is well resolved from the 602-nt fragment in the unrepaired DNA. In the case of the substrate Vhp50GG, the production of 602-nt repair products was reduced under conditions of limited DNA synthesis.

For palindromic loops, omission of dNTPs or addition of ddNTPs also resulted in accumulation of bands between 352 and ~400 nt (indicated by the SspI/HindIII marker and deduced by the G+A marker). Similarly for the random loop substrates bands appeared between 321 and 352 nt shown in Fig 5B, which represent the excision intermediates. For VhpGG, a second species of about 370 nt corresponding to a discontinuity in the C strand at the location of the heterology was evident, but as can be seen, this species was also produced from the standard reaction in the presence of dNTPs. Hence, the latter species may be unrelated to the nick-directed excision

described here, although it could be a resynthesis extension intermediate since the probe we used binds to the 5' end of the C strand fragment.

For ChpGG and 3'-ChpTT, the nicks were positioned 3293 bases 5' to the heterologies and 154 bases 3' to the hairpin respectively. Despite their differences in nick orientations, under restriction of DNA synthesis, both showed similar excision intermediates between 352 and ~400 nt.

Although a slightly different pattern of intermediate tracts was evident between these substrates, prominent DNA fragments with termini mapping to a region very close to the positions of the heterologies were clearly detected in all looped-heteroduplex substrates. These results indicate that the excision reaction is associated with nick-directed loop repair and that the bulk of excision occurs at the heterologies. For this study of repair intermediates, substrates were chosen according to their specific features: 3'-Chp50TT and Chp50GG are an insertion type of palindromic loops, Vhp50GG is a deletion type of palindromic loop, 3'-xC32 is an insertion type of random loop, and 3'-xV32 is a deletion type of random loop. Thus, from this experiment we conclude that for the substrates analyzed, no matter which loop types, the majority of repair intermediates were confined to an area immediately surrounding the loop site, and this occurred regardless of whether the heterology was an insertion type or deletion type of loop. Although these observations do not suffice to establish the mechanism for nick-directed repair of large loops, they clearly show that both palindromic and random loop repair occurs by a similar pathway as deduced by using the same method.

In vitro loop repair is independent of MMR, NER, and SbcCD proteins

Previously we had shown nick-directed large loop repair is genetically independent from mismatch repair. To determine whether several other major DNA repair pathways are involved in large DNA loop repair, we assayed *E. coli* strains deficient in various DNA repair pathways for their ability to process 3'-xC32, 3'-xV32, and ChpGG, which represent those insertion, deletion, random, and palindromic loop species. The NER deficient strains used in this analysis are AB1886 (*uvrA*), AB1883(*uvrB*), AB1884(*uvrC*), and NK7512(*uvrD*). Several MMR mutants deficient in exonuclease activity were also tested, including SMR839 (xonA), SMR690 (recJ), SMR3488(recJ, xseA), and SMR3481(xonA, recJ, xseA). A strain DL733 deficient in SbcCD protein is also included. All bacterial strains tested, regardless of deficiency in MMR, NER, or SbcCD pathway, showed some degree of activity for nick-directed loop repair pathway. Compared with the repair levels found in previous experiment, these DNA repair mutants had levels of repair comparable with those of NM522 cells. These results indicate that both random loops and palindromic loops repair are independent of the MMR and NER pathways, as well as SbcCD protein.

四、計畫成果自評

本研究與原申請計畫相符合，大致達成預期目標，本研究成果於 2007 年 4 月間曾整理投稿 Nucleic Acid Research 學術期刊發表, 但審稿人認為仍缺少若干生物學意義的實驗並未接受，目前則積極補實驗再投稿發表。

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