Strand Asymmetry of +1 Frameshift Mutagenesis at a Homopolymeric Run by DNA Polymerase III Holoenzyme of Escherichia coli

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We have recently shown that single-base frameshifts were predominant among mutations induced within the rpsL target sequence upon oriC plasmid DNA replication in vitro. We found that the occurrence of +1 frameshifts at a run of 6 residues of dAdT could be increased proportionally by increasing the concentration of dATP present in the in vitro replication. Using single-stranded circular DNA containing either the coding sequence of the rpsL gene or its complementary sequence, the +1 frameshift mutation by DNA polymerase III holoenzyme of Escherichia coli was extensively examined. A\textsubscript{6} \rightarrow A\textsubscript{7} frameshifts occurred 30 to 90 times more frequently during DNA synthesis with the noncoding sequence (dT tract) template than with the coding sequence (dA tract). Excess dATP enhanced the occurrence of +1 frameshifts during DNA synthesis with the dT tract template, but no other dNTPs showed such an effect. In the presence of 0.1 mM dATP, the A\textsubscript{6} \rightarrow A\textsubscript{7} mutagenesis with the dT tract template was not inhibited by 1.5 mM dCTP, which is complementary to the residue immediately upstream of the dT tract. These results strongly suggested that the A\textsubscript{6} \rightarrow A\textsubscript{7} frameshift mutagenesis possesses an asymmetric strand nature and that slippage errors leading to the +1 frameshift are made during chain elongation within the tract rather than by misincorporation of nucleotides opposite residues next to the tract.

Among the spontaneous mutations occurring in various organisms, deletions or additions of one or a few nucleotides are often observed. These sequence alterations are called frameshift mutations when they destroy a structural gene coding frame (1). The most abundant types of spontaneous frameshifts are single-base deletions (−1 frameshifts) and single-base additions (+1 frameshifts). Because the function of target genes is abolished or severely affected by frameshift mutations, these are deleterious mutations undesirable for maintaining genetic information and survival of cells. There have been a large number of reports on frameshift mutations found in tumor suppressor genes of cancer cells as well as genes relating to genetic diseases. Therefore, molecular mechanisms of frameshift mutagenesis and its suppression are fundamental subjects in carcinogenesis and other areas of medical science.

Whereas an incorrect recombination process at a run of the same nucleotide was hypothesized in the classical slippage model for frameshift mutagenesis (2), it has been suggested that most spontaneous frameshifts might be caused by DNA replication errors. This is mainly because the frequency of frameshift mutations was elevated in cells lacking the capacity for mismatch repair that can correct replication errors (3–6). In addition, frameshift mutations were found in products of in vitro DNA synthesis with weakly processive DNA polymerases or replicative DNA polymerases that lack proofreading capacities (7–10). However, in some cases when in vitro and in vivo data could be directly compared, the pattern of frameshift mutations in a given target sequence differed between the in vitro and in vivo systems. Thus, there was no direct evidence that the cellular replicative apparatus made errors leading to frameshift mutations. We have recently examined errors made during DNA replication in vitro using a reconstituted Escherichia coli replicative apparatus (11). Using the same target sequence in the mutation assay, the mutations derived from replication errors in vitro were compared with mutations occurring in a mismatch repair-deficient E. coli strain. In this previous study, +1 and −1 frameshift mutations appeared to be the most frequent types of mutations derived from errors caused by the replicative apparatus of E. coli. Furthermore, the site distribution of +1 or −1 frameshifts and their relative frequencies were the same in both in vitro and in vivo systems. This implies that in vivo frameshift mutagenesis largely depends on the biochemical nature of the replicative apparatus.

There were three sites within the coding region of the rpsL gene, a target sequence for our mutation assay, at which the frequency of single-base frameshifts was markedly increased relative to the rest of the sequence. These “hot spot” sites corresponded to runs of 4, 5, and 6 adenine nucleotides. About 66% of in vitro and 85% of in vivo single-base frameshifts occurred at these sites. Most of the other single-base frameshifts occurred at runs of 2 or 3 of the same nucleotide. These results clearly demonstrate the strong tendency of the replicative apparatus to make slippage errors at runs of the same nucleotide. Interestingly, the occurrence of +1 frameshifts was significantly different from that of −1 frameshifts in their dependence on the length of the runs. With an increase in run length, the frequencies of both +1 and −1 frameshifts increased. However, +1 frameshifts occurred infrequently at runs shorter than 3 nucleotides, and the frequency of +1
frameshifts was enhanced more sharply than that of –1 frameshifts at runs longer than 4 nucleotides. Therefore, +1 frame shift mutagenesis seems to occur through a mechanism somewhat different from that inducing –1 frameshifts.

DNA replication errors leading to either +1 or –1 frameshifts, often called slippage error, might be expected to involve a structure in which one nucleotide residue is looped out at the mutation site. As described above, generation of the single-base loop error has been extensively studied using in vitro DNA replication with DNA polymerases other than DNA polymerase III holoenzyme. In those studies, rates of the single-base loop errors were much higher than that observed with the E. coli replicative apparatus, which catalyzes highly processive chain elongation and possesses a high proofreading capacity. Although some but not all of the polymerases showed a tendency to induce frameshifts at nucleotide runs, –1 frameshifts predominated in these previous studies, and +1 frameshifts were very rarely found even at runs longer than 4 nucleotides residues.

These differences strongly suggest that the mechanisms of frameshift mutagenesis by processive and accurate DNA polymerases are different from those of less processive and more error-prone DNA polymerases. In the present study, we focused on +1 frameshift mutations within a da6/7T run present in the rpsl target sequence. These mutations represented 44% of all frameshift mutations induced in the target sequence by DNA replication in vitro and 48% of the mutations in the same sequence recovered from mismatch repair-deficient E. coli cells. We found a strong strand asymmetry in the generation of single-base loop errors leading to +1 frameshifts within the run by Pol III1 holoenzyme, which plays a central role in the E. coli replicative apparatus. On the basis of the effects exerted on +1 frameshift mutagenesis by substrate nucleotides for in vitro DNA replication and by template nucleotides surrounding the da6/7T run, biochemical nature of +1 single-base loop errors within a polynucleotide run is discussed.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**SL7284, a Salmonella typhimurium LT2 strain was used for preparing Pol III holoenzyme DNA (11). ST102 is a derivative of E. coli K12 strain NM554 (12) carrying an F plasmid pKP1133 (T. Miki, Kyushu University) and was used for propagation of single-stranded phagemid DNA. An E. coli K12 strain, MF101, was used for selecting the rpsl1 mutant plasmid (11). Plasmid pMOL3 carrying the oriC region and rpsl1 (amber) gene was used for oriC plasmid DNA replication in vitro (11). For ss→ds replication in vitro, we used the circular ss DNA of phagemids pMOL36 and pMOL37 in which the same rpsl1 (amber) target sequence was inserted into pTV118N and pTV119N (TAKARA), respectively. pMOL38 and pMOL39 were constructed by replacing T at position 126 in the rpsl1 region and pMOL39 or primer D for pMOL37, 200 ng of annealed template (as nucleotides), varied concentrations of each dNTP, 1.2 μg of SSB, 16 units of Pol III*, and 200 nM of Pol III holoenzyme (130 ng), and HU (40 ng). Replication mixtures were assembled at 0 °C and incubated at 30 °C for 2 h. The reactions were stopped by heating the mixture for 10 min at 65 °C in the presence of 0.5% SDS and 25 mM EDTA. The product DNA was processed as described elsewhere (11).

**ss→ds DNA Replication in Vitro**—Four hundred primer A and 91 ng of the ss circular DNA of either pMOL36, pMOL37, pMOL38, or pMOL39 was annealed in a buffer containing 0.2×SCC at 42 °C for 20 min and slowly cooled to room temperature. The sequence of primer A is complementary to the template sequence about 300 bases upstream of the rpsl1 region. The reaction (25 μl) contained 20 mM Tris-HCl (pH 7.5), 4% glycerol, 8 mM MgCl2, 1 mM ATP, 80 μg/ml bovine serum albumin, 140 pmol of annealed template (as nucleotides), varied concentrations of each dNTP, 1.2 μg of SSB, 25 μl of primase, and 30 units of Pol III*, and 200 nM of Pol III holoenzyme. The results from three or four independent dilutions were used to calculate the average number of transformants with mutant plasmids. The frequency of the rpsl1 mutation was calculated by dividing the number of Km+t Smr transformants by the number of Km1 transformants for pMOL3 or by dividing the number of Ap1 Smr transformants by the number of Ap1 Sm1 transformants for pMOL36, pMOL37, 38, and 39.

**Mutation Assay—**The determination of forward rpsl1 mutation frequency was carried out as described elsewhere (11, 17). 100 ng of DNA was used for each electroporation experiment with 40 μl of competent MF101 cells. To determine the number of the transformants, the cell suspension after outgrowth in SOC medium was diluted and plated on LB agar plates containing 100 μg/ml kanamycin (for pMOL3) or ampicillin (for pMOL36, 37, 38, and 39). Four (for pMOL3) or two (for pMOL36, 37, 38, and 39) sets of appropriate dilutions of the cell suspension made, and each was placed on a duplicate plate. Detection of cells transformed with rpsl1 plasmid was carried out on LB agar plates containing 100 μg/ml streptomycin and either 100 μg/ml kanamycin or 150 μg/ml ampicillin. The data from three or four independent dilutions were used to calculate the average number of transformants with mutant plasmids. The frequency of the rpsl1 mutation was calculated by dividing the number of Km1 Smr transformants for pMOL3 or by dividing the number of Ap1 Smr transformants by the number of Ap1 Sm1 transformants for pMOL36, 37, 38, and 39. The rpsl1 coding region and surrounding sequence (from position −130 to position 385) in each mutant plasmid was determined using an automated DNA sequencer (model 373A, Applied Biosystems).

**Determination of Detection Efficiency for Frameshift Intermediates—**The efficiency of detection of frameshift intermediates da6/7T and da6/7T was determined by transforming heteroduplex molecules containing these frameshift intermediates. These heteroduplex molecules were prepared by completion of ss→ds DNA synthesis using ss circular pMOL36 template DNA annealed to 40-mer-T or ss pMOL37 with 40-mer-T, Pol III holoenzyme was used for this reaction. The products were methylated by Dam methylase and introduced into MF101 cells. The products were methylated by Dam methylase and introduced into MF101 cells. The numbers of Ap1 Smr and Ap1 Sm1 transformants were determined. The detection efficiency was calculated by dividing the number of Ap1 Smr transformants by the number of Ap1 Sm1 transformants.
Slippage Errors at Mononucleotide Run by Pol III Holoenzyme

RESULTS

Effects of dATP on Generation of rpsL\(^{-}\) Mutations during oriC Plasmid DNA Replication in Vitro—Fidelity of DNA replication by E. coli replicative apparatus has been assessed using the fully reconstituted system for oriC plasmid DNA replication in vitro (11). During in vitro DNA synthesis by the replicative apparatus including Pol III holoenzyme, the frequency of forward mutations within the rpsL target sequence was increased to 1.9 \(\times\) \(10^{-4}\), 50-fold higher than the background level. Because about 70% of the forward mutations were single-base frameshifts and only a limited number of base substitutions were obtained, the nature of base substitution mutagenesis by the replicative apparatus remained unclear. In addition, base substitutions found in the replication products were mainly G:C \(\rightarrow\) T:A and A:T \(\rightarrow\) C:G transversions, both of which seemed to be caused by oxidative damage to guanine residues in template DNA and substrate nucleotides. To estimate the rate of generation of base-base mispairs during DNA synthesis, we carried out experiments in which unequal concentrations of dNTPs were provided. A similar approach was successfully used with a reversion assay for base substitution mutagenesis during \(\delta X174\) ss \(\rightarrow\) ds DNA replication (18, 19). As shown in Fig. 1, the frequency of rpsL\(^{-}\) forward mutations in the products of in vitro DNA synthesis was further increased severalfold when the concentration of dATP was increased relative to dGTP. On the other hand, however, higher concentrations of dTTP relative to dCTP showed no significant effect on the mutation frequency. It appeared that the increase of mutation frequency was not due to an imbalance between dATP and dGTP but was caused by the higher concentration of dATP in itself. The increased mutation frequency observed with 500 \(\mu\)M dATP and 50 \(\mu\)M dGTP was not changed when the concentration of dGTP was increased to 500 \(\mu\)M to balance dATP and dGTP. Furthermore, DNA synthesis with 50 \(\mu\)M dATP and dGTP resulted in a reduction of mutation frequency to 60% of that observed with all dNTPs present at 100 \(\mu\)M. From these results, we concluded that the concentration of dATP affects the generation of replication errors during the oriC plasmid DNA replication in vitro.

Higher Concentration of dATP Induced A\(_6\) Frameshifts as Well as G:C \(\rightarrow\) A:T Base Substitutions during in Vitro DNA Synthesis—To reveal the nature of replication errors specifically enhanced by higher concentrations of dATP, we determined sequence alterations in 31 rpsL\(^{-}\) mutant plasmids derived from the products of in vitro DNA replication with 1.5 mM dATP, 50 \(\mu\)M dGTP, 100 \(\mu\)M dTTP, and 100 \(\mu\)M dCTP (biased conditions). As a control, we also analyzed 100 rpsL\(^{-}\) mutant plasmids derived from the products of in vitro replication with all 4 dNTPs at 100 \(\mu\)M (balanced conditions). Surprisingly, 23 out of 30 mutations induced under the biased conditions were single-base frameshifts that added the same nucleotide at dA/dT tracts. Four G \(\rightarrow\) T and three G \(\rightarrow\) C base substitutions were also observed. Among the +1 frameshifts, A\(_6\) \(\rightarrow\) A\(_7\) mutations were predominant. The mutation frequency of each class of mutation is shown in Table I. It appeared that increased dATP concentrations in the in vitro DNA replication specifically enhanced +1 frameshifts at dA/dT tracts. A\(_6\) \(\rightarrow\) A\(_7\) frameshifts were also observed. The +1 frameshift mutagenesis using the oriC plasmid DNA replication system. To overcome this problem, we developed an assay for examining the frequency of A\(_6\) \(\rightarrow\) A\(_7\) frameshifts in the products of ss \(\rightarrow\) ds DNA replication by Pol III holoenzyme (Fig. 2). A set of ss circular DNAs containing either the coding (dA tract template, pMOL36) or noncoding (dT tract template, pMOL37) strand of the rpsL gene were used as the template DNA for ss \(\rightarrow\) ds DNA replication, in which a highly processive DNA synthesis starts from a defined primer and completes to produce a nicked ds DNA circle. The product DNA was methylated by Dam methylase and introduced into E. coli cells by electroporation to detect rpsL\(^{-}\) forward muta-

![Graph](image_url)

**Fig. 1. Effects of dNTPs on generation of rpsL\(^{-}\) mutations during oriC plasmid DNA replication in vitro.** Replication of pMOL3 DNA was performed using the concentrations of dNTPs indicated. The relative concentrations are expressed such that 1 denotes 100 \(\mu\)M.

| Class of mutation/conditions | Mutation frequency | Increase |
|-----------------------------|--------------------|---------|
| Base substitutions          |                    |         |
| G:C \(\rightarrow\) A:T     | 6.84               | 10.6    |
| G:C \(\rightarrow\) T:A     | 5.7                | 2.1     |
| Other types                 | 3.3                | <2.9    |
| Single-base frameshifts     |                    |         |
| A\(_6\) \(\rightarrow\) A\(_7\) | 5.7               | 10.9    |
| A\(_6\) \(\rightarrow\) A\(_6\) | 1.5               | 3.9     |
| Other types                 | 1.7                | 1.7     |
| Other classes               | 1.7                | <2.9    |
| Total                       | 21                 | 4.4     |

**TABLE I**

Class distribution of rpsL\(^{-}\) mutations induced during oriC plasmid DNA replication in vitro

100 and 31 rpsL\(^{-}\) mutant plasmids were chosen from the products of in vitro DNA replication under balanced (100 \(\mu\)M each dNTP) and biased (1.5 mM dATP, 50 \(\mu\)M dGTP, 100 \(\mu\)M dTTP, and 100 \(\mu\)M dCTP) conditions, respectively. The mutation frequency of each class was calculated by multiplying the total mutation frequency by the ratio of the number of mutants in each class to the total number of mutants in all classes.

**TABLE II**

Class distribution of rpsL\(^{-}\) mutations induced during in vitro DNA replication with 1.5 mM dATP, 50 \(\mu\)M dGTP, 100 \(\mu\)M dTTP, and 100 \(\mu\)M dCTP (biased conditions). As a control, we also analyzed 100 rpsL\(^{-}\) mutant plasmids derived from the products of in vitro replication with all 4 dNTPs at 100 \(\mu\)M (balanced conditions). Surprisingly, 23 out of 30 mutations induced under the biased conditions were single-base frameshifts that added the same nucleotide at dA/dT tracts. Four G \(\rightarrow\) T and three G \(\rightarrow\) C base substitutions were also observed. Among the +1 frameshifts, A\(_6\) \(\rightarrow\) A\(_7\) mutations were predominant. The mutation frequency of each class of mutation is shown in Table I. It appeared that increased dATP concentrations in the in vitro DNA replication specifically enhanced +1 frameshifts at dA/dT tracts. A\(_6\) \(\rightarrow\) A\(_7\) frameshifts were also observed. The +1 frameshift mutagenesis using the oriC plasmid DNA replication system. To overcome this problem, we developed an assay for examining the frequency of A\(_6\) \(\rightarrow\) A\(_7\) frameshifts in the products of ss \(\rightarrow\) ds DNA replication by Pol III holoenzyme (Fig. 2). A set of ss circular DNAs containing either the coding (dA tract template, pMOL36) or noncoding (dT tract template, pMOL37) strand of the rpsL gene were used as the template DNA for ss \(\rightarrow\) ds DNA replication, in which a highly processive DNA synthesis starts from a defined primer and completes to produce a nicked ds DNA circle. The product DNA was methylated by Dam methylase and introduced into E. coli cells by electroporation to detect rpsL\(^{-}\) forward muta-
pre-existing in the template DNA was also measured. When the dT tract template was used, \( \text{Ap} \rightarrow \text{A} \) mutations were detected more frequently in the product DNA than ss template DNA. On the other hand, \( \text{Ap} \rightarrow \text{A} \) mutations were not detected in the products of DNA replication with the dA tract template, even though approximately 200 \( \text{rpsL}^{-} \) mutant plasmids were examined in two independent experiments. It seemed likely that the mutation frequency in the product DNA was equal to or below the background level. Taking into account the different efficiencies of detection of \( \text{Ap} \rightarrow \text{A} \) frameshift intermediates in the replication products derived from dA and dT tract templates, the frequency of frameshift errors was estimated to be 20–90-fold higher in products derived from the dT tract (noncoding) template.

+1 Frameshift Mutagenesis during DNA Synthesis with dT Tract Template Was Sharply Stimulated by dATP—In a series of \( \text{Ap} \rightarrow \text{A} \) frameshift assays (Fig. 3), we found that dATP exerted the same effect on the +1 frameshift mutagenesis as on the \( \text{oriC} \) plasmid DNA replication in vitro. When dT tract template was used in the assay, the presence of 15-fold excess dATP (1.5 mM dATP, other dNTPs 100 \( \mu \text{M} \)) induced a 14-fold increase in \( \text{Ap} \rightarrow \text{A} \) mutation frequency relative to nucleotide-balanced conditions. This mutagenic effect was concentration-dependent. dTTP, dGTP, and dCTP did not show such a strongly stimulatory effect on the +1 frameshift mutagenesis. Interestingly, the stimulatory effect of excess dATP was also observed, although to a much lesser degree, on the reaction containing dA tract template. The compensated mutation frequency was about 1/80th of that observed with the dT tract template under the excess dATP conditions. From these results, we concluded that the specific induction of \( \text{Ap} \rightarrow \text{A} \) frameshifts during \( \text{oriC} \) plasmid DNA replication by increased concentrations of dATP was due to a strand asymmetry of +1 frameshift mutagenesis at the dA\(_{7}/\text{dT}_{6} \) tract.

Stabilization of Slipped Misalignment by Subsequent Deoxynucleotide Incorporation—To elucidate molecular mechanisms of the dATP-dependent +1 frameshift mutagenesis, we first examined the relevance of misinsertion of dAMP opposite the residue (dT) immediately 5’ to the dT tract, as expected from the misinsertion-misalignment model (20) (Fig. 4A). According to this model, increased concentrations of dATP would prompt misinsertion of dAMP opposite the dG residue, and the misinserted dA residue could make a base pair with a dT residue in the tract by misalignment because of spontaneous breathing of the growing chain. If such a misinsertion event triggers the +1 frameshift mutagenesis, the frequency of \( \text{Ap} \rightarrow \text{A} \) mutation should be decreased by the addition of excess dCTP. As described in the previous section, however, elevation of dCTP (1.5 mM) induced a 14-fold increase in the observed mutation frequency (Fig. 3). In fact, when 1.5 mM dATP was also present during the \( \text{in vitro} \) DNA synthesis, a higher concentration (0.8 mM) of dCTP actually stimulated the +1 frameshift mutagenesis (Fig. 3). The additional stimulation was diminished by a further increase in the concentration of dCTP, but the dATP-enhanced level of +1 frameshift mutagenesis was not significantly inhibited by 1.5 mM dCTP. The misinsertion-misalignment model therefore appeared to be insufficient to explain the \( \text{Ap} \rightarrow \text{A} \) frameshift mutagenesis and its stimulation by higher concentrations of dATP.

The effect of dATP on the +1 frameshift mutagenesis might be due to a general stimulatory effect of the dNTP complementarity to a tract on +1 frameshift mutagenesis within the tract. If this were the case, excess dTTP should enhance the frequency of \( \text{Ap} \rightarrow \text{A} \) frameshift during the \( \text{in vitro} \) DNA synthesis on the dA tract template. As shown in Table III, the frequency of +1 frameshift was increased at least 10-fold by the

![Fig. 2. Ap → A frameshift assay using ss → ds DNA replication and oligonucleotide hybridization. ss → ds DNA replication was carried out with ss pMOL36 (dA tract template) and pMOL37 (dT tract template) DNA under balanced conditions (100 \( \mu \text{M} \) each dNTP) and rpsL\(^{-} \) mutant plasmids were selected. PCR products derived from each of 90 mutant pMOL36 and pMOL37 plasmids were spotted onto nylon membrane together with positive (PC) and negative control (NC) DNA. Positive and negative controls were PCR products derived from each of 90 mutant pMOL36 and pMOL37 plasmids, respectively. 60, 15, and 1.5 \( \mu \text{g} \) of control DNA were spotted from left to right in the areas indicated by a bar. Amplified DNA containing the A6 frameshift was detected by Southern hybridization to the probes described under “Experimental Procedures.”](image_url)
addition of 1.5 mM dTTP. Therefore, it seemed very likely that 1 frameshift mutagenesis at mononucleotide runs is stimulated by incorporation of the correct nucleotide within the tracts. Replication errors leading to the 1 frameshift are probably generated by a slipped misalignment process during DNA chain elongation within the mononucleotide run, and a subsequent incorporation of nucleotide next to the replication error stabilizes the relatively unstable intermediate and promotes escape from decay or reversal of the misaligned structure (Fig. 4B). The stimulatory effect of 0.8 mM dCTP might be due to a similar stabilization of the +1 frameshift intermediate by extension of correctly base paired nucleotides next to the tract. This was supported by the observation that the frequency of +1 frameshift during in vitro DNA synthesis on the dA tract template was increased by the addition of 1.5 mM dATP, because the residue immediately 5' to the dA tract is dT (Fig. 3).

Effect of the Residue Immediately 5' to the dA6/dT6 Tract on +1 Frameshift Mutagenesis—Whereas the stimulatory effect of tract-complementary dNTP on +1 frameshift mutagenesis appeared to be basically the same during DNA synthesis with either strand of the mononucleotide run, the reason for the strand asymmetry of this +1 frameshift mutagenesis remained unclear. We found a clue to this problem in the course of analyses with modified rpsL target sequences, 126dC-dA tract and 126dG-dA tract, in which the 5'-neighboring residue, dT at position 126, was replaced with dC and dG, respectively. The residue immediately 5' to the dT tract could not be replaced because this would cause substitution of the proline residue specified by the codon next to the dA6/dT6 tract. As shown in Table III, 126dC-dA tract and 126dG-dA tract templates showed 15- and 36-fold higher frequencies of background 1 frameshift mutation, respectively. We were therefore unable to evaluate 1 frameshift mutagenesis occurring during in vitro DNA synthesis with the modified template under balanced conditions. However, when the concentration of dTTP was increased to 1.5 mM, the product DNA showed a much higher frequency of +1

| Table II | Strand asymmetry of +1 frameshift mutagenesis at dA6/dT6 run |
|----------|---------------------------------------------------------------|
| ss → ds DNA replication was carried out with ss pMOL36 (dA tract template) and pMOL37 (dT tract template) under balanced conditions, and the resulting plasmids were subjected to measurement of rpsL2 mutation frequency and A6 → A7 mutation assay. Background levels of A6 → A7 mutation were determined by the same analysis with the unreplicated template DNA. A6 → A7 mutation frequency was calculated by multiplying the rpsL2 mutation frequency by the ratio of A6 → A7 mutants to all mutants examined. The results were adjusted for the differing efficiencies of detection of dA and dT tract +1 frameshift intermediates by dividing the observed frequency by the measured detection efficiency, 0.12 for the dA tract template and 0.016 for the dT tract template.

| dA tract template | Experiment 1 | Background | 32 | 2/278 | 0.23 | 0.23 |
|-------------------|--------------|------------|----|-------|------|------|
|                   | Replication  product | 63 | 0/238 | <0.26 | <2.1 |
| Experiment 2      | Background   | 32 | 2/278 | 0.23 | 0.23 |
|                   | Replication  product | 140 | 0/180 | <0.26 | <6.3 |
| dT tract template | Experiment 3 | Background | 88 | 3/278 | 0.9 | 0.9 |
|                   | Replication  product | 110 | 12/458 | 2.9 | 180 |
| Experiment 4      | Background   | 46 | 0/180 | <0.26 | <0.26 |
|                   | Replication  product | 83 | 3/90 | 2.8 | 180 |

Fig. 3. Effects of dNTP on A6 → A7 frameshift mutagenesis with dT tract and dA tract templates. ss → ds DNA replication was carried out using the concentrations of dNTPs indicated. Product DNAs were examined for their rpsL2 mutation frequencies, and about 200 mutants of each product DNA were further examined by A6 → A7 mutation assay. A6 → A7 mutation frequency was calculated for each product DNA and adjusted for detection efficiency as described in Table II. The background levels were 2.3 × 10^-7 for pMOL36 and < 2.6 × 10^-7 for ss pMOL37.
Slippage Errors at Mononucleotide Run by Pol III Holoenzyme

In the present study, we demonstrated that the dATP-dependent +1 frameshift mutagenesis observed with oriC plasmid DNA replication in vitro is due to strand asymmetry of the frameshift mutagenesis occurring at a dA/dT tract in the target rpsL sequence. We also found that substrate nucleotides complementary to the tract template or its 5′ neighboring residue exert stimulatory effects on the +1 frameshift mutagenesis. It has been long known that the generation of DNA replication errors is affected by the concentration of dNTPs in in vitro DNA replication reactions. First of all, the generation of base-base mispairs at a given template residue during DNA synthesis depends on the concentrations of incorrect nucleotides relative to that of the correct nucleotide (18). Because the accuracy of base selection is determined mainly by the differential Km of DNA polymerase for correct versus incorrect dNTPs, higher concentrations of incorrect nucleotides increase the frequency of base-base mispairs (21). Secondly, higher concentrations of the next correct (“rescue”) dNTP also increase the frequency of base-base mispairs (22). This effect is relatively small and likely to be due to suppression of proofreading of the mispairs. Thirdly, unbalanced concentrations of dNTPs also stimulate frameshift mutagenesis by forming a base-base mispair next to the site of frameshift mutation (20, 23, 24). If the misincorporated nucleotide is complementary to the next template nucleotide, spontaneous misalignment between them can create a slippage error leading to a −1 frameshift. Therefore, higher concentrations of dNTPs complementary to the template residue next to a given site increase −1 frameshift mutagenesis at that site. The effect of substrate nucleotides on −1 frameshift mutagenesis at a dA/dT tract observed in this study is distinct from those previously reported and thus constitutes a fourth category. In this case, the effective nucleotide is primarily the one complementary to the tract, and the stimulatory effect is not diminished by increased concentrations of the dNTP that should suppress misinsertion at the template nucleotide next to the tract. Therefore, during DNA synthesis on a template consisting of a mononucleotide tract, the correct (tract-complementary) nucleotide is mutagenic for +1 frameshift within the tract.

From spectrum analyses of mutations induced during the oriC plasmid DNA replication in vitro, we proposed the melting-misalignment model for +1 frameshift mutagenesis at runs

| TABLE III |
| Effect of the residue immediately 5′ to the dA tract on +1 frameshift mutagenesis |

|           | rpsL+ mutation frequency | A6 → A7 mutations | A6 → A6 mutation frequency |
|-----------|--------------------------|-------------------|---------------------------|
|           | × 10^-6 positives/ examined |                     |                           |
| 126dT-dA tract template |                          |                    |
| Background |                         | 32                 | 2/278                     |
|           |                          | 0.23               | 0.23                      |
| Replication product |                         | 63                 | 0/238                     |
| Balanced conditions |                         | 62                 | 8/184                     |
| Biased conditions |                         | 62                 | 2.7                       |
| 126dC-dA tract template |                          |                    |
| Background |                         | 140                | 2/82                      |
|           |                          | 3.4                | 3.4                       |
| Replication product |                         | 460                | 1/72                      |
| Balanced conditions |                         | 560                | 8/90                      |
| Biased conditions |                         | 560                | 620                       |
| 126dG-dA tract template |                          |                    |
| Background |                         | 180                | 4/87                      |
|           |                          | 8.3                | 8.3                       |
| Replication product |                         | 280                | 0/90                      |
| Balanced conditions |                         | 280                | <3.1                      |
| Biased conditions |                         | 340                | 10/89                     |
|           |                          | 38                 | 210                       |

mid DNA replication in vitro is due to strand asymmetry of the frameshift mutagenesis occurring at a dA/ddT tract in the target rpsL sequence. From spectrum analyses of mutations induced during the oriC plasmid DNA replication in vitro, we proposed the melting-misalignment model for +1 frameshift mutagenesis at runs

DISCUSSION

In the present study, we demonstrated that the dATP-dependent +1 frameshift mutagenesis observed with oriC plasmid DNA replication in vitro is due to strand asymmetry of the frameshift mutagenesis occurring at a dA/dT tract in the target rpsL sequence. From spectrum analyses of mutations induced during the oriC plasmid DNA replication in vitro, we proposed the melting-misalignment model for +1 frameshift mutagenesis at runs...
of a single nucleotide (11). This model agrees well with the observations that +1 frameshifts at runs occurred much more frequently than base substitutions at any site and that their frequencies depended on the length of the run and sharply increased when the run was longer than 4 residues. In this model (Fig. 4B), a small loop formed during the proofreading process acts as an intermediate of +1 frameshift. A misalignment may occur upon setting back the growing chain after the exonucleolytic editing step. Although a mismatched base pair favors melting of the duplex DNA, a significant proportion of chains containing the correctly inserted nucleotide are also subject to the proofreading process. It has been shown that about 10% of correctly incorporated nucleotides are excised by Pol III holoenzyme or its core subassembly during DNA synthesis (25, 26). Therefore, the terminus of the growing chain is peeled off at least once for every 10 incorporation events by the replicative enzyme, and the frequency of terminal melting is about 1,000-fold higher than that of misinsertion events. Results obtained in the present study support the melting-misalignment model. Because high concentrations of the nucleotide complementary to the tract template do not suppress the +1 frameshift immediately, the results obtained in the present study support the melting-misalignment model. Because high concentrations of the nucleotide complementary to the template residue immediately 5’ to the tract did not suppress the +1 frameshift mutagenesis, most of the slippage errors are probably generated during chain elongation within the tract. Thus, the stimulative effect of nucleotides complementary to the tract template seems to be due to an enforcement of further elongation of the chain from the +1 frameshift intermediate. This is similar to the rescue effect of nucleotides complementary to the template residue 5’ to a base-base mispair (22).

The effect of substrate nucleotides on +1 frameshift mutagenesis was essentially the same when either strand of the da/dT tract was used as a template, but the basis for strand asymmetry in +1 frameshift mutagenesis at a da/dT tract in the rpsL target sequence remains unclear. There are several possibilities: 1) a difference in the frequency of misalignment; 2) a difference in the stability of the frameshift intermediate; and 3) a difference in the efficiency of the rescuing nucleotide. On the basis of analyses using modified sequences of rpsL, the third of these possibilities appeared to be at least a partial cause of the observed strand asymmetry. It seems probable that the rescuing effect of nucleotides forming a G:C pair would be stronger than that of nucleotides that form an A:T pair. On the other hand, it seems unlikely that the stability of frameshift intermediate is involved in the strand asymmetry. To assess the stability of frameshift intermediate, we measured melting temperature (Tm) of oligomer DNA containing da/dT6, da/dT7, or da/dT8, and calculated ΔTm for both types of +1 frameshift intermediate. No significant difference was found between these ΔTm. Finally, it should be noted that da/dT tracts are known to cause DNA bending (27, 28). Therefore, this unusual structure of da/dT tracts may make DNA synthe-

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2 M. Seki and H. Maki, unpublished data.

The strand asymmetry in +1 frameshift mutagenesis was observed during in vitro DNA synthesis by Pol III holoenzyme using physiological concentrations of dNTPs. The +1 frameshift mutations induced in oriC plasmid DNA replication in vitro closely resembled those produced in E. coli cells defective in the mismatch repair function (11). Thus, the characteristics of +1 frameshift mutagenesis at a da/dT tract found in the present study are probably the same as those of mutagenesis occurring in in vivo DNA replication.

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