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PROOFREADING OF A MUTAGENIC NUCLEOTIDE, N^4-AMINODEOXYCYTIDYLIC ACID, BY ESCHERICHIA COLI DNA POLYMERASE I

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N^4-Aminodeoxycytidine triphosphate, a putative metabolite of N^4-aminocytidine which is a potent mutagen, is incorporated, in vitro, into polynucleotides in place of dCTP and at a much lesser extent, but significantly, in place of dTTP by E. coli DNA polymerase I large fragment. The activity of the polymerase to proofread this unnatural nucleotide has now been investigated. The results indicate that the 3'- 5' exonuclease in the polymerase recognizes N^4-aminocytosine as an incorrect base when N^4-aminocytosine is incorporated opposite adenine but the enzyme cannot distinguish N^4-aminocytosine from cytosine when it is incorporated opposite guanine.

We have shown that N^4-aminocytidine, a nucleoside analog, is a potent mutagen in bacteria (1), phage (1), mammalian cells (2), and Drosophila (unpublished work). This base analog can be incorporated into cellular DNA (2), probably after being metabolized into $dC^{am}TP$ (2). It causes both AT to GC (3) and GC to AT transitions (unpublished results). N^4-Aminocytosine can take either the amino or the imino form by undergoing tautomerization (4). This change in the structure offers an explanation for its ambigous base-pairing property: N^4-aminocytosine should behave as cytosine in its amino form and as thymine in its imino form (Fig. 1). Previous studies using the assay for in vitro DNA synthesis have shown that N^4-aminocytosine is incorporated into DNA, efficiently in place of dCTP and much less so, but significantly, in place of dTTP (3). The N^4-aminocytosine incorporated in this way into the polymer is read as cytosine in the subsequent DNA daughter-strand synthesis (3). **Consistent with** these

Abbreviations used: $dC^{am}TP$, N^4-aminodeoxycytidine 5'-triphosphate; $dC^{am}MP$, N^4-aminodeoxycytidine 5'-phosphate.

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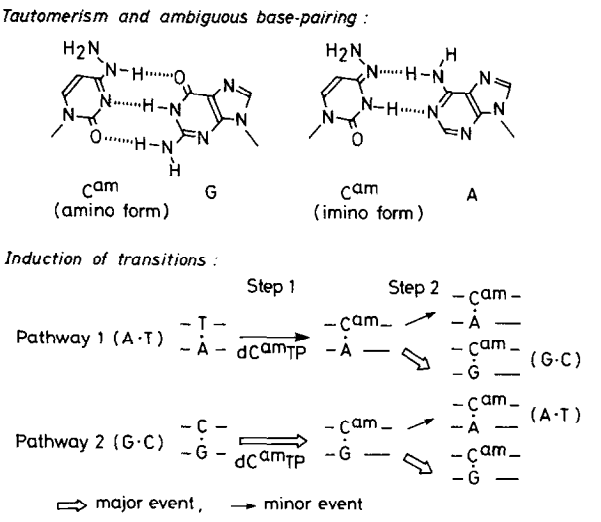

Fig. 1. Tautomerism of N^4-aminocytosine and possible mechanism of N^4-aminocytidine-induced transitions.

findings, incorporation of $dC^{am}TP$ into a replicative form DNA of phage ϕX174 am3, by use of nick translation, followed by transfection of the DNA to host bacterium resulted in the production of revertant phages (5). These observations gave a molecular basis for the AT to GC transition induced by N^4-aminocytidine. The mechanism is consistent with a hypothetical scheme proposed by Freese (6) for the nucleoside-analog mutagenesis. A scheme adapted to the N^4-aminocytidine mutagenesis is shown in Fig. 1.

A critical step for mutagenesis by a nucleoside- or base-analog is incorporation into DNA of the nucleoside triphosphate formed from the analog. It is known that in phage T4 the proofreading activity of T4 DNA polymerase greatly influences the incorporation extents of mutagenic nucleotides such as 2-aminopurine deoxyriboside triphosphate and 5-bromodeoxyuridine triphosphate (7-9). Genetic studies with phage T4 mutator and antimutator phenotypes have indicated that the proofreading by T4 DNA polymerase results in a reduced mutagenicity of 2-aminopurine (10). On the other hand, little is known about the effect of proofreading by bacterial nuclease on the nucleoside-analog induced mutagenesis.

Since N^4-aminocytosine nucleotide is efficiently utilized by E. coli DNA polymerase I, causing well-recognizable ambiguous base pairing, it is

expected that the question whether this unnatural nucleotide is subject to proofreading by the polymerase will be answered in a clear-cut manner. We have now examined if the incoming $dC^{am}TP$ is excised to give $dC^{am}MP$ by the 3'-5' exonuclease activity of E. coli DNA polymerase I large fragment. In this study, two copolymers of deoxyribonucleotides were used as template-primers to investigate the dependence of the rate of proofreading on the nature of the template-bases. Although the exonuclease activity of E. coli DNA polymerase was reported to be much weaker than that of T4 polymerase (11), we have found that this enzyme can proofread effectively the incoming $dC^{am}TP$.

MATERIALS AND METHODS

Materials Synthetic polynucleotides and oligonucleotides were purchased from Pharmacia (Sweden). Radioactive nucleotides were obtained from Amersham (England). [^{3}H]-$dC^{am}TP$ was synthesized from 1',2',5-[^{3}H]-dCTP by the hydrazine-bisulfite procedure (3). Non-radioactive $dC^{am}TP$ and $dC^{am}MP$ were similarly prepared from dCTP and dCMP, respectively. E. coli DNA polymerase I large fragment was obtained from Takara Shuzo (Japan). Polyethyleneimine cellulose plate was a product of Macherey-Nagel (W. Germany).

DNA polymerase assay Assay conditions for the E. coli polymerase I large fragment catalyzed polymerization were those used in our earlier studies (3).

Measurement of nucleoside monophosphate formation in the polynucleotide synthesis A tritium labeled nucleoside triphosphate was subjected to the polymerizing reaction, together with an unlabeled, another nucleoside triphosphate required. The radioactive nucleoside monophosphate formed in the reaction was separated from the triphosphates by thin layer chromatography on polyethyleneimine cellulose and quantitated. The procedure employed was the one described previously by Goodman (10), modified slightly in our hands. Thus, the reaction mixture consisted of 50 mM Tris-HCl (pH 7.5), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 20 units/ml of E. coli DNA polymerase I large fragment, 1 A_{260} unit/ml template-primer, and the substrate nucleoside triphosphates at 0.1 mM concentrations, in a total volume of 100 µl. The substrates were used at concentrations greater than those at which maximum incorporation rates were obtained. Incubation was done at 37°C, and 10 µl aliquots were taken in duplicate at appropriate intervals for measuring the monophosphate formation. In parallel, 10 µl single aliquots were taken to determine the incorporation of radioactivity into polynucleotides. For the measurement of mononucleotide formation, the aliquot was mixed with an equal volume of a marker solution which consisted of 50 A_{260} units/ml of the deoxyribonucleoside triphosphate, 50 A_{260} units/ml of the deoxyribonucleoside monophosphate and 20 mM EDTA. The mixture was placed on a polyethyleneimine cellulose plate which had been washed once with methanol. The plate was developed with 1 M LiCl. After drying, the spot of monophosphate marker was cut out, eluted with 1 ml of 0.6 N HCl at 37°C for 1 hr, and the radioactivity of the solution was determined by use of Triton X-100 fluor scintillation mix.

RESULTS AND DISCUSSION

We examined the activity of E. coli DNA polymerase I large fragment to proofread N^4-aminocytosine residues. Table I shows the results. First, we used poly(dG-dC)·poly(dG-dC) as the template-primer, and dGTP plus either ^{3}H-$dC^{am}TP$, ^{3}H-dCTP or ^{3}H-TTP as substrates. Consistent with the previous observation (3), $dC^{am}TP$ was incorporated at a rate half that of dCTP. The removal of N^4-aminodeoxycytidine monophosphate was slow, and the rate was similar to that for the removal of dCMP: about 10% of the nucleotides utilized were removed as the monophosphate. No utilization of dTTP was observed as expected.

This finding has shown that the DNA polymerase has no or little activity to excise the abnormal base, N^4-aminocytosine, when the base is situated opposite guanine. In other words, proofreading exonuclease of E. coli DNA polymerase I large fragment cannot distinguish $dC^{am}MP$ from dCMP in this situation.

Table 1

Incorporation and excision of $dC^{am}TP$ by E. coli DNA polymerase I large fragment

Template-primer and coexisting triphosphate	Incubation time and radioactive triphosphate	Utilization of radioactive triphosphate		Percent removal [b/(a+b)] x 100
		Incorporation (pmol) a	Monophosphate generated (pmol) b	
Poly(dG-dC)·poly(dG-dC) and dGTP	15 min			
	$dC^{am}TP$	11.4	0.9	7.4
	dCTP	27.1	2.99	9.9
	dTTP	< 0.4	< 0.2	-
	30 min			
	$dC^{am}TP$	17.1	2.1	10.8
	dCTP	40.8	5.2	11.2
	dTTP	< 0.4	< 0.2	-
Poly(dA-dT)·poly(dA-dT) and dATP	15 min			
	$dC^{am}TP$	2.8	11.2	80.2
	dTTP	116	8.6	6.9
	dCTP	< 0.4	2.2	∿ 100
	30 min			
	$dC^{am}TP$	3.1	18.7	86.0
	dTTP	196	21.3	9.8
	dCTP	< 0.4	3.9	∿ 100

We investigated the proofreading activity for $dC^{am}TP$ that was misinserted as a substitute for dTTP, using poly(dA-dT)·poly(dA-dT) as a template-primer. A high level of $dC^{am}MP$ formation was observed when [^{3}H]-$dC^{am}TP$ was used as a substrate together with dATP. The amount of $dC^{am}MP$ increased in a time dependent manner, while the incorporation of $dC^{am}TP$ into the poly(dA-dT)·poly(dA-dT) reached a saturation at 15 min. More than 80% of the incoming $dC^{am}TP$ was excised during the incubation for 30 min. In the control experiment with dCTP, essentially all the dCTP utilized was excised, giving the monophosphate. The experiment with dTTP also showed monophosphate generation, but the amount of dTMP formed was very small as expected.

It has been shown that the proofreading exonuclease of T4 DNA polymerase can efficiently reduce the incorporation of 2-aminopurine and 5-bromouracil during the *in vitro* DNA synthesis (7-10,12). When 2-aminopurine was inserted as adenine by the polymerase, it was removed several times more rapidly than adenine (10), although it is thought that 2-aminopurine pairs with thymine in its abundant form (12,13). In the case of the 5-bromouracil insertion opposite guanine, the 5-bromouracil nucleotide was excised at a high efficiency of 75-85%. It has been noted (8,11,12) that T4 DNA polymerase has a much higher proofreading activity than *E. coli* DNA polymerase I. Nevertheless, our experimental results have clearly shown that *E. coli* DNA polymerase I can remove the incoming abnormal nucleotide $dC^{am}MP$ by its proofreading activity, if the nucleotide was "misincorporated", that is, when it is incorporated opposite adenine.

It should be noted that *E. coli* DNA polymerase I large fragment does not always remove "unpaired" incoming nucleotide. For example, Singer recently showed that this enzyme did not proofread O^4-methyl dTMP opposite adenine (14).

Recently, template-mediated hydrolysis of deoxyribonucleoside 5'-triphosphate into its monophosphate by DNA polymerase was reported, and the phenomenon was termed "idling turnover" (15). In this phenomenon, the nucleoside triphosphate to be hydrolyzed bears a base unable to pair with

the template base. We examined the possibility that the formation of $dC^{am}MP$ in the present experiment with poly(dA-dT)·poly(dA-dT) as template might be due to this type of hydrolysis: $dC^{am}TP$ entering opposite thymine in the template (rather than opposite adenine) may be hydrolyzed into $dC^{am}MP$ by the idling turnover. For this, we used poly(dT)·oligo$(dA)_{12-18}$ as the template-primer and investigated whether $dC^{am}MP$ could be formed from $dC^{am}TP$ by catalysis with DNA polymerase I large fragment. No $dC^{am}MP$ was produced; thus the possibility of dC^{am} to enter opposite thymine in poly(dA-dT)·poly (dA-dT) was excluded (data not shown).

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