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Mutagenesis by N^4-Aminocytidine: Induction of AT to GC Transition and Its Molecular Mechanism†

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ABSTRACT: N^4-Aminocytidine is a potent mutagen toward *Escherichia coli* and *Salmonella typhimurium*. It induced reversion of an amber mutant of ϕX174 phage (am3) to the wild type. This reversion was shown to be exclusively due to the AT to GC transition. It is likely that N^4-aminocytidine is metabolized within the bacterial cells into N^4-aminodeoxycytidine 5′-triphosphate and this nucleotide is incorporated into DNA during the multiplication of the cells and the phages, thereby causing base-pair transitions. The molecular basis for this erroneous replication was obtained in studies of in vitro incorporation of N^4-aminodeoxycytidine 5′-triphosphate into polynucleotides catalyzed by the *E. coli* DNA polymerase I large fragment. The results have shown that this cytosine analogue can be efficiently incorporated as a substitute of cytosine and that it can also be incorporated as a substitute of thymine. The ratio in the rate of the N^4-aminocytosine nucleotide incorporation to that of natural nucleotide incorporation was 1/2 to cytosine and 1/30 to thymine. Furthermore, the N^4-aminocytosine residues in the polynucleotide templates can be read by the enzyme as efficiently as cytosines, and guanines were incorporated opposite to them.

Recently, we have found that N^4-aminocytidine can efficiently induce base-change mutations in bacteria and in phages (Negishi et al., 1983). Its high activity is exceptional for a nucleoside analogue and is comparable to that of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, one of the most potent mutagens. The activity is more than 1000 times stronger than that of 2-aminopurine in the reversion of *Salmonella typhimurium* TA100 as well as in the reversion of *Escherichia coli* WP2 *trp* (ocher). Thus, the numbers of revertants formed per nanomole of reagent (calculated from the linear dose–responses) were, N^4-aminocytidine/2-aminopurine/*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, in *S. typhimurium* TA100 60/0.02/300 and in *E. coli* WP2 uvr 160/0.012/10 (Negishi et al., 1983; unpublished work).

Probably, within the cells N^4-aminocytidine is converted to $dC^{am}TP$[1] and incorporated into DNA. The principal tautomeric structure of N^4-aminocytosine is the amino form (Brown et al., 1968; Takayanagi et al., 1980), and Brown et al. (1968) have estimated that about $^1/_{30}$ of the molecules is present in the imino form. A simple mechanism for the N^4-aminocytosine-induced mutagenesis would be that the imino form of N^4-aminocytosine pairs with adenine, while the major amino form pairs with guanine (Figure 1). Thus, during one replicational cycle, $dC^{am}TP$ is incorporated into DNA mostly as a substitute of dCTP, but it can also be incorporated to a certain extent as a substitute of dTTP (see step 1 in Figure 1). In the next cycle of replication, either dGTP or dATP can be incorporated opposite N^4-aminocytosine on the template DNA (step 2). This scheme is analogous to the classical one proposed by Freese (1959) for the base analogue mutagenesis.

The mechanism in Figure 1 predicts that N^4-aminocytidine should induce both the AT to GC and the GC to AT transitions and that no transversions should be caused. In the present work, we analyzed the mutational specificity in the N^4-aminocytidine-induced reversion of ϕX174 am3 phage and studied the in vitro utilization of $dC^{am}TP$ in the polynucleotide synthesis catalyzed by *E. coli* DNA polymerase I large fragment. The results obtained by sequencing the revertant phage DNAs have shown that a AT to GC transition took place in the am3 site. Furthermore, the study on the in vitro DNA synthesis gave strong support for the mechanism as illustrated in Figure 1.

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[1] Abbreviations: $dC^{am}TP$, N^4-amino-2′-deoxycytidine 5′-triphosphate; $dC^{oh}TP$, N^4-hydroxy-2′-deoxycytidine 5′-triphosphate; $imdC^{am}TP$ (and $imdC^{am}MP$), 6-(2′-deoxy-β-D-ribofuranosyl)-5-oxo-5,6-dihydro-*s*-triazolo[4,3-*c*]pyrimidine 5′-triphosphate (and 5′-monophosphate); HPLC, high-pressure liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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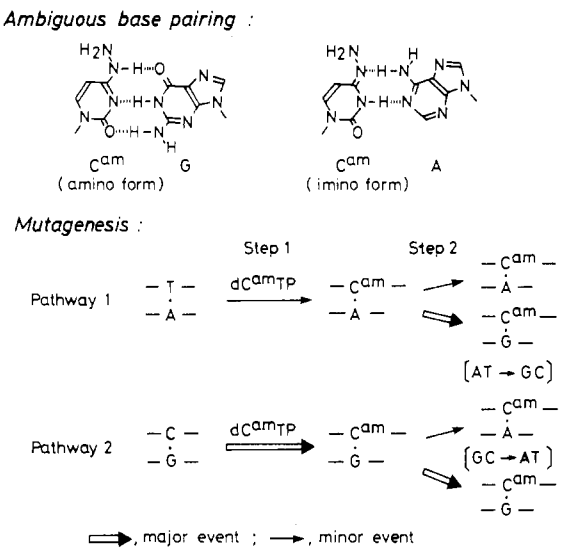

FIGURE 1: Mechanism of N^4-aminocytidine-induced base-pair transitions.

MATERIALS AND METHODS

Materials. Poly(dA), oligo(dT)$_{12-18}$, oligo(dA)$_{12-18}$, oligo(dG)$_{12-18}$, and the copolymers with alternating AT or GC sequences poly(dA-dT) and poly(dG-dC) were products of P-L Biochemicals. Activated calf thymus DNA was prepared according to Aposhian & Kornberg (1962). *E. coli* DNA polymerase I large fragment, terminal nucleotidyl transferase, and restriction endonucleases were obtained from Takara Shuzo. Radioactive chemicals were products of Amersham. dC^{oh}TP was synthesized from dCTP by treatment with hydroxylamine (Budowsky et al., 1972) and purified with HPLC.

Analysis of φX174 Phage Revertants. φX174 phage am3 was mutagenized by allowing the phage-infected *E. coli* HF4714 (sup$^+$) to be cultured in a medium containing 0.05 mM N^4-aminocytidine (Negishi et al., 1983). The revertants were detected by the growth on *E. coli* CI (sup$^-$). Among the revertants obtained, eight colonies were randomly selected. They were obtained in three independent experiments. Each colony was cultured to obtain RFI DNA (covalently closed circular replicative form) by the method of Ueda et al. (1981). The RFI DNA was digested with restriction endonuclease *Taq*I, and the DNA fragment containing am3 site (231 base pairs) was separated from other fragments by polyacrylamide gel electrophoresis. The fragment was further digested with *Hae*III, and a 179 base pair piece containing am3 site was isolated. The DNA fragment was sequenced by the Maxam–Gilbert (1977) procedure (see Figure 2). Only about a 60-nucleotide region encompassing the am3 site was sequenced.

Preparation of N^4-Aminocytosine Derivatives. N^4-Aminocytidine was prepared from cytidine as described (Negishi et al., 1983). dC^{am}TP and dC^{am}MP were similarly obtained from dCTP and dCMP, respectively. Thus, a solution containing 0.1 M dCTP, 4 M hydrazine, and 0.1 M sodium bisulfite at pH 7 (the pH was adjusted by addition of HCl) was incubated at 60 °C for 4 h. The reaction mixture was fractionated by HPLC on a column of Partisil 10SAX (4.6 × 250 mm) (15-μL reaction mixture for a run). The column was eluted with 0.3 M ammonium phosphate at pH 3.8 at a flow rate of 2 mL/min. The peak fraction detected by A_{254} was pooled and applied to a column of LiChrosorb RP18 (4.6 × 250 mm). The column was eluted with 10 mM triethylammonium bicarbonate–3% methanol at pH 6.3 at a flow rate of 1 mL/min. The dC^{am}TP fraction obtained was lyophilized and stored at −80 °C. [^{3}H]dC^{am}TP was prepared similarly from [1′,2′,5-^{3}H]dCTP. In this case, nonradioactive dC^{am}TP had been added as a carrier at the start of the second HPLC fractionation. Specific radioactivity of the [^{3}H]dC^{am}TP prepared was 500 Ci/mol. Purity of the dC^{am}TP was examined by derivatization with ethyl acetimidate to form the triazolopyrimidine compound imdC^{am}TP (Nitta et al., 1984; N. Nitta and H. Hayatsu, unpublished work). This was necessary because dC^{am}TP gave a tailing peak in the HPLC, a part of which overlapping with the peak for dCTP. HPLC analysis of the imdC^{am}TP on Partisil 10SAX with an eluting solvent, 0.3 M ammonium phosphate (pH 3.8)–acetonitrile, 10/1, showed that the [^{3}H]dC^{am}TP sample was more than 98.8% pure (contents of contaminating [^{3}H]dCTP and [^{3}H]dUTP were <0.8% and <0.4%, respectively). The retention time of nucleotides in this HPLC was 9.0 min for dC^{am}TP, 10.4 min for dCTP, 13.2 min for dUTP, and 17.1 min for imdC^{am}TP.

Incorporation of Radioactive Nucleoside Triphosphates into Polynucleotides in the DNA Polymerase Catalyzed Reaction. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, *E. coli* DNA polymerase I large fragment, appropriate template primers, and deoxyribonucleoside triphosphates containing a single radioactively-labeled substrate. The reaction was started by addition of the enzyme. The incubation was at 37 °C. The incorporation was measured by the DEAE paper method (Lindell et al., 1970). Ten-microliter aliquots from a reaction mixture were individually placed on 1.5-cm^2 square pieces of DE-81 paper (Whatmann). The paper was washed 6 times with 7% Na_2HPO_4 and then with water and dehydrated by soaking into ethanol and ether successively, and the radioactivity bound to the paper was measured with an Aloka liquid scintillation counter by use of a toluene-based scintillator. The counting efficiency was 40%, as determined for poly(dG-[^{3}H]dC). The zero-time samples were those taken immediately after the start of the reactions.

Identification of dC^{am}MP Incorporated into Polynucleotides. For identification of dC^{am}MP in the polynucleotides formed, the polymers obtained by using poly(dA-dT) and poly(dG-dC) as template primers were analyzed. In the synthesis directed by poly(dA-dT), a mixture of [^{3}H]-dC^{am}TP (0.03 mM, 270 Ci/mol) and dATP (0.03 mM) was allowed to react in the presence of 100 units/mL of the polymerase and 5 A_{260}/mL of the template primer. The volume of the reaction was 100 μL. The incubation was for 30 min at 37 °C. The reaction extent was monitored as described above, and a portion (50 μL) of the solution was subjected to the processing as follows. To the ice-cooled solution, 1 A_{260} of poly(dA-dT) as carrier, sodium chloride, and water were added. The final volume was 120 μL, and the sodium chloride concentration was 1 M. The solution was loaded on a column of Bio-Gel P-150 (0.6 × 14 cm), which had been maintained at 4 °C. The column was eluted with water (0.05 mL/min) to obtain the polynucleotide fraction, which was then concentrated and digested into mononucleotides by treatment with nuclease P1 (10 μg) in 0.05 M sodium acetate at pH 5 and 50 °C for 120 min. The mononucleotide mixture was treated with ethyl acetimidate, and the [^{3}H]imdC^{am}MP formed was detected by HPLC on Nucleosil 10$(CH_3)_2$NH (4.6 × 250 mm). The elution was done with 0.05 M ammonium phosphate, pH 3.3, at a flow rate of 1 mL/min.

The dC^{am}TP polymerization directed by poly(dG-dC) was carried out by using [^{3}H]dC^{am}TP (0.03 mM, 270 Ci/mol), dGTP (0.03 mM), 20 units/mL polymerase, and 1 A_{260}/mL poly(dG-dC). The incubation was 60 min, and the subsequent workup was done in the manner described above for the

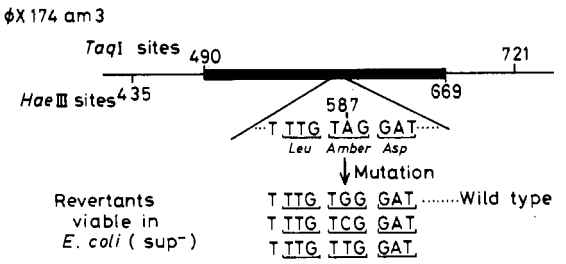

FIGURE 2: Possible nucleotide sequences at the amber codon in the revertants of phage φX174 am3. The sequence of φX174 am3 DNA is taken from Sanger et al. (1978).

poly(dA-dT)-directed synthesis.

Preparation of Poly(dA,dC^{am}). The template containing N^4-aminocytosine residues randomly distributed among adenine residues was prepared by use of terminal nucleotidyl transferase. The reaction mixture contained, in 100 μL, 0.08–0.24 mM dC^{am}TP or [^{3}H]dC^{am}TP, 2 mM dATP, 1 A_{260}/mL oligo(dA)$_{12-18}$, 150 units of enzyme, 8 mM $MgCl_2$, 1.2 mM 2-mercaptoethanol, and 0.2 M HEPES, pH 7.2. The incubation was at 37 °C for 8 h; 50 mM dATP (4 μL) was added, and the incubation was continued for additional 4 h. The mixture was treated at 75 °C for 15 min, and the insoluble material that precipitated was removed by centrifugation. The polynucleotide formed was purified by filtration through Bio-Gel P-150 as described for poly(dG-dC,[^{3}H]dC^{am}) in the above section.

For the characterization of the poly(dA,dC^{am}) and poly(dA,[^{3}H]dC^{am}), they were digested into mononucleotides, treated with ethyl acetimidate, and fractionated by HPLC, as in the characterization of poly(dA-dT,dC^{am}) and poly(dG-dC,dC^{am}) (see above). The dC^{am}MP content in these polynucleotides was estimated on the basis of ultraviolet absorbance of imdC^{am}MP found in the HPLC fractions. dUMP in the poly(dA,[^{3}H]dC^{am}) preparation was quantified from the radioactivity of the dUMP fraction in the HPLC of the digest.

Template Activity of Poly(dA,dC^{am}). The experiments to measure the poly(dA,dC^{am})·oligo(dT)-directed incorporation of tritiated nucleoside triphosphates into polynucleotides were performed as described for other templates. In this case, however, 1 A_{260}/mL poly(dA,dC^{am}) (or other polynucleotides), 0.2 A_{260}/mL oligo(dT)$_{12-18}$, and 0.05 mM nucleotide triphosphates were used. Nearest-neighbor frequency analysis was done as described (Singer et al., 1983). For this purpose, the polymerization was performed in an incubation mixture consisting of 0.3 A_{260}/mL poly(dA,[^{3}H]dC^{am}) or poly(dA), 0.06 A_{260}/mL oligo(dT), 0.05 mM each of dGTP, dCTP, dATP, and dTTP, and 250 μCi of [α-^{32}P]dTTP.

RESULTS

Nucleotide Sequences in Revertants of Phage φX174 am3. Phage φX174 am3 was mutagenized by treatment of the phage-infected *E.coli* (sup$^+$) with N^4-aminocytidine. Eight phage strains were chosen at random from the revertants obtained. Samples of RFI DNA were prepared from these revertants and treated with restriction endonucleases to produce fragments containing am3 site, and the fragments were sequenced (see Figure 2). All the revertant DNA samples showed the same sequences as that of the wild-type φX174 in the region surrounding the am3 site: all had TGG at the am3 site, and in both of the 30 base pairs before and after the am3 site no change was found. These results have shown that N^4-aminocytidine can induce the AT to GC transition.

Reversion of φX174 phage am3 has been extensively used to study the fidelity of DNA replication (Loeb & Kunkel, 1982; Kunkel et al., 1984), and phages whose nucleotide sequence at the am3 site is TTG or TCG have been reported to be viable in wild-type *E. coli* (Schaaper et al., 1983). The absence of such revertants among the randomly chosen eight strains suggests that N^4-aminocytidine does not induce transversions. In this experiment, the question whether N^4-aminocytidine can induce GC to AT transitions cannot be answered, because the detectable transition in the system is only the AT to GC mutation.

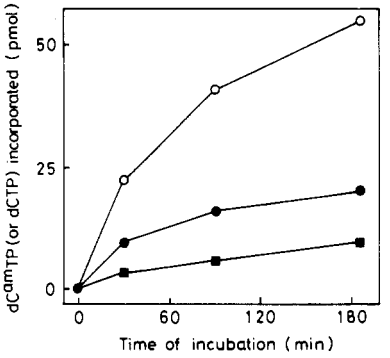

FIGURE 3: Incorporation of [^{3}H]dC^{am}TP into activated calf thymus DNA by catalysis of *E. coli* DNA polymerase I large fragment in the presence of native deoxyribonucleoside triphosphates. The reaction mixtures contained 0.016 mM each of [^{3}H]dC^{am}TP, dTTP, dGTP, and dATP (●), [^{3}H]dC^{am}TP, dCTP, dTTP, dGTP, and dATP (■), or [^{3}H]dCTP, dTTP, dGTP, and dATP [(○) control]. Each mixture also contained 5 units/mL of the polymerase and 10 A_{260}/mL of activated calf thymus DNA. The specific radioactivities of the nucleoside triphosphates used were 120 cpm/pmol.

Incorporation of dC^{am}TP into Polynucleotides by Catalysis of E. coli DNA Polymerase I Large Fragment. The AT to GC transition induced by N^4-aminocytidine can be explained in terms of the mechanism shown in pathway 1 of Figure 1. There are two steps leading to the transition: in the first replication, N^4-aminocytosine is incorporated opposite adenine, and in the second, guanine enters into the site opposite N^4-aminocytosine. To show that this pathway is indeed possible, we investigated the in vitro DNA synthesis with (1) dC^{am}TP as substrate and (2) polynucleotides bearing N^4-aminocytosine residues as template primer. The DNA-synthesizing enzyme employed was *E. coli* DNA polymerase I large fragment.

The experiments using activated calf thymus DNA as template primer (Figure 3) have shown that [^{3}H]dC^{am}TP can be efficiently incorporated into polynucleotide in the presence of dATP, dGTP, and dTTP. The rate of dC^{am}TP incorporation was about half that of dCTP incorporation in the control experiment. Significant incorporation of dC^{am}TP was observed even in the presence of equimolar dCTP.

We used three synthetic polynucleotides for studying further the specificity of the dC^{am}TP incorporation. Results from the experiments with an alternating GC copolymer poly(dG-dC)·poly(dG-dC) as the template primer have demonstrated that dC^{am}TP can be incorporated as a substitute of dCTP into the newly synthesized polynucleotide (Table I). This experiment also shows that N^4-aminocytosine enters at the site opposite to guanine of the template. The incorporation of [^{3}H]dC^{am}TP showed a time-dependent increase similar to that of the [^{3}H]dCTP incorporation, and the rate was again about half the rate for [^{3}H]dCTP. It is also shown that the incorporation of dC^{am}TP can proceed in the presence of dCTP. As expected, the poly(dG-dC)-directed incorporation of [^{3}H]-dCTP was inhibited by dC^{am}TP. These results have shown that dC^{am}TP behaves like dCTP, competing with it at the 3′-end of the growing polynucleotide chain where dCTP should enter for the elongation of the chain. In a separate experiment,

Table I: Utilization of $dC^{am}TP$ in Polynucleotide Synthesis Directed by Copolymers Having Alternating Dinucleotide Sequences[a]

template primer	dXTP used (radioactive dXTP is shown in italics)	radioactive nucleotide incorporated: pmol	radioactive nucleotide incorporated: time (min)	incorporation extent[b]
poly(dG-dC)·poly(dG-dC)	C^{am}, G	1.4	0	
		42	15	0.44
		61	30	
	C^{am}, C, G	0.83	0	
		15	15	0.16
		21	30	
	C, G (control)	96, 82[c]	15	1.0
	C, G, C^{am}	78[c]	15	0.95
	C, G, C^{am} (0.5 mM)	41[c]	15	0.50
poly(dA-dT)·poly(dA-dT)	C^{am}, A	1.5	0	
		5.6	15	0.035
		5.3	30	
	C^{am}, A, T (0.05 mM)	0.77	0	
		1.6	15	0.010
		4.2	30	
	C, A	0.5	0	
		1.1	15	0.007
	T, A (control)	160	15	1.0

[a] The template primer was used at 1 A_{260}/mL, the dXTP at 0.1 mM except where otherwise noted, and *E. coli* DNA polymerase I large fragment at 20 units/mL. The incubation was at 37 °C in 0.05 M Tris-HCl at pH 7.5 (containing 6.7 mM $MgCl_2$ and 1 mM 2-mercaptoethanol). [b] The extent is represented by the incorporation at 15 min relative to the normal base controls. [c] A separate experiment.

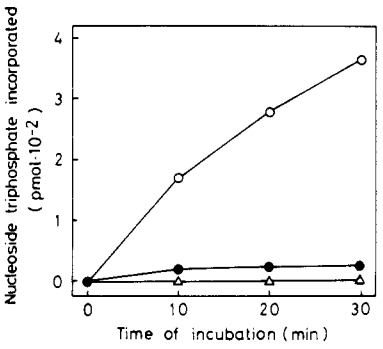

FIGURE 4: Incorporation of [^{3}H]$dC^{am}TP$ into the template-primer poly(dA)·oligo(dT)$_{12-18}$ by catalysis of DNA polymerase I large fragment. The reaction mixtures contained 1 A_{260}/mL poly(dA)·oligo(dT)$_{12-18}$, 5 units/mL of the polymerase and [^{3}H]$dC^{am}TP$ (160 cpm/pmol) (●), [^{3}H]dCTP (110 cpm/pmol) (△), or [^{3}H]dTTP (120 cpm/pmol) (○).

it was also shown that the poly(dG-dC)-directed incorporation of [^{3}H]dGTP (0.1 mM) in the presence of dCTP (0.1 mM) was not affected by the addition of $dC^{am}TP$ (0.05–0.1 mM)(data not shown). In this dGTP incorporation, the absence of stimulation by the added $dC^{am}TP$ could be ascribed to the saturating amounts of the nucleoside triphosphates present in the reaction mixture.

We analyzed the incorporation of N^4-aminocytosine in the site opposite adenine in the template: i.e., the incorporation as a substitute of dTTP into polynucleotides under the direction of poly(dA-dT)·poly(dA-dT) or poly(dA)·oligo(dT) as template primers. From the mutagenicity and the tautomeric properties of N^4-aminocytosine nucleosides, it can be anticipated that $dC^{am}TP$ would behave like dTTP, although its efficiency might be low. The results shown in the lower part of Table I and the results in Figure 4 indicate that small but significant amounts of $dC^{am}TP$ are incorporated into both of these primers. The incorporation extent was 3.5%, i.e., $^1/_{30}$

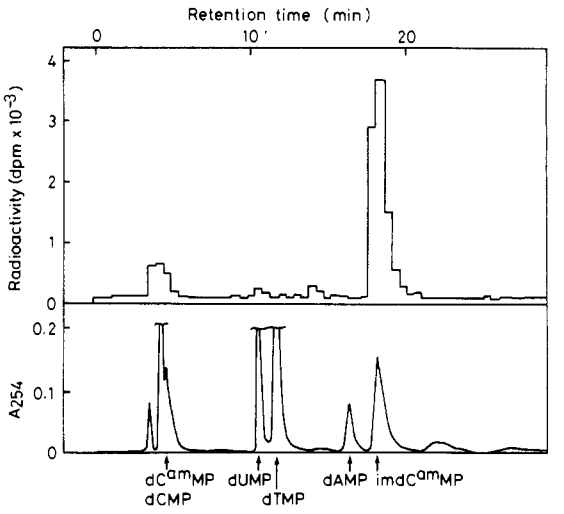

FIGURE 5: Identification of $dC^{am}MP$ in polynucleotide formed on the template-primer poly(dA-dT). Nuclease P1 digest of the polynucleotide was fractionated by HPLC on Nucleosil 10$(CH_3)_2NH$. $dC^{am}MP$, $imdC^{am}MP$, dUMP, and dCMP were added before chromatography as markers. Flow rate was 1 mL/min.

that of the dTTP-incorporation (Table I). In the poly(dA-dT)-directed synthesis, the incorporation in the presence of dATP appeared to stop at an early stage of the reaction (Table I). However, the addition of dTTP at an amount half that of $dC^{am}TP$ resulted in a slower but continuous increase in the amount of $dC^{am}TP$ incorporated (Table I). In contrast to the dCTP incorporation into poly(dG-dC) template, the dTTP incorporation into poly(dA-dT) template was not affected by $dC^{am}TP$, even with an addition of 5-fold equiv of $dC^{am}TP$ (data not shown).

All of these results indicate that $dC^{am}TP$ is incorporated at the site where dTTP should have been incorporated, namely, opposite adenine. This situation holds even if dTTP is present in the system. The efficiency of N^4-aminocytosine to substitute thymine, however, is much lower than that to substitute cytosine.

To evaluate the possibility of $dC^{am}TP$ to base pair with pyrimidines, incorporation of [^{3}H]$dC^{am}TP$ was measured with the use of poly(dT)·oligo(dA) or poly(dC)·oligo(dG) as a template primer. The experiment was carried out in the absence of other nucleoside triphosphates, and, as expected, no incorporation was detected (data not shown).

For identification of N^4-aminocytosine residues in the polynucleotides formed, the polynucleotides containing [^{3}H]-N^4-aminocytosine residues were freed from nucleoside triphosphates by gel filtration and digested with nuclease P1. The mononucleotides formed were treated with ethyl acetimidate for derivatization of the $dC^{am}MP$ into the triazolopyrimidine compound $imdC^{am}MP$, and the mixture was subjected to HPLC along with authentic markers. Results for the polynucleotide formed in the poly(dA-dT)-directed synthesis are given in Figure 5. Most of the radioactivity was eluted with the $imdC^{am}MP$ marker. Radioactivities in the dUMP and dCMP fractions were very small. When either poly(dG-dC)·poly(dG-dC) or poly(dA)·oligo(dT) was used as the template primer, the radioactive nucleotide in the products was also identified as $imdC^{am}MP$ (data not shown).

N^4-Aminocytosine in Template for DNA Synthesis. Samples of poly(dA,dC^{am}) were prepared in which N^4-aminocytosine residues as a minor component were present in a dispersed manner. Using oligo(dT) as primer, we investigated the activity of the poly(dA,dC^{am}) as template for the polynucleotide synthesis. The template activity was first assessed

Table II: N^4-Aminocytosine as Template for Guanine[a]

template primer	C^{am} (or C) content in template (%)[b]	incorporation of [^{3}H]dGTP (pmol)[c]
poly(dA,dC^{am})·oligo(dT)	8.7	29.8
	3.3	15.6
	0	0.6
poly(dA,dC)·oligo(dT)	8.6	30.5

[a] The concentration of poly(dA,dC^{am}) or poly(dA,dC) was 1 A_{260}/mL, oligo(dT) was at 0.2 A_{260}/mL, and the polymerase was at 20 units/mL. The incubation mixture contained [^{3}H]dGTP and dTTP each at 0.05 mM. Incorporation of [^{3}H]dTTP in the presence of dGTP was also examined, and for the four template primers in this table dTTP incorporation of 320–350 pmol at 30 min was observed. [b] Molar percentage of C^{am} or C in poly(dA,dC^{am}) or poly(dA,dC). [c] Incorporation at 15-min incubation.

Table III: Nearest-Neighbor Frequency Analysis of Polynucleotides Formed on Poly(dA,dC^a,m)·Oligo(dT) as Template Primer, Using [α-^{32}P]dTTP, dATP, dCTP, and dGTP as Substrates[a]

template primer	pyrimidine contents (% of total nucleotide)	^{32}P distribution (%)			
		dTpT	dGpT	dApT	dCpT
poly(dA,dC^{am})·oligo(dT)	dC^{am}, 5.3; dU, 0.16	94.6	5.3	0.13	0.007
	dC^{am}, 3.5; dU, 0.07	96.6	3.3	0.05	0.001
poly(dA,dC)·oligo(dT)	dC, 6.5	93.4	6.0	0	0
	dC, 3.8	96.6	3.4	0.003	0.005
poly(dA)·oligo(dT)		99.6	0.04	0.003	0.005

[a] The template primer used was 0.3 A_{260}/mL poly(dA,dC^{am}), poly(dA,dC), or poly(dA) in the presence of 0.06 A_{260}/mL oligo(dT)$_{12-18}$. The substrate dXTP was at 0.05 mM, with 250 μCi of [α-^{32}P]dTTP. The polymerase was at 10 units/mL, and the incubation was for 60 min.

by the incorporation of [^{3}H]dTTP in the presence of dCTP, dATP, and dGTP. Poly(dA,dC^{am}) containing 0, 3.3, or 8.7% N^4-aminocytosine and poly(dA,dC) containing 8.6% cytosine showed approximately equal activities to incorporate [^{3}H]-dTTP (see legend to Table II). This observation suggests that the DNA polymerase can read through the N^4-aminocytosine sites on the templates. Next, [^{3}H]dGTP incorporation was examined. As Table II shows, the extent of the dGTP incorporation was dependent on the N^4-aminocytosine content of the template. Obviously, the N^4-aminocytosine residues in the templates directed the dGTP incorporation as efficiently as the cytosine residues. The template specificity of the N^4-aminocytosine residues was analyzed more precisely, by determining the nearest-neighbor frequencies of the polymer synthesized. As Table III shows, the net amount of GpT in the product was equal to the N^4-aminocytosine content in the template. These results indicate that the dGTP was incorporated in a dispersed manner into the newly synthesized poly(dT,dG) and have shown that N^4-aminocytosine residues in the template that are located adjacent to adenines can participate in base-pair formation with dGTP.

A small but significant amount of adenylate incorporation was observed (Table III). However, it is difficult to evaluate this incorporation because the template poly(dA,dC^{am}) contained a small amount of deoxyuridylate residues, which were probably formed by spontaneous hydrolysis of the N^4-aminocytosine moieties during the process of the template preparation.

Comparison of $dC^{am}TP$ and $dC^{oh}TP$ as Substrates for DNA Synthesis. Since N^4-hydroxycytosine has been a focus of interest in laboratories studying base analogue induced mutagenesis, we compared the ability of $dC^{am}TP$ and $dC^{oh}TP$ to support DNA synthesis in the absence of natural pyrimidine nucleotides. The results are shown in Table IV. On removal of dCTP from the complete system (i.e., dTTP, dGTP, and dATP only), the rate of synthesis on activated DNA decreased to 33%. Addition of $dC^{am}TP$ restored the rate to about 70%. Addition of $dC^{oh}TP$ also restored the rate, but to a lesser extent. Furthermore, the strong ability of $dC^{am}TP$ to replace dCTP in the poly(dG-dC)-directed reaction is not found in $dC^{oh}TP$. The lowered syntheses observed when dTTP was subtracted were only slightly restored by addition of either $dC^{oh}TP$ or $dC^{am}TP$. These results indicate that $dC^{oh}TP$ can be only poorly utilized by the enzyme system in contrast to $dC^{am}TP$, which can be efficiently used when appropriate templates are present.

Table IV: Effect of Replacing Normal Nucleotides with $dC^{am}TP$ or $dC^{oh}TP$ on the Rate of DNA Synthesis in Vitro[a]

nucleoside triphosphate	relative rate (%) of DNA synthesis directed by		
	activated calf thymus DNA	poly(dG-dC)·poly(dG-dC)	poly(dA-dT)·poly(dA-dT)
complete[b]	100	100	100
–dCTP	33	2.3[c]	
+$dC^{am}TP$	69	44	
+$dC^{oh}TP$	52	4.7	
–dTTP	28		1.8
+$dC^{am}TP$	31		2.4
+$dC^{oh}TP$	35		3.8

[a] Incorporation extents were measured by using [^{3}H]dATP for activated calf thymus DNA and poly(dA-dT)·poly(dA-dT) and by using [^{3}H]dGTP for poly(dG-dC)·poly(dG-dC). The conditions in the activated calf thymus DNA reactions were the same as those given in Figure 3 except that 0.2 unit/mL enzyme was used. The conditions in the copolymer reactions were as shown in Table I except that 15 units/mL enzyme was used. The addition of $dC^{am}TP$ and $dC^{oh}TP$ was at concentrations equal to those of other nucleoside triphosphates. The rate in the table is the incorporation extent at 15 min of reaction relative to that of the complete system. [b] The complete system contained dCTP, dTTP, dGTP, and dATP for the activated calf thymus DNA template, dCTP and dGTP for the poly(dG-dC)·poly(dG-dC) template, and dTTP and dATP for the poly(dA-dT)·poly(dA-dT) template. [c] In this experiment, dTTP was added in place of dCTP.

Discussion

Here we have shown that N^4-aminocytidine can induce the AT to GC transition. We propose that this AT to GC transition takes place through pathway 1 of Figure 1. The results in studies of in vitro DNA synthesis are consistent with this scheme. In the incorporation experiments, $dC^{am}TP$ mimics dCTP, and in a small but significant extent it also behaves like dTTP. This dual character of N^4-aminocytosine must be responsible for the mutations occurring in cells. When N^4-aminocytidine is administered into *E. coli*, in which ϕX174 am3 phages are growing, $dC^{am}TP$ would be formed and used in the phage DNA synthesis. This $dC^{am}TP$ can be incorporated into the middle position of the am3 amber codon at a certain frequency during the (–) strand synthesis. The N^4-aminocytosine incorporation would direct incorporation of dGTP during the viral (+) strand synthesis. In this way, the reversion of the phage can be accomplished.

The results have also indicated that $dC^{am}TP$ formed in cells would be incorporated into dC sites at a high frequency. If N^4-aminocytosine residues in the template can direct incorporation of dATP, a GC to AT transition would result (pathway 2 in Figure 1). We have attempted to show that this is possible. Unfortunately, our poly(dA,dC^{am}) samples contained small amounts of deoxyuridylate (2–3% of dC^{am}), and the extent of dATP incorporation observed (Table III) was almost coincident with the deoxyuridylate content of the template. It was difficult, therefore, to evaluate the results.

However, the efficiency with which the template N^4-aminocytosine directs the incorporation of dATP cannot be greater than a few percent of that with which it directs the incorporation of dGTP. It would be an important subject to investigate whether pathway 2 can indeed proceed in vivo and in vitro.

The dual-pairing property of N^4-aminocytosine is very likely due to its tautomeric forms as shown in Figure 1. The efficiency of $dC^{am}TP$ incorporation opposite adenine was $^1/_{30}$ of the dTTP incorporation (Table I). This value is consistent with the imino/amino ratio, 1/30, determined for 1-methyl-N^4-aminocytosine by Brown et al. (1968). It is desirable to measure the tautomeric equilibrium constant for $dC^{am}TP$ itself to provide firmer ground for this interpretation.

The incorporation of N^4-aminocytosine nucleotide into the template-primer poly(dA-dT)·poly(dA-dT) appears to slow down at an early stage of the synthesis (Table I and Figure 4). This might suggest that the analogue has a chain-terminating effect. The other possibility is that the analogue is subject to proofreading by the enzyme. This aspect is to be clarified by further studies.

Modification of nucleobases in DNA by actions of chemical agents can change base-pairing properties of the bases, thereby leading to mutations (Singer & Kuśmierek, 1982; Singer & Grunberger, 1983). Mechanistically, the mutagenesis by a nucleobase analogue is expected to be similar to that by these base-modifying agents, and it appears that experimental evidence has now accumulated to support this view. Thus, the mechanism in the mutagenesis induced by 2-aminopurine and 5-bromouracil has been extensively investigated and shown to be explicable in terms of schemes analogous to that in Figure 1 (Watanabe & Goodman, 1981; Mhaskar & Goodman, 1984; Lasken & Goodman, 1984). Our present results provide strong support for this concept.

An important question is why the mutagenicity of N^4-aminocytidine is very high as compared with other nucleoside or base analogues. When N^4-aminocytosine is compared with 5-bromouracil, the former can take the minor but crucial tautomeric form far more frequently than the latter. The fraction of the enol form in 5-bromouracil has been estimated to be $10^{-3.3}$ that of the keto form (Katritzky & Waring, 1962). Clearly, 5-bromouracil would produce ambiguous base pairing much less frequently than N^4-aminocytosine: an explanation for the weak mutagenicity of 5-bromo-2′-deoxyuridine compared to N^4-aminocytidine (in the *Salmonella* and *E. coli* systems examined, 5-bromodeoxyuridine was nonmutagenic). On the other hand, in the case of N^4-hydroxycytosine, its imino/amino ratio has been estimated to be 10/1 (Brown et al., 1968). The poor utilization of $dC^{oh}TP$ in the DNA synthesis as a substitute for either dTTP or dCTP offers an explanation for the much weaker mutagenicity of N^4-hydroxycytidine compared to N^4-aminocytidine (Negishi et al., 1983).

A feature in N^4-aminocytosine is that it is structurally similar to cytosine. It takes the amino form and has only a small-sized additional amino group as compared to cytosine. The pK_a of N^4-aminocytidine is 4.7 (A. Nomura and H. Hayatsu, unpublished work), which is also similar to that of cytidine. It is likely, therefore, that N^4-aminocytidine can be metabolized within cells to its 5′-triphosphate as efficiently as cytidine. Furthermore, the formed triphosphate can be incorporated into DNA irrespective of its neighboring nucleotides (Table III). It should be recalled that the incorporation of $dC^{oh}TP$ between two purine nucleotides is low (Topal et al., 1982). Our present experiments have also shown that the incorporation of $dC^{oh}TP$ into either poly(dG-dC)·poly(dG-dC) or poly(dA-dT)·poly(dA-dT) is very low (Table IV). $dC^{oh}TP$ has been used as an agent for the in vitro site-specific mutagenesis (Wieringa et al., 1983). Hopefully, $dC^{am}TP$ may serve as an excellent reagent in this respect, because it can be incorporated into DNA more uniformly and more efficiently than $dC^{oh}TP$ in the in vitro synthesizing system.

Registry No. $dC^{am}TP$, 90335-46-9; N^4-aminocytidine, 57294-74-3.

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