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Induction of Mutation in Mouse FM3A Cells by N^4-Aminocytidine-Mediated Replicational Errors

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To explore the potential use of a nucleoside analog, N^4-aminocytidine, in studies of cellular biology, the mechanism of mutation induced by this compound in mouse FM3A cells in culture was studied. On treatment of cells in suspension with N^4-aminocytidine, the mutation to ouabain resistance was induced. The major DNA-replicating enzyme in mammalian cells, DNA polymerase α, was used to investigate whether the possible cellular metabolite of N^4-aminocytidine, N^4-aminodeoxycytidine 5′-triphosphate ($dC^{am}TP$), can be incorporated into the DNA during replication. Using [^{3}H]$dC^{am}TP$ in an in vitro DNA-synthesizing system, we were able to show that this nucleotide analog can be incorporated into newly formed DNA and that it can serve as a substitute for either dCTP or dTTP. $dC^{am}TP$ in the absence of dCTP maintained the activated calf thymus DNA-directed polymerization of deoxynucleoside triphosphates as efficiently as in its presence. Even in the presence of dCTP, $dC^{am}TP$ was incorporated into the polynucleotide. When $dC^{am}TP$ was used as a single substrate in the poly(dA)-oligo(dT)-directed polymerase reaction, it was incorporated into the polynucleotide fraction. The extent of incorporation was 4% of that of dTTP incorporation when dTTP was used as a single substrate. Even in the presence of dTTP, $dC^{am}TP$ incorporation was observed. A copolymer containing N^4-aminocytosine residues was shown to incorporate guanine residues opposite the N^4-aminocytosines. However, we were unable to observe adenine incorporation opposite N^4-aminocytosine in templates. These cell-free experiments show that an AT-to-GC transition can take place in the presence of $dC^{am}TP$ during DNA synthesis, strongly suggesting that the mutation induced in the FM3A cells by N^4-aminocytidine is due to replicational errors.

N^4-Aminocytidine (Fig. 1) is strongly mutagenic to procaryotes, e.g., *Escherichia coli*, *Salmonella typhimurium*, and bacteriophages (21, 22). This compound also shows mutagenicity in eucaryotes, such as Chinese hamster lung V79 cells in culture (23) and *Drosophila* sp. (T. Negishi, K. Negishi, H. Ryo, S. Kondo, and H. Hayatsu, Mutagenesis, in press). Many nucleoside and nucleobase analogs are known to be mutagenic (3, 4, 9, 13, 15, 19), and N^4-aminocytidine is exceptionally potent. It is generally assumed that nucleobase analogs can cause replicational errors. Studies with procaryotic polymerases on the incorporation of nucleotide analogs into DNA in vitro have been carried out in many laboratories (12, 17, 22, 26, 27, 31, 34). In contrast, there are only a few reports regarding the utilization of base analogs by eucaryotic DNA polymerases (10, 11, 33). Because of its high potency, N^4-aminocytidine offers an excellent opportunity for studying the mechanism of base analog mutagenesis. Using *E. coli* DNA polymerase I large fragment in an in vitro DNA-synthesizing system, we have previously shown, that N^4-aminodeoxycytidine 5′-triphosphate ($dC^{am}TP$) can be incorporated ambiguously, giving rise to a base pair transition, AT to GC (21, 28) (Fig. 2). With Chinese hamster lung V79 cells in culture, it was possible to show that N^4-aminocytosine residues are formed in cellular DNA following exposure of the cells to N^4-aminocytidine (23). Although 6-thioguanine-resistant mutants arose in the treatment of V79 cells with N^4-aminocytidine, whether replicational errors were responsible for this mutagenesis remained unknown. In addition, experiments with an in vitro DNA-synthesizing system (22) may not necessarily reflect the in vivo situation because *E. coli* DNA polymerase I is not a cellular primary replicase.

We have recently purified the replication enzyme DNA polymerase α from a mouse mammary tumor cell line, FM3A (7). Since the mutagenesis of FM3A cells can also be measured, it should be possible to test whether this enzyme can bring about erroneous replication of DNA in the presence of N^4-aminocytosine nucleotide and at the same time to examine whether mutations can be induced by treatment of the cells with N^4-aminocytidine. In this communication, we report that erroneous incorporation of $dC^{am}TP$ occurs in this in vitro system, with which an AT-to-GC transition can be explained, and that the mutation of the cells to ouabain resistance can be induced with N^4-aminocytidine treatment.

MATERIALS AND METHODS

Materials. N^4-Aminocytidine was obtained from Funakoshi Chemicals, Tokyo, Japan. N^4-Aminodeoxycytidine was prepared as described earlier (21). These compounds were dissolved in water and diluted with phosphate-buffered saline to obtain appropriate concentrations for treating the cells. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Radioactive compounds were obtained from Radiochemical Centre, Amersham, England. [^{3}H]$dC^{am}TP$ was synthesized from [1′,2′,5′-^{3}H]dCTP by the hydrazine-bisulfite procedure (22). Hooked template primers, poly(dA,dC^{am})-dT and poly-(dA,dC)-dT, were prepared by the following two-step reactions: first, N^4-aminocytosine (or cytosine) and adenine nucleotides were added to the poly(dA) tail randomly by using terminal deoxynucleotidyl transferase as described earlier (22). After heat inactivation of the enzyme and gel

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filtration to collect the polynucleotides, thymine nucleotide residues were added in a similar way to these polynucleotide tails. The N^4-aminocytosine and cytosine residues in these templates were determined by high-pressure liquid chromatography analysis (22) to be 4.2 and 7.0%, respectively.

Cell culture. Mouse mammary carcinoma cell line FM3A, clone 28 (20), was maintained in suspension culture in RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.). Incubation was with 5% CO_2 in air.

Effect of N^4-aminocytidine on growth of FM3A cells. Cells were inoculated with 2 ml of growth medium into 30-mm petri dishes at a density of 5.0×10^4 cells per ml with various concentrations of N^4-aminocytidine. Incubation was at 33°C. Viable cell numbers in duplicate cultures were counted every 24 h for 5 days, using a hemacytometer, the viable cells having been distinguished by dye exclusion treatment with 0.04% erythrosine (Merck & Co., Inc., Rahway, N.J.).

Assay for mutagenic activity. Mutation to ouabain resistance was measured. Logarithmically growing cells were inoculated into the medium at a density of 5×10^4 cells per ml. The culture was incubated at 37°C for 1 day; then the drug was added and the cell suspension was incubated for 16 h. The medium was changed, and the cells were cultured for an additional 3 days for expression. Then the cells were inoculated into plates of soft agar consisting of 0.33% Noble agar (Difco Laboratories, Detroit, Mich.) in RPMI 1640 medium supplemented with 10% calf serum, layered over 0.5% agar in the same medium. The number of cells inoculated into each dish was 5×10^5. Ouabain had been added in a 2.0 mM concentration to the agar layers on the plates. The plates were cultured for 10 days at 37°C, and ouabain-resistant colonies formed were counted.

Assay for DNA polymerase activity. FM3A DNA polymerase α was prepared as described earlier (7). The assay conditions were also described before (7, 22). The reaction mixture consisted of 20 mM Tris hydrochloride (pH 8.0), 5 mM $MgCl_2$, 3.3 mM 2-mercaptoethanol, 0.2 mg of bovine serum albumin per ml, either 1 A_{260} unit of synthetic polynucleotide or 0.5 mg of activated DNA per ml as template primer, and substrate nucleotides in 100 μM concentrations which contained a single tritium-labeled substrate (specific radioactivities, approximately 150 Ci/mol, unless otherwise noted). No ATP was added. FM3A DNA polymerase α was added to this mixture, and incubation was at 37°C. Samples were taken at appropriate periods and placed on DE81 papers, and incorporation of labeled triphosphates was measured as described previously (22).

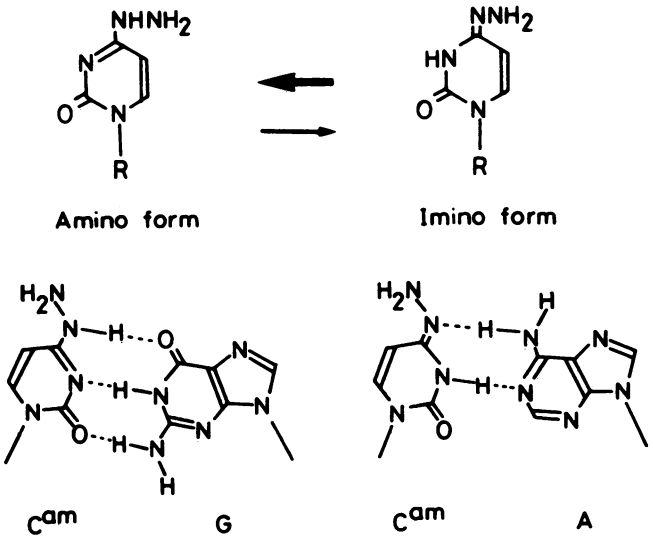

FIG. 1. Tautomerism of N^4-aminocytosine nucleotide and the possible base-pairing modes.

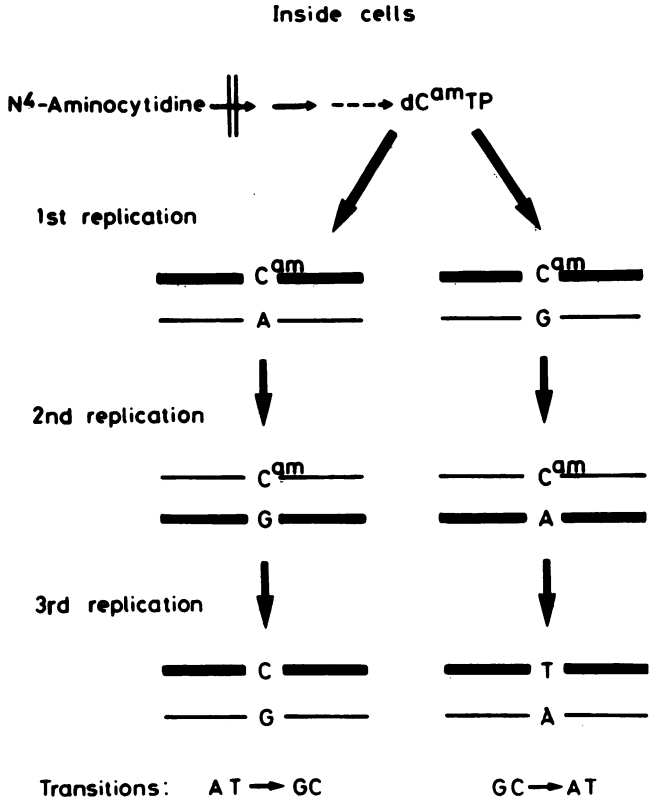

FIG. 2. Possible mechanism of N^4-aminocytidine mutagenesis. Thick lines represent newly synthesized strands.

RESULTS

Cytotoxicity and mutagenicity of N^4-aminocytidine in FM3A cells. The effect of N^4-aminocytidine on growth of FM3A cells was examined (Fig. 3). This reagent showed no cytotoxicity at a 30 μM concentration, but it showed 50% inhibition of cell growth at a 300 μM concentration. N^4-Aminodeoxycytidine showed no toxicity up to 300 μM (data not shown). The mutagenic activities of these compounds to FM3A cells were investigated by measuring mutations to ouabain resistance. It is known that ouabain resistance behaves as a codominant mutation and involves alterations in the properties of the Na^+,K^+-activated plasma membrane ATPase (3). Both N^4-aminocytidine and N^4-aminodeoxycytidine showed dose-dependent mutagenicity (Table 1). N^4-Aminocytidine at 300 μM (80 μg/ml) showed a potency close to that of the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 0.25 μg/ml. The mutagenicity of N^4-aminodeoxycytidine was 1 order of magnitude lower than that of N^4-aminocytidine. The lower mutagenicity in N^4-aminodeoxycytidine has been observed previously for Chinese hamster V79 cells (23) and for *Drosophila* sp. (Negishi et al., in press). The colony-forming ability of the cells did not

greatly decrease after these treatments, ranging from 61 to 86%.

Effect on cellular DNA synthesis. Using Chinese hamster V79 cells, we recently showed that N^4-aminocytosine can be incorporated into cellular DNA: N^4-aminocytosine residues were found in the DNA at an extent of 0.1% of the cytosine residues (23). These results suggest that N^4-aminocytidine mutagenesis in mammalian cells will involve its metabolic conversion into the DNA precursor deoxynucleoside triphosphate $dC^{am}TP$ and utilization of the nucleotide as a substrate during cellular DNA replication. This incorporation would lead to mutagenesis due to ambiguous base pairing of this base analog (Fig. 1 and 2). Thus, the effect of N^4-aminocytidine on FM3A DNA synthesis in vivo was first examined. For this purpose, the cells in suspension culture were treated with N^4-aminocytidine for 30 min at 33°C; then they were treated with [^{3}H]thymidine for 30 min to measure incorporation of the label into acid-insoluble materials. N^4-Aminocytidine exhibited no significant inhibition of DNA synthesis up to a 300 μM concentration, a concentration at which a high mutation frequency was observed. Cellular DNA synthesis decreased to 50% at 3 mM N^4-aminocytidine (data not shown). Similarly, effects of both N^4-aminocytidine and N^4-aminodeoxycytidine on cellular DNA synthesis were examined in HeLa S3 cells in culture. Again, neither of these chemicals showed significant effects at 300 μM concentrations (data not shown).

Incorporation of $dC^{am}TP$ by FM3A DNA polymerase α. We have recently shown that N^4-aminocytidine can cause, in phages, not only AT-to-GC (22) but also GC-to-AT transitions (unpublished results). To induce these transitions, $dC^{am}TP$ is expected to be incorporated in place of either dTTP or dCTP in cellular DNA replication (Fig. 2). We used DNA polymerase α of FM3A cells for the DNA-synthesizing reaction in vitro. First, we used activated calf thymus DNA as a template primer (Table 2). When dCTP was omitted from the standard four-nucleotide mixture, the incorporation of [^{3}H]dTTP decreased to 34.6% of the original (cf. experiments 1 and 2 in Table 2), and an addition of $dC^{am}TP$ to the cytosine-minus mixture restored the [^{3}H]dTTP incorporation to the original value (experiment 3). When [^{3}H]$dC^{am}TP$ was used in place of [^{3}H]dCTP, its incorporation was 88% of that of [^{3}H]dCTP (experiments 4 and 5). These results indicate that $dC^{am}TP$ can be a good substitute for dCTP. When both dCTP and $dC^{am}TP$ were present in the reaction mixture, $dC^{am}TP$ incorporation was one-third that of dCTP (experiments 6 and 7); i.e., $dC^{am}TP$ can compete with dCTP in being utilized by the enzyme.

Next, we used poly(dA)-oligo$(dT)_{12-18}$ as the template primer and examined the incorporation of $dC^{am}TP$ in place of dTTP. If the $dC^{am}TP$ is incorporated, it should mean that

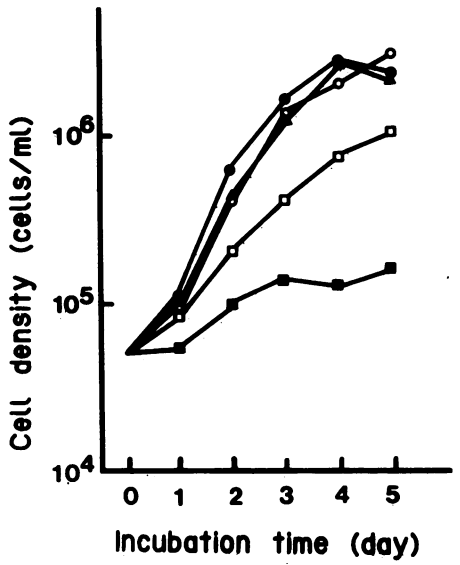

FIG. 3. Effect of N^4-aminocytidine on growth of FM3A cells. Concentrations of N^4-aminocytidine used were 0 (●), 3 μM (○), 30 μM (▲), 300 μM (□), and 3 mM (■).

N^4-aminocytosine is used in place of thymine, entering opposite adenine on the DNA strand. Table 3 shows the results. $dC^{am}TP$ was incorporated in a time-dependent manner. The amount of incorporation was about 4% of that of dTTP. Even in the presence of dTTP, a significant incorporation of $dC^{am}TP$ was observed. In a study with *E. coli* DNA polymerase I large fragment, $dC^{am}TP$ was incorporated at 3% of the dTTP incorporated (22). Thus, procaryotic and eucaryotic DNA polymerases show similar $dC^{am}TP$ incorporation patterns, incorporation in place of both dCTP and dTTP. With *E. coli* DNA polymerase I large fragment, we previously observed that the enzyme can proofread efficiently the incoming $dC^{am}TP$ when this analog nucleotide is incorporated in place of dTTP, excising it as $dC^{am}MP$. Thus, the net incorporation of $dC^{am}TP$ opposite adenine was only 20% of the total utilization (29). Since mammalian DNA polymerase α preparations have been reported not to show 3′→5′ exonuclease activity (25), we tested whether the FM3A DNA polymerase α also lacked this proofreading activity. For this purpose, we used poly(dA)-oligo(dT) as the template primer and attempted to detect monophosphate generation during polymerization (29). No removal as monophosphate was observed with either a correct (dTTP) or an incorrect (dCTP or $dC^{am}TP$) substrate (data not shown). Thus, $dC^{am}TP$ was incorporated in place of dTTP without being proofread.

TABLE 1. Mutagenic activity of N^4-aminocytidine and N^4-aminodeoxycytidine on FM3A cells

Reagent (μM)	No. of ouabain-resistant mutants scored/total no. of original cells	Mutation frequency ($Oua^s \rightarrow Oua^r$)[a]	Colony-forming ability (%)
None	$4/2.6 \times 10^7$	1.8×10^{-7}	85
N^4-Aminocytidine			
30 μM	$87/9.5 \times 10^6$	1.1×10^{-5}	86
300 μM	$359/1.3 \times 10^7$	4.0×10^{-5}	69
N^4-Aminodeoxycytidine			
30 μM	$2/1.4 \times 10^7$	1.9×10^{-7}	76
300 μM	$39/1.4 \times 10^7$	3.7×10^{-6}	75
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine, 0.25 μg/ml	$1,046/2.2 \times 10^7$	7.8×10^{-5}	61

[a] (Number of mutants)/(number of original cells × colony-forming ability).

TABLE 2. Incorporation of $dC^{am}TP$ by FM3A DNA polymerase α in vitro, using activated calf thymus DNA as template primer

Expt	dNTP added[a]	Labeled nucleotide incorporated (pmol)[b]
1	C, [^{3}H]T, G, A	131.5
2	[^{3}H]T, G, A	45.5
3	[^{3}H]T, G, A, C^{am}	130.1
4	T, G, A, [^{3}H]C^{am}	78.2
5	[^{3}H]C, T, G, A	88.6
6	[^{3}H]C, T, G, A, C^{am}	70.2
7	C, T, G, A, [^{3}H]C^{am}	23.8

[a] The concentration of each dNTP was 100 μM. In experiments 6 and 7, both dCTP and $dC^{am}TP$ were present at 100 μM.
[b] Incubation was for 30 min, and 30-μl samples were examined.

Recognition by polymerase of N^4-aminocytosine in template. To induce a mutation, N^4-aminocytosine residues incorporated into a newly formed DNA strand must be recognized and read by the polymerase in the next replication step (Fig. 2). We prepared synthetic hooked template primers poly(dA,dC^{am})-dT (aminocytosine content, 4.2%) and poly(dA,dC)-dT (cytosine content, 7.0%) and then examined their template activities in the DNA polymerase α-catalyzed reactions. The results are presented in Table 4. Both poly(dA,dC^{am})-dT and poly(dA,dC)-dT were good templates for incorporating thymine nucleotides, which took place in a time-dependent manner. The rate of thymine nucleotide incorporation on the template with N^4-aminocytosine residues randomly dispersed among adenines was half that on the template with cytosines among adenines. Importantly, dGTP was incorporated on poly(dA,dC^{am})-dT. The amount of dGTP incorporated on poly(dA,dC^{am})-dT was about 5% of the sum of the amounts of dTTP and dGTP incorporated, both at 15 and 30 min of incubation. This value was close to the content of aminocytosine residues in the template. For poly(dA,dC)-dT, the guanine nucleotide incorporated was 7.0% of the T-plus-G incorporation, a value correlating well with the cytosine content in the template. These results clearly show that the N^4-aminocytosine residues in the template are read as cytosine, with guanines being incorporated as their base-pairing counterparts. The same template action was previously observed in *E. coli* DNA polymerase I large fragment-catalyzed DNA polymerization (22). In the present experimental system, we were unable to obtain positive incorporation of dATP. Therefore, it remains to be explored whether or not aminocytosine templates can be read by the mammalian enzyme as thymine.

DISCUSSION

N^4-Aminocytidine is mutagenic in mouse FM3A cells in culture (Table 1). A feature of this mutation is that this mutagen shows only a low toxicity to the cells at concentrations at which significant mutagenesis takes place (Fig. 3). Also, cellular DNA synthesis, as measured by the incorporation of [^{3}H]thymidine administered to the cells after mutagen treatment, is not greatly reduced by the action of the mutagen.

TABLE 3. $dC^{am}TP$ as a substitute for dTTP in poly(dA)-oligo$(dT)_{12-18}$-directed polynucleotide synthesis

dNTP added[a]	Incubation time (min)	Radioactive nucleotide incorporated (pmol)[b]
[^{3}H]T	15	136.7
	30	219.2
[^{3}H]C^{am}	15	5.0
	30	10.0
[^{3}H]C^{am} + T	15	1.3
	30	1.6
[^{3}H]C	15	0.1
	30	0.2

[a] The concentration of each dNTP was 100 μM.
[b] 10-μl samples were examined.

TABLE 4. Activity of N^4-aminocytosine-containing polynucleotide as template primer in an FM3A DNA polymerase α-catalyzed reaction

Template primer	[^{3}H]dNTP used[a]	Time of incubation (min)	Nucleotide incorporated (pmol)[b]	G/[T + G] (%)
Poly(dA,dC^{am})-dT	T	15	14.9	5.6
	G	15	0.88	
	T	30	24.4	5.6
	G	30	1.44	
Poly(dA,dC)-dT	T	15	34.6	6.9
	G	15	2.57	
	T	30	57.7	7.0
	G	30	4.33	
Poly(dA)-oligo $(dT)_{12-18}$	T	15	47.8	0.16
	G	15	0.08	
	T	30	80.6	0.26
	G	30	0.21	

[a] All four dNTPs were present in the reaction mixture, at 100 μM concentrations. A single nucleotide among them, as indicated, was radioactive. The specific radioactivity of [^{3}H]dTTP was 124 Ci/mol and that of [^{3}H]dGTP was 1,000 Ci/mol. The time course results for a single set of reactants were those obtained in a single reaction mixture.
[b] 15-μl samples were examined.

Our present data show clearly that the eucaryotic replication enzyme of FM3A can use $dC^{am}TP$ as a substitute for both dCTP and dTTP in DNA synthesis (Tables 1, 2, and 3). Utilization of $dC^{am}TP$ in poly(dA)-oligo-$(dT)_{12-18}$-directed polymerization was a few percent that of dTTP with both DNA polymerase α (Table 3) and *E. coli* DNA polymerase I large fragment (22). This similarity between the eucaryotic and procaryotic polymerases was somewhat unexpected. The error rate in DNA synthesis by DNA polymerase α is approximately 1 in 30,000 nucleotides incorporated (1, 16, 18), whereas that by *E. coli* DNA polymerase I is much lower, 1 in 10^8 nucleotides (18). We have shown previously that the *E. coli* polymerase can proofread the incorporated deoxyaminocytidine nucleotide (29), and the present experiments show that the polymerase α cannot do so. Therefore, with polymerase α, a much higher incorporation of $dC^{am}TP$ in place of dTTP might have been expected, but it was not the case. Thus, mechanisms such as a selective recognition of substrate deoxynucleoside triphosphate (dNTP) or a selection at the primer terminus may be invoked. Indeed, models for such actions of eucaryotic DNA polymerases have been reported (2, 8).

Structurally, N^4-aminocytidine is very similar to cytidine; in this compound, a hydrogen atom in the $-NH_2$ at position 4 is replaced by $-NH_2$. Therefore, it is likely that this ribonucleoside analog, once it enters the cell, can be metabolized by the cellular enzymes, which usually metabolize normal ribonucleosides, into the DNA precursor $dC^{am}TP$. The "misincorporation" of $dC^{am}TP$ in place of dTTP into the DNA may be expected because of the ease with which this compound tautomerizes between the amino and imino forms. In the imino form, it can base pair with adenine (Fig. 1), and the proportion of the imino form in the tautomerism of N^4-aminocytidine has been estimated to be 1/30, a signif-

icant proportion (5). This high proportion of a tautomer is in contrast to the situation with 5-bromodeoxyuridine, which is often referred to as having a significant tautomeric shift, but its tautomeric equilibrium constant between the keto and enol forms is $10^{-3.3}$ (14). Once N^4-aminocytidine is incorporated opposite adenine in the parental DNA strand, the next two replications would fix the base pair into GC, and the net result is an AT-to-GC transition (Fig. 2). We were able to show that N^4-aminocytosine in the parental strand can direct the incorporation of guanine opposite it. However, it was difficult to demonstrate that N^4-aminocytosine as a template can behave as thymine, thereby incorporating adenine opposite it. This ambiguous template action may be required for the GC-to-AT transition to occur (Fig. 2). GC-to-AT transitions do take place in phage M13 when *E. coli* infected with the phage is treated with N^4-aminocytidine (unpublished results). The property of N^4-aminocytosine as a template base in DNA replication requires further investigation.

Another aspect of interest is the fate of the incorporated N^4-aminocytosine residues. To cause mutation in FM3A cells, the residue must survive until the second replication starts, a period of at least 15 h. Whether the N^4-aminocytosine nucleotides in DNA are subject to cellular repair mechanisms or not remains to be seen.

Recently, DNA polymerase δ was isolated from mouse, human, and calf cells, and this polymerase, having the 3′→5′ exonuclease activity, was implicated as participating in cellular replication (6, 24, 30, 32). It would be important to investigate the action of DNA polymerase δ on utilization of $dC^{am}TP$ in polymerization.

It is remarkable that N^4-aminocytidine is mutagenic in a variety of systems, including both procaryotes and eucaryotes. Its use as a standard mutagen for causing base pair transitions may facilitate production of mutants in many studies. Unlike reactive mutagens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, ethyl methanesulfonate, and 4-nitroquinoline 1-oxide, N^4-aminocytidine would not damage the existing DNA strands. A way of using N^4-aminocytidine for inducing mutations in a specific gene of eucaryotes would be a pulse administration of this nucleoside analog at a specific time in the DNA-synthesizing period of the cell cycle.

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