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Mechanism of Degradation of 2′-Deoxycytidine by Formamide: Implications For Chemical DNA Sequencing Procedures

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Abstract—We describe the reaction of formamide with 2′-deoxycytidine to give pyrimidine ring opening by nucleophilic addition on the electrophilic C(6) and C(4) positions. This information is confirmed by the analysis of the products of formamide attack on 2′-deoxycytidine, 5-methyl-2′-deoxycytidine, and 5-bromo-2′-deoxycytidine, residues when the latter are incorporated into oligonucleotides by DNA polymerase-driven polymerization and solid-phase phosphoramidite procedure. The increased sensitivity of 5-bromo-2′-deoxycytidine relative to that of 2′-deoxycytidine is pivotal for the improvement of the one-lane chemical DNA sequencing procedure based on the base-selective reaction of formamide with DNA. In many DNA sequencing cases it will in fact be possible to incorporate this base analogue into the DNA to be sequenced, thus providing a complete discrimination between its UV absorption signal and that of the thymidine residues. The wide spectrum of different sensitivies to formamide displayed by the 2′-deoxycytidine analogues solves, in the DNA single-lane chemical sequencing procedure, the possible source of errors due to low discrimination between C and T residues. © 1997 Elsevier Science Ltd.

Introduction

The order of the four bases along given DNA fragments can be established according to two different procedures. The first uses chemical reagents that react with specific bases to break DNA preferentially at given nucleotides (the Maxam and Gilbert methodology[1]), the second is based on the analysis of the products of DNA polymerization selectively interrupted by chain terminating deoxyribonucleotides (the Sanger methodology[2]).

Of the two DNA sequencing methods, the chemical analysis has the higher potentiality for data compression: if one could obtain unambiguous and complete sequence information in a single lane, then four or more different DNAs, each labeled with a different fluorophore, could be analyzed in the same electrophoretic lane (*four fluorophores/four DNAs*). The methods developed so far, which aim to simplify the sequencing procedures by introducing chemical alternatives to the classical Maxam and Gilbert protocols[1] have been reviewed.[3–5] As for the Sanger approach, partial sequence data compression has been reported[6,7] and a single-lane sequencing procedure is currently available. This method (*four fluorophores/four bases*) cannot a priori be compressed further, contrary to the DNA sequencing procedures based on a purely chemical rationale.

A DNA sequencing method based on the selective degradation of nucleic acids by amides at high temperature was reported[8–11] and recently improved by the use of *N*-methyl-formamide.[12] The chemistry and the dynamics of the cleavage of the phosphodiester bonds has been determined[12] and the method has been applied to several biotechnological problems (analysis of cloned DNA sequences, localization of DNA mutations in lower eukaryotes, analysis of oligonucleotides).[12] The sensitivity of the method is such that it can be used to detect mutations of single bases in single-copy human genes and has been applied to compare wild-type and mutant β-globin genes.[13] The DNA degradation by amides provides complete sequence information in a single electrophoretic lane, on the basis of the intensity of the signal corresponding to each cleaved sequence position.[8–11] This property and the possibility of analyzing several differently labeled samples in the same lane offers the possibility of accurate comparative familial analyses.[13] The reliability of the analysis is based on the possibility of unambiguous discrimination among the four signals. The order of sensitivity observed in the formamide reaction is G>A>C>T, where the reactivity ratio G/A ranges from 1 to 1.5 depending on the reaction conditions; the reactivity ratio C/A is 0.15, and that one T/C is between 0.7 and 0.5. To abolish the possibility of errors in the

Key words: 2′-deoxycytydine, formamide, DNA, sequencing procedures.

determination of G and A residues, purine analogues can be used.[11-14] These could be incorporated into the DNAs to be analyzed by either the Polymerase Chain Reaction (PCR)[15] or the Primer Extension (PE)[16] procedures. Their degradation occurs at different rates, therefore increasing the sensitivity intervals.[11-14] The purine compounds to be used in this approach have been defined following a rational approach derived from the determination of the mechanism of degradation of purine nucleosides by formamide.[14]

The low discrimination between C and T residues remains as a problem, is a potential source of errors and must be solved in order to reach complete reliability for this novel chemical sequencing method. Thus, knowledge of the degradation pathway of pyrimidine nucleosides by formamide is necessary to select the analogues to be incorporated and then selectively degraded in order to solve possible ambiguities between C and T residues.

Cleavage of the pyrimidine ring of the nucleic acid bases can occur through the action of a number of reagents. Some reactions take place through nucleophilic reagents (usually strong bases), attacking the most electrophilic C-2, C-4, and C-6 atoms.[16] The pyrimidine ring, in ordinary pyrimidine nucleosides and nucleotides, is resistant to the action of alkalies, although sufficiently vigorous alkaline treatment (1 N KOH, 100 °C) opens the ring by hydroxyl ion attack on the C-4 position.[17] Since the primary products are rapidly decomposed further, the only degradation products so far identified are urea and ammonia.[17] Certain pyrimidine derivatives exhibit high reactivity toward nucleophilic reagents such as NaSH,[18] Na_2SO_3,[19] $NaHSO_3$,[20] RSH,[21] ROH,[22] RNH_2, [23]NaCN,[24] and NaN_3.[25] These reactions are known to occur via initial attack of a nucleophile on the pyrimidine C-6 position, giving rise to a 5,6-dihydropyrimidine intermediate from which the respective product is formed. Moreover, cytosine reacts with 4 M hydrazine solution at pH 6 and 80 °C to give, after 60 h, two principal substitution products: 4-exo-*N*-aminocytosine and *N,N'*-bis-[2-keto-1,2-dihydropyrimidyl-4]-hydrazine. The reaction also proceeds under analogous conditions with monomethylhydrazine, in which case only *N,N'*-bis-[2-keto-1,2-dihydropyrimidyl-4]-hydrazine is formed.[26] If the reaction is carried out in alkaline medium[27] or with anhydrous hydrazine,[28] it is accompanied by opening of the pyrimidine ring.[28] In particular, cytosine derivatives[26,29] react with hydrazine to give urea, 5-aminopyrazole, and 3-ureido-pyrazole.[30] Under similar experimental conditions, the cleavage of the pyrimidine ring in uracil and thymine derivatives takes place through nucleophilic attack on the C-6 atom and gives rise to urea and 5-pyrazolinone derivatives.[26] Hydroxylamine reacts with pyrimidine residues of nucleic acids in a manner similar to hydrazine, but more specifically.[31] Freese[32] and Schuster[33] reported that this reagent is capable of inducing mutations in bacteriophages, through specific modification of cytosine bases in DNA.[34] As shown by Blackburn,[35] two products are produced simultaneously in a ratio which varies with pH and with the concentration of hydroxylamine: direct substitution of the cytosine amino-group gives *N*(4)-hydroxycytosine while an alternative process, involving also the hydroxylamine addition across the pyrimidine 5,6-double bond, yields 5,6-dihydro-*N*(4)-hydroxy-6-hydroxyaminocytosines. Although the degradation pathway of pyrimidine nucleosides and nucleotides with hydroxyl ion, hydrazine, and hydroxylamine is widely described, the reaction with a weak base as formamide has not been previously studied, with the exception of the results reported by Hirota et al.[36] relating to the reaction of uracil derivatives with guanidine, urea, and thiourea.

In this paper we decribe the reaction of thymidine **1** and 2′-deoxycytidine **2** with formamide. The degradation pattern for 2′-deoxycytidine, as defined by this analysis, indicates the positions of the pyrimidine ring whose structural modification modifies the sensitivity towards formamide, thereby allowing substantial improvement in the DNA chemical sequencing procedure.

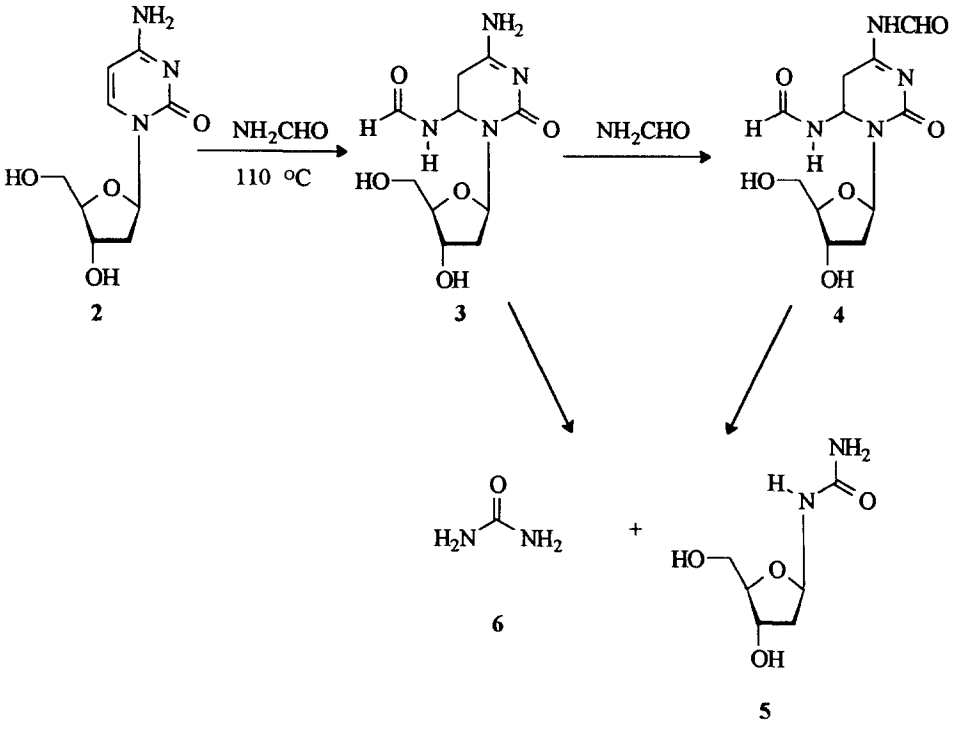
Results and Discussion

The degradation of 2′-deoxycytidine by formamide

In order to obtain information on the degradation pathway of pyrimidine nucleosides by formamide, we have analyzed the reaction of compounds **1** and **2**, using the experimental conditions described for the formamide DNA sequencing method.[11] Thymidine **1** did not react with excess aqueous formamide at 110 °C over a prolonged reaction time. This observation is in agreement with the reported low reactivity shown by thymidine residues in the chemical DNA sequencing procedure.[8] In comparison with the reaction of **1** with formamide, compound **2** showed significantly greater reactivity. In this case, 6-formylamino-5,6-dihydro-4-amino-1[β-D-ribofuranosyl]2-(1*H*)-pyrimidone (**3**) and 4,6-di(formylamino)-5,6-dihydro-1[β-D-ribofuranosyl]2-(1*H*)-pyrimidone (**4**) were obtained in isolated form by analytical TLC purification (reverse-phase; water: methanol (1:1)) of a small amount of the reaction mixture and characterized by capillary gas chromatography–mass spectrometry (GC–MS) after silylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA),[37] ^{1}H NMR and elemental microanalysis (products are numbered as reported in Scheme 1).

Compounds **3** and **4** were found to be unstable even when recovered under a nitrogen atmosphere and give 2′-deoxy-β-D-ribofuranosyl-urea (**5**) and urea **6** as the main isolated degradation products. Moreover, partial conversion of **3** to **4** by further treatment with formamide was revealed through gas chromatographic analysis. The remaining reaction mixture was distilled under high vacuum to eliminate the excess formamide and purified following the usual procedures, to yield compounds **5** and **6**.

On the basis of these data it is reasonable to suggest that formamide reacts with **2** by nucleophilic addition at



Scheme 1.

the C-6 atom of the cytosine ring, probably followed by substitution of the labile amino group in the dihydrocytosine derivative thus formed. Under vigorous conditions (i.e., removal of excess formamide), further degradation involving the C-6 pyrimidine ring opening may occur to yield **5** and **6** (Scheme 1). This hypothesis is in agreement with the distribution of electron density in the cytosine ring, according to which the C-6 and C-4 atoms must be under nucleophilic attack,[38] even though other degradation pathways cannot be completely excluded because of the lack of characterization of all possible intermediates. Moreover, a similar reaction pathway has already been described for the reaction of cytosine derivatives with hydroxylamine and *O*-methylhydroxylamine to yield 4,6-di(hydroxylamino)-5,6-dihydro-2-oxo-derivatives and 4-hydroxylamino-2-oxo-derivatives.[39] The indication that formamide degrades 2′-deoxycytidine by C-6 pyrimidine ring opening may provide the chemical clues to predict the variation of reactivity for 2′-deoxycytidine analogues. In fact, structural modifications that enhance or reduce the electrophilic character of the C-6 position of the cytosine ring might drastically change its reactivity towards a weak nucleophile such as formamide.

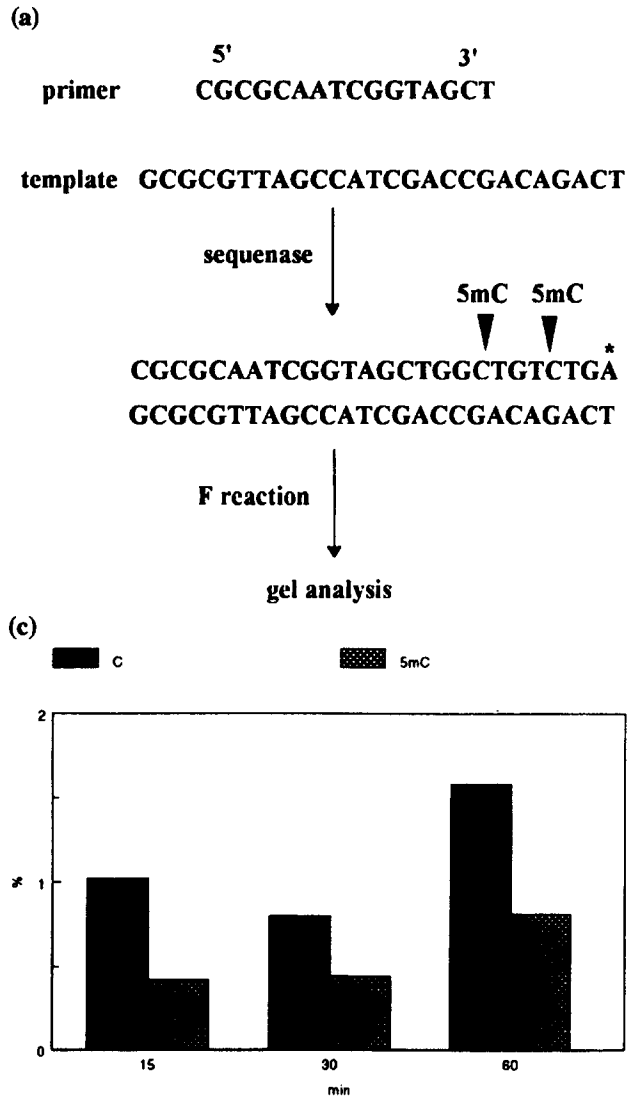
In order to test this hypothesis and to apply this rationale to the DNA chemical sequencing procedure, we have analysed the sensitivity to formamide of 5-bromo-2′-deoxycytidine and 5-methyl-2′-deoxycytidine residues when incorporated into polynucleotides.

Degradation of 5-bromo-2′-deoxycytidine and 5-methyl-2′-deoxycytidine inserted into polynucleotides

The DNA chemical sequencing procedure used on 3′-radioactively labeled oligonucleotide fragments is the 'formamide protocol' performed as previously described.[8] The procedure is based on the degradation of purine and pyrimidine bases by formamide at high temperature (>100 °C) followed by scission of the glycosidic linkages and by cleavage of 3′ and 5′ phosphodiester bonds through β-elimination reactions. In the presence of a weak base such as formamide, only the 3′β-elimination occurs efficiently;[8] efficient β-elimination at 5′ (leading to unbiased sequencing of 5′-labeled DNAs) requires the use of piperidine[9,10,14] as a second reaction step. In addition, we have observed that efficient cleavage of the 5′ phosphodiester bond is carried out by *N*-methyl-formamide in the presence of Mn^{+2}.[12] Irrespective of the specific protocol used for the phosphodiester bond breakage, the selective (i.e., base-specific) part of the reaction is carried out by formamide.[8,11,14]

Figure 1(a) shows the molecular construct used to synthesize double-stranded DNA molecules carryng 5-methyl-2′-deoxycytidine residues. The indicated 26-mer template segment (lower strand) was annealed to the 16-mer oligo (upper strand) and used as a template-primer substrate for a Sequenase-driven polymerization. The resulting upper strand contains (beyond the initial primer) sites which incorporate 5mC residues only at the positions 19 and 23 and was synthesized

using alternatively dCTP and 5mdCTP. The reaction of the two differently substituted types of oligonucleotides with formamide was analyzed on a sequencing gel (Fig. 1b). The presence of the methyl group as substituent at the C-5 position of the pyrimidine ring causes slight retardation in the migration of all degradation products containing the methylated residue. As a result the distance between the band produced by cutting at 5mC and the upper bands is consistently increased compared to the control lane. The lower part of the gel is not reported because of artifacts generated by the overlap of DNA molecule bands with the migration front.



The results show that 5 mC modification decreases the susceptibility to formamide attack: as shown in Figure 1(c), cleavage at residue #19 expressed as a percentage of the total cleavage is reduced by a factor of two when methylated.

5-Bromo-2′-deoxycytidine was incorporated directly into the primer oligonucleotide according to the phosphoramidite procedure routinely used for chemical DNA synthesis.[40] Figure 2(a) shows the molecular construct used to synthesize double-stranded DNA molecules carrying 5BrC residues. The indicated 26-mer template segment (lower strand) was annealed to the 16-mer oligo (containing sites which carry 5BrC residues only at the positions 9 and 14) and used for a Sequenase-driven polymerization. Figure 2(b–c) reports the results of this analysis and shows that 5BrC modification more than doubles the susceptibility to formamide attack. These data are in agreement with the previously hypothesized reactivity relationship between

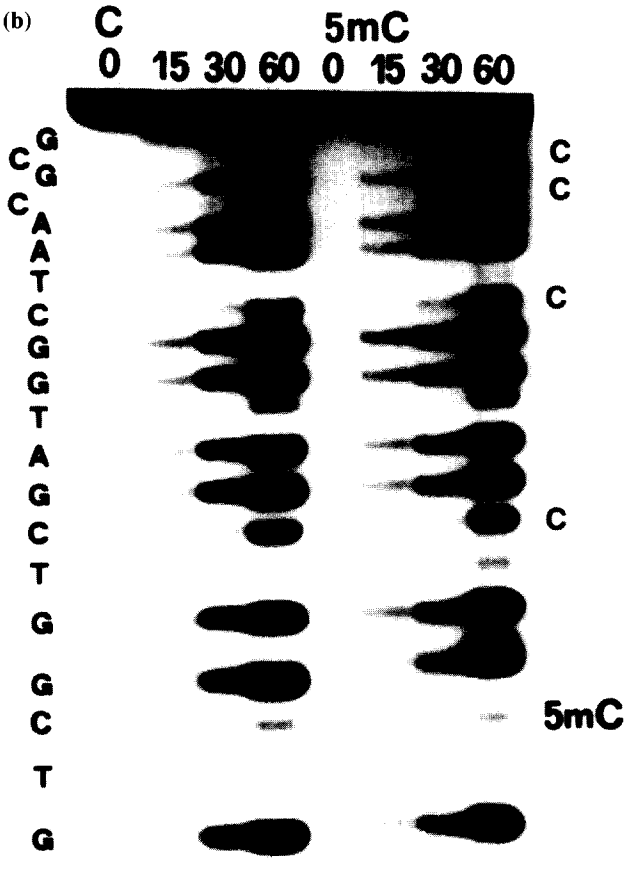


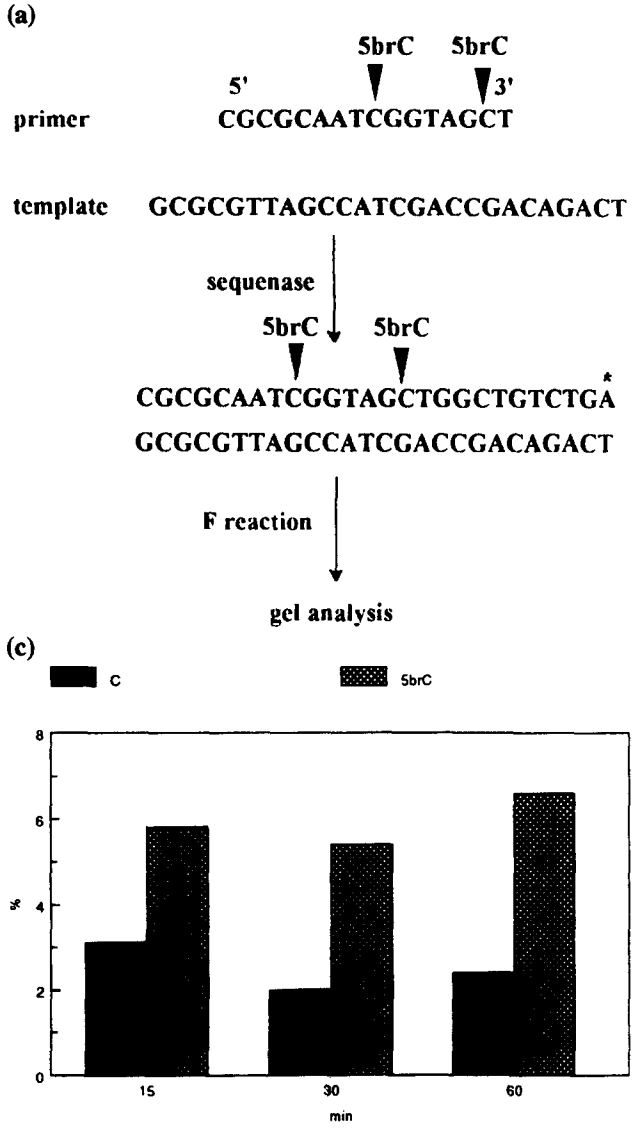
Figure 1. Degradation of 3′-labelled DNA by formamide. Comparison of the reactivity of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine. (**a**) The DNA construct used to insert the base-analogue in two defined locations (as indicated in the lower molecule, see text). The scheme of the procedure used for the analysis described in the following panels is indicated. * = labeled position. F reaction = formamide degradation, see text. (**b**) Gel analysis (18% acrylamide, 7 M urea) of the product of the attack by formamide on the DNA carrying substitutions. Each group of samples shows the products of formamide degradation on molecules in which 2′-deoxycytidine (C) or 5-methyl-2′-deoxycytidine (5mC) were incorporated at position 19 and 23, as indicated in the scheme in panel (a). Reactions were carried out for 0, 15, 30 and 60 min, as indicated. Left panel: 2′-deoxycytidine; right panel: 2′-deoxy-5-methyl cytidine. The methyl group causes slight retardation in the migration. The lower part of the gel is not reported because of artifacts generated by the overlap of DNA bands with the migration front. (**c**) Cleavage at the residue #19 of the template strand (indicated in **a** as 5mC), reported as a percentage (ordinata) of the total degradation products, as a function of the reaction time (abscissa). Data obtained by scanning densitometry of the gel autoradiography.

the electronic effect exerted by the substituent at the C-5 position of the pyrimidine ring and the susceptibility to formamide attack.

Conclusions

Summarizing the practical effects of the use of cytosine analogues in the formamide-based chemical DNA sequencing procedure, we observe the following: as previously described, formamide sensitivity of purines (A and G residues) is high and rather homogenous, with a slightly (10–15%) higher reactivity for Gs. Setting the sensitivity of G to a value of 100, this bias is completely solved by enhancing the G signal by substituting it with Inosine,[11] up to a comparative intensity of 200 and/or decreasing the A signal to the desired intensity by mixing at the desired ratios A with deazaA residues in the amplification reaction. [14] The intensity order of G (=100) ≥ A (=85) > C (=10–15) ≥ T (=5),[8] where ≥ indicates a possible source of errors, thus becomes: Inosine (=200) > A (=85) > C (=10–15)) ≥ T = 5.[11,14]

The present study offers a solution for the remaining source of ambiguity: that among pyrimidines. Formamide sensitivity is low for pyrimidines and the signal for C is ≈15% of that of the nearest G.[14] By substituting C with 5brC, as determined above, its signal can be brought from ≈15% to ≈35% (relative to G) (Fig. 2). Keeping the T signal at its original low value (≈ 8%), the intensity order of sensitivity to formamide in a base-substituted, PCR-amplified DNA becomes Inosine (=200) > A (=85) > 5brC (=35) > T (=8). In addition, 5mC can be used instead of C when lowering of the C intensity is preferred over its increase (i.e., in sequencing DNA tracts or oligonucleotides devoid of T residues).



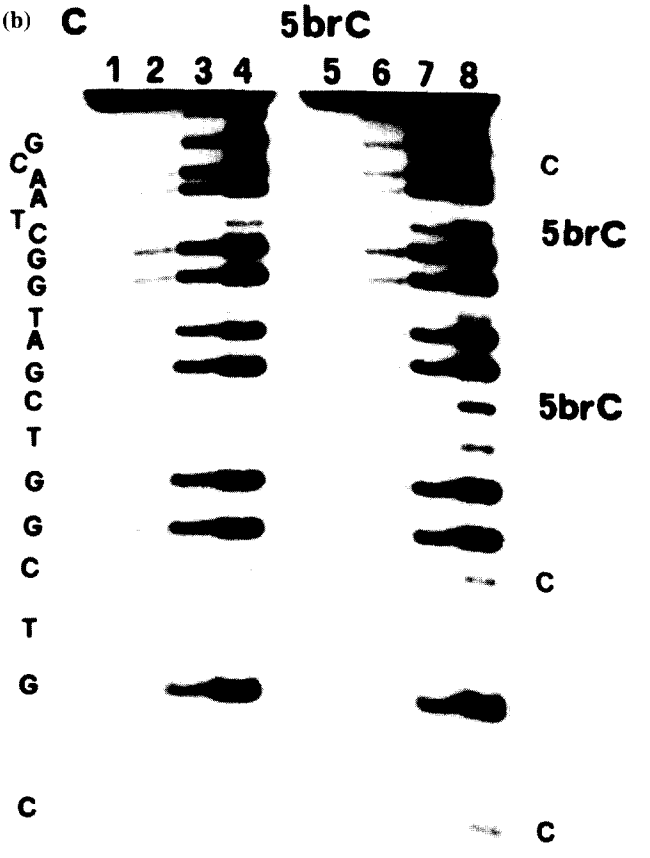


Figure 2. Degradation of 3′-labeled DNA by formamide. Comparison of the reactivity of 2′-deoxycytidine and 5-bromo-2′-deoxycytidine. The analysis was conducted and the data are presented as in Figure 1. (**a** and **b**) The higher reactivity of 5-bromo-2′-deoxycytidine is evident. (**c**) Cleavage at residues # 9 and # 15 of the primer strand (indicated in **a** as 5brC), reported as a percentage (ordinata) of the total degradation products, as a function of the reaction time.

In conclusion, based on the hypothesized mechanism of degradation of C residues by formamide, the use of cytosine analogues provides large intensity differences among signals and allows unambiguous sequence determination. The same procedures can be applied to both 5′-radioactively- or fluorescently-labeled DNA using *N*-methyl-formamide to generalize the method further.

Experimental

General procedures

Abbreviations: G, guanine; A, adenine; C, cytosine; T, thymine; 5mC, 5-methyl-cytosine; 5BrC, 5-Bromo-cytosine; dCTP, 2′-deoxycytidine triphosphate; 5mdCTP, 5-methyl-2′-deoxycytidine triphosphate; 5BrdCTP, 5-bromo-2′-deoxycytidine-triphosphate. PCR, polymerase chain reaction; PE, primer extension; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

Thymidine, 2′-deoxycytidine (Aldrich, Co.), 5-methyl-2′-deoxycytidine-5′-triphosphate, and 5-bromo-2′-deoxycytidine-3′-phosphoramidite (Boehringer) were used without further purification. All solvents used were ACS reagent grade and were redistilled according to standard procedures. Formamide was from IBI, piperidine (puriss. p. a.) from Fluka.

^{1}H NMR spectra were recorded with a Bruker AC-200 spectrometer (^{1}H frequency: 200.13 MHz). Data processing was performed with an Aspect 3000 computer using UXNMR software. 5-mm Probe was as follows: solvent DMSO-d_6 (Aldrich); temperature, 273 K.

The application of capillary gas chromatography–mass spectrometry (GC–MS) to chemical characterization of altered DNA bases is well established.[41,42] The relative non-volatility of pyrimidine derivatives was improved by the use of 2′-deoxy-3′,5′-di-*O*-trimethylsilyl-β-D-ribosyl derivatives. Gas-chromatographic analyses and mass spectra were performed on samples derivatized with BSTFA[43] in pyridine at 25 °C by use of a HP GC–MS 5972 spectrometer equipped with a HP-5MS column (0.25 mm, 30 m). The spectra were recorded at 70–250 °C with the following program: isothermal at 70 °C for the first min, then 10 °C/min, finally isothermal at 250 °C for 40 min. Mass spectra were recorded on a Kratos MS80 spectrometer. Melting points were obtained on a Reichert Kofler apparatus and are uncorrected.

Elemental microanalyses were recorded on a Carlo Erba elemental analyzer EA 1108, and were all within 0.4% of calculated values for carbon, hydrogen and nitrogen. Reverse-phase TLC was performed using Merck 15682 RP-8 $F_{254}S$ glass sheets (water:methanol, (9:1)). All the oligonucleotides were synthetized on a ABI 394 DNA synthesizer by standard protocols. Scanning densitometry was performed with a Biorad Imaging Densitometer GS 670.

Degradation of 2′-deoxycytidine 2 with formamide. 2′-Deoxycytidine (454 mg, 2 mmol) was dissolved in wet formamide (10 mL) and heated at 110 °C. A small portion (0.5 mL) of the crude reaction mixture was analyzed by capillary gas chromatography–mass spectrometry (GC–MS) after silylation with BSTFA and directly chromatographed on phase-reverse analytical thin-layer plates using water:methanol (9:1) as eluent to give 4-formylamino-1-[β-D-ribofuranosyl]2-(1*H*)-pyrimidone (**3**) and 4,6-di(formylamino)-5,6-dihydro-1-[β-D-ribofuranosyl]2-(1*H*)-pyrimidone (**4**). The remaining mixture was evaporated under high vacuum to dryness, the residue was dissolved in chloroform and purified by flash-chromatography (chloroform:methanol, 9.5:0.5) to give 2′-deoxy-β-D-ribofuranosylurea (**5**) and urea **6.**

6-Formylamino-5,6-dihydro-4-amino-1-[β-D-ribofuranosyl]2-(1*H*)-pyrimidone (3). IR (KBr) 3400–3350 (OH and NH), 2825 (CHO), 1712 (CO),1678 (CO), 1637 (C=C), 1280, 970 cm^{-1}. Oil; ^{1}H NMR (Me_2SO-d_6) δ 2.12–2.43 (2H, m), 4.10–4.87 (5H, m), 4.09–4.25 (1H, m), 4.89–5.46 (2H, m), 9.10 (1H, s), 9.35 (2H, b.s.); MS after silylation with BSTFA *m/z* 344 (M^+ as mono-trimethylsilyl derivative), 314 (M-30 [2 CH_3]), 299 (M-45 [3 CH_3]), 270 (M-74 [$HSi(Me)_3$]), 225 (M-139 [$HSi(Me)_3$ and NH_2CHO]). Anal. calcd for $C_{10}H_{16}N_4O_5$: C, 44.12; H, 5.92; N, 20.50. Found: C, 44.07; H, 5.95; N, 20.53.

4, 6-Di(formylamino)-5,6-dihydro-1-[β-D-ribofuranosyl]2-(1*H*)-pyrimidone (4). IR (KBr) 3492–3350 (OH and NH), 2850 (CHO), 1723 (CO), 1669 (CO), 1629 (C=C), 1276, 975 cm^{-1}; mp 178–181 °C (with decomposition); ^{1}H NMR (Me_2SO-d_6) δ 2.23–2.57 (2H, m), 4.10–4.23 (2H, m), 4.63–4.95 (3H, m), 4.16–4.25 (1H, m), 4.68–5.41 (3H, m), 9.10 (1H, s), 9.21 (1H, s); MS after silylation with BSTFA *m/z* 516 (M^+ as tris-trimethylsilyl derivative), 413 (M-103 [2 CH_3 and $HSi(Me)_3$]), 367 (M-148 [2 $HSi(Me)_3$]), 297 (M-219 [3 $Si(Me)_3$]), 255 (M-249 [3′,5′-bis-trimethylsilyl-1-β-D-ribofuranosyl]). Anal. calcd for $C_{11}H_{16}N_4O_6$: C, 44.00; H, 5.37; N, 18.66. Found: C, 44.10; H, 5.39; N, 18.79.

2′-Deoxy-β-D-ribosyl urea (5). IR (KBr) 3400 (OH and NH), 1700 (C=O), 1075 (C-OH) cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 1.71–2.01 (2H, m), 3.65 (3H, m), 5.22–5.65 (2H, m), 4.62 (3H, b.s.); MS *m/z* 176(M^+). Anal. calcd for $C_6H_{12}N_2O_4$: C, 40.91; H, 6.86; N, 15.90. Found: C, 40.83; H, 6.87; N, 15.93.

The formamide sequencing protocol. The DNA sequencing procedure used on 3′-radioactively labeled DNA fragments is the 'formamide protocol' performed as previously described,[8] consisting essentially of heating DNA in formamide at 110 °C for 10 min without further handling. Labeling, chemical treatment, and gel analysis were performed as described.[8]

Incorporation of cytidine analogues into polynucleotides. 5-Bromo-2′-deoxycytidine was incorporated into the primer oligonucleotide used in the experiment reported in Figure 2 according to the chemical procedure routinely used for in vitro chemical DNA synthesis.[40] 5-Methyl-2′-deoxycytidine was incorporated during the construction of double-stranded DNAs (see below). The K_m of Sequenase for dCTP was found to be similar to that of 5mdCTP.

Construction of double-stranded DNAs in vitro. The nucleotide sequence of the DNA fragments and the strategy employed for the insertion of base derivatives into polynucleotides and for testing their sensitivity to formamide are reported in Figures 1 and 2. The procedure used for the preparation of the DNA molecules used for this analysis is the following: 260 picomoles of the in vitro synthesized 26-mer template (see Fig. 1a) were mixed with 260 picomoles of 16-mer primer in 50 mM Tris–HCl (pH 7.5), the annealing mixture (20 μL) was heated 5 min at 95 °C, then slowly cooled to 30 °C and divided in four aliquots. Each aliquot was incubated 30 min at 37 °C with 0.2 U of Sequenase[44] (purchased from USB) in 10 μL final volume of 20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 250 μM TTP, 250 μM dGTP, 1 μM [α-^{32}P] dATP (3000 Ci/mM) and one of: 250 μM dCTP or d5mCTP. The reaction was stopped by addition of 20 μL of 90% formamide—10 mM EDTA, heated 2 min at 90 °C and directly loaded on a 6% denaturing polyacrylamide gel for purification of the labelled 26-mer (Fig. 1a, upper strand). End-labeling was achieved by terminal selective incorporation of [α-^{32}P] ATP at the 3′ extremity in the unique A position (indicated by a star in Fig. 1a).

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