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Transcriptional errors and ambiguity resulting from the presence of 1,N^6-ethenoadenosine or 3,N^4-ethenocytidine in polyribonucleotides

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ABSTRACT

1,N^6-Ethenoadenosine (εA) and 3,N^4-Ethenocytidine (εC) in copolymers with unmodified nucleosides were transcribed using DNA-dependent RNA polymerase in the presence of Mn^{2+}. Nearest neighbor analysis of the products showed that εA directed incorporation of A>U>C while εC directed the incorporation of U≥A≫C. Neither directed G into the complementary polymer. Such misincorporations resulting from εA and εC, compounds that are formed *in vivo* by the carcinogen vinyl chloride, may have a biological role as promutagens.

INTRODUCTION

Vinyl chloride is one of the few human carcinogens[1,2] where there have been detailed studies of the chemical reactions of mutagenic and carcinogenic metabolites with nucleic acids. Chloroethylene oxide has been identified as a product formed from vinyl chloride by metabolic activation using microsomal cytochrome P-450-dependent monooxygenases[3]. Chloroethylene oxide behaves like all epoxides and the ring opens easily to form chloroacetaldehyde, also identified as a vinyl chloride metabolite[3]. Both chloroethylene oxide and chloroacetaldehyde are mutagenic[4], but tumorigenicity has been reported only for chloroethylene oxide[5].

Because chloroacetaldehyde reacts with nucleosides to produce chemically useful fluorescent derivatives, these reactions were studied some time before it became evident that they were of biological interest. Chloroacetaldehyde reacts readily in aqueous solution with cytidine (optimum pH 3.5) to form 3,N^4-ethenocytidine and with adenosine (optimum pH 4.5) to form 1,N^6-ethenoadenosine[6,7]. In addition, guanosine forms 1,N^2-ethenoguanosine (optimum pH 6.5)[8] and 2-thiouridine forms the hydroxyetheno or etheno derivative at pH 3[9]. At pH 6, both 2-thiouridine and 4-thiouridine are converted to uridine[9]. Although the final reaction product of chloroacetaldehyde with adenosine, cytidine or guanosine is an etheno derivative, stable intermediates have been isolated and it is postulated

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that there may be different mechanisms with different substrates[10]. None of these studies has been carried out on polymers and it is conceivable that chloroacetaldehyde modification of homopolynucleotides may yield more than a single derivative if the intermediates are of sufficiently great stability.

When nucleotides are base-paired in a double-strand, none should react with chloroacetaldehyde since the positions to be modified are hydrogen bonded. Even with high concentrations of chloroacetaldehyde used for 42 hr, less than 0.5% of the adenine residues in "double-helical" DNA were modified[11]. Later experiments using calf thymus DNA with slightly milder conditions indicated that about 0.36% εdC and 0.16% εdA were found[12]. This extremely low level of modification is probably due to thermal denaturation even at 37°[13] or to reaction in single-stranded segments. Nevertheless, even with such low reactivity Green and Hathway were able to detect and identify both εdC and εdA in the liver DNA of rats given 250 ppm vinyl chloride in their drinking water for two years[12].

Studies of fidelity in transcription have shown that many modified nucleosides lead to misincorporation or mispairing[14-18]. Such ambiguity can be shown in a number of cases to be directly responsible for *in vivo* mutagenesis[16]. Both εA and εC are products of a carcinogen and it was of interest to investigate whether they behaved as promutagens when transcribed in our system.

MATERIALS AND METHODS

Materials. Sources were: Nucleoside diphosphates (PL Biochemicals), [α^{32}P]-GTP (20-30 Ci/mM) (New England Nuclear), M. luteus polynucleotide phosphorylase for preparing polymers (PL Biochemicals), E. coli DNA-dependent RNA polymerase for transcription (Miles), and snake venom phosphodiesterase and bacterial alkaline phosphatase for HPLC analysis (PL Biochemicals).

Preparation and Analysis of Polyribonucleotides. All polymers were prepared as described by Singer and Kröger[19] and analyzed using HPLC. The composition was determined using integration of the UV peaks[18]. Although it has been reported that the etheno group makes εA and εC in polynucleotides resistant to nucleolytic cleavage[20,21], in our experiments the large amounts of enzymes used were capable of completely hydrolyzing copolymers containing up to 27% εC or εA. Similarly, chloroacetaldehyde modified DNA is completely hydrolyzed[11].

Transcription of Polyribonucleotides and Nearest Neighbor Analysis. The 625 μl standard incubation mixture contained 0.15 absorbancy units of polyribonucleotide and was 0.4 mM each in ATP, CTP, UTP and GTP [α^{32}P]

labeled (15 μCi), 4 mM $MnSO_4$, 0.8 mM K_2HPO_4, 40 mM β-mercaptoethanol and contained 15 μg RNA polymerase. In "non-competitive" experiments, only one other nucleoside triphosphate was used in addition to the [$\alpha^{32}P$] GTP. The concentration was then 0.8 mM for each triphosphate in order to maintain a constant 1.6 mM triphosphate concentration. After 2 hr incubation at 37°, 75 μL was spotted on DEAE paper disks and washed seven times with 7% Na_2HPO_4, briefly twice with H_2O and twice with ethanol. After drying, the radioactivity on the disk was counted using toluene scintillation fluid. The count in the aliquot gives a measure of total [^{32}P] GMP incorporation.

Nearest neighbor analysis was performed as described by Kröger and Singer[19]. Using poly (C) as template there is always some non-specific incorporation of [$\alpha^{32}P$] in CMP, AMP and UMP, attributed to streaking from the high GMP radioactivity. The percent of each counts compared to the total incorporation of GMP was found to be similar regardless of the amount of poly (C) transcribed (over 2 orders of magnitude). Therefore even when polymers containing modified bases are poorly transcribed, the same percent non-specific radioactivity as in polymers with more transcription can be subtracted as background. In each nearest neighbor experiment, a poly (C) transcript is included to give this background. Only radioactivity more than three times background is considered to represent significant incorporation resulting from the modified base in the polymer. When using C polymers and [$\alpha^{32}P$] GTP, radioactivity in ApG sequences indicates that the modified nucleoside simulated the presence of U. CpG and UpG radioactivity indicates simulation of G or A, respectively.

RESULTS

Fidelity of Transcription in Copolymers with Cytidine.

a) Competitive conditions. Three polymers were used for each modification, containing approximately 7, 15 and 25 percent εC or εA. As the amount of etheno derivative increased, the total transcription decreased (Tables 1,2) but was always of sufficient magnitude to determine fidelity. When all four nucleoside triphosphates were present in equal amounts (GTP being [$\alpha^{32}P$] labeled), εC directed incorporation of U, A and C (Tables 1,2). When poly (C, 7.3% εC) was transcribed only Ap and Up were found, and approximately in equal amounts. As the proportion of εC was increased, Cp was also found. The total misincorporation represented about 55% of the εC present. Thus, at least half of the modified residues directed A, U or C in the complementary polymer.

Table 1. Example of Data Obtained by Nearest Neighbor Analysis of Transcription Products From Polynucleotides[a]

Template[b]	$[^{32}P]$ cpm x 10^{-3} in 2'(3') Nucleotide			
	Cp	Ap	Up	Gp
Poly (C, 7.3% εC)	2.1	18.0	18.9	636
Poly (C, 15% εC)	1.4	12.0	14.6	236
Poly (C, 6% εA)	2.8	16.9	6.5	552
Poly (C, 12.7% εA)	2.4	12.8	6.0	306
Poly (C, 10% G)	36.9	5.5	9.0	467
Poly (C, 17% A	4.1	7.3	29.0	822
Poly (C, 24% A)	1.2	4.7	54.3	723
Poly (C, 8.3% U)	3.0	111.0	9.3	1054
Poly (C)	1.5	8.3	6.7	1097

[a]All four nucleoside triphosphates were present in equal amounts. GTP was $[\alpha^{32}P]$ labeled. See Methods and Materials for other experimental conditions.

[b]The composition of all polymers was analyzed by HPLC. There was no detectable U in any polymer containing εC or εA.

εA, on the other hand, exhibited a distinct preference for directing Ap incorporation, but with increasing amounts of εA in poly (C), Up and Cp were also found (Tables 1,2). There appeared to be more Cp incorporation directed by εA than by εC. If all nucleotides except Gp are considered as misincorporations then the total misincorporation directed by εA is about 35% of the εA in the polymer. Although εA is apparently less efficient in transcription than is εC, the resulting misincorporation is certainly not infrequent.

b) Non-competitive conditions. These experiments are designed to amplify or "force" misincorporation by using only two nucleoside triphosates in equal amounts but with the same total molarity as in competitive experiments. $[\alpha^{32}P]$ GTP is always present with one other triphosphates in each experiment.

Using εC polymers and $[\alpha^{32}P]$ GTP plus CTP or UTP it was clear that there was a considerably higher proportion of CpG or UpG sequences in the transcript than when all four NTP's were used. ApG was not noticeably increased in transcription with $[\alpha^{32}P]$ GTP and ATP (Table 3).

Table 2. Effect of 3,N^4-Ethenocytidine (εC) and 1,N^6-Ethenoadenosine (εA) on Transcription of Poly (C)[a]

Template	Minor Base %	[^{32}P] Radioactivity (%) in Nearest Neighbor Sequence[b] CpG	ApG	UpG	Total Transcription[c] % of Poly (C)
Poly (C,εC)	7.3	n.d.	1.9	1.8	59
	15	0.2	3.7	4.6	23
	27	0.55	6.2	8.0	12
Poly (C,εA)	6	0.35	2.1	n.d.	51
	12.7	0.5	3.2	n.d.	29
	25	0.85	5.1	3.1	15
Poly (C,A)	24	n.d.	n.d.	6.3	70
Poly (C,G)	10	6.8	n.d.	n.d.	42
Poly (C,U)	8	n.d.	7.8	n.d.	122
Poly (CAGU)[d]		6.7	5.8	4.9	17

[a] See footnotes *a* and *b*, Table 1.

[b] The non-specific incorporation directed by the Poly (C) carrier is subtracted (see Table 1 for type of data). Only values that are three times higher than background are given. Lower values are indicated by n.d.

[c] Total transcription includes GpG sequences which are not shown on the Table but such data is in Table 1. When the polymers contain a high percent of C, most of the radioactivity is found in G^{32}p. Poly (C) as transcribed in these experiments results in approximately 1.3 x 10^6 cpm in G^{32}p (Table 1).

[d] Data from Singer and Spengler[18]. The polymer composition was 70:5:15:9.

No nucleotide incorporation was increased when poly (C, εA) was transcribed using two NTP's (data not shown). Transcription of poly (C, 24% A) or poly (C, 10% G) using "forced" conditions does not cause misincorporation or increase the amount of complementary nucleotide incorporation. In this respect, εA is like an unmodified base or one having a specific base-pairing capability as is also indicated by the preference of εA to substitute for U and direct Ap incorporation.

Table 3. "Forced" Misincorporation of Nucleotides Directed by Poly (C) Containing εC[a]

Poly (C) Containing	$[\alpha^{32}P]$GTP + NTP	$[^{32}P]$ Radioactivity (%) in Nearest Neighbor Sequence[b] CpG	ApG	UpG
7.3% εC	CTP	1.5 (n.d.)		
	ATP		0.6 (1.9)	
	UTP			3.8 (1.8)
15% εC	CTP	3.1 (0.2)		
	ATP		5.5 (3.7)	
	UTP			8.2 (4.6)
27% εC	CTP	3.2 (0.55)		
	ATP		7.6 (6.2)	
	UTP			11.8 (8.0)

[a] $[\alpha^{32}P]$ GTP and one other nucleoside triphosphate were present in equal amounts, conditions equivalent to those used in "non-competitive" experiments[17]. The total NTP concentration was 1.6 mM which is the same as for experiments where all four NTPs are used.

[b] The non-specific incorporation directed by the Poly (C) carrier is subtracted. The numbers in parenthesis are the percent incorporation under competitive conditions i.e., all four NTPs (Table 2). Significant numbers are underlined. n.d. indicates that no significant incorporation occurred. See Materials and Methods for method of evaluating significance of data.

Transcription of εA and εC in Copolymers with A. Although the presence of 6-7% εA or εC in C polymers did not depress Gp incorporation dramatically (Table 2), the only direct way to determine whether either etheno compound directed G incorporation was to transcribe A or U "carrier" polymers. "Carrier" refers to the unmodified major component of polynucleotides. Using poly (A, εC) and poly (A, εA) in transcription experiments again with $[\alpha^{32}P]$ GTP and all NTP's, there was no transfer of the $[^{32}P]$ to U indicating that neither derivative could substitute for C. The appropriate control, poly (A, C) clearly directed G and $U^{32}pG$ was found as a consequence of poly (U, G) being formed.

DISCUSSION

Chloroacetaldehyde, a mutagenic metabolite of vinyl chloride, has been used to modify poly (A) and poly (C) as a simple method for the preparation of poly (A, εA) and poly (C, εC)[22,23]. Although no thorough investigation has been made regarding products other than εA or εC it is probable that no other derivatives are formed except intermediates that are hydrates[10]. However, for a higher degree of certainty that a polymer contains only εA or εC residues, polymerization of the appropriate nucleoside diphosphate is preferable. Such polymers have been prepared[21,24,25] and, in principle, the modified and synthesized polyribonucleotides possess similiar physical properties. For our studies, we used several copolymers including poly (C, εA) and poly (A, εC) which can only be prepared by synthesis.

Poly (εA) has no distinct organized secondary structure at neutral pH[21] but there is evidence that the planar ε-adenosine bases are involved in stacking interactions[20,22]. Poly (εC) also lacks organized secondary structure but Ludlum et al.[25] find evidence for short stretches of helicity stabilized by base stacking. However, the introduction of relatively small amounts of εA or εC into polymers does not greatly perturb the structure of the "carrier" polymer [22,23]. In our present work we find that the rate and extent of transcription are not affected by $<10\%$ εA or εC, again indicating that the secondary structures of these etheno-containing polymers resemble that of copolymers with unmodified bases, e.g., poly (C, A) or poly (C, G).

The misincorporations observed have some similiarity to those found with polymers containing m^1A, m^3C and m^3U[17]. In all these cases an essential Watson-Crick hydrogen-bonding site is blocked. The etheno group of εA or εC is almost planar, based on x-ray crystallography[26,27], and certainly shields two normal hydrogen-bonding positions. However, in the case of εC, the second ring gives the molecule the dimensions of adenine and the possibility to base-pair with U. In our experiments, the simulation of A by εC is the predominent behavior in transcription as is also reported by Barbin et al.[28]. In addition, εC simulates U and to a lesser extent, G (Table 4). The comparable derivative, m^3C simulates A, U and G[17] but differs from εC in one respect, since m^3C simulates G much more frequently. εA behaves much like m^1A[17] in transcription but not identically since it does not direct any incorporation of G as does m^1A (Table 4).

Barbin et al.[28] transcribing chloroacetaldehyde modified poly (dA) and poly (dC), find some of the same misincorporations and some which differ. Their transcription is with DNA-dependent DNA polymerase in the presence of

Table 4. Effect of εC and εA on Transcription of Polyribonucleotides Using DNA-Dependent RNA Polymerase[a]

Modified Nucleoside	Simulates the Presence Of			
	A	G	U	C
εC	+++	±	+++	-
εA	+	±	+++	-

[a]Data on which this summary is based are in Tables 1, 2 and in the text.

Mg^{2+}. Mg^{2+} has been shown to decrease the stacking interactions in poly (A, εA) and poly (εC), possibly by electrostatic interaction with the etheno bases[22,25] and this may account for some of the differences between the two systems.

We conclude that substitution involving the N-3 of C or the N-1 of A does not stop transcription but the modified nucleotide does not specifically base-pair, thus leading to considerable ambiguity. The etheno ring, although relatively bulky, resembles a methyl substituent blocking one of the base-pairing sites.

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