REACTION OF *O*-METHYLHYDROXYLAMINE WITH ADENOSINE SHIFTS TAUTOMERIC EQUILIBRIUM TO CAUSE TRANSITIONS

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1. Introduction

O-Methylhydroxylamine, like hydroxylamine, is a mutagen in many systems [1–4]. It can directly substitute a methoxyamino group for the amino group of cytidine [5,6] or adenosine [7] when these nucleosides are not hydrogen bonded, forming N^4-methoxycytidine (mo^4C) and N^6-methoxyadenosine (mo^6A). While the reaction with adenosine with *O*-methylhydroxylamine is very slow it is comparable to that of 5-methylcytidine (m^5C) [8]. The cytidine reaction has been found to result in a complete change in the tautomeric equilibrium so that N^4-methoxycytidine substitutes for U in transcription [9] and is hydrogen bonded to A in the double-stranded transcript [10].

We have now prepared the diphosphate of the reaction product of *O*-methylhydroxylamine and adenosine, mo^6ADP, and copolymerized it with CDP or ADP. Transcription of the resulting copolymers showed that mo^6A can act like A or G, but not U or C. The specific changed basepairing is considered to be due to a tautomeric shift from the amino to the imino form. On the basis of transcription data, it is estimated that the K_T for the amino form is $\simeq$10. This is the first direct demonstration of a substitution reaction by a mutagen changing the tautomeric equilibrium of a purine. In considering the mechanism of mutation of hydroxylamines, this reaction should also be considered as a contributing factor.

2. Materials and methods

[α-^{32}P]GTP (20–30 Ci/mmol) was purchased from New England Nuclear. Unmodified nucleoside diphosphates and triphosphates were purchased from P-L Biochemicals. Polynucleotide phosphorylase (P-L Biochemicals), RNA polymerase *Escherichia coli* K12 (Miles Labs), snake venom phosphodiesterase and bacterial alkaline phosphatase (Worthington) were all commercial preparations. TLC was on Eastman cellulose sheets no. 6065.

2.1. *Preparation of mo^6ADP*

N^6-Methoxy ADP (mo^6ADP) was prepared by reacting 300 mg ADP with 5 ml 3 M *O*-methylhydroxylamine (pH 5) for 17 days at 37°C, essentially as in [11]. The diphosphate was freed of reagent by precipitation with 3 vol. ethanol and then washed 4 times with ethanol. Separation of mo^6ADP from ADP and small amounts of contaminating AMP, mo^6AMP, and 2 other faint UV absorbing bands was by descending chromatography on Whatman 3MM paper using two solvents. Solvent 1 was 1% $(NH_4)_2SO_4$–isopropanol (1:2) and solvent 2 was butanol–ethanol–H_2O (80:10:25). In solvent 1, mo^6ADP was seen as a pale yellow area and moved between ADP and AMP. The area containing mo^6ADP was re-chromatographed in solvent 2 in 5 days where it moved ahead of the markers; ADP, AMP and mo^6AMP. Further verification of the identity and purity of mo^6ADP was obtained by comparison of the R_F of a sample dephosphorylated by bacterial alkaline phosphatase with authentic mo^6Ado, using cellulose TLC developed with isopropanol–H_2O-saturated $(NH_4)_2SO_4$ (18:2:80). mo^6Ado prepared directly [7] and by dephosphorylation of mo^6ADP had identical R_F-values ($R_{Ado} \simeq 1.4$) and both were pale yellow. The spectrum of mo^6ADP is shown in fig.1. The yield of mo^6ADP represented 8% of the starting material.

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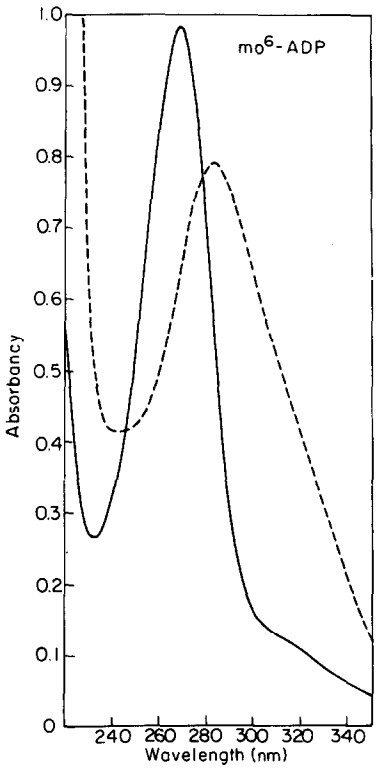

Fig.1. UV spectrum of mo^{6}ADP: pH 6 (——) λ_{max} 268 nm, λ_{min} 233 nm; pH 13 (– – –) λ_{max} 283 nm, λ_{min} 243 nm; no spectral change occurs in acid as compared to neutral pH.

2.2. *Polymerization, analysis and transcription of copolymers containing mo^{6}A*

Copolymers were prepared from mo^{6}ADP and CDP or ADP as in [12]. mo^{6}ADP polymerized well and the input and output ratios were almost identical. Polymer composition of mo^{6}A,C copolymers was determined, after enzyme digestion to nucleosides, by integration of HPLC peaks as in [13]. mo^{6}Ado and Ado did not separate on the HPLC cation exchange column (Aminex HP-C) using 0.4 M ammonium formate (pH 7) and were therefore separated on cellulose TLC using ethanol–1 M ammonium acetate (pH 7.5) (75:30) and the amount of each nucleoside quantitated by UV absorbance.

Transcription of polymers by *E. coli* DNA dependent RNA polymerase in the presence of Mn^{2+} and nearest neighbor analyses of the products have been detailed in [13].

3. Results

Ribopolynucleotides containing mo^{6}A were good templates for transcription by DNA-dependent RNA polymerase. Copolymers of C with 17–33% mo^{6}A directed the incorporation of U and C but not A (table 1). UpG sequences result when mo^{6}A has the

Table 1
Effect of mo^{6}A on fidelity of transcription of poly(C)[a]

Template[b]	^{32}P radioactivity (mol fraction of total transcription in nearest-neighbor sequence)[c]			Total transcription[d]	
	CpG	ApG	UpG	cpm × 10^{-6}	% of poly(C)
poly (C, 17% mo^{6}A)	0.6	–	5.9	1.1	46
poly (C, 21% mo^{6}A)	0.6	–	5.3	0.83	35
poly (C, 27% mo^{6}A)	1.0	–	9.7	0.6	25
poly (C, 33% mo^{6}A)	1.4	–	11.4	0.42	18
poly (C, 14% A)	–	–	3.2	1.6	67
poly (C, 6% G)	9.9	–	–	0.84	35
poly (C, 10% U)[e]	–	6.6	–		87
poly (C)				2.4	(100)

[a] All 4 nucleoside triphosphates were present in equal amounts; GTP was α-^{32}P-labeled
[b] Percent second nucleoside was determined by HPLC or TLC as in section 2; no A was present in mo^{6}A polymers
[c] The non-specific incorporation or streaking of ^{32}P when poly(C) is transcribed is subtracted. This amounted to ≃0.1% Cp, 0.6% Ap and 0.4% Up. Values less than double these backgrounds are shown as blanks. Radioactivity in CpG sequences indicates that the modified nucleoside simulated the presence of G. Similarly, ApG and UpG radioactivities indicate simulation of U and A, respectively
[d] Total transcription includes GpG sequences which are not shown
[e] Data from [9]

same base-pairing capability as A while CpG sequences result from base-pairing as G. With increasing mo^{6}A content, the amounts of UpG and CpG increase, with CpG radioactivity always ~10% that of UpG. The data for control polymers indicate that when unmodified A or G or U are incorporated into poly(C) and transcribed, no anomalous incorporations occur (table 1).

When transcribing copolymers with a high percentage of C using [^{32}P]GTP, it is obvious that there will always be a large amount of radioactivity transferred to Gp (table 1) and thus no direct information can be obtained on the ability of a modified nucleoside to simulate C. Therefore copolymers of A and mo^{6}A were prepared and transcribed using [^{32}P]GTP with all 4 NTPs present. In this case, the presence of C is indicated by transfer of ^{32}P to a neighboring U. Copolymers containing A and 13% or 18% mo^{6}A had no significant radioactivity in UpG, while poly (A, 20% C) directed appreciable incorporation of G (not shown).

These results thus show that mo^{6}A does not substitute for either pyrimidine but for both purines.

4. Discussion

When *O*-methylhydroxylamine is used as a mutagen, there is evidence that AT → GC transitions occur [4,11], as well as GC → AT which are expected on the basis that substitution of the N^4 of cytidine is the mutagenic reaction. Modification of adenosine to form mo^{6}A could also be mutagenic if mo^{6}A has a changed tautomeric equilibrium to favor the imino form (fig.2) [11]. N^6-Hydroxyadenosine was used as a base analog for phage T_4 rII mutants [14] and the compound found to induce base-pair transitions in both directions indicating that the hydroxylamine derivative could substitute for G as well as A.

The transcription data (table 1) clearly show that mo^{6}A can direct incorporation of C into the transcript

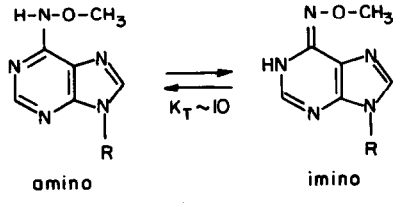

Fig.2. Tautomeric forms of mo^{6}A. The methoxyamino group is shown as being *anti* to the base-pairing side. In the amino form, mo^{6}A has the same base-pairing capability as A, while in the imino form it can base-pair like G (table 1).

~10% as often as U which indicates a K_T for the amino form of ≃10 (fig.2). The only other mutagen-substituted derivative to show specifically changed base-pairing is mo^{4}C which only acts as U [9,10], in contrast to ho^{4}C which apparently can substitute not only for U and C, but also causes misincorporation of A and G as a result of the substituent rotating *syn* to the Watson-Crick base-pairing side [9]. The methoxy substituent in mo^{6}A is held *anti* to the Watson-Crick side (fig.2), at least in transcription, since rotation would lead to complete ambiguity [9,15].

The dual functional specificity of mo^{6}A would make it useful for site-specific mutagenesis of a purine.

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