CORRECTION FOR NON-UNIFORM PHOSPHATE LABELING OF E. COLI AND PHAGE RNAs SYNTHESIZED IN VIVO

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Summary

E. coli cells grown to phosphate starvation incorporate $^{32}PO_4$ unequally into the α position of the four ribonucleotide triphosphates during a short period of labeling. A method for determining the relative specific activities of nucleotides in RNA molecules synthesized under these conditions and correcting sequence data is described.

To isolate and study short-lived RNA species such as messenger RNAs and precursors to stable RNAs, growing cells are usually exposed to radioactive compounds for only a fraction of a generation time. If RNA molecules labeled with $^{32}PO_4$ in this way are to be subjected to sequence analysis by standard techniques, such as those developed by Sanger and colleagues (3), it is essential that all four nucleotides be uniformly labeled in the α position.

Examination of several E. coli and bacteriophage T7 RNAs, prepared after $^{32}PO_4$ labeling of cells approaching phosphate starvation, revealed significant differences (up to four-fold) in the relative specific activities of the incorporated nucleotides. However, using the approach described here, it is possible to determine experimentally specific activity ratios for the 4 nucleotides and thus correct data obtained during sequence analysis.

Methods

E. coli SY106 was incubated with shaking at 30°C in TG medium supplemented with 1% Casamino acids (1). Doubling times of about 50 minutes were observed until growth halted due to phosphate starvation at a density of approximately 3 x 10^8 cells/ml. Chloramphenicol at 400 µg/ml was added where indicated and cells shaken for five minutes before addition of isotope. To label T7 phage mRNAs, the cells were infected with T7 at a multiplicity of 10 five minutes after chloramphenicol treatment and shaken for an additional 4 minutes. $^{32}PO_4$ was added in amounts indicated in Table 1 and incorporation allowed for 7 to 24 minutes. The cells were rapidly chilled and harvested; RNA was extracted and fractionated on polyacrylamide gels according to Summers (2).

T_1 ribonuclease digests of RNA species were fingerprinted using the methods of Sanger, et al. (3). The appropriate spots were eluted, hydrolyzed with alkali, and subjected to electrophoresis at pH 3.5 for base analysis (3).

Results

To determine the relative specific activities of the four nucleotides in various ^{32}P-labeled RNAs, T_1 ribonuclease fingerprints were first prepared. The alkaline hydrolysis products of the UCG, CUG, UAG and AUG spots - all of whose sequences are uniquely determined by position on the fingerprint - were then counted. Due to transfer of the α phosphate of each nucleotide to its 5' neighbor in the RNA chain, the counts observed in the first two bases of the above oligonucleotides provide a measure of the C/G, U/G, A/G and U/G specific activity ratios, respectively. For example, the products of UCG are Up, Cp and Gp, where the Up phosphate was originally the α phosphate of CTP and the Cp phosphate is derived from GTP; thus the Up/Cp ratio determines the relative specific activity of CTP/ GTP.

This type of analysis was performed on E. coli and T7 messenger RNAs labeled with $^{32}PO_4$ under several conditions. Specific activities of the incorporated nucleotides relative to GTP are shown in Table 1. In each

Table 1 - Conditions and Results

Experiment #	Time (min.)	CM	T7	^{32}P (mC/ml)	RNA	C/G	U/G	A/G
1	8	+	+	0.1	B4	0.39	0.77 ± 0.02	1.51
					B5	0.52	0.83 ± 0.04	1.16
2	7	+	+	0.1	B1	0.48	0.92 ± 0.06	1.00
					B4	0.65	0.88 ± 0.10	1.05
3	17	+	+	0.1	p16S	0.57	0.92 ± 0	1.16
					B4	0.53	0.94 ± 0.02	1.24
4a	8	-	-	0.02	Bulk	0.24	0.65 ± 0.10	1.09
4b	24	-	-	0.02	Bulk	0.48	0.83 ± 0.02	1.23
5a	8	+	-	0.002	Bulk	0.47	1.38 ± 0.02	1.91
5b	20	+	-	0.002	Bulk	0.58	1.32 ± 0.10	1.58
5c	8	+	+	0.002	Bulk	0.28*	1.18 ± 0.02	1.27
5d	20	+	+	0.002	Bulk	0.51*	1.30 ± 0.14	1.83*
6	17	+	+	0.2	B2	0.69	1.03 ± 0.06	1.01

Time - duration of labeling period
+ CM - chloramphenicol added
+ T7 - infected with T7
RNA - T7 or E. coli RNAs separated on 2.5% acrylamide - 0.5% agarose gels (2) or bulk extracted RNA. Nomenclature for T7 early RNAs from Summers, et al. (1). (B1 contaminated with p23S rRNA).
C/G, U/G, A/G - As explained in Results; U/G is average of data from CUG and AUG spots.
In all cases, except where noted by *, greater than 30 counts per minute above background were detected.

of the RNA samples, the labeling is non-uniform, with ATP consistently exhibiting the highest specific activity and CTP the lowest. Such deviations in specific activity - up to four-fold differences - are significantly greater than the usual $\pm$ 0.1 observed in sequence analysis of uniformly labeled RNAs.

The exact conditions of $^{32}PO_4$ labeling obviously affect the specific activity ratios observed, and it should be noted that in all cases reported in Table 1 isotope was added after cell growth had slowed as a result of phosphate starvation. Greater uniformity of labeling is observed with longer pulses. However, results vary from preparation to preparation; minor differences in specific activity ratios are detected when comparing various RNA species from the same preparation. Treatment of cells with chloramphenicol or infection by T7 phage before labeling appears to have no systematic effect.

Discussion

We have observed that in E. coli cells approaching phosphate starvation, exposure to $^{32}PO_4$ for 1/7 to 1/2 of a generation time yields RNA molecules in which the 4 nucleotides are not uniformly labeled. Since erroneous sequences may therefore be deduced, specific activity ratios should be determined (as by the method described here) when analyzing bacterial RNAs synthesized under similar labeling conditions. Unequal specific activities of the 4 nucleotides may also explain anomalous sequence data obtained for RNAs isolated from mammalian cells labelled with $^{32}PO_4$ in culture (S. Dube, A. E. Smith, pers. commun.).

It seems likely that the unequal nucleotide labeling observed here results from a depletion of nucleotide pools which, upon addition of small amounts of radioactive phosphate, are not reestablished coordinately with RNA synthesis. The specific activity ratios observed are consistent with known precursor-product relationships in the biosynthesis of the

purine and pyrimidine nucleotides (5). Lutkenhaus, et al. have shown that cells not starved for phosphate incorporate $^{32}PO_4$ in the α position of ATP and GTP equally even after short labeling times (4). Thus, it is possible that more equal labeling of the 4 nucleotides with $^{32}PO_4$ could be achieved by supplementing the medium with unlabeled phosphate or by exposure to isotope well before the onset of phosphate starvation.

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