ENZYMATIC INCORPORATION OF 5-BROMO CYTIDYLIC ACID INTO THE TERMINAL SEQUENCE OF S-RNA

R. L. Soffer, S. Uretsky, L. Altwerger, and G. Acs

Institute for Muscle Disease, Inc., New York, N. Y. 10021

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In connection with other studies on the metabolism of nucleotide analogs in mammalian tissue, we have examined the effect of incorporating 5-bromo CMP into the terminal sequence of S-RNA in place of CMP. The results reported in this communication demonstrate (1) that Br-CMP can be incorporated into the terminal sequence of S-RNA in place of CMP, and (2) that such S-RNA functions effectively in accepting amino acids and in transferring them into peptide linkage.

Materials and Methods: S-RNA was isolated from rabbit liver according to Acs and Lipmann (1966). The terminal S-RNA pyrophosphorylase was purified from rabbit liver by the method of Daniel and Littauer (1963). Conditions of incubation for pyrophosphorolysis and reconstitution of S-RNA were as described by those authors. A dialyzed 105,000 X g supernatant fraction from rabbit liver was used as a source of activating enzymes. 5-Br-CTP (a gift of Mr. Alan Kapuler) was made by bromination of CTP. After purification by chromatography on Dowex-1-Cl, the product was free of CTP as judged by paper electrophoresis and U. V. absorption. In experiments on the transfer of phenylalanine from S-RNA into protein, ribosomes

(Nirenberg and Matthaie, 1961) and transfer enzyme from E. coli were employed. The latter was purified through the ammonium sulfate fractionation described by Nishizuka and Lipmann (1966).

Results: The terminal sequence of crude S-RNA, pyrophosphorolyzed as described, can be reconstituted enzymatically with ATP in conjunction with either Br-CTP or CTP. As seen in Table I, the incorporation of AMP is 80% dependent upon the presence of CTP or of its brominated analog.

TABLE I

Incorporation of AMP from ATP-H^3 into S-RNA

Addition	mμmoles AMP-H^3/mμmole S-RNA
ATP	0.06
CTP+ATP	0.358
Br-CTP+ATP	0.398

The reaction mixtures (1.0 ml) contained: glycine buffer, (pH 9.8) 80 μmoles; $MgCl_2$, 6 μmoles; PEP, 10 μmoles; PEP kinase, 40 μgrams; ATP-H^3, 800 mμmoles (3 X 10^3 CPM/mμmole ATP); CTP, 460 mμmoles or Br-CTP, 458 mμmoles; pyrophosphorolyzed rabbit liver S-RNA, 36 mμmoles; S-RNA pyrophosphorylase, 2 mg. The reaction was stopped after 30 minutes incubation at 37°C by the addition of an equal volume of 0.5 N PCA and the precipitate was washed with 0.25 N PCA, alcohol-ether (3:1 v/v) and ether. It was then dissolved in concentrated formic acid and radioactivity was determined in a scintillation counter.

To demonstrate that AMP was actually incorporated next to Br-CMP the reaction was performed using alpha-P^{32}-ATP as a substrate. The fragments produced by alkaline hydrolysis of the product were analyzed by paper electrophoresis and 80% of the radioactivity was associated with the spot corresponding to Br-CMP. This is equivalent to the fraction of AMP incorporation which is dependent upon the presence of cytidine nucleotide and indicates that pyrophosphorolysis of the CMP moieties was probably incomplete.

Equal aliquots of a preparation of pyrophosphorolyzed S-RNA were reconstituted with Br-CTP+ATP-H^3 and with CTP+ATP-H^3, respectively. Each aliquot was purified on Sephadex G-75 according to Röschenthaler and Fromageot (1965). The fractions showing radioactivity were pooled and assayed for amino acid acceptor activity as shown in Table II. The ratio of mμmoles ATP per mμmole S-RNA for these fractions was 0.8.

TABLE II

Amino Acid Acceptor Activity of Reconstituted S-RNA

	Pyrophosphorolyzed S-RNA	RNA Reconstituted with ATP+CTP	RNA Reconstituted with ATP+Br-CTP
Phenylalanine	38	750	710
Arginine	--	884	690
Valine	--	2040	1920
Lysine	--	1000	1070
Tyrosine	--	3620	2240
Algal Hydrolysate	44	870	725

Values are expressed as CPM/mμmole S-RNA. Reaction mixtures (1.0 ml) contained: Tris-HCl, (pH 7.2) 20 μmoles; $MgCl_2$, 10 μmoles; KCl, 50 μmoles; ATP, 4 μmoles; C^{14} amino acid, 4 μcuries (specific activity 150 μcuries/μmole); liver supernatant fraction, 1 mg. After 15 minutes incubation at 37°C, the samples were treated as described in Table I save that radioactivity was determined in a gas flow counter.

These data indicate that reconstitution with either CTP or Br-CTP results in acceptor activity for all the amino acids tested. In the case of tyrosine the analog-containing S-RNA possesses somewhat less acceptor activity than the control preparation. Other experiments indicate that this is true for both the rate and end point of the reaction. This suggests that the interaction of the terminal C-C-A of S-RNA molecules with their respective activating enzymes is probably not identical for all enzyme S-RNA pairs. We have obtained similar results with preparations of S-RNA in which the terminal AMP is replaced by various analogs of adenosine. These will be described fully in the near future.

The effect of substitution of Br-CMP in the terminal sequence of S-RNA upon the transfer of amino acid into polypeptide was investigated using phenylalanyl S-RNA. Table III illustrates the results of these experiments.

TABLE III

The Transfer of Phenylalanine from S-RNA into Polypeptide

		μμmoles L-phenylalanine transferred per ml	
		Br-CMP-containing S-RNA	CMP-containing S-RNA
$MgCl_2$	10 μmoles/ml	11	6
$MgCl_2$	13.3 μmoles/ml	34	19
$MgCl_2$	17.5 μmoles/ml	114	54
$MgCl_2$	17.5 μmoles/ml Minus Poly U	3	3

The reaction mixtures (150 μl) contained, in μmoles/ml: Tris-HCl, (pH 7.8) 50; NH_4Ac, 30; 2-mercaptoethanol, 10; C^{12} L-phenylalanine, 1; polyuridylic acid, 300 μgrams; transfer enzyme, 2 mg; ribosomes, 1.5 mg; and Br-CMP S-RNA, 16.5 mμmoles containing 180 μμmoles C^{14} L-phenylalanine (specific activity, 375 μcuries/μmole) or CMP S-RNA 16.0 mμmoles containing 200 μμmoles of L-phenylalanine. Incubation was at 37° for 30 minutes and duplicate 50 μl samples were processed on filter paper discs for hot TCA-insoluble material as described by Bennett et al. (1965).

These results indicate a very efficient transfer of phenylalanine from S-RNA containing Br-CMP into hot TCA-insoluble material. At the three magnesium concentrations tested, this transfer was greater than with control S-RNA. The reaction with either S-RNA was highly dependent upon the synthetic template, polyuridylic acid.

Discussion: Other investigators (Yu and Zamecnik, 1963; Weil et al., 1964) have employed chemically brominated S-RNA to study amino acid activation and transfer. They have provided evidence that the recognition site for amino acid activating enzymes is not identical with the anticodon. Use of the S-RNA pyrophosphorylase, as described in the present report, provides a means of introducing nucleotide analogs into a known locus in the S-RNA molecule, namely the CCA terminus. Since this is a common feature

of different S-RNA molecules, it is unlikely to be part of either the recognition site for specific amino acyl synthetases or of the anticodon. Our results indicate that the diminished acceptor activity of brominated S-RNA for phenylalanine and lysine (Yu and Zamecnik, 1963) is not a consequence of bromination of the CMP residues in the terminal sequence and thus are consistent with the notion that this is an effect at the recognition sites for the amino acyl S-RNA synthetases. However, the data presented in this report, as well as results obtained with S-RNA preparations containing adenosine analogs in place of the terminal AMP, indicate that substitution in the terminal sequence of S-RNA can somewhat modify both its acceptor and transfer activities. Such considerations may be of importance in interpreting the effect on protein synthesis of S-RNAs which contain nucleotide analogs.

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