THE ROLE OF MESSENGER RNA AND PEPTIDYL-tRNA IN THE SYNTHESIS OF THE GUANINE NUCLEOTIDES MS I AND MS II BY RIBOSOMES *IN VIVO*

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Received 11 January 1973

1. Introduction

Amino acid starvation inhibits the synthesis of stable RNA in all wild-type bacteria. This "stringent" control is determined by the RC-gene; RC^{rel} (RC^-) strains do not respond by diminuition of ribosomal and transfer RNA synthesis to lack of an amino acid [1]. Circumstantial, but convincing evidence based on the restoration of stable-RNA synthesis by several antibiotics has shown that the ribosomes or (a) factor(s) acting on the ribosomes are responsible for this control phenomenon [2,3]. Cashel and Gallant [4] discovered the unusual nucleotides ppGpp and pppGpp (MS I and MS II, respectively) and established a connection between the production of these nucleotides and the inhibition of RNA synthesis. While a causal relationship and a mechanism remain to be proven the experiments in several laboratories [2–4, 11] tend to show that there is no production of these nucleotides without concomitant inhibition of stable RNA synthesis. Likewise, there is no case of inhibition of RNA synthesis which can be fully relieved by chloramphenicol, sparsomycin, tetracycline or other inhibitors of protein synthesis with similar action, which is not accompanied by the strongly increased formation of these nucleotides.

Based on *in vitro* experiments [5], trimethoprim has been considered to inhibit specifically initiation of protein synthesis. *In vivo*, however, the synthesis of purines and certain amino acids is inhibited primarily [6]. When purines and amino acids are supplied to the growth medium MS formation in stringent strains becomes spurious as was found in different laboratories [7,8], and also by us. A partial inhibition of stable-RNA synthesis may occur not only in stringent, but also in relaxed strains. This inhibition cannot be relieved by chloramphenicol. Thus it is highly unlikely that this inhibition of stable-RNA synthesis is controlled in the usual way by the RC gene.

Haseltine et al. [9] have recently demonstrated the formation of the unusual nucleotides *in vitro* under conditions which *in vivo* would result in the inhibition of stable-RNA synthesis and production of MS. They have, moreover, shown that this *in vitro* reaction is under the control of the RC gene. Very probably then, the formation of the guanine nucleotides is always linked to the stringent response.

In this study, we have investigated whether the formation of these nucleotides *in vivo* occurs only on ribosomes when these ribosomes are still on the mRNA, and thus have a peptidyl-tRNA in their P-site, or whether the ribosomes will form these nucleotides when they have terminated peptide synthesis, and have run off.

It is clear that ribosomes still on mRNA can produce these nucleotides since immediately after depletion of an amino acyl-tRNA, independent of the type of amino acid, synthesis of the unusual nucleotides proceeds at a maximal rate. On the other hand, polysome disintegration, following amino acid starvation of a stringent strain is a slow process [10].

The rationale for our experiments was to use rifampicin to inhibit initiation of RNA synthesis. We added rifampicin to bacteria under conditions of a somewhat "leaky" amino acid (or amino acyl-tRNA) starvation. The ribosomes will be arrested at a codon for the amino acid lacking, but will be able to resume their movement as the amino acyl-tRNA involved is still being available, albeit to a much lower extent than under non-starvation conditions. After some

time they will terminate the peptide chain they were synthesizing, and leave "their" mRNA molecule. As no new mRNA chains are initiated the number of transiently arrested ribosomes on mRNA carrying peptidyl-tRNA will drop with a rate inversely related to the severity of starvation. If the unusual nucleotides are only formed by ribosomes on mRNA carrying peptidyl-tRNA their concentration should fall after rifampicin addition concurrently with the running off of these ribosomes.

2. Experimental

Escherichia coli NP29, a mutant containing a temperature sensitive valyl-tRNA synthetase [12] and a *Salmonella typhimurium* stringent relaxed pair [18] were used. All bacteria were grown in a Tris-glucose medium with low phosphate as described by Cashel [19]. The medium for NP29 was supplemented with 0.05% yeast extract and 0.2% Difco casamino-acids. To the medium for *S. typhimurium* 20 μg/ml carrier L-leucine was added.

Small cultures (about 3 ml) were labeled with 50 μCi/ml carrier free ^{32}P-orthophosphate (Philips Duphar, Amsterdam, The Netherlands), 20 min prior to first treatment in order to measure nucleotide pools and stable RNA accumulation. To measure protein synthesis, the same culture was labeled with 50 μCi/ml [5-^{3}H]L-leucine (The Radiochemical Centre, Amersham, U.K.).

The measuring of the MS accumulation was carried out as described by Cashel [19]. The separated ^{32}P-labeled nucleotides on the polyethylene-imine cellulose plates were cut out and counted in a Philips proportional counter with about 20% efficiency. The ordinate of the figures always represents the activity in 10 μl culture at a cell density of $A_{450\,nm}^{1\,cm}$ is 0.3. Throughout all experiments samples were taken for estimation of protein and RNA accumulation in all cultures. The well-known figures are, for shortness, not shown. The drug concentrations used were: 100 μg/ml 5-DL-methyltryptophan (Sigma Chem. Co., St. Louis, Mo., USA), 200 μg/ml Rifampicin (Schwarz-Mann, Orangeburg, New York) and 50 μg/ml Trimethoprim (2, 4-diamino-5 (3′, 4′, 5′-trimethoxybenzyl) pyrimidine), kindly provided by Dr. H.G. Hitchings, Burrougis Wellcome, Amsterdam, The Netherlands. Rifampicin and trimethoprim were put into the incubation vessels as solutions in ethanol. The ethanol was evaporated before addition of the bacterial suspension.

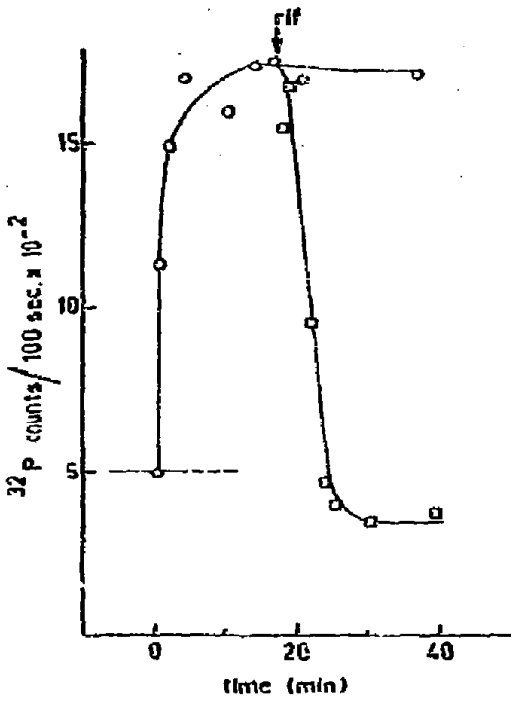

Fig. 1. Effect of rifampicin on MS accumulation caused by tryptophan starvation in *Salmonella typhimurium*. (o—o—o) 5-MT added at t_0; (□—□—□) rifampicin added to the tryptophan-starved culture at t_{18}.

3. Results and discussion

Fig. 1 shows our results with a 5-methyltryptophan-induced tryptophan starvation in *S. typhimurium*. This starvation is not complete as protein synthesis still proceeds at a rate of 10 to 20% of the uninhibited culture. It is evident from fig. 1 that the concentration of the unusual nucleotides drops quickly after addition of rifampicin as would be expected if "empty" ribosomes do not contribute to the formation of ppGpp and pppGpp. Addition of rifampicin prior to starvation results in a diminished formation of the unusual nucleotides; the larger the interval, the less is formed, as was also found by Lund and Kjeldgaard [11].

Fig. 2 shows a similar experiment with *E. coli* NP29 which possesses a temperature-sensitive valyl-

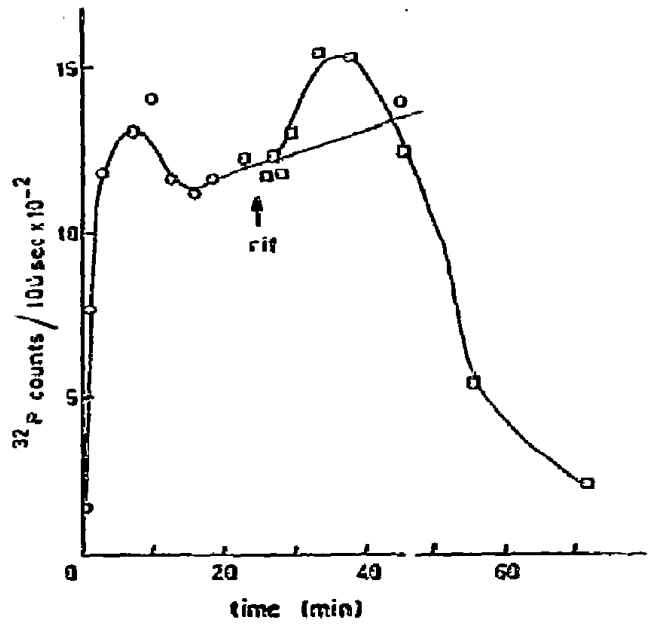

Fig. 2. Effect of rifampicin on MS accumulation induced by inactivation of valyl-tRNA synthetase at 42° in *E. coli* NP29. (o—o—o) Control brought to 42° at t_0; (□—□—□) rifampicin added 25 min after the shift to 42°.

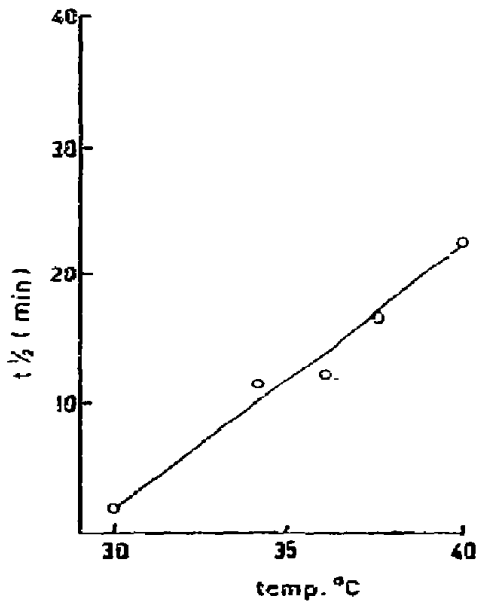

Fig. 3. Relationship of half-life of MS I after rifampicin treatment at different temperatures in NP29. The plot is composed from figures like fig. 2, except that rifampicin was added 2 min after the temperature shift.

tRNA synthetase [12]. After the temperature shift the unusual nucleotides accumulate, but now their concentration starts dropping very slowly after the addition of rifampicin. We investigated this phenomenon at different temperatures and again found the expected relation between the severity of inhibition and the rate of the ppGpp drop (fig. 3).

In another approach experiments were carried out with trimethoprim under conditions where it will generalize the amino acid starvation already induced by 5-methyltryptophan, will lead to purine depletion, but now also inhibits polypeptide chain initiation. If our view is correct, trimethoprim should transiently increase the production of the unusual nucleotides by arresting ribosomes still travelling on mRNA, but should then cause a decline because no new initiation can take place while the ribosomes can still, at a reduced rate, terminate their peptide chains and run off. Fig. 4 shows that these expectations are borne out. It also shows that the decline in MS is not caused by the lack of the precursors GDP and GTP which remain relatively constant during this period. The limited decline in ATP concentration is not the cause of the decline in the nucleotides as an increase of the unusual nucleotides can occur with a simultaneous decline in ATP (and even GTP) as was shown in other experiments, and also by Cashel et al. [13].

Altogether, our experiments show that only ribosomes arrested on a messenger RNA molecule and thus possessing peptidyl-tRNA are able to cause formation of unusual nucleotides *in vivo*. In other words, for the production of an unusual nucleotide an occupied P-site is a requirement while the A-site must, in view of starvation, be empty or occupied by an uncharged tRNA [14]. The results of Wong and Nazar [15], Gallant [16], Watson and Yamazaki [17] and Lund and Kjeldgaard [11] are all in agreement with our view, although in part differently interpreted by these authors. In the *in vitro* system of Haseltine et al. [9] the presence of peptidyl-tRNA and (parts of degraded) mRNA on the ribosomes cannot be excluded. Further experiments will have to show whether *in vitro*, too, only messenger-bound ribosomes with an occupied P-site produce the unusual nucleotides.

Acknowledgements

The authors would like to express their thanks to Miss J. Brands for her skillful technical assistance and

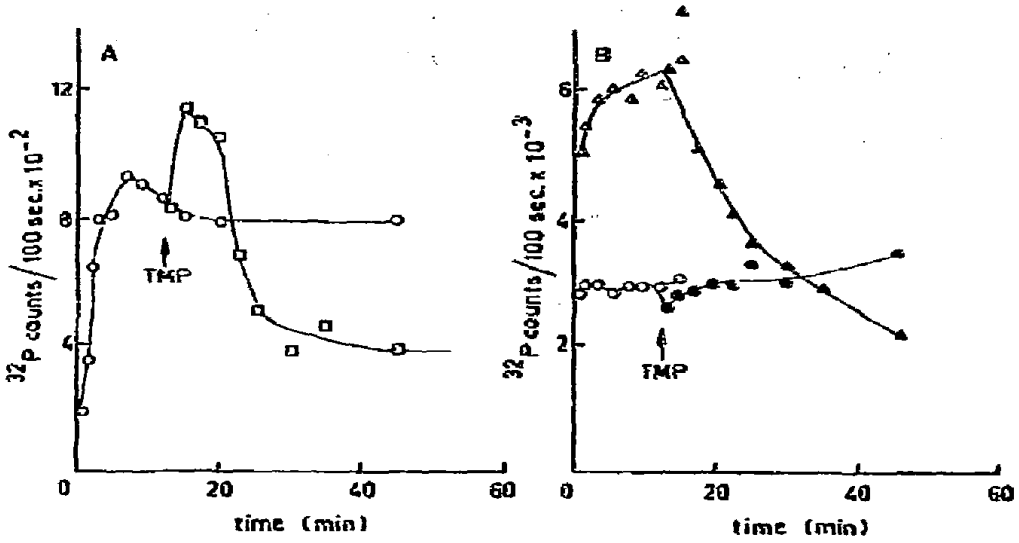

Fig. 4. A) Effect of trimethoprim on MS accumulation induced by 5-MT in *S. typhimurium* R C^{str}. 5-MT was added at t_0; trimethoprim at t_{14}. (o—o—o) 5-MT only; (□—□—□) 5-MT plus trimethoprim. B) Correspondent effects of trimethoprim on GTP and ATP pool. (o—o—o) GTP, and (△—△—△) ATP, both after 5-MT only. (●—●—●) GTP, and (▲—▲—▲) ATP, both after 5-MT plus trimethoprim.

J.A. van den Berg for his interest, suggestions and encouragement. The present investigations have been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for Advancement of Pure Research (Z.W.O.).

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