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In vivo incorporation of the intrinsic photolabel 4-thiouridine into *Escherichia coli* RNAs

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The *in vivo* incorporation of the photoactivable uridine analogue 4-thiouridine into the RNAs of an *Escherichia coli* K12 *pyrD* strain has been optimised. s^4Urd uptake in RNAs appears to be strikingly dependent upon the age of the preculture, i.e. the number of generations the cells have undergone immediately before dilution in the thiolation medium. Conditions have been set up where efficient RNA thiolation occurs in cells growing exponentially at 50 to 70% the rate of the control. The substitution level s^4U/U is maximal after growth for 9 to 10 generations in the thiolation medium and reaches 17±3% in tRNA and bulk RNA. Most of ribosomal derived ribonucleoproteins, 65 ± 5%, sediment as 70S ribosomes (s^4U/U=7±2%) on a high Mg^{2+} sucrose gradient. The thiolated RNAs were characterized by their migration on a thiol-specific affinity electrophoretic gel.

The construction of three-dimensional (3D) models of a complex ribonucleoprotein particle such as the ribosome (or the spliceosome) is a first step towards an understanding of its detailed functioning. In the case of the 30S ribosomal particle immunoelectron microscopy and neutron diffusion studies (1) have allowed the low resolution spatial localisation of most of the 21 ribosomal protein components. In spite of recent advances (2-6) the folding of the 16S RNA chain within the 30S particle remains an open question. A particularly useful approach to this question are RNA-RNA and RNA-proteins crosslinking methods. Currently available techniques have limitations such as the inaccessibility of strategic regions of the native particle to extrinsic reagents and their inability in most cases to yield direct contacts. Ultraviolet light at low intensity leads to a high level of undesired reactions so that only the cross-links obtained at low fluence can be seriously considered. This approach can be improved by using high-intensity light which is able to trigger biphotonic processes. The RNA-protein crosslinking efficiency is increased by a factor of 20 to 100, but as yet the ratio of crosslinks over local RNA lesions has not been studied. It is claimed that an analysable number of cross-links can be produced by a single 10 ns pulse (7).

ABBREVIATIONS: APM, N-acrylaminophenyl mercuric chloride; s^4U, 4-thiouracil.

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We have developed an alternative approach based on the *in vivo* incorporation of the uridine specifically photoactivable analogue, 4-thiouridine, into *E. coli* ribosomes. We currently obtain 70S ribosomes with 4 to 5% substitution of uridine by 4-thiouridine. These particles retained 50% of their biological activity in the Poly U, Poly Phe test. About two ribosomal proteins were found covalently linked to rRNAs before illumination : s^4U plays the role of a dark affinity probe. 366 nm photoactivation increases the RNA-protein crosslinking yield by a factor of 2 to 3 i.e. up to six or seven proteins per 70S particle (8). The proteins chemically and photochemically crosslinked to 16S and 23S RNA respectively have been identified (9). Using specific DNA hybridization probes it was shown that in the 30S particle proteins S7, S9, S13 have multiple crosslinking sites (10). The incorporation conditions used so far, suffered from two main limitations : i) the cells grow linearly indicating that some critical step(s) in their metabolism is (are) impaired. Accordingly only 25 to 35% of the ribonucleoproteins are in the form of stable ribosomes ii) substitution is clearly not random : the ratio s^4U/U is found to be 12 % in bulk ribonucleoprotein but only 4 to 5 % in the isolated stable 70S fraction. Here we describe *in vivo* thiolation conditions allowing exponential cell growth, a significantly higher yield of stable 70S particles and a higher thiolation level.

MATERIALS AND METHODS

A) Strains and growth medium : Strain AB 1157 is an *E. coli* K12 F^- *Leu, Pro, His, Arg, Thr, Str, Gal, Xyl, Man, Lac.*. The strain used is a derivative of AB 1157 carrying the *SfiA, PyrD* mutations (gift from R. D'Ari). The preculture growth medium was 63 B1 (8) complemented with a final concentration of 0.2 % glucose, 0.4 % casamino acids, 40 µg/ ml uridine. The same medium complemented with 100 µg/ml 4-thiouridine -except when explicitly stated- was used for *in vivo* thiolation experiments. All precultures and thiolation cultures were performed at 30°C.

B) Products and buffers : 4-thiouridine was from Sigma. Buffer A : 10mM triethanolamine/HCl pH 7.5 ;10 mM Mg $(OAc)_2$; 50 mM KCl ; 10mM 2-mercaptoethanol or 1mM dithiothreitol. Buffer B or TBE buffer : 90mM Tris pH 8.3; 90mM HBO_3 ; 2mM EDTA Na_2.

C) Cell extraction and ribosome preparation : Cells were extracted in a French pressure cell at 1.400 kg/cm^2. 70S ribosomes were routinely separated from non-associated 50S and 30S particles in 5-20 % sucrose gradients.

D) Affinity gel analysis : The organo-mercurial compound, N-acryloylaminophenylmercuric chloride (APM), was used as a covalently linked component in a polyacrylamide gel. The tRNAs (0.2µg/sample) were analysed on a 12% vertical polyacrylamide gel, 2µg/ml APM, 7M urea, 50°C and the gels were run during 1h at 20V/cm. rRNAs (1µg/sample) were analysed on a 3% vertical polyacrylamide gel with a basis of 15% polyacrylamide, 1µg/ml APM, 7M urea , 50°C and the gels were run for 1h at 24V/cm. Both gels were run with buffer B. They were stained with ethidium bromide and photographed on a transilluminator.

E) Determination of the s^4Urd substitution level : This was achieved as established earlier (8) by measuring the absorbances at 260 nm, A_{260}, and 330 nm, A_{330}, of substituted ribonucleic particles or RNAs. The percent uridine substituted by s^4Urd is given by : $x = 282\ (A_{330} / A_{260}) + 1.5$

RESULTS

In our standard procedure, a preculture grown overnight at 30°C in absence of s^4Urd, was diluted to A_{650} = 0.05 into fresh medium supplemented with 100 µg/ml s^4Urd (thiolation culture) and growth was allowed to continue at 30°C until A_{650} reached 0.5. Under these conditions, the yield of stable 70S ribosomes remains low (30%). In one instance unexpectedly, a much higher yield (60 %) of thiolated (x~7%) stable 70S ribosomes was obtained from cells growing quasi-exponentially. This led us to reexamine closely the incorporations conditions.

1. State of cells and s^4Urd incorporation.

Since the temperature and medium conditions have been optimised previously (8), they were kept invariant and peculiar attention was payed to the state of cells immediately before thiolation. The importance of this parameter was emphasized by the following experiment. A standard overnight preculture was diluted, A_{650}=0.05, in medium without s^4Urd and allowed to grow at 30°C. The addition of s^4Urd 100 µg/ml when A_{650} reached 0.15 (sample 1) or 0.23 (sample 2) corresponding to N=1.6 and 2.2 generations is shown to hardly affect growth in strong contrast to cell behaviour under classical thiolation conditions. More than 85 % of the ribonucleic particles, RNP, extracted when A_{650}=0.5 are in the form of stable 70S ribosomes which exhibit a low thiolation level (respectively 1.5 and 0.7%). This result led us to carry out a systematic investigation. An overnight preculture was diluted in the s^4Urd less medium (A_{650}=0.05) and grown for a defined number N of generations, $1 \leq N \leq 6$. Each preculture is in turn diluted, A_{650}=0.05, in the standard thiolation medium and grown until A_{650} reaches 0.5. Cells were then collected and ribosomal particles extracted as described (8). The total amount of ribonucleoproteins, RNP, was determined by absorption measurement at 260 nm. The fraction of these RNP that remain associated as stable 70S particles was measured after sucrose gradient centrifugation performed in the presence of 10 mM Mg^{2+} and their thiolation level x was obtained as described in Material and Methods. For $1 \leq N \leq 2$ growth is unaffected, the fraction of stable 70S particles is higher than 90 % and substitution remains at an undetectable level (Fig. 1). A sharp transition is observed for $2 \leq N \leq 3$. Compared to the control, the growth-rate is reduced by ~40% and efficient thiolation occurs. The thiolation level x is maximal for N ~ 3.5 to 4. Clearly there is a tight correlation between x and the fraction of RNP in the stable 70S form.

The behaviour above could in principle be related either to N and/or to the cell concentration in the preculture. By realizing appropriate dilutions, precultures were prepared with N varying from 2 to 5.25 (when A_{650} reached 0.2). Each of these precultures was diluted into the thiolation medium at A_{650} = 0.05 and growth was continued until A_{650} reached 0.5. As shown in Table I both the fraction of stable 70S particles and their thiolation

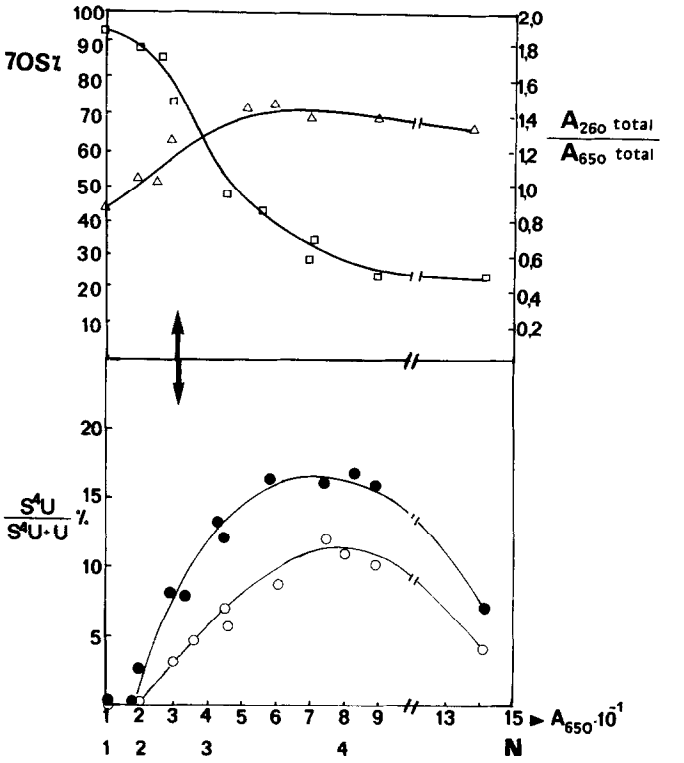

Fig.1 Thiolation of stable RNAs as a function of the state of the preculture :
- upper part : □ percent stable 70S (left scale) ; Δ yield of ribonucleoproteins determined from the ratio of RNP recovered (A_{260}) from a given mass of cells (A_{650}).
- lower part : thiolation levels of RNA in ●-●-● bulk RNP particles ; O-O-O stable 70S ribosomes.

level are in agreement with the data obtained in the previous experiment. Hence N is the predominant parameter governing the *in vivo* thiolation of RNA.

2. Optimization of thiolation conditions.

In our previous thiolation conditions (8), a preparation of "thiolated" stable 70S particles is expected to contain ~20% unsubstituted ribosomes originating directly from the

TABLE I

Thiolation level of 70S stable RNAs as a function of N

N	2	3	4.3	5.3
% stable 70S	90	55	25	25
% s^4U/U	<1	5.2	8	4

Overnight grown cells were diluted in the s^4Urd less medium at $A°_{650}$=0.05, 0.025, 0.01, 0.005 respectively and allow to grow until A_{650}=0.2. N is given by N.LN2=LN(0.2/$A°_{650}$).

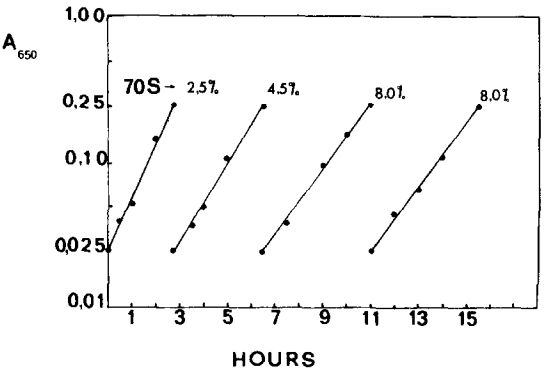

Fig.2 Growth of the pyrD strain, N~2.5, at 30°C in the presence of 100 µg/ml s^4Urd in the medium (cycle I) and 72 µg/ml in the following cycles. The s^4U/U level in stable 70S is indicated.

preculture. To "dilute" these non-thiolated ribosomes, incorporation was performed by successive growth cycles under the optimal conditions defined above. We noticed that cell growth in the s^4Urd containing medium remains fully exponential at $A_{650} \leq 0.3$. Hence, the preculture (N~2.5 to 3, A_{650} ~0.25) is diluted ten times in the thiolation medium and growth allowed to continue until $A_{650} = 0.25$ is reached. At this point, another dilution cycle is begun (cycle II). In general, the s^4Urd concentration at cycles II and III is lowered to keep the growth rate equal or higher than 50% of the control rate (Fig 2).

Table II shows that x increases in parallel in bulk tRNA, bulk RNP and stable 70S and that the maximal level of thiolation is reached after three cycles. For routine preparation of thiolated ribosomes, the thiolation cultures which were respectively 200 ml at cycle I and II were scaled up at 2 liters for cycle III to yield approximately 500 A_{260} units of bulk RNP. Under optimal conditions, x can reach 17 % in bulk RNP 26 % in the RNA from non associated particles and 10 to 15 % in the RNA from stable 70S. Dissociation and separation

TABLE II

s^4U/U levels (%) in various RNAs during a preparative thiolation experiments

cycle	0	1	2	3	4
tRNA	4	5	6	16	16
bulk RNP	0	4	9	17.5	18
stable 70S	0	2.5	4.5	8	8

400 ml thiolation culture were used for each cycle. The presence of s^4U in bulk tRNA is due to the naturally occuring s^4U_8 residue.

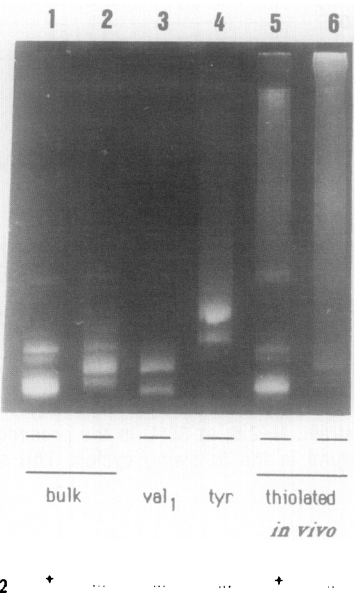

Fig.3 Electrophoresis of thiolated tRNAs on an affinity gel, polyacrylamide 12%, APM 1µg/ml. Lanes 1 and 2 : bulk *E.coli* tRNA, lane 3 $tRNA^{Val}$, lane 4 $tRNA^{Tyr}$, lanes 5 and 6 tRNAs obtained from an *in vivo* thiolation experiment (s^4U/U=16%). Conversion of s^4Urd into Urd was achieved by H_2O_2 treatment (2M) at pH7 for 2 hours at 40°C and the tRNAs recovered by precipitation with ethanol.

of 30S and 50S particles on sucrose gradient generally decreases x. Currently, we obtain (80±20) A_{260} units of 50S particles, (45±15) A_{260} units of 30S with x values in the range 5 to 10 %.

3. Affinity electrophoresis of thiolated RNAs.

In order to characterize the *in vivo* thiolated RNAs, we have used the affinity electrophoresis technique developed by Igloi (11). The polyacrylamide gel is polymerised in the presence of an organomercurial derivative of acrylamide (APM). The mercury atoms specifically interact with the ARN thiol groups slowing down their migration. The migration of *in vivo* thiolated tRNAs and rRNAs (obtained from 70S stable particles) after three thiolation cycles have been determined together along with controls (Fig.3). As shown in Fig.3A, removal by H_2O_2 treatment of the s^4U residues found at position 8 in the sequence of 70 % of the molecules in bulk tRNA clearly increases the migration rate as expected. The $tRNA^{val}_1$ sample exhibits two bands, the faster one being presumably due to a small fraction of non-thiolated or 8-13 crosslinked tRNA. $tRNA^{Tyr}$ in principle contains two s^4U in position 8 and 9 respectively and, for this reason, the major band migrates slowly on the gel (11). The minor faster band is certainly due to $tRNA^{Tyr}$ molecules thiolated only in one of the positions 8 or 9. The *in vivo* s^4Urd substituted tRNA sample exhibits a highly heterogenous behaviour, the major fraction being retained at the upper part of the gel with practically no molecules migrating to the position of the control. H_2O_2 treatment restores

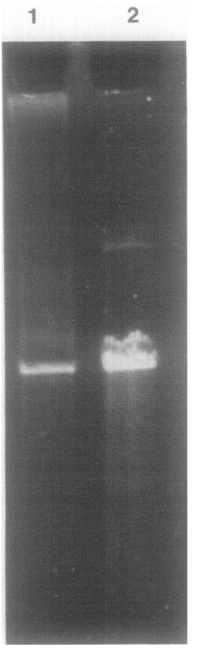

Fig.4 Electrophoresis of 16S RNA on an affinity gel, polyacrylamide 3%, APM 1µg/ml. lane 1 *in vivo* thiolated RNA; lane 2 control RNA.

the behaviour of the control tRNA. The retarded fractions which remain are presumably due to incomplete dethiolation or formation of intramolecular crosslinks. The migration of independant preparations of 16S (and 23S RNA) was checked on the APM gel. All thiolated samples exhibit a highly heterogeneous behaviour as shown in Fig. 4 for 116S RNA.

DISCUSSION

The *in vivo* incorporation of 4-thiouridine into *E.coli* rRNAs in our thiolation conditions appears to be critically dependent upon the "age" of the preculture, i.e. the number N of exponentially growing generations undergone by the cells before contact with the thiolation medium. When $1 \leq N \leq 2$, the growth behaviour remains unaffected and no incorporation can be detected. It is not yet clear whether s^4Urd can be transported (or efficiently reexported) under these conditions or whether the nucleoside is rapidly converted *in vivo* into Urd or into derivatives that do not affect cell metabolism. One known example of an enzymatic activity able to modify s^4Urd is *in vitro* conversion into the disulfide form by horseradish peroxidase (12). For $2.5 \leq N \leq 4$ i.e. with fully exponentially growing cells significant incorporation occurs in rRNAs. At the same time, both the cell RNP content and the proportion of non associated RNP increase. It appears likely that s^4Urd incorporation into rRNAs prevents some maturation and/or rRNA-r protein interaction step or transconformations leading to a block in 50s and 30s particle association (8). Hence, the

concentration of functional ribosomes within the cell would become limiting with a corresponding decrease of the rate of protein synthesis. A limited amount of functional ribosomes would also result in derepression of rRNA neosynthesis and would provide an explanation for RNP accumulation. In any case a stringent relA dependent effect due to inactivation of an eventual tRNA species can be excluded. Finally when the thiolation culture is initiated with cells in the prestationnary state, growth becomes linear in spite of less efficient s^4Urd incorporation (Fig.1). This could be due at least in part to the initially low supply of functionnally active ribosomes.

From these observations we have selected "optimal" conditions which associate an appreciable yield of stable 70S ribosomes (~ 65%) with efficient thiolation of RNAs and exponential growth. After several growth cycles (Fig.2), the thiolation level reaches 8(±2)% in 70S particles, corresponding to the incorporation of 70-75 s^4Urd residues. As shown in Table II, the degree of thiolation increases in parallel in tRNAs and bulk rRNAs in agreement with the suggestion that *E.coli* RNA polymerase incorporates s^4U residues at random. This view also fits with the migration behaviour of bulk thiolated tRNA on the affinity electrophoretic gel (Fig.3). Clearly, few tRNA chains have less than two s^4U residues as expected from the random distribution of s^4U, x~16%, in a "mean" tRNA having 17 positions available for substitution. On the other hand, the s^4U distribution is definitively not random in rRNAs as shown by i) the widely distinct thiolation levels in non-associated ribosomal particles and in 70S ribosomes (Table II) ; ii) the behaviour of thiolated 16S (23S) RNA on the APM gel. In different experiments between 30 to 50% of the 16S RNA molecules extracted from stable particles were found to migrate at the control positions. In view of the tRNA behaviour discussed above it is difficult to understand how these rRNA chains could completely escape *in vivo* thiolation. Rather it is likely that they contain a few s^4U residues at positions which in the folded RNA conformation are not available for interaction with the mercury atoms in the gel. From our previous results there are at least two sources of heterogeneity in rRNA thiolation. First substitution at RNA key positions certainly prevents some rRNA precursors maturation and protein association steps, leading to highly thiolated non associated particles (8). Second chemical dethiolation during ribosomes reconstitution may well occur in line with the formation of s^4U mediated "dark" covalent RNA-protein crosslinks in stable 70S ribosomes (8).

Photoactivation (335 or 366 nm) increases the yield of crosslinking by a factor of 2.5 (8) and 30S particles prepared as described here have been used to study RNA-protein interactions in domains 1 and 2 of 16S RNA (13). The procedure certainly allows thiolation of all *E. coli* cellular RNAs and can be extended to RNAs directed by exogenous genomes such as plasmids and bacteriophages. Subsequent near-ultraviolet light irradiation may result in *in situ* fixation of RNA-protein interactions. The procedure can be extended to eucaryotic cells. s^4Urd is readily incorporated in germinating radish seeds (14) and animal cells in

culture (15-16) and efficient RNA-protein photocrosslinking has been demonstrated in CV-1 cells (16).

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