THE RABBIT RETICULOCYTE LYSATE AS A SYSTEM FOR STUDYING mRNA

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Introduction

In this article we describe the use of the rabbit reticulocyte lysate as an assay system for added mRNA. Some of the techniques we describe are familiar, but we have recently realised that the system can be used in rather subtile ways to provide additional information about the RNA under study. Although our own studies with viral RNA are in their early stages, we believe that the approaches we suggest here will be generally useful to a wide range of workers in all fields of virology. We are taking the opportunity of describing our methodology in considerable detail in the hope that it may be practically useful.

Preparation of reticulocyte lysates

We use rabbits weighing 2 – 3 kg and make them anaemic by injecting them subcutaneously with 2.5 ml of 1.25 % w/v acetylphenylhydrazine on four successive days. We bleed them by cardiac puncture on the ninth day of the schedule. The long gap between the last injection of phenylhydrazine and the collection of the blood allows the damage caused by the phenylhydrazine to be repaired so that although we obtain a lower proportion of reticulocytes the haematocrit is almost 100 % higher, and the reticulocytes are much more consistently active in protein synthesis.

The blood is collected with heparin as anticoagulant, and the reticulocytes collected by centrifugation in a refrigerated centrifuge. They are washed three times with ice-cold saline (0.13 M NaCl, 5 mM KCl, 7.5 mM $MgCl_2$) and lysed with 1.5 volumes of ice-cold water per volume of packed cells. The debris are removed by centrifugation at 30,000 g for 15 min at 4°. The supernatant from this spin is stored as 1–2 ml aliquots under liquid nitrogen. Such a preparation seems to be stable indefinitely if stored like this.

Incubation conditions for protein synthesis

The lysate is thawed carefully by rolling in the palm of the hand, and is made 20 μM in haemin before it has completely melted. It is very important not to let the lysate warm up before adding the haemin, and it is also vital to make the haemin solution up correctly – the source of the haemin is not critical. It is made up at a final concentration of 1 mM in approximately 90 % ethylene glycol at pH 8–8.5 by taking a weighed amount of haemin and dissolving it in a small volume (0.2 ml) of

0.5 N KOH together with enough Tris-Cl pH 8.0 to give a final concentration of 50 mM. About 3/4 of the final volume of ethylene glycol is added, and the pH adjusted to around 8 with 1N HCl by rapid swirling or stirring on a pH meter. It is important to avoid local excess of acidity which precipitates the haemin, and to keep the final pH above 7.5. The final volume is adjusted with ethylene glycol, and the concentration checked spectroscopically by adding a sample to 10 mM KCN and reading the absorbance at 540 nm; 1 mM cyanmethaem has an absorbance of 11.1 at this wavelength.

To get protein synthesis by the lysate it is necessary to do three things: adjust the K^+ and Mg^{++} concentrations, add extra amino acids (while the pools of e. g. arginine and alanine are very large, others like leucine, phenylalanine and methionine are present at only a few μmoles/1. Failure to add amino acids causes a serious impairment of the rate and extent of protein synthesis by the lysate). Lastly, a source of ATP and GTP is necessary. For years we added ATP, GTP and creatine phosphate, but in fact creatine phosphate alone is sufficient and much more convenient, since its solutions are neutral, and it does not chelate Mg^{++}. The extent of protein synthesis is linearly dependent on added creatine phosphate up to about 6 mM, and we now add 10 mM to be safe.

So a 'standard incubation mixture' contains the following components: 0,8 ml of lysate, 0.05 ml of salts solution (2 M KCl, 10 mM $MgCl_2$), 0.05 ml of 0.2 M creatine phosphate, 0.05 ml of labelled amino acid solution, and 0.05 ml of an amino acid mixture with the following composition: Ala 3 mM; Arg 0.5 mM; Asn 0.5 mM; Asp 2.0 mM; Cys 0.5 mM; Gln 0.5 mM; Glu 2.0 mM; Gly 2.0 mM; His 2.0 mM; Ile 0.5 mM; Leu 3.0 mM; Lys 2.0 mM; Met 0.5 mM; Phe 1.5 mM; Pro 1.0 mM; Ser 2.0 mM; Thr 1.5 mM; Try 0.5 mM; Tyr 0.5 mM; Val 3.0 mM. One or more of these will be omitted according to the label being used. This mixture is neutralised with KOH to pH 7.5 and is made 10 mM in dithiothreitol to keep Cys and Met in good shape. It is stored, as is the creatine phosphate solution at −20°. Besides these carefully measured components, creatine kinase is added as 'a few crystals' – about 0.1 mg/ml final concentration.

When additions are to be made, one can either add 5–10 % of the final volume and trust that this minor dilution of the standard mixture will have only a slight effect, or alternatively use less lysate and make up the volume with the solution of inhibitor or mRNA. Either way works perfectly well.

Analysis of synthetic products

It is usually wise to monitor the time-course of incorporation of label into TCA insoluble material, since this is often an indication of the nature of problems when they arise (as they often do, particularly with unknown mRNA). Samples of 2–10 μl are removed with a Hamilton microsyringe into 0.2–1.0 ml of distilled water, and the syringe washed up and down 3 or 4 times in the water. This allows one to take successive samples from an array of tubes quickly and accurately with hardly any cross-contamination between samples; the dilution with water stops incorporation completely. When sampling is over, 0.5 ml of 1N NaOH containing roughly 1 mg/ml unlabelled amino acid is added to each sample, and after 15 min at 37° 1 ml of 25 % TCA is added. The samples are filtered into Whatman GF/C

filters, washed with 8 % TCA and glued to cardboard discs for counting in a gas-flow counter. If it is necessary to count the samples in a scintillation counter, the colour of the haemoglobin has to be removed, either by organic solvents like acidified acetone, or by bleaching with H_2O_2. The latter is more convenient, and can be achieved by adding 0.1 ml of 30 % H_2O_2 to the samples before adding the NaOH. After 5 minutes or so, while there is some evolution of O_2, the samples go colourless.

Analysis of samples by SDS-polyacrylamide gel electrophoresis is straightforward if the system of Laemmli is used (1). The interesting thing about these gels is that they allow excellent resolution of proteins with higher molecular weight than globin despite the outrageous overloading with globin (2, 3).

Sucrose gradient analysis

In order to obtain sharp patterns of polysomes and ribosomal subunits from the lysate it is vital to dilute the sample first. Our typical system is to make 50 µl incubations and to stop the reaction by adding 200 µl of ice-cold SMISH buffer (25 mM KCl, 10 mM NaCl, 10 mM Tris-Cl, 1 mM $MgCl_2$, 0.25 mM DTT, pH 7.5). The whole samples is then layered over a 5 ml 15–30 % w/v sucrose gradient in SMISH buffer and spun at 50,000 rpm in the SW 50.1 rotor for periods ranging from 30 min for resolution of polysomes to 2.5 hours for looking at subunits. The temperature is normally 2°. After the run, usually with the brake off (our experience is that faulty or excessive braking is the commonest cause of bad results), the gradients are pumped through a recording spectrophotometer into a fraction collector. The fractions are usually counted by precipitation with 1 % cetyltrimethylammonium bromide (CTAB) in the presence of 0.25 M Na acetate pH 5.1 (the low pH minimises discharge of tRNA) and about 500 µg of carrier RNA. A solution of 2 % CTAB in 10 mM NaAc buffer is added first, followed by an equal volume of 0.5 M NaAc buffer containing the carrier RNA. A precipitate forms at once, and is allowed to clot slightly before filtering and washing with water. This procedure has the advantage of selectively precipitating all RNA species (hence aminoacyl-tRNA and peptidyl-tRNA) while solubilising most proteins, including globin. This gets round the serious problem of colour quenching at the top of the gradients.

Shift assays

The rationale of these assays is described below; this section simply gives some critical experimental details. A lysate is incubated under the standard conditions except that a solution of 2 mM sparsomycin is substituted for the labelled amino acid. The first incubation is usually for 5 min at 30°. The tube is then chilled on ice, and 2 µl of ^{35}S-met-tRNA$_f$ (10,000–100,000 cpm) added, followed by 1–5 µl of mRNA dissolved in water, or an equivalent volume of water for the control. The tubes are incubated for a further 2 min at 30° and are then analysed on sucrose gradients as described above (typically a 2 hr centrifugation). This assay can be modified by using 50 µg/ml diphtheria toxin and 0.15 mM NAD with 0.1 mM puromycin instead of the sparsomycin. The latter system gives a higher yield of

active 40S subunits and hence of initiation complexes, and also allows met-X dipeptides which sparsomycin tends to prohibit (because it inhibits peptide bond formation; diphtheria toxin + NAD is a translocation inhibitor). Another modification, which does away with the need for purified met-tRNA$_f$ is described below.

Translation of added viral RNA

The classic mRNA for bacterial cell-free systems is the genome of the small RNA phages. These studies have allowed many insights into viral physiology to be gained. We presume that similar studies with the genomes of eukaryotic RNA phages will also be helpful in understanding their biology. But we note that eukaryotic cell-free systems translate bacterial mRNA poorly if at all, and feel strongly that studies in which bacterial cell-free systems are used to translate eukaryotic mRNA are irrelevant if not misleading from the true goal.

Most published studies of the translation of viral RNA in eukaryotic systems concern the translation of EMC RNA in a cell-free system from krebs II ascites cells. They have not been terribly illuminating in terms of understanding either protein synthesis or viral physiology; the same can be said for most successful efforts at translating added mRNA in heterologous systems. One succeeds chiefly in adding support to the concept of mRNA, a task once vitally important but rather hackneyed by now. There are several interesting points still to be clarified, however. First, the lysate system is strongly inhibited by added EMC RNA, whereas the ascites system is stimulated (4, 5). The inhibition is relieved somewhat by adding S100 from ascites cells to the lysate, and such an addition also allows some translation of the RNA. This phenomenon is not at all understood; apparently a protein and an RNA are responsible for the effect (4). The inhibition of the lysate by added RNA is common; sometimes it is due to trivial effects such as contamination of the preparation with phenol or heparin, which can be removed by washing with 3M Na Acetate pH 6 or 2M LiCl (6). In other cases it may be that the viral RNA competes effectively for ribosomes with the endogenous globin mRNA, but that factors necessary for the completion of translation are missing – this may be the case with EMC RNA. Another common cause of inhibition is the presence of double stranded RNA. The lysate is sensitive to as little as 0.01 ng/ml dsRNA (7), so that amounts of contamination with viral RF, RE or RI too low to be detected by conventional means can cause trouble. A good example of this was provided by McDowell *et al.*(8) who obtained good synthesis of viral proteins specified by *in vitro* synthesised reovirus RNA, but who also found that synthesis in the lysate stopped abruptly after only 10 min when they added the RNA. Inhibition by added RNA is not necessarily a disaster, therefore. In principle it is possible to abolish this inhibition by treating the RNA with RNase III from E. coli (9), but this is not a commercially available enzyme and is reputedly difficult to purify. It may be that if an RNA inhibits the lysate with the characteristic kinetics of dsRNA (a brief uninhibited phase followed by an abrupt cessation of synthesis) this is the best possible evidence that the RNA does contain true dsRNA. We have tested Sendai RNA in the lysate, and found that it behaved as if it were mostly dsRNA. Of course it would be naive ever to expect the lysate to be stimulated by added mRNA unless a protein

with an unusual content of the amino acid used as label were being synthesised. Most of the ribosomes are already active in protein synthesis, and the most one can hope for is therefore to wean them off globin mRNA and into the added message.

We have recently done some experiments with TMV RNA as a model for this kind of work, and have been impressed with a number of somewhat disturbing features. It is easy to prepare both TMV and extract the RNA from the virions with phenol and SDS, but although the RNA obtained was in our hands about 75 % pure 27S, it inhibited synthesis overall rather strongly by inhibiting initiation of protein synthesis. However, as more TMV RNA is added, viral products predominate over globin. We have not detected the synthesis of coat protein and the products are a collection of proteins with a modal MW of about 70,000; unless that is one uses doses of RNA below 100 μg/ml, in which case there is only one product, a band at 140,000 daltons. At concentrations above 100 μg/ml there is no detectable synthesis of this band. At first we thought that this might be due to preferential use of the smaller RNA fragments present in the preparations of RNA over the intact RNA, but this was shown to be unlikely by adding pure 27S RNA, and finding that it behaved exactly the same way. We do not yet know whether we are seeing false initiation, premature termination or some kind of specific proteolysis. A further finding was that the purified TMV RNA did not inhibit endogenous synthesis at all, even at inputs of 200 μg/ml, at which level the crude material inhibited by 90 %. We cannot account for these findings, but if they have any generality it means that one should probably try to use added mRNA at more physiological input levels, and also interpret the data obtained by this kind of experiment very cautiously.

The shift assay for mRNA

Since a protein cannot be made without initiating its synthesis, one can use the relative numbers of proteins made as a measure of the relative effectiveness of two mRNA species at competing for a limited supply of ribosomes. However, because proteins can be modified – at the extreme destroyed – after their synthesis, and because factors other than the initiation of their synthesis may affect their production it would be very nice to have an assay for initiation which did not rely on protein synthesis. We have recently stumbled on such an assay in the course of studies of the mechanism of initiation of protein synthesis in the lysate. We found that native 40S subunits can be labelled with ^{35}S met-tRNA$_f$ even though protein synthesis is completely inhibited by a wide variety of inhibitors which do not affect the initiation of synthesis – e. g. sparsomycin, cycloheximide and diphtheria toxin. Such 40S/met-tRNA$_f$ complexes are natural intermediates in the initiation of protein synthesis, and the next step in the pathway is the binding of mRNA, followed by attachment of the 60S subunit to give an 80S initiation complex. Thus if one adds labelled met-tRNA$_f$ to a standard incubation which has been preincubated with an inhibitor of synthesis and analyses the reaction on a sucrose gradient, one finds label only at the top of the gradient and on the 40S subunits. When mRNA is added, however, there is a shift of counts from the 40S subunits to the 80S region, and the degree of shift is proportional to the amount of mRNA added. This provides an extremely sensitive assay for mRNA – we can easily detect 0.2 μg of globin mRNA (1 pmole) contained in 1–5 μl of solution. Besides this

sensitivity, the assay has the advantage that one can immediately tell whether the RNA in question has only one or more than one binding sites for ribosomes, since a length of RNA which has two binding sites will cause a shift of label from 40S to the dimer region of the gradient. We have some suspicions about TMV in this respect, but it is quite certain that we can detect such goings-on because random AUG can cause polysome formation when assayed at the appropriate input level. Besides watching the shift and measuring its extent, one can also take the shifted counts and analyse them to see what the met-X dipeptides are. Here again one may pick up multiple initiation sites by finding more than one new dipeptide (though one has to be sure that tripeptides are not being formed). This suggests the possibility of doing direct competition experiments between different mRNA species. Such experiments can be done very easily if the second amino acid specified by each of two mRNAs is known, since one can add labelled tRNA corresponding to these amino acids instead of met-tRNA$_f$. This procedure is complicated by the need to correct for the different pool sizes of each species of tRNA, and yet another way of doing competition experiments is to use labelled mRNA. We have shown that one can use the shift assay to detect binding of labelled sea-urchin histone mRNA and globin mRNA, but so far we have not pursued these studies to their natural conclusion. Neither have we tried the other obvious experiment of using this assay as a means of purifying mRNA from mixtures with neutral RNA.

Finally, it is worth pointing out that one can do shifts without the need for making met-tRNA$_f$, which is the limiting step in these experiments. The problem is to inhibit globin synthesis but still get charging of the tRNA pools with added ^{35}S methionine; the solution is to incubate the lysate with 0.1 mM puromycin before adding the label, and later adding either sparsomycin or diphtheria toxin and NAD followed by mRNA. The background of counts on ribosomes is higher than in the classic shift (10), but is perfectly acceptable, as can be seen in figure 1. We have not yet analysed the dipeptides formed in this reaction.

Conclusion

The reticulocyte lysate has two great advantages over other existing systems which can be used for studying protein synthesis by added mRNA. It is easier to use and prepare than the ascites cell-free system, the Xenopus oocyte system, or the system of Schreier & Staehelin (11). It is much more active than any of these systems, and although its endogenous activity is high, at least this activity is well characterised so that the possibility of confusion of endogenous synthesis with the new synthesis is in practice not much of a problem unless the new products are exceedingly diverse. Its low nuclease content is a further advantage, though we would be wrong in claiming that no nuclease activity exists.

People often accuse the reticulocyte of being an atypical cell and therefore a poor model system for the more interesting aspects of control of protein synthesis. We believe this to be an envious and empty criticism; it may be true that the lysate lacks the ability to translate EMC RNA efficiently, and that intact reticulocytes fail to respond to amino acid starvation in the way that most cells do, but these failures actually make it a better system for studying these effects, since it constitutes an

assay system for the factors involved. In no case, not even the case of the requirement for added haemin to prolong linear protein synthesis, is there an example of the reticulocyte doing something that other cells do not also do, except yield highly active cell-free systems.

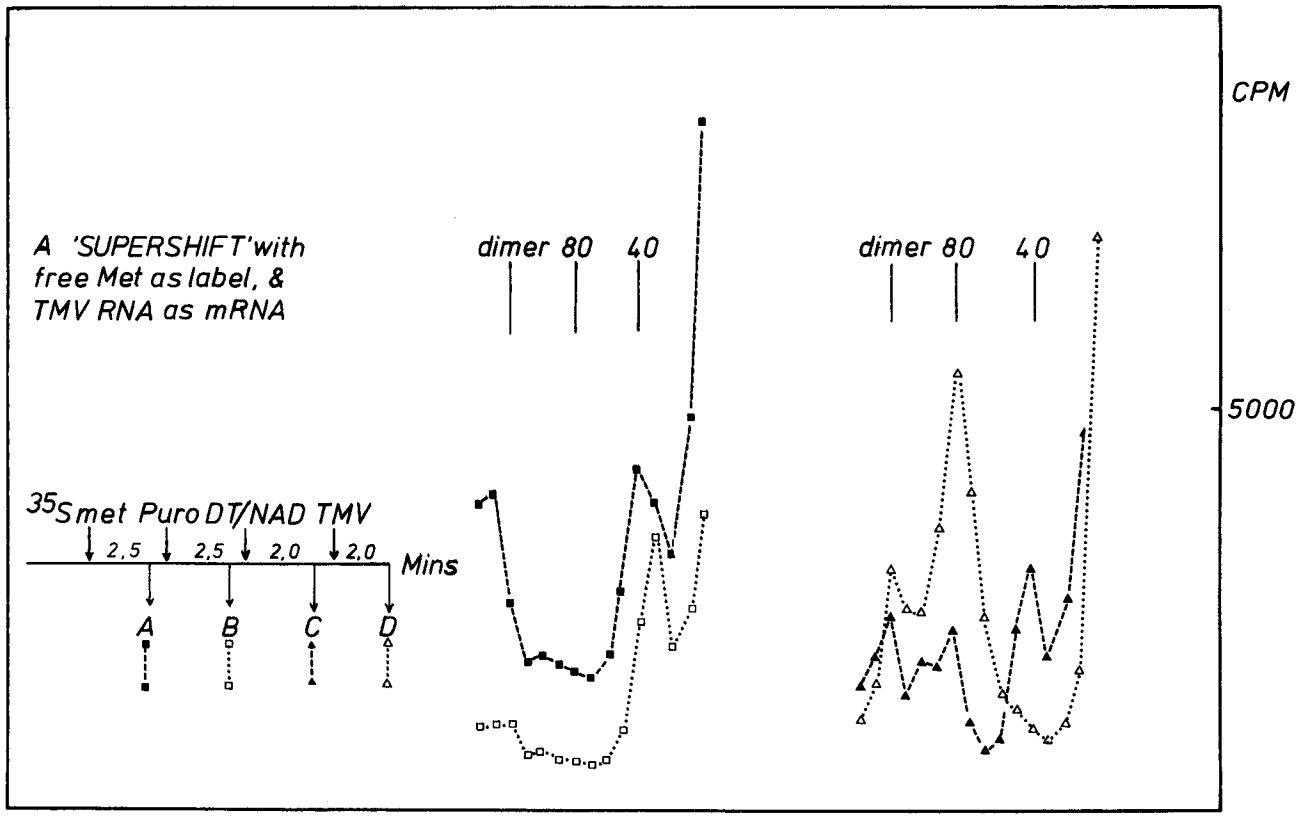

Fig. 1:
A standard incubation mixture was incubated at 30 ° with ^{35}S methionine. After 2mins incubation a sample was removed and diluted with SMISH buffer as described in the methods section. At $2\frac{1}{2}$ min, the mixture was made 1 x 10^{-4}M in puromycin, and another sample of the mixture removed for gradient analysis at $4\frac{1}{2}$min. At 5 min, the mixture was made 50 μg/ml in diphtheria toxin and 0.15 mM in NAD, and a samples removed for analysis at 7 min. Finally, 5 μg of TMV RNA was added to the remaining 50 μl of mixture, and the last sample taken 2 min after this addition. The four samples were then analysed by sucrose gradient centrifugation for 1.5hr on a 10 % – 30 % gradient in SMISH buffer at 45,000 rpm and 2 ° in the spinco 50.1 rotor. The gradients were fractionated and the radioactivity determined by CTAB precipitation.

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