DNA-RNA HYBRIDISATION OF 9S MESSENGER RNA FOR MOUSE GLOBIN

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Summary: Milligram amounts of 9S messenger RNA for mouse globin have been isolated from mouse reticulocytes, and are estimated to be 90% pure using polyacrylamide gel electrophoresis. The 9S RNA has been methylated with tritiated dimethylsulphate, to a specific activity of approximately 7,000 dpm/μg. The methylated RNA has been hybridised in 50% formamide/2xSSC at 37°C to caesium chloride-purified DNA. Hybridisation occurs to 0.5% of the DNA under conditions of RNA excess (RNA:DNA ratio of 6). The hybridised RNA can be competed to 100% with unlabelled 9S RNA, and only gives a small amount of competition with ribosomal RNA. The hybridisation is complete in less than ten minutes. Several alternative explanations for these findings are discussed.

There is now considerable evidence that the 9S RNA component found in mammalian reticulocyte polysomes is the messenger RNA for globin. It has different labelling kinetics from ribosomal RNA (1,2) and a different oligonucleotide fingerprint pattern (3). It is the only component to disappear after disruption of polysomes to monosomes with low levels of RNase (4) or with sonication (5). 9S RNA has been reported to stimulate mRNA-specified globin synthesis in heterologous protein synthesizing systems from rabbit/*E. coli* (6), two mutant strains of rabbit (7), and most convincingly, mouse/rabbit (8).

Experimental:

We have found that mouse reticulocytes give reproducible preparations of 9S RNA. Reticulocytes were collected from phenylhydrazine-treated mice, and the buffy coat discarded. The reticulocytes were lysed in two volumes of 0.001M $MgCl_2$ and the lysate centrifuged at 10,000 rpm for 15 minutes. RNA was prepared from the supernatant using the 4-aminosalicylic acid/phenol/m-cresol/-8-hydroxyquinoline method of Kirby (9). The 9S RNA was separated from the 28S, 18S and 5S ribosomal components and the 12S RNA found in reticulocytes (3,5,6) using a combination of zonal ultracentrifugation and preparative polyacrylamide gel electrophoresis (10). The detailed preparative procedure and properties

of the 9S RNA will be described elsewhere (11).

Since reticulocytes are anucleate, cell cultures cannot be used to obtain radioactive RNA at high specific activity in this system. Unlabelled 9S RNA was therefore methylated with tritiated dimethylsulphate (Radiochemical Centre, Amersham, Batch 4) using the following modification of the method of Smith et al. (12). 500 μg of RNA were dissolved in 0.2 ml water and 0.3 ml of an ether-benzene solution containing 1 mC dimethylsulphate was added. The mixture was shaken at room temperature for 2 hours. The RNA obtained has a specific activity of 7-9,000 dpm/μg, and is unaltered in behavior on sucrose gradients and polyacrylamide gel electrophoresis. There is evidence that the behavior of methylated RNA in DNA:RNA hybridisation is the same as that of identical RNA labelled in vivo (12).

9S RNA was also prepared from the polysomes of 14½-day mouse embryonic liver after disaggregation and culture in the presence of tritiated uridine. The RNA obtained had a specific activity of 20,000 dpm/μg, but could not be prepared in as pure a state as the RNA isolated from reticulocytes.

Total 17-day mouse embryo DNA used for hybridisation was purified by banding in CsCl. The hybridisation method was that of Gillespie and Spiegelman, as modified by McConaughy et al. (13). 20 μg of DNA were used per

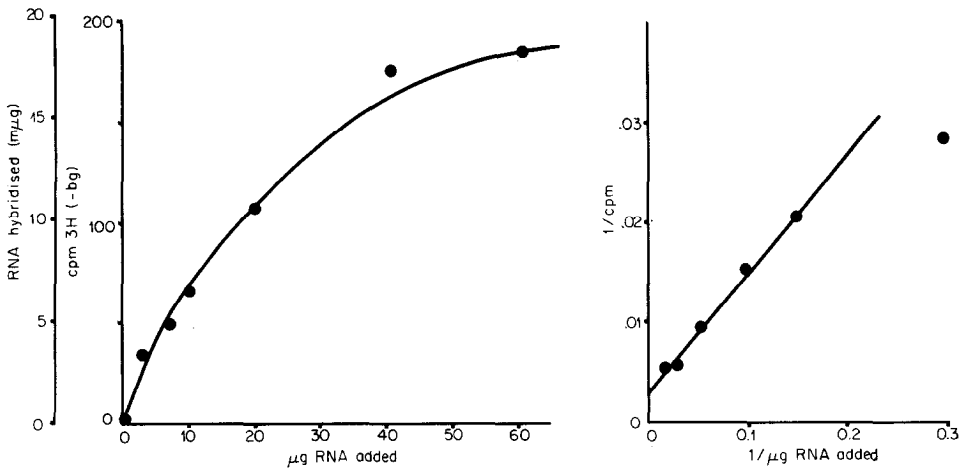

Figure 1: Saturation curve and 'double-reciprocal' plot of hybridisation of mouse 9S mRNA with total mouse embryo DNA. 3H-methyl labelled 9S RNA (2,940 cpm/μg) incubated with 13 mm filters, each containing 20 μg DNA, in 0.2 ml 50% formamide/2xSSC at 37°C for 16 hours. Counts corrected for blank filter backgrounds. At saturation 0.47% of the DNA hybridises to RNA, given by the intercept of the statistical regression line (P = 0.025).

filter. The hybridisation was for 16 hours in 50% formamide/2xSSC at 37°C. After hybridisation the filters were treated with RNase and exhaustively washed. Tests were incorporated using tritiated DNA to ensure that under our conditions the DNA was both initially retained on the filter and remained on the filter after hybridisation. A saturation curve and 'double-reciprocal' plot (to give an estimate of the RNA hybridised at infinite RNA input) is shown in Fig. 1; the saturation value corresponds to 0.5% of the DNA complementary to RNA. This is equivalent to 50,000 copies of a gene for a 9S RNA (mol.wt.= 150,000). The RNA hybridised can be competed to 100% with unlabelled 9S RNA (Fig. 2), but only to 10-15% with ribosomal RNA. The backgrounds for filters containing either no DNA or heterologous (E. coli) DNA are only 1-2 counts above machine background. Similar saturation levels were obtained for 14½ day embryonic liver RNA labelled in cell culture.

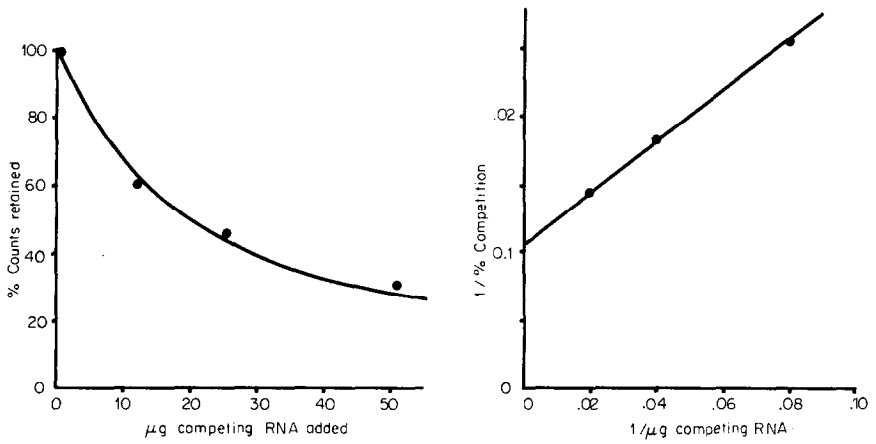

Figure 2: Competition of 3H-methyl labelled 9S RNA (2,179 cpm/μg) with unlabelled 9S RNA. Hybridisation conditions as in Fig. 1. The experimental points (corrected for non-saturation conditions) in the left hand figure fall on the theoretical competition curve. The intercept of the 'double-reciprocal' plot shows greater than 95% homology between the methylated and unlabelled 9S RNAs, indicating that methylation does not affect hybridisation under these conditions.

The time course of the hybridisation is shown in Table 1. Even though this experiment was not carried out at fully saturating conditions, the hybridisation was completed in less than ten minutes.

Time (min.)	cpm hybridised (duplicates corrected for blank filter bg.)
10	60, 57
30	70, 76
120	52, 48
480	68, 62
3,000	68, 66

TABLE 1: Time Course of Hybridisation

Each vial contained, in 0.2 ml 50% formamide/2xSSC, 20.3 μg 3H-methyl-9S RNA, 2,938 cpm/μg, two 13 mm filters containing 20 μg mouse embryo DNA and 1 blank 13 mm filter.

In an attempt to determine the stability of the hybrid (a measure of the exactness of base pairing between the DNA and the RNA) hybridised filters which had not been treated with RNase were incubated at increasing temperatures and the counts released were measured. The melting curve is both polydisperse and the Tm well below that expected for hybrid with an exact DNA:RNA base pairing. It was found that the Tm of the hybrid after RNase treatment was very low, indicating that the RNase had reduced the size of the hybridised lengths very considerably, probably by nicking at badly paired regions of the RNA.

We have found little difference in hybridisation saturation level for total mouse embryo DNA and DNA isolated from 14½ day mouse liver, an erythropoietic tissue.

Discussion:

The interpretation of hybridisation data from mammalian sources is made more complicated by the existence of 'families' of DNA which differ in their multiplicity and relatedness (14,15). Under our hybridisation conditions, for purely kinetic reasons, most of the RNA will hybridise only to reiterated sequences of DNA. It is thus not only impossible to draw conclusions from hybridisation data about the absolute number of sequences transcribed, but also very difficult to determine what fraction of the measured hybridisation values represent annealing to exactly complementary DNA sequences or to

slightly different sequences of the same family. It is not yet known whether the members of families of DNA sequences have any functional relationship to one another, or are fortuitously similar enough to cross-hybridise.

The combination of very rapid hybridisation kinetics (indicating that at least one of the components is present at very high multiplicity) and the gradual saturation curve requiring high RNA:DNA ratio for saturation is difficult to reconcile. We would suggest the following alternatives:

a) There is a minor component present in the RNA which hybridises to a DNA of very high multiplicity. The kinetics would allow for such a situation, but not for a mixture of contaminating RNAs each hybridising to a DNA family of relatively low multiplicity (say, 100-500). Thus the possibility that the result is due to contamination with a variety of mRNAs for the other enzymes made in very low amounts by reticulocytes is excluded by the kinetics, but not contamination by one or two minor RNA species which hybridise to a DNA sequence repeated at a multiplicity of tens of thousands.

It has been previously reported that nuclear RNA from duck reticulocytes thought to contain globin mRNA precursor hybridises to 7% of the DNA, also a high value (16).

b) Exact gene reduplication for globin is contra-indicated by the low melting temperature of the hybrid. If the hybridised DNA contained only copies of reduplicated globin genes, the hybrid would be expected to give a sharp melting curve similar to that for ribosomal RNA. However, it is known that unrelated proteins often have similar short stretches of amino acid sequences, some 8-10 residues long (17). It is possible that the high level of hybridisation is due to pairing with short stretches of genes specifying other proteins. Poor complementarity would then be expected.

c) It has been suggested, using data obtained from polyacrylamide gel electrophoresis (3,11) that the molecular weight of the 9S mRNA for globin exceeds that required to code for the globin polypeptide chain by as much as 25%. If such additional sequences exist, and are not an artifact caused by the absence of secondary structure in the mRNA as compared with the

ribosomal RNA standards, they might be involved in initiation or completion sites, in a 'ticketing' control mechanism (18), or be sequences transcribed from 'control' DNA contiguous with the 'messenger' DNA (19,20). Any of these types of sequences might be expected to hybridise to many regions of the genome apart from that specifying globin. However, there is evidence from sedimentation equilibrium experiments on 9S RNA, which are less sensitive to secondary structure effects, that the molecular weight of the messenger may be exaggerated when determined by polyacrylamide gel electrophoresis (21).

Experiments are in progress to distinguish between these possibilities.

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References:

1. Chantrenne, H., Burny, A. and Marbaix, G., Prog. Nucl. Acid Res. Mol. Biol., 7, 173 (1967).
2. Evans, M.J. and Lingrel, J.B., Biochemistry 8, 3000 (1969).
3. Labrie, F., Nature, 221, 1217 (1969).
4. Marbaix, G. and Burny, A., Biochem. Biophys. Res. Comm., 16, 522 (1964).
5. Williamson, R., Lanyon, G. and Paul, J., Nature, 223, 628 (1969).
6. Laycock, D.G. and Hunt, J.A., Nature, 221, 1118, (1969).
7. Schapira, G., Dreyfus, J.C. and Maleknia, N., Biochem. Biophys. Res. Comm., 32, 558 (1968).
8. Lockard, R.E. and Lingrel, J.B., Biochem. Biophys. Res. Comm., 37, 204 (1969).
9. Kirby, K.S., Biochem. J., 96, 266 (1965).
10. Lanyon, G., Williamson, R. and Paul, J., FEBS Letts., 1, 279 (1968).
11. Williamson, R., Morrison, M., Lanyon, G. and Paul, J., manuscript in preparation.
12. Smith, K.D., Armstrong, J.L. and McCarthy, B.J., Biochim. Biophys. Acta, 142, 323 (1967).
13. McConaughy, B.L., Laird, C.D. and McCarthy, B.J., Biochemistry, 8, 3289 (1969).
14. Britten, R.J. and Kohne, D.E., Science, 161, 529 (1968).
15. Davidson, E.H. and Hough, B.R., Proc. Natl. Acad. Sci., 63, 342 (1969).
16. Scherrer, K. and Marcaud, L., J. Cell Physiol., 72 Suppl. 1, 181 (1968).
17. Sorm, F. and Keil, B., Adv. Protein Chem., 17, 167 (1962).
18. Sussman, M., Nature, 225, 1245 (1970).
19. Britten, R.J. and Davidson, E.H., Science, 165, 349 (1969).
20. Georgiev, G.P., Ann. Rev. Genetics, 3, 155 (1969).
21. Lingrel, J.B. and colleagues, personal communication.