A Method for Measuring the Content of a Specific Messenger Ribonucleic Acid in *Escherichia coli*

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A method is described for measuring the proportion of a specific messenger RNA in the total RNA extracted from pulse-labelled cells. A model system consisting of total ribosomal RNA and *Escherichia coli* DNA is used to validate the method and to define the conditions under which it can be used.

Several different hybridization methods have been used in attempts to measure the concentration of a specific type of mRNA in *Escherichia coli* (Imamoto *et al.* 1965; Hill & Echols, 1966; Attardi, Naono, Rouvière, Jacob & Gros, 1963; Edlin, Stent, Baker & Yanofsky, 1968; Stubbs & Hall, 1968; Lavallé & de Hauwer, 1968). In most cases the underlying assumption was made that all of the specific mRNA available becomes hybridized with the DNA (usually purified episomal DNA). In one case, the assumption was made that the hybridization reaction reaches an equilibrium state. Both of these assumptions have been questioned and shown most probably to be in error (Bishop, 1969*a*, 1970). The present paper presents the results of experiments designed to find an accurate method for measuring the amount of a specific mRNA, and to test it directly. The main difficulties are (1) that any specific mRNA forms only a very small proportion of the total cellular RNA, and (2) when RNA is isolated from pulse-labelled cells, the specific radioactivity of the unstable RNA cannot be measured. The method described here was developed by using total ribosomal RNA from *E. coli* as a model. The amount and specific radioactivity of the ribosomal RNA can be measured accurately, and consequently the proportion which has become hybridized can be determined directly. Under appropriate conditions an extrapolation of the hybridization measurements predicts the hybridization of all of the ribosomal RNA present. In the case of mRNA a similar extrapolation measures the radioactivity present in the specific mRNA that is able to hybridize. A device is described that allows the specific activity of the mRNA to be estimated in certain cases. A combination of the two estimates measures the concentration of the specific mRNA.

MATERIALS AND METHODS

³²P-labelled rRNA and ¹⁴C-labelled DNA were prepared from *E. coli* K12, strain M.R.E. 600, as described by Bishop (1969*b*, 1970). The membrane-annealing method of Gillespie & Spiegelman (1965) was used for DNA-RNA hybridization with the modifications described by Bishop (1969*b*), except that the annealing period was 40 h, and two loaded membranes plus one blank membrane were rolled together in each sample tube.

RESULTS AND DISCUSSION

Previous papers have dealt with the analysis of the type of hybridization experiment in which an excess amount of RNA is annealed with membrane-bound denatured DNA (Bishop, 1969*b*; Bishop, Robertson, Burns & Melli, 1969). When the annealing time was kept constant, and the RNA concentration varied, it was found that the reciprocal of the relative hybridization (μg of DNA/ μg of RNA hybridized) was a linear function of the reciprocal of the RNA concentration. The relationship may be written:

$$1/r = K/h + 1/r_s$$

where r and h represent respectively the relative hybridization and the RNA concentration ($\mu\text{g}/\text{ml}$). K is a constant which varies with the annealing conditions and the genetic complexity of the DNA and RNA, and r_s is an estimate of the relative hybridization that would occur at infinite concentration of RNA. For the present purpose it is more convenient to use the ratio of RNA input to DNA input (H) rather than the RNA concentration. For a constant volume and constant DNA input the relationship is unaffected and:

$$1/r = K/H + 1/r_s$$

The equation may also be rearranged:

$$H/r = K + H/r_s \quad (1)$$

and K and r_s may be estimated from the intercept and slope respectively of a plot of H/r against H . The relationship was found to be obeyed when the RNA under study is a single molecular species or a mixture of different sequences that are equal in concentration.

In the case of mRNA the specific radioactivity of the RNA that becomes hybridized with the DNA is not known, and the radioactivity in the hybrid consequently cannot be used to calculate the amount of RNA hybridized. By using the radioactivity in hybridized RNA without conversion (c , c.p.m./ μ g of DNA) we can write:

$$H/c = K' + H/c_s \quad (2)$$

where c_s is the estimated radioactivity in hybridized RNA after annealing with RNA at infinite concentration.

Clearly, if the amount of RNA in μ g that will hybridize with each μ g of DNA at infinite RNA concentration can be determined by some other means, the specific radioactivity of the mRNA (s , c.p.m./ μ g) can be estimated from:

$$s = c_s/r_s \quad (3)$$

Further, if we can determine the concentration of the radioactivity contained in RNA molecules complementary to the DNA (the radioactive RNA potentially able to hybridize), say in the stock RNA solution used to prepare the different dilutions for the annealing experiment (c.p.m./ml), the concentration of the complementary RNA in μ g/ml can be obtained by dividing by s . Then, knowing the total RNA concentration in the same solution (h_o) the proportion of the total RNA that is formed by the mRNA species in question (α) is given by:

$$\alpha = (\text{concn. of messenger radioactivity})/h_o \cdot s$$

In the plot of H/c against H , the intercept K' can be considered to be the value to which H/c tends when $H = 0$, that is, when the ratio of DNA input to RNA input tends to infinity. If conditions can be found that allow the hybridization of all or nearly all of the complementary RNA present, K' should approximate the ratio $h_o/(\text{concentration of messenger RNA radioactivity})$, since corresponding values of H and c are divided by the same number of μ g of DNA. Thus:

$$\alpha = 1/K's \text{ and } s = 1/K'\alpha \quad (4)$$

The condition most likely to produce this result is a combination of a high DNA input and low RNA input with a long annealing time. Since the RNA will be limiting over at least part of the range,

it cannot be assumed that there will be a linear relationship between H/c and H . The linearity of the relationship must first be examined. An experiment was therefore carried out with *E. coli* DNA and rRNA, with an annealing time of 40h, 40 μ g of membrane-immobilized DNA per ml of annealing solution, and from 0.03 to 1 μ g of rRNA/ml. Since about 0.4% of *E. coli* DNA is able to hybridize with rRNA, this is a range from a fivefold excess of DNA over RNA to a sixfold excess of RNA over DNA. The specific radioactivity of the rRNA was measured directly, so that the amount of RNA hybridized could be calculated from the radioactivity hybridized. The appropriate equation in this case is eqn. (1) and $\alpha = 1/K$. Since all or nearly all of the rRNA is complementary to the DNA, the correct value of α is close to 1. The result is shown in Fig. 1. Fig. 1(a) shows directly the dependence of the amount of RNA hybridized (per μ g of DNA) on the RNA/DNA input ratio. Fig. 1(b) shows a reciprocal plot. The results fall close to a straight line with intercept (K) equal to 1.09. Thus $\alpha = 0.92$, which is close to the correct value. The reciprocal of the slope of the line is 0.00362. This is about the value of r_s expected for *E. coli* rRNA. A single experiment can evidently give plausible estimates of both r_s and α , and we can expect similar experiments with mRNA to give estimates of c_s and $s\alpha$ to the same degree of accuracy.

A series of experiments was carried out with different amounts of DNA to see whether there is a lower limit to the amount that can be used. The results are shown in Fig. 2. Fig. 2(b) shows that four of the five experiments fall very close together in both slope and intercept. Only at the lowest

Fig. 1. Hybridization of *E. coli* ribosomal RNA with *E. coli* DNA. Each sample (1ml) contained one blank membrane and two membranes each loaded with approximately 20 μ g of denatured *E. coli* DNA. Results for the two membranes were calculated separately. Annealing was for 40h at 65°C in 0.3M-NaCl-0.03M-sodium citrate buffer, pH7. (a) Plot of hybridization, r (μ g of RNA hybridized/ μ g of DNA) against H (RNA/DNA ratio). (b) Plot of H/r [(RNA/DNA ratio)/hybridization] against H (RNA/DNA ratio).

Fig. 2. Hybridization of *E. coli* ribosomal RNA with *E. coli* DNA. Effect of DNA input. The conditions of the experiments were as described for Fig. 1. (a) Plot of r against H . (b) Plot of H/r against H . The average amounts of DNA/sample in the different series were 42.3 (■), 38.2 (●), 27.5 (○), 12.9 (□) and 6.6 (Δ) μg.

Table 1. Analysis of the hybridization of ribosomal RNA with *E. coli* DNA

The volume of each sample was 1 ml. The range of RNA concentrations used in each case was from one-fifth of the amount of DNA to six times the amount of DNA, approximately. The results are shown in Fig. 2.

Expt. no.	DNA/sample (μg)	No. of determinations	$(1/r_s) \pm \text{s.e.m.}$	$10^2 \times r_s$	K'	$1/K'$
1	42.3	12	271 ± 18	0.362	1.09	0.92
2	38.2	12	274 ± 10	0.365	0.88	1.14
3	27.5	12	249 ± 10	0.402	0.95	1.05
4	12.9	12	236 ± 9	0.424	1.29	0.78
5	6.6	12	255 ± 13	0.392	1.88	0.53
1+2+3+4		48	255 ± 5	0.392	1.06	0.94

DNA input (6.6 μg) is there an obvious deviation. The results are analysed in Table 1. Pooling the four experiments with a range of DNA inputs from 13 to 42 μg gives values of 0.00392 for r_s and 0.94 for α , in good agreement with expectation.

The five saturation curves in Fig. 2(a) fall quite close together. If r were plotted against h instead of H they would clearly fall much further apart. Since there is some variation in the amount of DNA retained on different membrane filters, it is advantageous to analyse the results in terms of H rather than h . It should be made clear, however, that this is true only under the present conditions; that is, when the RNA tends to be limiting, the DNA sequence concentration is high, and the annealing time is long.

In making measurements on a specific pulse-labelled RNA, we plot H/c against H (eqn. 2) and obtain estimates of c_s and $s\alpha$ ($s\alpha = 1/K'$). To

estimate α , it is necessary to use c_s to estimate s . This is possible only if the proportion of the DNA with which the mRNA can hybridize is known independently. In the experiments described in the following paper, galactose-specific mRNA (gal-mRNA) is hybridized with the DNA of bacteriophage λ dg which carries the galactose operon. The molecular weight of λ dg DNA is known, and the molecular weight of the sense-strand DNA sequence of the galactose operon can be estimated approximately from the molecular weights of the polypeptide chains specified by the three cistrons of the operon. This gives $M_{\text{gal}}/M_{\lambda}$, the proportion of the DNA that is complementary to gal-mRNA. It was previously found that the amount of single-stranded RNA that can be estimated to hybridize with DNA at infinite RNA concentration (r_s) is less than the complementarity by a factor of 1.75 (Bishop *et al.* 1969). This suggests $M_{\text{gal}}/1.75M_{\lambda}$

as the best available estimate of r_s . By using this value the specific radioactivity of gal-mRNA is given by eqn. (3) and the proportion it forms of the total RNA by eqn. (4):

$$s = c_s/r_s = 1.75c_s M\lambda / Mgal \quad (5)$$

$$\alpha = 1/K's = Mgal / 1.75K'c_s M\lambda \quad (6)$$

Two approaches may be taken to calculate the average number of copies of the gal-mRNA per cell (n) or per gene (m) in the growing culture. If the average amount of RNA per growing cell (R) is known:

$$n = RN_A / Mgal \quad (7)$$

where N_A is Avogadro's number. If the RNA/DNA ratio (Q) in the culture is known, the amount of gal-mRNA/unit of DNA is αQ and the average number of copies per genome equivalent of DNA is $\alpha Q Mb / Mgal$, where Mb is the size of the bacterial genome. If the average number of genes (o) per genome equivalent and the average number of genome equivalents of DNA per cell (l) are known:

$$m = \alpha Q Mb / o Mgal = Q Mb / 1.75K'c_s o M\lambda \quad (8)$$

$$n = \alpha l Q Mb / o Mgal = l Q Mb / 1.75K'c_s o M\lambda \quad (9)$$

Since $Mgal$ appears in the denominator of relationships (7), (8) and (9) and also in the numerator of the expression for α (eqn. 6), the estimates of m and n are independent of the value assumed or calculated for $Mgal$.

Lastly, the proportion of the total RNA radioactivity contained in gal-mRNA (β) is given by:

$$\beta = 1/K'S \quad (10)$$

where S is the overall specific radioactivity of the total RNA.

In choosing appropriate DNA concentrations for particular situations, account must be taken of the likelihood that the important consideration is the specific DNA sequence concentration. λ dg DNA contains 1 copy of the galactose operon and its molecular weight is 30×10^6 . About 0.4% of the DNA of *E. coli* harvested in exponential-phase growth in minimal medium hybridizes with a saturating amount of ribosomal RNA. This corresponds to 10 copies of the ribosomal DNA cistrons per replicating chromosome (1.4 genome equivalent,

or $1.4 \times 2700 \times 10^6$ daltons of DNA), or 17.5 copies if we apply the hybridization factor referred to above. In terms of the sequences being hybridized in the two different cases, 1μ g of *E. coli* DNA is equivalent to $(30 \times 10 \times 1.75) / (1.4 \times 2700) = 0.14 \mu$ g of λ dg DNA.

The proportion of rRNA estimated to hybridize with DNA is noteworthy. The observed value for Expts. 1-4 (Table 1) is 0.94. Since the membranes were treated with ribonuclease, this shows that the RNA-DNA duplex made under these circumstances is virtually insensitive to ribonuclease attack, in agreement with the results of Avery & Midgley (1969). In some cases, partial ribonuclease-sensitivity of hybridized RNA has been suspected under similar conditions of treatment. The sensitive RNA in such cases is most probably non-specifically bound, or is in the form of tails caused by overlapping sequences. Both of these effects would be expected to be more frequent when a large excess of RNA is used during the annealing period.

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