Estimation of Gene Reiteration from Hybridization Kinetics in Moderate Deoxyribonucleic Acid Excess

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Theoretical calculations were carried out to clarify how the DNA/RNA or the DNA/cDNA (complementary DNA) ratio in the hybridization reaction mixture affects the kinetics of DNA–RNA or DNA–cDNA reassociation, and theoretical formulae were derived as a function of these ratios. From these formulae, it was found that the DNA/RNA or the DNA/cDNA ratio did not much affect the initial reaction rates of hybridization, but greatly affected the terminal value for the extent of hybrid formation. Therefore the results obtained when one normalizes the experimental data for hybridization and derives the reiteration frequency from a number called the ‘half C_0t ’ ($C_0t_{1/2}$) are not accurate, especially in the presence of a moderate excess of DNA. A simple method for the estimation of gene reiteration was demonstrated that did not use the half C_0t value in the determination. This simple method is useful even if DNA–RNA or DNA–cDNA hybridizations are done with a moderate excess of DNA. With mouse myeloma cells as a model system, the gene reiteration of the 28S rRNA cistron was determined.

The technique of DNA–RNA or DNA–cDNA* reassociation is a powerful tool for the characterization and determination of the proportion of the genome specific for a class of RNA. Many studies on DNA–RNA or DNA–cDNA reassociation have been reported (Britten & Kohne, 1968; Packman *et al.*, 1972; Straus & Bonner, 1972). The method of DNA–RNA hybridization in vast DNA excess was described by Melli *et al.* (1971). However, in order to use their technique, RNA with an extremely high specific radioactivity would be required. As it is often impossible to obtain such highly labelled RNA or cDNA, the conditions of hybridization would not be ideal. Therefore the question arises of how the RNA/DNA or cDNA/DNA ratio in the reaction mixture affects the kinetics of hybridization.

The present paper discusses the mathematical formulations needed to describe the reassociation kinetics, taking the RNA/DNA or cDNA/DNA ratio into consideration, and demonstrates simple methods for the estimation of gene reiteration, which work even though the hybridizations are carried out with moderate excess of DNA.

The rRNA of mouse myeloma cells, which is an example of an RNA species produced by transcription of a very limited region of DNA, was used as a model system in the study of quantitative aspects of DNA–RNA reassociation kinetics.

* Abbreviation: cDNA, complementary DNA.

Materials and Methods

Materials

Eagle's minimum essential medium (Eagle, 1959) was from Daigo Chemical (Osaka, Japan) and bovine serum from Chiba Serum Institute (Chiba, Japan). Nonidet P.40 was from Shell Kagaku (Tokyo, Japan). Ribonuclease (from ox pancreas) was from Worthington Biochemical Co. (Freehold, NJ, U.S.A.), and ribonuclease T₁ (from *Aspergillus oryzae*) was from Boehringer Mannheim G.m.b.H. (Mannheim, Germany). Both enzymes were preheated at 100°C for 5 min just before use (Gillespie & Spiegelman, 1965). Pronase was obtained from Calbiochem (San Diego, CA, U.S.A.), and Soluene-350 was from Packard Instrument Co. Inc. (La Grange, IL, U.S.A.).

Cells

Tumour-cell line X5563 (Muto & Morita, 1971), producing immunoglobulin G myeloma protein, was maintained by subcutaneous transplantation in C3H mice. A myeloma-cell suspension was prepared from the solid tumour in Eagle's minimum essential medium supplemented with 5% (v/v) bovine serum and cultured in roller tubes.

Labelling conditions

For the labelling of rRNA with [³H]uridine, myeloma cells (10⁶ cells/ml) were incubated for 24 h

in the presence of 22 μ Ci of [5- 3 H]uridine/ml (The Radiochemical Centre, Amersham, Bucks., U.K.; 40 Ci/mmol).

Isolation of rRNA

The cells were homogenized in 0.25 M-sucrose/3 mM-MgCl₂/50 mM-KCl/50 mM-Tris/HCl (pH 7.4)/0.4% Nonidet P-40 by using a glass homogenizer. The postmitochondrial supernatant was obtained by centrifugation at 20000g for 20 min, and the ribosome/polyribosome fraction was pelleted by centrifuging the postmitochondrial supernatant after treatment in 0.5% sodium deoxycholate in a type 65 rotor of a Spinco L ultracentrifuge for 2 h at 160000g (r_{av} 5.7 cm) at 4°C. The pellet was resuspended in 1 ml of 0.05 M-Tris/HCl (pH 7.4)/0.025 M-KCl. To prepare the 60 and 40S ribosomal subunits, the ribosome/polyribosome suspension was adjusted to 1 mM-EDTA by adding 0.5 ml of 0.05 M-Tris/HCl (pH 7.4)/0.025 M-KCl/3 mM-EDTA and dialysed for 2 h against 500 vol. of 0.05 M-Tris/HCl (pH 7.4)/0.025 M-KCl/0.001 M-EDTA (four changes) (Amaldi & Attardi, 1968; Girard *et al.*, 1965). The dialysed sample was applied to a linear 15–30% (w/v) sucrose gradient (in the same buffer as that used for dialysis) and centrifuged in a SW 25-1 rotor of a Spinco L ultracentrifuge for 16 h at 54000g (r_{av} 9.1 cm) at 4°C. The two major rRNA species were isolated from the ribosomal subunits by the sodium dodecyl sulphate/phenol extraction method (Muto & Morita, 1971). The concentrations of RNA solutions were determined on the basis of ultraviolet absorption measurements, by using a value of 214 for $A_{1\text{cm}}^{1\%}$ at 260 nm (Hall & Doty, 1959). An appropriate sample of RNA solutions was dissolved in 1 ml of Soluene-350/water (9:1, v/v) and counted for radioactivity in a toluene-based scintillator system (Mizuno *et al.*, 1969). The specific radioactivities of the 28 and 18S rRNA species were 1.47×10^5 and 1.39×10^5 c.p.m./ μ g respectively.

Isolation of DNA

DNA was extracted from the nuclei of myeloma cells by the Marmur (1961) procedure, by treatment with ribonuclease (100 μ g/ml, 37°C, 1 h), Pronase (50 μ g/ml, 37°C, 1 h) and seven deproteinization steps with sodium dodecyl sulphate/phenol (Gillespie & Spiegelman, 1965). The aqueous extract was precipitated with 2 vol. of cold ethanol and the DNA strands were spooled on a glass rod. The DNA was dissolved in 0.3 M-KOH and incubated at 37°C for 18 h. The DNA solution was dialysed extensively in the cold against 1 mM-EDTA solution and then against 0.15 M-NaCl/0.015 M-sodium citrate.

The DNA was fragmented to a size of 5–7S by sonication (UR-150P, Tominagaworks, Tokyo,

Japan), as estimated by centrifugation in a linear 5–20% alkaline sucrose gradient, containing 0.7 M-NaCl/1 mM-EDTA/0.3 M-NaOH. DNA was precipitated with 2 vol. of ethanol, pelleted by centrifugation at 1400g (r_{av} 10 cm) for 25 min and dissolved in a minimum volume of 0.015 M-NaCl/1.5 mM-sodium citrate.

DNA–RNA hybridization

The hybridization mixture, containing 5 mg of myeloma DNA/ml and 2100 or 21000 c.p.m. of 3 H-labelled 28S rRNA/ml in 0.15 M-NaCl/15 mM-sodium citrate and 0.1% sodium dodecyl sulphate, was heated to 100°C for 5 min in a glycerol bath and quickly transferred to a water bath at 65°C. The hybridization was carried out at 65°C in silicon-stoppered tubes. Samples were taken at various times and cooled rapidly in an ice/water bath. The reaction mixture was diluted to a DNA concentration of 50 μ g/ml with 0.3 M-NaCl/30 mM-sodium citrate and divided into halves. One half was incubated with pancreatic ribonuclease (15 μ g/ml) and ribonuclease T₁ (15 units/ml) for 1 h at 37°C. The other half of the sample was incubated similarly in the absence of ribonuclease. Ice-cold trichloroacetic acid was added to give a final concentration of 5% (w/v). After incubation at 0–4°C for 1 h, the precipitate was collected by centrifugation at 1400g (r_{av} 10 cm) for 25 min, and washed with 5% (w/v) cold trichloroacetic acid. The precipitate was then dissolved in 1 ml of Soluene-350/water (9:1, v/v) and counted for radioactivity in a toluene-based scintillator (Mizuno *et al.*, 1969).

Results

Theory

When denatured DNA and RNA are mixed together in solution under conditions which promote DNA–RNA hybridization, DNA renaturation occurs at the same time. Therefore derivations of the differential equations needed to create a mathematical model of the system were performed by considering the following reactions:

where D_1 represents those DNA bases in single-stranded sequences at any time which are complementary to cellular RNA, D_2 represents those DNA bases in single-stranded sequences which are complementary to D_1 , R represents the RNA bases in single-stranded sequences at any time, and their concentrations are indicated by D_1 , D_2 and R ; k_{+1} is the rate constant of renaturation and k_{+2} is the rate constant of DNA–RNA hybridization.

The rate equations of the above reactions are:

$$-\frac{dD_1}{dt} = k_{+1} D_1 D_2 + k_{+2} D_1 R \quad (1)$$

$$-\frac{dD_2}{dt} = k_{+1} D_1 D_2 \quad (2)$$

$$-\frac{dR}{dt} = k_{+2} D_1 R \quad (3)$$

$$R + D_2 - D_1 = mC_0 \quad (4)$$

where m is the ratio of RNA/DNA and C_0 is the total concentration of DNA bases complementary to the RNA. Eqns. (1), (2) and (3) were also described by Straus & Bonner (1972). Eqn. (4) is obtained since mC_0 shows the total RNA concentration in the reaction mixture, and R is the RNA concentration that does not hybridize with DNA and $D_2 - D_1$ is equal to the RNA concentration that hybridizes with DNA.

On substituting $D_2 = mC_0 - R + D_1$ into eqn. (1) we get:

$$-\frac{dD_1}{dt} = k_{+1} D_1 (mC_0 - R + D_1) + k_{+2} D_1 R \quad (5)$$

On inserting

$$D_1 = -\frac{1}{Rk_{+2}} \cdot \frac{dR}{dt}$$

that derived from eqn. (3), into eqn. (5), we obtain:

$$\frac{d^2 R}{dt^2} = \frac{k_{+1} + k_{+2}}{k_{+2} R} \left(\frac{dR}{dt} \right)^2 + (k_{+1} - k_{+2}) R \frac{dR}{dt} - k_{+1} mC_0 \frac{dR}{dt} \quad (6)$$

Using the notation:

$$Z = \frac{dR}{dt}$$

$$\frac{dZ}{dR} = \frac{d \left(\frac{dR}{dt} \right)}{dR} = \frac{d^2 R}{dt^2} \cdot \frac{dt}{dR}$$

eqn. (6) can be written in the form:

$$\frac{dZ}{dR} - \frac{(k_{+1} + k_{+2})Z}{k_{+2} R} + [(k_{+2} - k_{+1})R + k_{+1} mC_0] = 0 \quad (7)$$

Since this differential eqn. (7) is a first-order linear equation, it has a general solution (Simmons, 1972):

$$Z = \exp \left[\int \left(\frac{k_{+1} + k_{+2}}{k_{+2} R} \right) dR \right] \cdot \left\{ \int [(k_{+1} - k_{+2})R - k_{+1} mC_0] \cdot \exp \left[- \int \left(\frac{k_{+1} + k_{+2}}{k_{+2} R} \right) dR \right] dR + B \right\}$$

where B is a constant. Therefore we can obtain a solution that satisfies the initial conditions $R = mC_0$ and $dR/dt = -0.5k_{+2}mC_0^2$ at $t = 0$:

$$Z = \frac{dR}{dt} = k_{+2} mC_0 R - k_{+2} R^2 - 0.5k_{+2} C_0 R \left(\frac{R}{mC_0} \right)^{(k_{+1})} \quad (8)$$

By using the notation $y = R/mC_0$, eqn. (8) may be written in the form:

$$\frac{dy}{dt} = k_{+2} C_0 y [m - my - 0.5y^{(k_{+1}/k_{+2})}] \quad (9)$$

The value of $(1-y)$ denotes the proportion of RNA bases hybridized.

Eliminating D_1 and R from eqns. (1), (2) and (4) as described above, we also obtain:

$$\frac{d^2 D_2}{dt^2} = \frac{k_{+1} + k_{+2}}{k_{+1} D_2} \left(\frac{dD_2}{dt} \right)^2 + (k_{+2} - k_{+1}) D_2 \frac{dD_2}{dt} - k_{+2} mC_0 \frac{dD_2}{dt} \quad (10)$$

Integrating eqn. (10) with the initial conditions such that $D_2 = C_0/2$ and $dD_2/dt = -k_{+1}C_0^2/4$ at $t = 0$, we obtain:

$$\frac{dD_2}{dt} = -k_{+1} D_2^2 + k_{+1} mC_0 D_2 - k_{+1} mC_0 D_2 \left(\frac{D_2}{0.5C_0} \right)^{(k_{+2})} \quad (11)$$

By using the notation $a = D_2/0.5C_0$ we may also write eqn. (11) in the form:

$$\frac{da}{dt} = k_{+1} C_0 a [m - 0.5a - ma^{(k_{+2}/k_{+1})}] \quad (12)$$

The value of $(1-a)$ denotes the proportion of DNA-DNA renaturation. If we can solve eqn. (9) for y , we are able to understand how the ratio of RNA/DNA and rate constants k_{+1} and k_{+2} affect the DNA-RNA hybridization. If, as in the ideal case, $m \rightarrow 0$ and $k_{+1}/2 \rightarrow k_{+2}'$ [rate constant described by Melli *et al.* (1971)] and integrating eqns. (9) and (12), we can easily obtain the same solutions as were derived by Melli *et al.* (1971).

Since eqns. (9) and (12) could not be solved analytically, I chose to obtain a numerical solution, by using digital computers. These numerical solutions were obtained by the Euler-Romberg method (McCalla, 1967). To illustrate the numerical solutions obtained, the proportions of DNA-RNA hybridization are shown as an example in Fig. 1,

Fig. 1. Theoretical curves of hybridization at various ratios of DNA/RNA obtained by using eqns. (9) and (12)

Curve (1) is the ideal DNA renaturation curve when the DNA/RNA ratio is 10^4 , and curves (2), (3) and (4) are the ideal DNA-RNA hybridization curves for DNA/RNA ratios of 10^4 , 10^2 and 10 respectively. The rate constants are $k_{+1} = 0.462$ and $k_{+2} = 0.128$.

under the conditions when the rate constant $k_{+1} = 0.462$ and $k_{+2} = 0.128$ at RNA/DNA ratios of 10^{-4} , 10^{-2} and 10^{-1} . These solutions show that under the chosen conditions the amount of DNA-RNA hybridization after the infinitely long time of annealing does not reach 100%. For instance, under the condition when RNA/DNA = 10^{-2} , only 69.5% of the RNA would be hybridized and the remaining 30.5% of the RNA would not react with the DNA. This is a result of the fact that DNA-DNA renaturation competes with DNA-RNA hybridization. We can also account for this fact as follows. From eqn. (9), we may write:

$$\int \frac{dy}{y[m - my - 0.5y^{(k_{+1}/k_{+2})}]} = k_{+2} C_0 \int dt \quad (13)$$

If there exists $y = y_1$, which satisfies the following equations:

$$1 \geq y > 0 \quad (14)$$

$$m - my - 0.5y^{(k_{+1}/k_{+2})} = 0 \quad (15)$$

eqn. (13) is divergent at $y = y_1$, because the denominator of eqn. (13) becomes zero. Therefore even if the annealing time of the DNA-RNA hybridization was infinitely long, the proportion of hybridization will not increase beyond the value of $(1 - y_1)$. Since the proportion of DNA-RNA hybridization does not become 100% at finite ratios of RNA/DNA, results are not accurate when one normalizes the experimental data of DNA-RNA hybridization (in which the hybridization from the single-stranded to the duplex form is plotted against $\log C_0 t$) and derives the reiteration frequency from $C_0 t_{\frac{1}{2}}$, the value of the product of the initial DNA concentration and the annealing time at which the mid-point of the transition is reached. The values of $C_0 t_{\frac{1}{2}}$ determined

by this method will be an underestimation, especially if approximately equivalent amounts of reactive DNA and RNA sequences are present.

Simplified alternative method for estimation of gene reiteration

Since the gene reiteration is generally determined by using an RNA species transcribed from a very limited region of DNA or using its cDNA, one has to modify the above formulae slightly. From eqn. (13), the proportion, x , of the RNA bases in single-stranded sequences at time t is given by:

$$\int_1^x \frac{dy}{y[m - my - 0.5y^{(k_{+1}/k_{+2})}]} = k_{+2} C_0 \int_0^t dt \quad (16)$$

where C_0 is the concentration of DNA bases that are complementary to the RNA. If the proportion of C_0 to the concentration of total cellular DNA bases (C) is defined to be P , the ratio of C to that of the RNA to be M , and the ratio of C_0 to that of the RNA to be w , then:

$$C_0 = PC \quad (0 < P \leq 1) \quad (17)$$

and

$$w = PM \quad (18)$$

$$w = 1/m \quad (19)$$

From eqns. (17) and (18), we obtain:

$$C_0 = \frac{w}{M} C \quad (20)$$

From eqns. (16), (19) and (20), we may also write:

$$\log(Ct) = \log \left\{ \frac{M}{k_{+2}} \int_1^x \frac{dy}{y[1 - y - 0.5wy^{(k_{+1}/k_{+2})}]} \right\} \quad (21)$$

In this formula, w , k_{+1} and k_{+2} are unknown values.

(i) *DNA-cDNA hybridization.* When the cDNA (probe), which is, for example, synthesized from an RNA species by RNA-dependent DNA polymerase, is used instead of the RNA and the value:

$$w' = \frac{\text{cDNA (the amount of genome) in total cellular DNA}}{\text{cDNA (probe) instead of an RNA species}}$$

(total cellular DNA is the amount of cellular DNA in the hybridization mixture) is used instead of w , the formula (21) can be solved analytically since $k_{+1}/k_{+2} = 1$, and we obtain:

$$x = \frac{\frac{2}{w' + 2}}{1 - \left(\frac{w'}{w' + 2} \right) e^{-(k_{+1} Ct/M)}} \quad (22)$$

Fig. 2. Time-course of ideal DNA-cDNA hybridization

The value of $(1-x)$ denotes the proportion of DNA-cDNA hybridization. D is the terminal value for the extent of hybrid formation (saturation value).

The value of $(1-x)$ denotes the proportion of DNA-cDNA hybridization. Therefore even if the annealing time of the DNA-cDNA hybridization was infinitely long, the proportion of hybridization will not increase beyond the value of $[1-2/(w'+2)]$ (Fig. 2). If the saturation value (D) is obtained from the experimental data, we can estimate the gene reiteration as follows. Since $D = 1 - [2/(w'+2)]$, we obtain;

$$w' = \frac{2D}{1-D} \quad (23)$$

We may also write from definition:

$$w' = \left[\frac{\text{cDNA (the amount of genome) in total cellular DNA}}{\text{total cellular DNA}} \right] \times \left[\frac{\text{total cellular DNA}}{\text{cDNA (probe)}} \right] \quad (24)$$

If the cDNA as probe has the DNA bases of a whole cistron and the molecular weight of this cDNA (as single-stranded DNA) is G , and if we let f be the gene reiteration of this cistron:

$$w' = \frac{2Gf}{Q} \cdot M \quad (25)$$

$$w = \left[\frac{\text{cDNA (the amount of genome) in total cellular DNA}}{\text{total cellular DNA}} \right] \times \left(\frac{\text{total cellular DNA}}{\text{RNA}} \right)$$

where Q (daltons) is the average DNA content of a cell (diploid). Therefore, we may write:

$$f = \frac{Qw'}{2GM} = \frac{Q}{2GM} \cdot \frac{2D}{1-D} \quad (26)$$

(ii) DNA-RNA hybridization. Since the value of k_{+1}/k_{+2} is not equal to 1 in DNA-RNA hybridization, eqn. (21) could not be solved analytically.

However, if two series of DNA-RNA hybridizations with different DNA/RNA ratios are carried out, we can estimate the gene reiteration from their saturated values. We assume that two series of hybridizations are carried out under one of the following conditions. (a) (Concentration of total cellular DNA bases/concentration of RNA bases) = M ; (concentration of DNA bases which are complementary to the RNA/concentration of RNA bases) = w . We let R_1 be the saturated value of the hybridization under this condition. (b) The concentration of the RNA bases is n times that in case (a), i.e. (concentration of total cellular DNA bases/concentration of RNA bases) = M/n and (concentration of DNA bases which are complementary to the RNA/concentration of RNA bases) = w/n . Let us also assume that the saturated value of the hybridization in this case is R_2 . From eqns. (15), (19) and (21), we obtain the following relationships between w and k_{+1}/k_{+2} :

$$1 - (1 - R_1) - 0.5w(1 - R_1)^{k_{+1}/k_{+2}} = 0 \quad (27)$$

$$1 - (1 - R_2) - 0.5 \frac{w}{n} (1 - R_2)^{k_{+1}/k_{+2}} = 0 \quad (28)$$

Eliminating w from eqns. (27) and (28), k_{+1}/k_{+2} may be written in the form:

$$\frac{k_{+1}}{k_{+2}} = \frac{\log\left(\frac{R_1}{nR_2}\right)}{\log\left(\frac{1-R_1}{1-R_2}\right)} \quad (29)$$

From eqn. (27), we can obtain w :

$$w = \frac{2R_1}{(1 - R_1) \left[\log\left(\frac{R_1}{nR_2}\right) / \log\left(\frac{1-R_1}{1-R_2}\right) \right]} \quad (30)$$

We may also write from definition:

If the molecular weight of the RNA is G :

$$w = \frac{2Gf}{Q} \cdot M \quad (31)$$

where Q (daltons) is the average DNA content of a cell (diploid), f is the gene reiteration and M is the ratio of the concentration of cellular DNA to that of the RNA in the hybridization mixture of case (a).

Fig. 3. Hybridization of myeloma-cell DNA with myeloma 28S rRNA

○, DNA/RNA ratio 3.5×10^5 ; △, DNA/RNA ratio 3.5×10^4 . The hybridization was carried out as explained in the Materials and Methods section. —, Theoretical curves of hybridization by using eqn. (21) with rate constants $k_{+1} = 206.5 \text{ mol}^{-1} \cdot \text{s}^{-1} \cdot \text{litre}$, $k_{+2} = 41.3 \text{ mol}^{-1} \cdot \text{s}^{-1} \cdot \text{litre}$, $w = 135.1$, $C = 5 \text{ mg/ml}$ and $M = 3.5 \times 10^5$ (curve 1) or $M = 3.5 \times 10^4$ (curve 2). w was calculated from the saturation values ($R_1 = 0.61$, $R_2 = 0.425$) of curve (1) and curve (2) by using eqn. (30).

Therefore we can also estimate the gene reiteration f from eqns. (30) and (31):

$$f = \frac{Q}{2GM} \cdot \frac{2R_1}{(1 - R_1) \left[\log\left(\frac{R_1}{R_2}\right) / \log\left(\frac{1 - R_1}{1 - R_2}\right) \right]} \quad (32)$$

As a model system, the DNA-RNA hybridization of myeloma DNA and 28S rRNA was carried out under conditions such that the ratio of myeloma DNA/28S rRNA was (a) 3.5×10^5 and (b) 3.5×10^4 respectively. In case (b), since the concentration of the RNA bases is 10 times that in case (a), I estimated from Fig. 3 that $n = 10$, $R_1 = 0.61$ and $R_2 = 0.425$. Therefore, by using $M = 3.5 \times 10^5$, $G = 1.6 \times 10^6$ and $Q = 3.6 \times 10^{12}$ (Sober & Harte, 1970), eqn. (31) gives $f = 434$ (diploid). It is necessary to know the values of w , k_{+1} and k_{+2} to calculate the theoretical hybridization curves from eqn. (21). Since the values of w and k_{+1}/k_{+2} are known and only the value of k_{+2} is unknown, eqn. (21) may be written as follows:

$$Y = k_{+2} \cdot X \quad (33)$$

where

$$Y = M \int_1^x \frac{dy}{y[1 - y - 0.5wy^{(k_{+1}/k_{+2})}]}$$

and $X = Ct$. The values of these formulae Y and X can be calculated from the experimental data of the proportion x_t of the hybridization at the annealing time t_t . Therefore we can calculate the optimum value of k_{+2} by using the method of least squares. From the experimental data of the hybridization of myeloma DNA and 28S rRNA, I obtained rate constants k_{+1} and k_{+2} of 206.5 and $41.3 \text{ mol}^{-1} \cdot \text{s}^{-1} \cdot \text{litre}$ respectively. These theoretical curves are shown in Fig. 3.

Discussion

From the theoretical considerations, it was found that when the ratio of DNA to its complementary RNA sequences was a finite value, 100% hybridization of the RNA to DNA could not be achieved, even if the time of incubation was infinitely long. Figs. 1 and 3 show that the DNA/RNA ratio had little effect on the initial reaction rates of DNA-RNA hybridization, but the DNA/RNA ratio greatly affected the terminal value for the extent of hybrid formation. Therefore, the reiteration frequency derived from the $C_0t_{1/2}$ value of a normalized hybridization curve is not correct, as it would be an underestimate. The differential equations (1)–(3) were also analysed by Straus & Bonner (1972) with a computer, and in their formulae, they had used R_0 , which was the ratio of the initial concentration of RNA to the initial concentration of either of the DNA strands that was complementary to the RNA. However, for hybridizations as generally carried out, this value was unknown and I could not apply their formulae directly, because an RNA species is produced in general by transcription of a very limited region of the cellular DNA.

Since it was found that saturated values of the hybridization were sensitive to the extent of the gene reiteration, I calculated the gene reiteration from the saturated values. This method is useful even if the hybridizations are done with a moderate excess of DNA. As a model system, I have used myeloma DNA and purified 28S rRNA labelled with [^3H]uridine. RNA-DNA hybridization experiments were carried out with conditions such that the DNA/RNA ratio was (a) 3.5×10^5 and (b) 3.5×10^4 . The results obtained as shown in Fig. 3 are consistent with the theoretical considerations. By using the fact that the saturation value changed markedly with the DNA/RNA ratio, I calculated the rate constants and the gene reiteration. From these results, I concluded that this myeloma cell line (diploid) has about 430 28S ribosomal cistrons, which is within the range of published values (Amaldi *et al.*, 1969; Brimacombe & Kirby, 1968).

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