

# HYBRIDIZATION PROPERTIES OF DNA SEQUENCES DIRECTING THE SYNTHESIS OF MESSENGER RNA AND HETEROGENEOUS NUCLEAR RNA

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## ABSTRACT

The relationship of the DNA sequences from which polyribosomal messenger RNA (mRNA) and heterogeneous nuclear RNA (NRNA) of mouse L cells are transcribed was investigated by means of hybridization kinetics and thermal denaturation of the hybrids. Hybridization was performed in formamide solutions at DNA excess. Under these conditions most of the hybridizing mRNA and NRNA react at values of  $D_0t$  (DNA concentration multiplied by time) expected for RNA transcribed from the nonrepeated or rarely repeated fraction of the genome. However, a fraction of both mRNA and NRNA hybridize at values of  $D_0t$  about 10,000 times lower, and therefore must be transcribed from highly redundant DNA sequences. The fraction of NRNA hybridizing to highly repeated sequences is about 1.7 times greater than the corresponding fraction of mRNA. The hybrids formed by the rapidly reacting fractions of both NRNA and mRNA melt over a narrow temperature range with a midpoint about 11°C below that of native L cell DNA. This indicates that these hybrids consist of partially complementary sequences with approximately 11% mismatching of bases. Hybrids formed by the slowly reacting fraction of NRNA melt within 4°–6°C of native DNA, indicating very little, if any, mismatching of bases. Hybrids of the slowly reacting components of mRNA, formed under conditions of sufficiently low RNA input, have a high thermal stability, similar to that observed for hybrids of the slowly reacting NRNA component. However, when higher inputs of mRNA are used, hybrids are formed which have a strikingly lower thermal stability. This observation can be explained by assuming that there is sufficient similarity among the relatively rare DNA sequences coding for mRNA so that under hybridization conditions, in which these DNA sequences are not truly in excess, reversible hybrids exhibiting a considerable amount of mispairing are formed. The fact that a comparable phenomenon has not been observed for NRNA may mean that there is less similarity among the relatively rare DNA sequences coding for NRNA than there is among the rare sequences coding for mRNA.

## INTRODUCTION

It is possible to distinguish heterogeneous nuclear RNA from polyribosomal messenger RNA by several characteristics (see review by Darnell, 1968). The NRNA<sup>1</sup> is of larger mean size, has a

broader size distribution, and turns over much more rapidly than mRNA. Kinetic studies have

<sup>1</sup> Abbreviations used: EDTA, ethylenediaminetetraacetate; mRNA, messenger RNA; NRNA, hetero-

geneous nuclear RNA; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M sodium citrate; TCA, trichloroacetic acid;  $T_m$ , temperature at which 50% of a nucleic acid duplex is denatured.

shown that not more than 10–20% of labeled NRNA is destined to become mRNA (Attardi et al., 1966; Scherrer et al., 1966; Penman et al., 1968; Soeiro et al., 1968). Furthermore, on the basis of hybridization competition experiments it has been generally concluded that all of the nucleotide sequences present in mRNA are also present in NRNA, but that there may be many sequences present in NRNA which are not present in mRNA (Shearer and McCarthy, 1967; Georgiev, 1967; Church and McCarthy, 1967; Drews et al., 1968; Church et al., 1969; Church and McCarthy, 1970; Soeiro and Darnell, 1970).

However, the discovery by Britten and Kohne (1968) that the genomes of eukaryotes contain DNA sequences of various degrees of repetition pointed out the serious shortcomings of the competition experiments. Competition had been carried out under conditions in which only transcripts from the highly repeated sequences formed hybrids, whereas the transcripts from nonrepeated or rarely repeated sequences were not detected (Gelderman et al., 1969; Davidson and Hough, 1969). Furthermore, since the members of a family of redundant DNA sequences are similar, but not identical, competition between two RNA fractions derived from redundant sequences does not necessarily mean that the RNA fractions are identical. Consequently the competition results can give only minimum estimates of the difference between the competing RNAs.

Therefore, we decided to explore the relationship between NRNA and mRNA, employing conditions in which hybridization of RNA transcribed from DNA sequences of all degrees of repetition would be expected to occur. In agreement with previous reports we have found that a fraction of both mRNA and NRNA is transcribed from highly reiterated DNA sequences. However, the majority of both mRNA and NRNA is transcribed from DNA sequences of a low degree of repetition. Under certain conditions of formation the mRNA hybrids of this class have a lower thermal stability than the NRNA hybrids, suggesting that there may be more similarity among the relatively rare DNA sequences coding for mRNA than there is among the rare sequences coding for NRNA.

## MATERIALS AND METHODS

### *Cell Culture and Labeling*

Mouse L cells were cultivated as described previously (Perry and Kelley, 1968 *a*; 1968 *b*). Suspend-

tion cultures in exponential growth at a density of about  $3.5 \times 10^5$  cells/ml were treated with 0.08  $\mu$ g/ml actinomycin D. Labeling was stopped by pouring cells over frozen, crushed, balanced salt solution. DNA was labeled by growing cells overnight in the presence of 0.05- $\mu$ Ci/ml uridine-2- $^{14}$ C (50 mCi/mole). Actinomycin D was a gift from Merck, Sharp & Dohme, West Point, Pa. Radioactive precursors were purchased from New England Nuclear Corp., Boston, Mass.

### *Cell Lysis and Fractionation*

L cells were lysed and fractionated into nucleus and cytoplasm by stirring gently in cold isotonic buffer with 0.05% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) as described previously (Perry and Kelley, 1968 *a*; 1968 *b*). Polyribosomes were further purified by centrifuging through a discontinuous sucrose gradient (Wettstein et al., 1963; Perry and Kelley, 1970).

### *RNA Purification and Preparation for Hybridization*

Polyribosome pellets were suspended in SDS buffer (Penman, 1966) and deproteinized at room temperature with a 1:1 (v/v) mixture of chloroform and buffer-saturated phenol. Nuclei were lysed with high salt buffer and DNase after purification with a mixture of Tween 40 (Atlas Chemical Industries, Inc., Wilmington, Del.) and sodium deoxycholate (Penman, 1966). The nuclear lysate was precipitated with 2 vol of ethanol, resuspended in SDS buffer, and deproteinized as above. After precipitation with ethanol the nuclear nucleic acids were dissolved in 0.1 M NaCl, 0.01 M  $MgCl_2$ , 0.01 M Tris (pH 7.6) and treated for 1 or 2 hr at 37°C with 100  $\mu$ g/ml electrophoretically purified DNase (Worthington Biochemical Corp., Freehold, N.J.). After addition of EDTA to 0.01 M and of SDS to 0.5% the nuclear extract was again deproteinized with phenol-chloroform.

To prepare for hybridization the polysome and nuclear RNAs were precipitated with ethanol, and the precipitates were pelleted, washed with ether, dried, and dissolved in water. The RNA was hydrolyzed 45–50 sec by addition of 1 vol of freshly prepared 2 M NaOH with rapid mixing. Hydrolysis was stopped by neutralization with 3 M acetic acid. The RNA was then chromatographed on a  $1 \times 7$  cm column of G-75 Sephadex (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) in 0.1 M  $NH_4HCO_3$ , pH 7.4–7.5. The fractions corresponding to the void volume were pooled, lyophilized, and the RNA was dissolved in the hybridization solution.

### *DNA Preparation*

The purification of DNA was based on the method of Britten et al. (1970). Detergent-purified L cell

nuclei were homogenized with a Servall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) at 14,000 rpm for 1 min at room temperature in a solution containing 0.24 M sodium phosphate buffer (pH 6.8), 1% SDS, 0.01 M EDTA, 8 M urea, and 1 M NaClO<sub>4</sub>. The resulting clear solution was deproteinized twice with chloroform-phenol, then dry Bio-Rad HTP hydroxylapatite crystals (Bio-Rad Laboratories, Richmond, Calif.) were added at 0.4 g/mg DNA. After stirring a few minutes the hydroxylapatite and adsorbed DNA were collected by filtration and washed on the filter five times each with equal volumes of 0.18 M phosphate buffer-8 M urea and 0.012 M phosphate buffer. Finally, the DNA was eluted with 0.4 M phosphate buffer. The DNA solution was concentrated and dialyzed extensively against buffer (0.1 M NaCl, 0.01 M sodium acetate at pH 6.0, 0.001 M EDTA), using a Diaflo ultrafiltration apparatus (Amicon Corp., Lexington, Mass.). The DNA was next sheared at 10,000 psi with a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.), ethanol precipitated, desiccated *in vacuo*, and dissolved in the hybridization solution at  $\geq 20$  mg/ml. This was done by first dissolving the DNA in 75% formamide, 0.125% SDS, and then adding 1/4 vol of 25  $\times$  SSC. The DNA was adjusted to the desired <sup>14</sup>C specific radioactivity of 20-250 cpm/ $\mu$ g by mixing labeled and unlabeled DNA.

#### Preparation of Bacterial DNA and RNA

*Bacillus subtilis* 168M was obtained from Dr. Betsy Ohlsson-Wilhelm (Department of Biophysics, University of Chicago) and grown as described by Gage (1969). The bacterial cells, growing with a doubling time of 45 min, were labeled for 2 min at a density of 10<sup>8</sup> cells/ml with 1  $\mu$ Ci/ml uridine-5-<sup>3</sup>H (30 Ci/mmol), then poured over crushed frozen saline with 10 mM NaN<sub>3</sub>. The cells were lysed as described by Gage (1969). RNA and DNA were prepared exactly as for L cell nuclear RNA and DNA.

#### Electrophoresis

Polyacrylamide gel electrophoresis of high molecular weight RNA was carried out as described by Perry and Kelley (1968 b).

#### Hybridization

All reactions were carried out at 40°C in a solution containing 60% formamide (Matheson, Coleman, and Bell, Cincinnati, Ohio, 99% purity), 5  $\times$  SSC (pH 6.2), and 0.1% SDS. Under these conditions L cell DNA has a T<sub>m</sub> of about 55°C. The use of formamide makes it possible to carry out prolonged hybridization at relatively low temperatures to avoid thermal degradation of nucleic acids, while at the

same time achieving a high rate of reaction and a high degree of sequence specificity in hybrid formation (McConaughy et al., 1969).

Mixtures of DNA and RNA in 6  $\times$  50 mm stoppered glass tubes were heated to 65°C for 5 min to denature DNA-DNA and DNA-RNA duplexes, then immediately transferred to a 40°C water bath. At various times samples were taken for assay of hybridization by diluting at least 50-fold with prewarmed 0.1  $\times$  SSC. The diluting solution also contained purified low molecular weight yeast RNA as an inhibitor of nonspecific DNA-RNA interactions in the ratio of 10 parts of yeast RNA to one part of DNA. The samples were quickly chilled, brought to 6.6  $\times$  SSC, and passed through Millipore HAWG filters (Millipore Corp., Bedford, Mass.) (Nygaard and Hall, 1964) which were thoroughly washed with 2  $\times$  SSC and dried before counting. The recovery of DNA bound to the filter by this method was 70-80% of the input estimated by TCA precipitation of samples of the hybridization mixture, and did not vary as a function of incubation time. Per cent hybridization was calculated as RNA-<sup>3</sup>H/DNA-<sup>14</sup>C (in hybrid)  $\times$  100  $\div$  RNA-<sup>3</sup>H/DNA-<sup>14</sup>C (TCA insoluble). D<sub>0t</sub> (in moles  $\cdot$  sec/liter) was calculated as ( $\mu$ g/ml DNA)  $\times$  hours/83 (Britten and Kohne, 1968), using A<sub>260</sub> = 20 for a 1 mg/ml solution of native DNA.

#### Thermal Denaturation of Hybrids and Native DNA

Reaction mixtures were diluted at least 50-fold with appropriate solutions to give a final composition of 0.1  $\times$  SSC, 1.2% formamide, and 0.005 M Tris (pH 7.5) and a 10-fold excess of yeast RNA over DNA. The diluted hybrids were kept ice cold until denaturation, which was accomplished by placing aliquots in a thermostatted water bath for 5 min at each temperature. The temperature was raised in 5°C steps. After heating, the mixtures were chilled in ice, and the surviving hybrid was assayed by trapping on Millipore filters as described above.

The thermal denaturation of native DNA was followed in a temperature-controlled recording ultraviolet spectrophotometer (Gilford Instrument Company, Oberlin, Ohio).

#### RESULTS

##### Use of Actinomycin D in Preparing mRNA and NRNA

In the hybridization of mRNA and NRNA the noise level is minimized when one uses RNA preparations which do not have a high proportion of radioactivity in ribosomal RNA. The selective labeling of mRNA and NRNA can be accom-

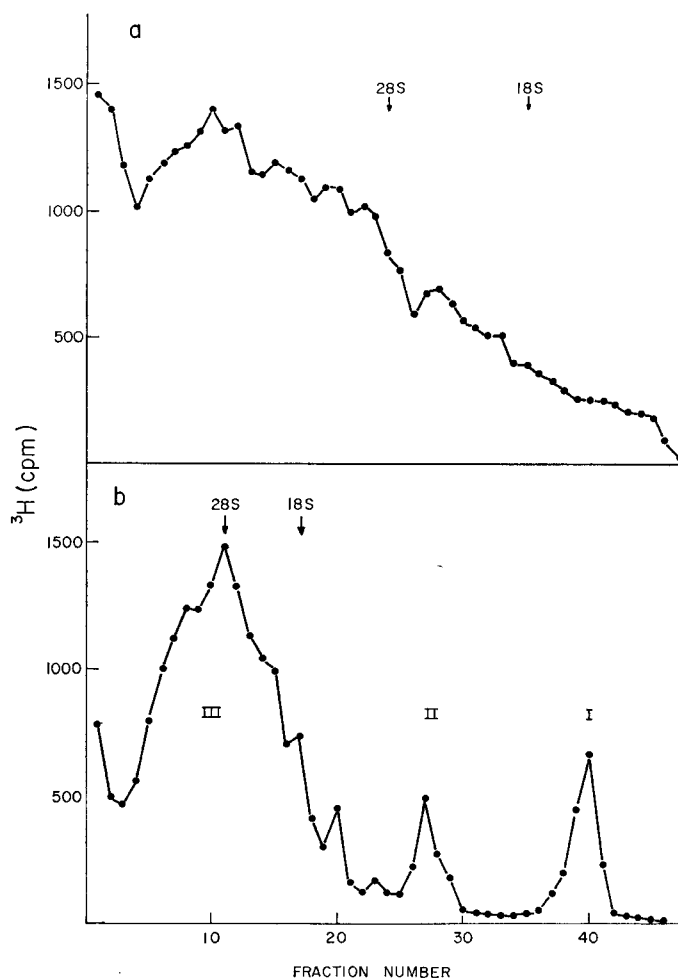


FIGURE 1 Electrophoretic analysis of nuclear RNA and polyribosome RNA. Fig. 1 *a*, nuclear RNA extracted from cells incubated 30 min with 0.08  $\mu\text{g}/\text{ml}$  actinomycin D without uridine- $^3\text{H}$ , and then 60 min in the presence of the drug with 10  $\mu\text{Ci}/\text{ml}$  uridine- $^3\text{H}$ . Fig. 1 *b*, polyribosome RNA from cells labeled as in Fig. 1 *a*. Electrophoresis was carried out for 2.5 hr (Fig. 1 *a*) or 5 hr (Fig. 1 *b*) at 5 ma/gel. The arrows indicate the positions of molecular weight markers of unlabeled L cell ribosomal RNA included in the gels. The origin is at the left.

plished either by labeling for short intervals, or by specifically inhibiting the synthesis of ribosomal RNA. The use of a short labeling time has the disadvantages that RNA of relatively low specific activity is obtained and that different classes of mRNA and NRNA may differ in specific activity. On the other hand, the specific inhibition of ribosomal RNA synthesis makes it possible to obtain mRNA and NRNA which have a high specific activity and which are more uniformly labeled than would be possible in a short labeling time.

From previous studies (Perry and Kelley, 1968 *b*; 1970) it was known that incubation of L cells with 0.08  $\mu\text{g}/\text{ml}$  actinomycin D for 0.5 hr before labeling inhibits the synthesis of the 45S ribosomal precursor RNA with virtually no effect on the synthesis of other types of RNA. Use of these conditions in the present experiments, followed by 1- or 2-hr labeling periods, gave mRNA and NRNA preparations which had no detectable radioactivity in ribosomal RNA.

## The Size Distributions of NRNA and mRNA

Fig. 1 shows characteristic profiles from polyacrylamide gel electrophoretic analyses of the NRNA and mRNA preparations used in these studies. The size distributions are basically similar to those reported by others for mammalian and avian cells (Attardi et al., 1966; Scherrer et al., 1966; Penman et al., 1968; Latham and Darnell, 1965). In Fig. 1 *a* it may be seen that NRNA is of very large size ( $> 5 \times 10^6$  daltons), and very little is smaller than 18S ( $0.7 \times 10^6$  daltons). It also has quite a heterogeneous size distribution. The polyribosomal RNA, on the other hand, is of smaller average size, and appears to consist of three main classes of components. These are a 4S peak (component I) presumably corresponding to amino acid transfer RNA, a peak of molecular weight 130,000 (component II), and a broad peak with a modal size of about  $10^6$  daltons (component III). Component II may correspond to histone messenger RNA (Borun et al., 1967; Kedes and Gross, 1969), while component III includes other species of mRNA. The gel pattern of NRNA is highly reproducible. However, the proportions of the three main components of polyribosomal RNA may vary with different batches of cells or different labeling times.

The maximum possible contamination of the polyribosomal RNA with other types of RNA was estimated by isopycnically banding the polyribosome preparations in CsCl gradients, and measuring the proportion of radioactivity which failed to band coincidentally with the ribosomes (Perry and Kelley, 1968 *a*). Measurements of several different preparations indicated that contamination with nonpolyribosomal components does not exceed 16% (Schochetman, 1970).

For the hybridization experiments the mRNA and NRNA were degraded to a fairly uniform size of about  $10^5$  daltons by brief alkaline hydrolysis. This was done to circumvent any possible effect of size on the reaction rate and to minimize the possible formation of structures containing short base-paired regions together with long unpaired regions.

### Kinetics of Hybridization

The renaturation of DNA, whether followed by adsorption of double-stranded DNA to hydroxylapatite, or by increase in hypochromicity, is a second order reaction (Britten and Kohne, 1968).

The second order rate constant is inversely proportional to the complexity of the genome (number of nucleotide pairs in nonrepeated sequences) (Britten and Kohne, 1968; Wetmur and Davidson, 1968). Some fractions of eukaryote DNA renature at rates greatly in excess of those expected on the basis of genome complexity, and it has been inferred that these fractions consist of highly repeated sequences (Britten and Kohne, 1968).

It seems reasonable to assume that the hybridization of RNA with DNA is physically similar to DNA renaturation and that it has the same or a very similar rate constant. These assumptions are supported by the findings of Nygaard and Hall (1964). Therefore, when denatured DNA and RNA transcribed from that DNA are mixed under conditions favorable for reassociation two competing reactions will occur, that of DNA with DNA, and that of DNA with RNA. If DNA is in large excess over RNA, then the change of DNA concentration with time will be largely due to DNA renaturation. Under these conditions the hybridization of RNA with DNA should follow the equation

$$R/R_0 = 1/(1 + kD_0t) \quad (1)^2$$

where  $t$  is the time of incubation,  $R$  is the RNA concentration at time  $t$ ,  $R_0$  is the initial RNA concentration,  $D_0$  is the initial DNA concentration, and  $k$  is the second order rate constant (applicable to both DNA-DNA and DNA-RNA annealing according to the above assumptions). Equation (1) is expected to be strictly valid only for the case of perfect hybrid formation, and is not expected to account fully for the complex situation occurring where hybrids of relatively low stability are formed as a result of similarities among the different reacting sequences.

<sup>2</sup> Equation (1) is derived as follows: Given that  $D \gg R$ , then

$$-dR/dt = k D R \quad (2)$$

$$-dD/dt = k D^2 \quad (3)$$

Equation (3) is integrated and substituted in equation (2) to give

$$-dR/dt = k R D_0/(1 + kD_0t)$$

which, upon integration and taking the antilogarithm gives equation (1).

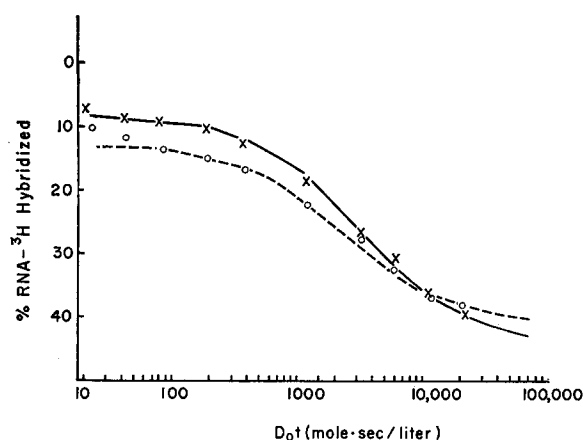


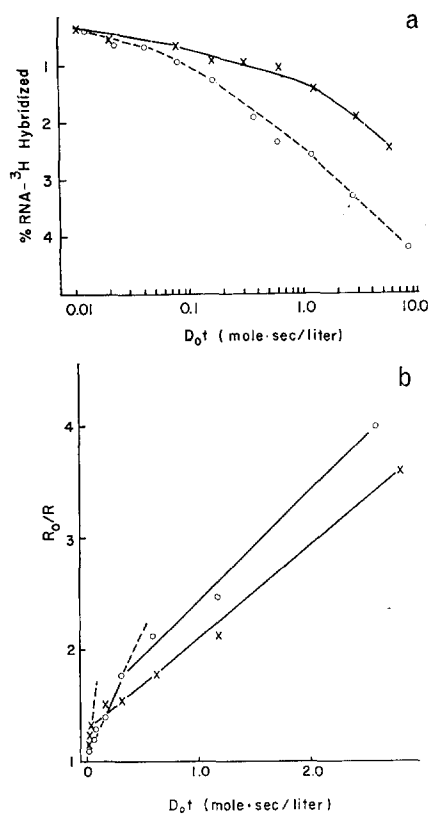
FIGURE 2 Kinetics of hybridization of nuclear RNA and polyribosome RNA at high  $D_0t$ . The reaction volumes were  $10 \mu\text{l}$  at times less than 24 hr, and  $40 \mu\text{l}$  at times  $\geq 24$  hr. The experimental details are given in Table I, exp No. 1. X, mRNA. The solid line represents a theoretical second order reaction for a component with a rate constant of  $3.3 \times 10^{-4}$  liter/mole-sec comprising 82% of the reaction, O, NRNA. The dashed line represents a theoretical second order reaction with a rate constant of  $4.0 \times 10^{-4}$  liter/mole-sec for a component comprising 68% of the reaction.

Fig. 2 shows the hybridization of L cell DNA with NRNA and mRNA at constant inputs of DNA and RNA. The results are plotted in the way adopted by Britten and Kohne (1968) for plotting DNA renaturation kinetics; namely the extent of reaction is plotted against  $D_0t$  (in moles per liter of DNA nucleotides multiplied by seconds) on a logarithmic scale. The lines drawn through the data points represent theoretical second order rate equations (equation [1]) fitted by trial and error. It was assumed that the total hybridization reaction consists of two or more components and that the reaction is nearly complete at the highest values of  $D_0t$ . The theoretical lines represent a single component comprising 68% of the over-reaction in the case of NRNA and 82% of the over-all reaction in the case of mRNA. The rate constants for the fitted equations are  $4.0 \times 10^{-4}$  liter/mole-sec and  $3.3 \times 10^{-4}$  liter/mole-sec for the NRNA and mRNA, respectively. Except for the two earliest time points, none of the experimental data deviates from the fitted equations by more than 5%. Therefore, the hybridization reaction seems to follow equation (1) with respect to time dependence. The observed rates of reaction are similar to published data for the renaturation rate of the nonrepeated sequences of mouse DNA (McConaughy et al., 1969).

A fraction of both NRNA and mRNA appar-

ently reacted at such a high rate that the reaction could only be followed at much lower values of  $D_0t$  than those of Fig. 2. The results of a low  $D_0t$  experiment are shown in Fig. 3 a. The data could not be fitted by a single second order equation. In order to establish whether they could be fitted by more than one second order reaction, and whether the rapidly hybridizing fractions of mRNA and NRNA differ with respect to their rate of reaction, the data were plotted in such a way that, according to equation (1) a straight line segment should be obtained for each second order component of the reaction ( $R_0/R$  vs.  $D_0t$ ). In order to do this, it was assumed that the reaction was essentially complete ( $R_0/R \rightarrow \infty$ ) at  $D_0t = 6$  for the rapidly annealing fractions of both NRNA and mRNA. As seen in Fig. 3 b, the data for both NRNA and mRNA are best fitted by two straight line components. The mRNA and NRNA hybridizing at low  $D_0t$  react at very similar rates, although unlike our previous results (Perry et al., 1970), the curves for mRNA and NRNA are not identical. The apparent rates of reaction for the mRNA and NRNA hybridizing at low  $D_0t$  are thousands of times greater than for the mRNA and NRNA hybridizing at the high  $D_0t$ , and, therefore, must correspond to the highly repeated DNA sequences of the mouse genome (Britten and Kohne, 1968).

A summary of data from three high  $D_0t$  experi-



**FIGURE 3 a** Kinetics of hybridization of nuclear RNA and polyribosome RNA at low  $D_0t$ . The RNA was prepared as described in Materials and Methods. The DNA concentration was  $10 \mu\text{g/ml}$ , and the specific activity was  $245 \text{ cpm}/\mu\text{g}$ .  $\times$ , mRNA. The input was  $18,400 \text{ cpm}/\mu\text{g}$  DNA.  $\circ$ , NRNA. The input was  $6400 \text{ cpm}/\mu\text{g}$  DNA. The reaction volume was  $0.1 \text{ ml}$ . The curves drawn through points are for convenience only; they do not represent fits to a theoretical equation. **FIGURE 3 b** The data of Fig. 3 a were plotted according to equation (1) as a linear function of  $D_0t$ . It was assumed that  $R_0/R \rightarrow \infty$  at  $D_0t = 6$ .  $\times$ , mRNA.  $\circ$ , NRNA.

ments utilizing different preparations of mRNA and NRNA is shown in Table I. There is relatively good agreement in the values of  $k$  and in the proportions of rapidly and slowly annealing RNA.

#### Thermal Stability of the Hybrids

The thermal denaturation behavior of renatured DNA and DNA-RNA hybrids can provide a measure of the extent of base mismatching within the reassociated regions. When each DNA se-

quence has only one complement per genome, then the reassociated sequences will have no base mismatching, and, therefore, a thermal stability similar to that of native DNA. This is also true for the case in which a DNA sequence has multiple identical complementary sequences. On the other hand, where partially complementary sequences exist the reassociated sequences may have some mismatched bases, and, therefore, a reduced thermal stability. We assume, for the purpose of this discussion, that variation in GC content and the size of the hybridized molecules does not greatly affect the  $T_m$ .

As check on our experimental procedures, we determined the thermal denaturation behavior of DNA-RNA hybrids containing essentially no mismatched bases. Hybrids were made between *Bacillus subtilis* DNA and pulse-labeled *B. subtilis* RNA. The DNA of microorganisms has no repeated sequences other than in the genes for ribosomal and transfer RNA (Britten and Kohne, 1968), and *B. subtilis* provides an especially favorable standard of comparison for mammalian DNA-RNA hybrids since the base composition of its DNA is similar to that of mammalian DNA (Church and McCarthy, 1968). With the labeling conditions and DNA to RNA ratio used, the hybridized RNA may be assumed to consist essentially of mRNA (Kennell, 1968). Fig. 4 a shows the thermal denaturation of *B. subtilis* DNA and of DNA-RNA hybrids. It may be seen that the hybrids melt sharply (although somewhat less sharply than the DNA) with a  $T_m$   $4^\circ\text{C}$  below that of the DNA. DNA-RNA hybrids melt at a slightly lower temperature than the corresponding DNA even in the absence of mismatched sequences (Kohne, 1968).

The thermal denaturation of various L cell DNA-RNA hybrids and L cell DNA is shown in Figs. 4 b and 4 c. The most sharply melting hybrids are those formed by mRNA and NRNA at  $D_0t = 6$  (Fig. 4 b), which presumably consist of RNA transcribed from highly repeated sequences. Assuming (from Fig. 4 a) that a hybrid with no mismatching melts  $4^\circ\text{C}$  below the corresponding DNA, and that 1.5% mismatching causes a  $1^\circ\text{C}$  drop in  $T_m$  (Laird et al., 1969), the  $T_m$ 's of these hybrids are indicative of about 11% mismatching of bases. The hybrids formed by NRNA at  $D_0t = 10,000$  melt over a somewhat broader range than the hybrids formed by NRNA at  $D_0t = 6$ , and are composed largely of more heat-stable material (Fig. 4 c).

TABLE I  
Kinetic Behavior of mRNA-DNA and NRNA-DNA Hybrids

Exp		DNA	DNA	RNA	k (major component)	% slowly annealing component	% rapidly annealing component
No.		mg/ml	$\frac{cpm\ ^{14}C}{\mu g}$	$\frac{cpm\ ^3H}{\mu g\ DNA}$	liter/mole·sec		
1	NRNA	10.5	18.6	77.5	$4.0 \times 10^{-4}$	68	32
1	mRNA	10.2	18.6	69.0	$3.3 \times 10^{-4}$	82	18
2	NRNA	16.5	22.1	121	$5.5 \times 10^{-4}$	67	33
2	mRNA	16.5	22.1	74.7	$20 \times 10^{-4}$	81	19
3	NRNA	9.72	18.6	67.1	$5.0 \times 10^{-4}$	69	31
3	mRNA	9.66	18.6	69.8	$4.5 \times 10^{-4}$	79	21

Each experiment represents a separate batch or batches of cells. In exp No. 1 and No. 3 the mRNA and NRNA were from the same batch of cells labeled 60 min with  $10\ \mu\text{Ci/ml}$  uridine- $^3\text{H}$  in the presence of  $0.08\ \mu\text{g/ml}$  actinomycin D. In exp No. 2 the mRNA was from a batch of cells labeled as in exp No. 1 and No. 3, but the NRNA was from cells labeled for 120 min. The inputs of mRNA were 0.26, 0.49, and  $0.079\ \mu\text{g}/\mu\text{g}$  DNA, and the maximum proportions of mRNA and NRNA hybridized at the highest values of  $D_0t$  were 40%, 45%, and 70% for exp No. 1, 2, and 3, respectively.

The thermal stability of the hybrids formed by mRNA at  $D_0t = 21,000$  was unexpectedly low (Fig. 4c). Although these hybrids were formed at a temperature  $15^\circ\text{C}$  below the  $T_m$  of DNA, many of them melt at a temperature more than  $15^\circ\text{C}$  below the  $T_m$  of DNA. This suggests that some of the hybrids are unstable, or reversible, at their temperature of formation.<sup>3</sup> The reason for this phenomenon became apparent when we investigated the effect of RNA input on hybrid stability (*vide infra*).

#### Basis for the Low Thermal Stability of mRNA Hybrids Formed at High $D_0t$

The low melting temperature of the mRNA hybrids formed at high  $D_0t$  is paradoxical. These hybrids are formed at a rate consistent with a very low degree of redundancy in the DNA sequences from which mRNA is transcribed, and one does

<sup>3</sup> It is important to note that the hybrids were formed in 60% formamide-5 × SSC at  $15^\circ\text{C}$  below the  $T_m$  of native DNA, and were collected for  $T_m$  determination in  $0.1 \times \text{SSC}$  at  $40^\circ\text{C}$ , which is  $29^\circ\text{C}$  below the DNA  $T_m$ . They were therefore collected under conditions favorable for the preservation of unstable hybrids. If the hybrids had been collected under more stringent conditions, then presumably their measured  $T_m$ 's would have been higher.

not expect mispairing of bases among unique sequences.

Two plausible explanations that could account for this behavior were tested. One is that some property of the mRNA preparations, perhaps the presence of a large amount of ribosomal RNA or some other contaminant, interferes with hybrid formation. This possibility was tested by mixing labeled NRNA with unlabeled mRNA at various inputs comparable to those used in the experiments of Table I, hybridizing at  $D_0t \approx 20,000$  and melting the hybrids. It was found that the presence of  $0.2\ \mu\text{g}$  of the mRNA-containing preparation per  $\mu\text{g}$  DNA reduced the  $T_m$  of the NRNA hybrids from  $66^\circ$  to  $63^\circ\text{C}$ . This effect is too slight to account for the large difference in thermal stability seen in Fig. 4c.

Another possibility is that the low thermal stability of the mRNA hybrids formed at high  $D_0t$  results from the existence of partial homology in the relatively rare DNA sequences from which mRNA is transcribed. In this case there would be some DNA sequences capable of hybridizing stably with mRNA, namely, those sequences from which the mRNA was actually transcribed, and other DNA sequences capable of forming hybrids with mRNA, which are of such low stability that they are reversible at their temperature of formation. At any particular RNA input the sites capable



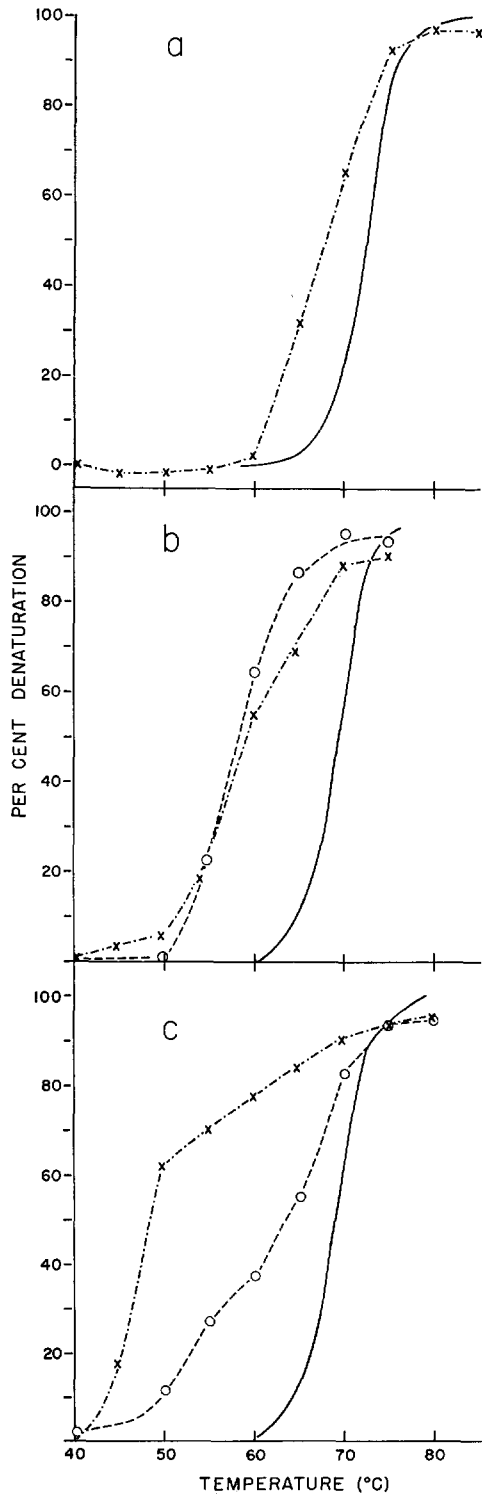


FIGURE 4 *a* Thermal denaturation of *B. subtilis* DNA and DNA-RNA hybrids. RNA was extracted from

of hybridizing stably with mRNA should be preferentially filled. When these sites are saturated, then partially homologous sequences may also be filled.

If the foregoing argument is correct, it may be predicted that as the input of mRNA is reduced, the stability of the hybrids would increase. Similarly, since the stability of the NRNA hybrids is relatively high to begin with, it should be little affected by reducing the input. Fig. 5 shows the results of an experiment in which we measured the thermal stability of NRNA and mRNA hybrids formed at high  $D_{ot}$  with various inputs of RNA. It is apparent that over a fourfold range of inputs the stability of the NRNA hybrids is unchanged. On the other hand, the stability of the mRNA hybrids depends strongly on the RNA input. At sufficiently low inputs the mRNA hybrids are as stable as the NRNA hybrids.

## DISCUSSION

### *Interpretation of Hybridization Kinetics*

The pattern of the transcription of the L cell genome obtained by hybridization kinetics closely reflects the organization of the mouse genome elucidated by Britten and Kohne (1968) on the basis of DNA renaturation kinetics. We have consistently observed that 70–80% of the hybridizing NRNA and mRNA reacts only at high  $D_{ot}$ 's corresponding to those required for the renaturation of the nonrepeated DNA fraction, and that the remainder reacts at  $D_{ot}$ 's comparable to those

cells labeled 2 min with uridine- $^3$ H and hybridized at DNA/RNA = 1 and a DNA concentration of 100  $\mu$ g/ml to  $D_{ot}$  = 23. The DNA contained 2% L cell DNA- $^{14}$ C to monitor DNA recovery. 25% of the labeled RNA was hybridized. —, DNA,  $T_m$  = 72°C. X, hybrid,  $T_m$  = 68°C.

FIGURE 4 *b* Thermal denaturation of L cell DNA and DNA-RNA hybrids at low  $D_{ot}$ . The DNA and RNA used for hybridization and DNA and RNA concentrations were those of Fig. 3. O—O, NRNA ( $D_{ot}$  = 6;  $T_m$  = 58°C). X—X, mRNA ( $D_{ot}$  = 6;  $T_m$  = 58°C). —, DNA ( $T_m$  = 69°C).

FIGURE 4 *c* Thermal denaturation of L cell DNA and DNA-RNA hybrids at high  $D_{ot}$ . The DNA and RNA used for hybridization and DNA and RNA concentrations were those of Table I, exp No. 1 (mRNA) and No. 2 (NRNA). O—O, NRNA ( $D_{ot}$  = 10,000;  $T_m$  = 63°C). X—X, mRNA ( $D_{ot}$  = 21,000;  $T_m$  = 49°C).

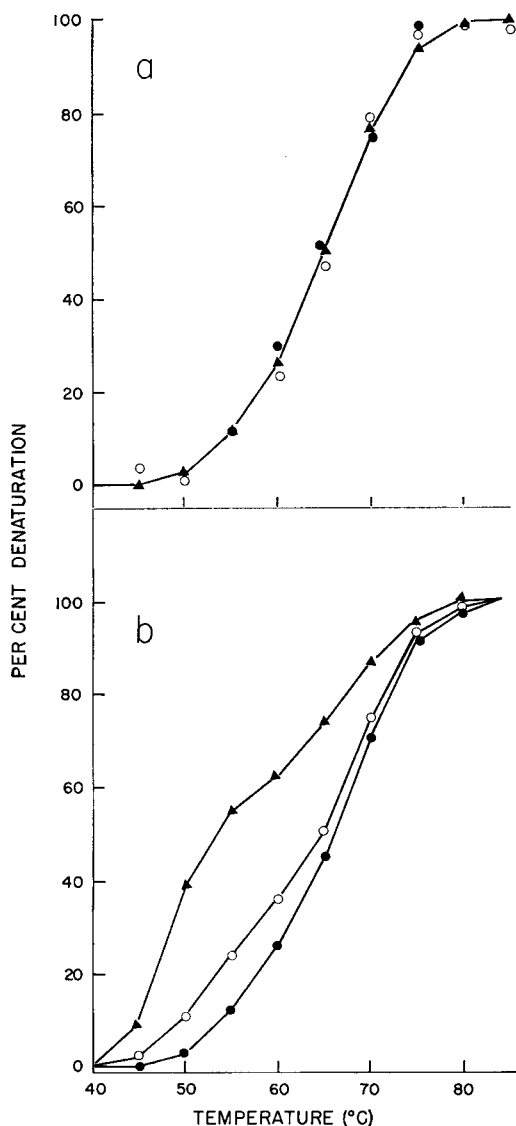


FIGURE 5 Effect of RNA input on the thermal stability of NRNA and mRNA hybrids at high  $D_{0t}$ . Reactions were prepared containing 10 mg/ml L cell DNA- $^{14}C$  (18.0 cpm/ $\mu g$ ) and various inputs of NRNA (24,300 cpm/ $\mu g$ ) and mRNA (882 cpm/ $\mu g$ ), and hybridized to  $D_{0t} \approx 20,000$ . Fig. 5 a, NRNA. ●—●,  $8 \times 10^{-4}$   $\mu g/\mu g$  DNA,  $T_m = 64.5^\circ C$ . ○—○,  $16 \times 10^{-4}$   $\mu g/\mu g$  DNA,  $T_m = 65.5^\circ C$ . ▲—▲,  $32 \times 10^{-4}$   $\mu g/\mu g$  DNA,  $T_m = 65.5^\circ C$ . Fig. 5 b, mRNA. ●—●, 0.016  $\mu g/\mu g$  DNA,  $T_m = 66.0^\circ C$ . ○—○, 0.04  $\mu g/\mu g$  DNA,  $T_m = 65.0^\circ C$ . ▲—▲, 0.13  $\mu g/\mu g$  DNA,  $T_m = 53.5^\circ C$ . The curves have been normalized to 100% denaturation at 85°C to facilitate comparison. The RNA preparations were those of Table I, exp No. 3. The per cent hybridization at 40°C was 66–99% for NRNA, and 44–65% for mRNA.

required for the renaturation of highly redundant DNA sequences. If the rate constant for DNA-RNA hybridization, like the rate constant for DNA renaturation, is inversely proportional to the size of the genome and proportional to the redundancy, then the reaction of mRNA and NRNA at low  $D_{0t}$  would correspond to on the order of 10,000 copies per genome of each transcribed sequence and the reaction at high  $D_{0t}$  would correspond to one or a few copies of these sequences per genome. Since, under certain conditions a significant amount of mispairing can be observed in mRNA hybrids formed at high  $D_{0t}$ , it follows that some of the rare DNA sequences are not strictly unique. It is not known to what extent mispairing affects the relationship between rate constant and degree of repetition, but the nucleotide sequencing studies of Southern (1970) on the guinea pig  $\alpha$ -satellite DNA suggest that nucleic acid reassociation kinetics give only a minimal estimate of redundancy.

It is unlikely that the fraction of mRNA hybridizing at low  $D_{0t}$  represents NRNA lost from the nucleus in cell fractionation. Our estimate of the maximum contamination of mRNA with NRNA is 16%, and the maximum fraction of NRNA which hybridizes at low  $D_{0t}$  is 32%. If all rapidly reacting mRNA represented nuclear contamination, then the maximum amount of mRNA reacting rapidly would be 16% of 32% or, 5%. This figure is considerably lower than the 18% of mRNA which we estimate to hybridize at low  $D_{0t}$ .

It should be noted that no RNA was found to hybridize at a  $D_{0t}$  corresponding to the renaturation of the most highly repeated fraction of the mouse genome, i.e., the mouse satellite DNA. This result is in agreement with the findings of Flamm et al., (1969) who were unable to detect any RNA capable of hybridizing with the purified mouse satellite DNA.

The maximum percentage hybridization obtained at the highest values of  $D_{0t}$  varied among the different experiments from about 40 to 70% for both mRNA and NRNA. It is not clear why the remaining labeled RNA did not hybridize. Since there was little or no label in ribosomal RNA and not more than 15% of the label in transfer RNA, one cannot attribute this result to the presence in the RNA preparations of these species, which are known to saturate the complementary DNA at very low inputs. Moreover, the extent of hybridization was not detectably affected by increasing or reducing the ratio of DNA to RNA by a factor of three compared with that routinely used in the

TABLE II  
Thermal Denaturation of mRNA-DNA and  
NRNA-DNA Hybrids

Conditions of hybridization	$T_m$	
	NRNA	mRNA
$D_0t$	$^{\circ}C$	$^{\circ}C$
6	58	58
10,000–25,000	63*	49–66†

Data were taken from Figs. 4 and 5 and similar experiments.  $T_m$  values given are experimentally determined temperatures at which 50% of the DNA-RNA duplexes are denatured. Under the conditions used, native DNA melts at 69°C.

\* Average for the three RNA preparations of Table I. Values are 60°, 63°, and 65°C for experiments 1, 2, and 3, respectively. No correction was made for the contribution of the minor fraction of rapidly annealing components present in the hybrid samples. Therefore, the  $T_m$  of the slowly forming hybrids may be expected to be slightly higher than the values given.

†  $T_m$  depends on RNA input (see Fig. 5). Lowest and highest  $T_m$  values correspond to inputs of 0.49 and 0.016  $\mu g$  RNA/ $\mu g$  DNA, respectively.

experiments, and therefore it would appear that this ratio was not the limiting factor.

### Thermal Denaturation

The  $T_m$ 's of the hybrids formed at both low and high  $D_0t$  are summarized in Table II. The melting behavior of the L cell DNA-RNA hybrids formed at low  $D_0t$  is similar to that of mammalian DNA renatured at low  $D_0t$ . The depressed  $T_m$  of the hybrids is characteristic of the reassociation products formed by sequences which are only partially complementary (Britten and Kohne, 1968).

The bulk of the NRNA hybrids formed at high  $D_0t$  have a thermal stability higher than that of the NRNA which hybridizes at low  $D_0t$ . In some experiments the NRNA hybrids formed at high  $D_0t$  exhibited  $T_m$ 's approaching those expected for perfectly base paired L cell DNA-RNA hybrids. This result indicates that a high proportion of NRNA is most likely transcribed from the truly unique sequences of L cell DNA.

At sufficiently low inputs of RNA the mRNA formed at high  $D_0t$  also have a high thermal stability indicative of little or no mismatching of bases. However, at higher inputs of mRNA hybrids are formed which are not completely stable at their

temperature of formation. The formation of such reversible hybrids containing mismatched bases has also been observed by others (Church and McCarthy [1968]; Soeiro and Darnell [1969]). The fact that these hybrids are part of the slowly annealing fraction, i.e. they are formed only at high  $D_0t$ , implies that the participating DNA sequences must be relatively rare. However, there appears to be sufficient homology among these DNA sequences to allow a particular mRNA segment, when reacted under appropriate conditions, to hybridize to a DNA sequence other than that from which it was transcribed. The extent of homology is apparently not great, however, because the stability of such hybrids is quite low. By comparison the highly repetitious DNA sequences which are transcribed into mRNA have a measurably greater homology among themselves, as reflected by the higher  $T_m$  of their hybrids. Perhaps the partial homology among the rare sequences coding for mRNA should not be considered too surprising in view of the extensive amino acid sequence similarities known to occur among mammalian proteins of differing function (Dayhoff, 1969).

The question arises as to why NRNA and mRNA differ with respect to the input dependence of the thermal stability of their respective hybrids. It may be that we simply did not use sufficiently high inputs of NRNA to obtain hybrids of low stability. On the other hand, it is possible that the relatively rare DNA sequences which transcribe NRNA have less similarity among themselves than do those which code for mRNA. In this regard it is of interest that Hahn (1970), using mouse brain nuclear RNA, obtained hybrids of high stability at truly saturating inputs. Thus, the input dependence of hybrid stability may be a distinctive property of mRNA.

Another question of interest is why duplexes of low stability formed at high  $D_0t$  have not been detected in DNA renaturation experiments. If unstable hybrids are formed predominantly by the small fraction of the genome involved in the transcription of mRNA, then the hybrids formed by this small fraction may go undetected in DNA renaturation experiments. Another important difference between DNA renaturation and DNA-RNA hybridization is that in DNA renaturation the complementary sequences are always present in exactly equivalent proportions, whereas in DNA-RNA hybridization an RNA sequence may be

present in excess over the DNA sequence to which it is exactly complementary.

### Biological Implications

The fact that the proportion of NRNA transcribed from the highly repeated DNA sequences of the L cell genome is greater than the proportion of mRNA transcribed from the highly repeated sequences is consistent with the idea that some of the highly redundant sequences may be used for transcribing RNA which is restricted to the nucleus and which functions as a regulator of gene activity (Britten and Davidson, 1969). However, we have not been able to detect any fraction of NRNA which is transcribed from DNA sequences more highly repeated than those from which mRNA is transcribed. Moreover, it should be noted that the proportion of NRNA which is transcribed from highly repeated sequences is only about one-third. In view of the fact that some mRNA is also transcribed from highly repeated sequences, this means that the fraction of NRNA which is both transcribed from highly repeated sequences and is also nucleus restricted must be less than one-third. When this fraction is compared to estimates indicating that 4/5 or more of the NRNA is nucleus restricted (Soeiro et al., 1968), we are led to conclude that much of the nucleus-restricted RNA must be derived from relatively rare or unique DNA sequences. This would be the case if all nucleus-restricted RNA molecules contain major portions which are complementary to rare sequences, or alternatively, if there are some nucleus-restricted RNA molecules which are complementary to highly repeated sequences and some which are complementary to rare sequences. Experiments currently in progress may allow us to choose between these possibilities.

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