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Sequence Composition of Rat Nuclear Deoxyribonucleic Acid and High Molecular Weight Nuclear Ribonucleic Acid†

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ABSTRACT: Rat ascites nuclear DNA has been characterized by its reassociation profile [$(Na^+) = 0.18$ at 62°] as judged by chromatography on hydroxyapatite. Single copy DNA ($C_0t_{1/2}^{obsd} = 1.5 \times 10^3$) comprises 65% of the genome. Sequences repeated an average 1800 times (middle repetitive DNA, $C_0t_{1/2}^{obsd} = 1$) comprise 19% of the genome while 9% of the genome (highly repetitive DNA) reassociates faster than is measured in these experiments ($C_0t_{1/2}^{obsd} < 2 \times 10^{-2}$). Middle repetitive and single copy DNA have been isolated and characterized with respect to their reassociation kinetics and melting profiles. Both isolated classes reassociate with the kinetics describing these components in total nuclear DNA. Reassociated single copy DNA has high thermal stability indicative of fidelity in base pairing; reassociated middle repetitive DNA has a lower thermal stability that may be attributed, in part, to base-pair mismatch, and, in part, to the shortness of the duplex regions. Rat ascites heterogeneous nuclear RNA (HnRNA, 5–10 $\times 10^6$ daltons) has been sheared and hybridized in excess to isolated single copy or middle repetitive DNA [$(Na^+) = 0.18$ at 62°]. HnRNA hybridizes to a minimum of 4.5% of the single copy and a minimum of 7.8% of the middle repetitive DNA. The T_m of single copy or middle repetitive hybrids is 1–2° lower than that of the reassociated homologous DNA. DNA isolated from single copy or middle repetitive hybrids reassociates with kinetics similar to the input DNA. Sheared HnRNA has been hybridized to an excess of total nuclear DNA. 38% of the HnRNA hybridizes with kinetics ($C_0t_{1/2} = 2 \times 10^3$) similar to single copy DNA and 12% hybridizes with kinetics ($C_0t_{1/2} = 5.6$) a little more slowly than the major reassociating component of middle repetitive DNA. The remaining 50% of the HnRNA failed to hybridize.

In a previous paper (Holmes and Bonner, 1973) we showed that it was possible to isolate high molecular weight, rapidly labeled heterogeneous nuclear RNA (HnRNA) from rat ascites cells. Apparently a substantial proportion (*ca.* 30%) of the radioactivity in HnRNA, after a 30-min pulse of [^{3}H]-uridine, is in molecules of 5–10 $\times 10^6$ daltons as determined under denaturing conditions. This paper analyzes the composition of such high molecular weight HnRNA with respect to various classes of hybridizable single copy and repetitive sequences. In a subsequent paper we examine the physical organization of such sequences within high molecular weight HnRNA molecules (Holmes and Bonner, 1974).

Materials and Methods

Isolation of DNA and RNA. DNA was isolated from the chromatin of rat Novikoff ascites cells by the method of Dahmus and McConnell (1969). rRNA was isolated from rat Novikoff ascites cells as described previously (Holmes and Bonner, 1973). HnRNA was prepared either by sedimentation in aqueous sucrose gradients and RNA >60 S selected or by sedimentation in Me_2SO gradients and RNA between 5 $\times 10^6$ and 10^7 daltons selected as described by Holmes and Bonner (1973).

Shearing and in Vitro Labeling of RNA. HnRNA or rRNA samples were dissolved in degassed 1 mM EDTA (pH 8) at a concentration of about 0.25 mg/ml and sonicated for a total of 2 min (10-sec pulses with 10-sec intervals for cooling in ice) using a Branson micro-tip sonicator. Argon was bubbled through the solution during sonication. Sheared RNA was extracted with an equal volume of phenol–chloroform (1:1), precipitated at −18° for 4 hr in 2% (v/v) sodium acetate (pH 5)–2 vol of 95% ethanol, and dissolved in 0.01 M sodium acetate (pH 6)–0.1 M NaCl–1 mM EDTA–0.2% sodium dodecyl sulfate; 1-ml samples of the RNA at a concentration of *ca.* 0.5 mg/ml were loaded onto 5–20% sucrose gradients (Mann ultrapure sucrose) made in the same buffer

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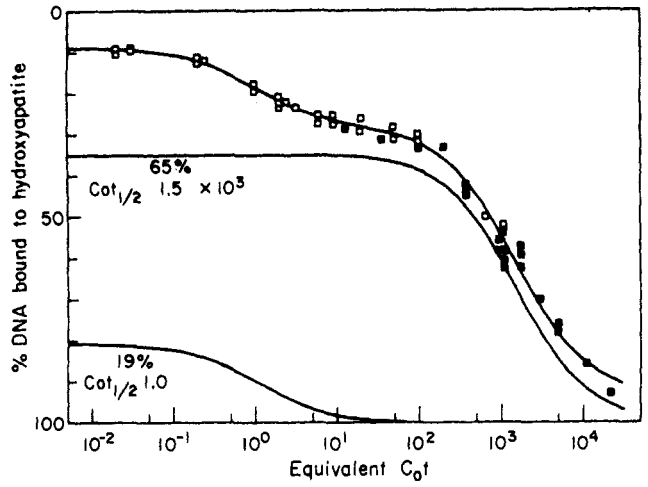

FIGURE 1: Computer analysis of the renaturation profile of total nuclear rat ascites DNA. Denatured sheared DNA (*ca.* 350 nucleotides in length) was reassociated in either 0.12 M PB (□), or 0.48 M PB (■), at 62 or 69°, respectively. Reassociation was measured by passage of the reacted DNA through hydroxyapatite as described by Britten and Kohne (1967). The computer program was supplied by Dr. R. J. Britten. The root mean square of the line describing the reassociation of total DNA is 0.025; reassociation of the DNA goes to 94% completion.

and centrifuged for 16 hr at 25,000 rpm in the Spinco SW 25.2 rotor at 18°. Following centrifugation the tubes were dripped and the A_{260} of the fractions was determined. The sheared RNA formed a reasonably symmetrical peak (modal value of 6 S) with some tailing on the high molecular side. There was no evidence of nonrandom shearing. Fractions corresponding to RNA of 6–8 S (comprising about 40% of the RNA) were pooled and precipitated as described above. (*Escherichia coli* 23 S and 16 S [^{3}H]rRNA were used as markers.) The RNA was dissolved in 0.1 M potassium phosphate buffer (pH 7.4) at a concentration of *ca.* 1 mg/ml and labeled *in vitro* with [^{3}H]dimethyl sulfate (New England Nuclear, 2300 Ci/mol), essentially by the method of Smith *et al.* (1968); 200 μl of the RNA was added to 5 mCi of [^{3}H]-dimethyl sulfate in the same vial as the isotope was purchased (care was taken to ensure that all [^{3}H]dimethyl sulfate on the walls and top of the vial was dissolved in buffer), and incubated overnight at 4°. Unreacted [^{3}H]dimethyl sulfate was removed by Sephadex G-50 chromatography followed by precipitation of the excluded RNA with ethanol as described above. The resulting specific activities were 110,000 cpm/μg (4% of the total bases methylated) for rRNA and 76,000 cpm/μg (3% of the total bases methylated) for HnRNA (% methylation corrected for quenching).

Purification of DNA Components and Labeling of DNA. DNA (about 1 mg/ml) was dissolved in 0.06 M PB[1] and sheared to about 350 nucleotides (single-strand length judged by electron microscopy) by three passages through a Ribi-Sorvall cell fractionator at 50,000 psi. Purified DNA components were prepared by incubation of the DNA in PB (for conditions see legend to Figure 1) to an appropriate equivalent C_0t followed by separation of double- from single-stranded DNA by hydroxylapatite chromatography as described by Britten and Kohne (1967). Single copy DNA was prepared by isolating DNA that remained single stranded both at a C_0t of 2×10^3 on a first cycle of reassociation and at a C_0t of 10^3 on a second cycle of reassociation. Middle repetitive DNA was isolated by separation of DNA that reassociated between C_0t 2×10^{-2} and C_0t 10^2. Highly repetitive DNA that reacted before C_0t 2×10^{-2} (*ca.* 9% of the total) was discarded.

Purified single copy and middle repetitive DNA were labeled *in vitro* with ^{125}I by a modification of the method of Commerford *et al.* (1971). In initial experiments different iodinated DNAs were found to have remarkably different abilities to retain ^{125}I during subsequent hybridization reactions. Losses from 10 to 99% of "apparently" bound ^{125}I occurred during the course of hybridization reactions lasting several days at high temperature. We found that loss of ^{125}I could be minimized by observing the following precautions. (Several of these precautions were suggested by our colleagues Drs. Roy Britten, Jerry Johnson, Mr. Joel Gottesfeld, and Mr. Glen Galau.) (1) The DNA must be free of minor contaminants such as protein, EDTA, and insoluble residues present after lyophilization of a large volume. We routinely resuspended isolated DNA in 7 M urea–0.35 M NaCl–0.001 M EDTA–2% sodium dodecyl sulfate–0.01 M Tris (pH 8) and extracted with a mixture of equal amounts of redistilled phenol–chloroform as described in Holmes and Bonner (1973). The aqueous phase was dialyzed extensively against freshly prepared glass distilled water and lyophilized to dryness. The DNA was resuspended in a small volume of water and re-extracted with phenol–chloroform and redialyzed against water. The DNA was denatured by boiling for 5 min. Usually about 25 μg of DNA in 50 μl of water was labeled at one time. (2) All solutions were prepared from deionized freshly boiled, cooled water. (3) Solutions of sodium acetate, potassium acetate, and sodium sulfite were deaerated before use. (4) Thallium chloride was prepared within 1 min before use. (5) Care was taken to ensure that the reaction mixture was pH 5.0. (6) The two incubations of ^{125}I with DNA reported by Commerford (1971) have been changed to 20 min at 60° followed by 30 min at 40°. (7) At the end of the reaction the DNA was separated from unbound ^{125}I by passage over Sephadex G-50. The column buffer was 0.1 M NaCl–0.03 M PB. DNA was placed in a boiling water bath for 5 min, cooled in ice, and passed over a column containing hydroxyapatite equilibrated at 60° with 0.1 M NaCl–0.03 M PB. The column was washed with 6 column volumes of the same buffer at 60° to remove unbound ^{125}I and with 6 column volumes of 0.48 M PB at 60° to remove [^{125}I]DNA. Generally about 10% of ^{125}I was found in the 0.1 M NaCl–0.03 M PB wash and about 5% of the ^{125}I remained bound to the column after washing with 0.48 M PB. During the course of an extensive incubation at high temperature (*e.g.*, 72 hr in 0.48 M PB at 69°) between 30 and 50% of the ^{125}I was lost from the DNA and was determined as described above.

Hydroxyapatite Chromatography. Chromatography of DNA or RNA–DNA mixture on hydroxyapatite was carried out by the method of Britten and Kohne (1967); 1 ml of packed hydroxylapatite (Bio-Rad) was used per 100 μg of nucleic acid applied and the DNA eluted with 6 column volumes of either 0.12 or 0.48 M PB. Eluted samples were precipitated in cold 10% Cl_3CCOOH at 4° for 10 min. If necessary yeast soluble RNA (CalBiochem) was added to bring the final concentration of total nucleic acids to 50 μg/ml. The filters were washed with cold 10% Cl_3CCOOH followed by 60% ethanol, then dried and dissolved in 1 ml of ethyl acetate, and counted in a toluene scintillation cocktail.

[1] Abbreviations used are: PB, sodium phosphate buffer pH 6.8 (made from equimolar amounts of mono- and disodium phosphate); HnRNA, heterogeneous nuclear RNA.

TABLE I: Composition of Rat Nuclear DNA with Respect to Components of Various Degrees of Repetition.[a]

Class of DNA	% Total DNA	$C_0t_{1/2}^{Obsd}$	Average $C_0t_{1/2}$ pure (estimated)[b]	Average kinetic complexity (daltons) relative to *E. coli*[c]	Daltons per Genome[d]	Reiteration Frequency
Nonrepetitive	65	1.5×10^3	9.7×10^2	1.1×10^{12}	1.17×10^{12}	1.1
Middle repetitive	19	1.0	0.19	2.0×10^6	0.35×10^{12}	1.8×10^3
Highly repetitive	9				0.16×10^{12}	

[a] Calculated from the data of Figure 1. [b] Average $C_0t_{1/2}$ pure (estimated) = fraction of DNA $\times$ $C_0t_{1/2}^{obsd}$. [c] The complexity of *E. coli* is assumed to be 2.8×10^9 daltons (Cairns, 1963). The effect of GC content on the rate of renaturation of rat DNA (41% GC) relative to *E. coli* DNA (50% GC) is to reduce the rate by 0.83 (Wetmur and Davidson, 1968) and assuming the GC content of each major reassociating class of rat DNA is the same. [d] Assuming the rat haploid genome consists of 1.8×10^{12} daltons of DNA (Sober, 1968).

Quenching was monitored and estimation of radioactivity adjusted accordingly.

An extinction coefficient of 6600 was assumed for double-stranded DNA and 8850 for single-stranded DNA.

RNA Excess Hybridization. Unlabeled sheared HnRNA was added to single copy [^{125}I]DNA and denatured by placing in a boiling water bath for 7 min and hybridization carried out in either 0.12 M PB–1 mM EDTA at 62° or in 0.48 M PB–1 mM EDTA at 69°. The concentration of RNA and DNA and the length of incubation are recorded in Table II. At the end of incubation the reaction mixes were diluted to 0.12 M PB and passed through a column containing hydroxylapatite equilibrated with 0.12 M PB at 62°. The column was washed with 0.12 M PB to remove single-stranded DNA followed by 0.48 M PB to remove DNA–RNA hybrids. The radioactivity associated with DNA was measured as described above. Loss of ^{125}I from DNA was monitored as described in a previous section and data have been corrected accordingly. Zero time binding of [^{125}I]DNA was estimated by heat denaturing solutions followed by rapid cooling in a Dry Ice–ethanol bath and the double- and single-stranded DNA content estimated by chromatography on hydroxylapatite as described above. If large volumes (*e.g.*, 5.0 ml) were used then they were applied in several steps to the hydroxylapatite to avoid the possibility of DNA reassociation prior to adsorption onto hydroxylapatite. DNA–RNA duplexes formed during the course of the reaction were measured as described by Hough and Davidson (1973). DNA was isolated from the RNA–DNA hybrids and its reassociation kinetics estimated as described by Hough and Davidson (1973).

DNA Excess Hybridization. Sheared, *in vitro* labeled, [^{3}H]-HnRNA or [^{3}H]rRNA were reacted with total nuclear DNA (sheared to about 350 bases single-strand length). The DNA to rRNA ratio was about 319,000:1 and the DNA to HnRNA ratio was about 170,000:1. The conditions of the reaction are described in the legend to Figure 6. At the end of incubation the solution was divided into two aliquots. One aliquot was adjusted to 0.24 M PB and subjected to treatment with 20 μg/ml of ribonuclease A and 20 units/ml of ribonuclease T1 at 37° for 15 min. The solution was precipitated with Cl_3CCOOH and collected on filters as described by Melli *et al.* (1971). The filters were washed and the radioactivity was estimated as described above. The other aliquot was used to measure DNA reassociation by chromatography on hydroxylapatite as described above, using the A_{260} of the eluent to estimate DNA content.

Melting Profiles. Purified middle repetitive DNA was reassociated to a C_0t of 10^2 and single copy DNA to a C_0t of 2×10^4 as described above and applied to 0.12 M PB to hydroxylapatite, equilibrated at 55°. The column temperature was raised at 5° increments and allowed to equilibrate for 5 min at each step. Single-stranded DNA was eluted with 0.12 M PB, precipitated with Cl_3CCOOH onto filters and counted as described above. The melting profile of RNA–DNA hybrids was estimated in the same manner. Hybrids for melting were taken from equivalent reactions that determined the final hybridization points shown in Figure 4a,c.

Results

Sequence Composition of Nuclear DNA. The point of departure of these studies is an analysis of the sequence composition of rat nuclear DNA. This analysis is a necessary prerequisite to the identification and isolation of kinetic components of nuclear DNA which will be described in the next section. Figure 1 shows the reassociation profile of sheared nuclear DNA determined by the method of Britten and Kohne (1967). Second-order reaction curves have been fitted to the major reassociating components. Table I lists the parameters that describe these reaction curves. These parameters depend, in part, on the conditions used to determine duplex stability (Britten and Kohne, 1968; McCarthy and Duerksen, 1970). At the chosen criterion of reassociation [0.18 (Na^+), 62°, T_m −23°] about 65% of the input DNA reassociates with kinetics indicating that each sequence is present about once per haploid genome and 19% reassociates with kinetics indicating that each sequence of the major component of this class is repeated, on average, about 1800 times per haploid genome. We define this fraction of the DNA (reassociating between a C_0t of 2×10^{-1} and 10^2) as "middle repetitive." About 9% of the total DNA reassociates faster than can be measured in this experiment. This component may represent DNA with internal strand homology and/or may correspond to the fast-reassociating satellite found in other organisms (Waring and Britten, 1966; Kram *et al.*, 1972).

Isolation of Single Copy and Middle Repetitive DNA Sequences. The strategy for the isolation of single copy and middle repetitive DNA is based on the reassociation of total DNA to a particular C_0t followed by the separation of single- from double-stranded DNA. The success of this strategy depends in part on the size of the DNA fragments which are allowed to reassociate and in part on the magnitude of the difference in the reaction rate between the particular components. In these experiments the DNA was sheared to an

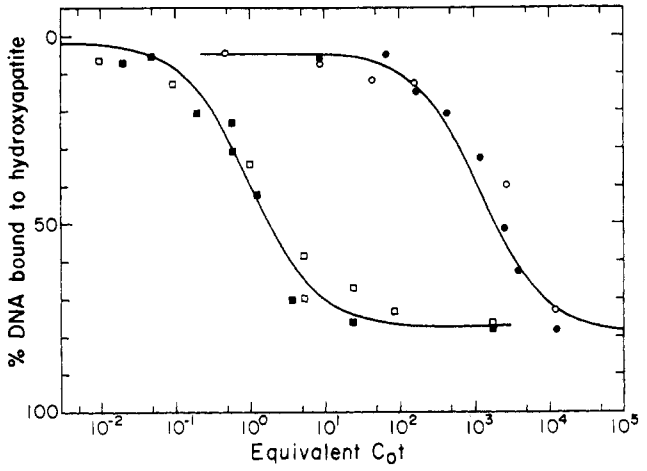

FIGURE 2: C_0t plot of trace quantities of single copy and middle repetitive [^{125}I]DNA in the presence of unlabeled total nuclear DNA. A ratio of about 1300:1 total nuclear DNA to [^{125}I]DNA was used. Reassociation was carried out in 0.12 M or 0.48 M PB at 62 or 69°, respectively and the extent of reassociation measured by passage of the reaction mixture through a hydroxyapatite column as described by Britten and Kohne (1967): purified middle repetitive [^{125}I]DNA (■); purified middle repetitive [^{125}I]DNA isolated from DNA:HnRNA hybrids (□); purified single copy [^{125}I]DNA (○); purified single copy [^{125}I]DNA isolated from DNA:HnRNA hybrids (●); second-order reaction curves have been fitted to the data. Sucrose-purified HnRNA was used to produce the hybrids. Hybridization was carried out as described for the last points displayed in Figure 4a, c. The [^{125}I]DNA was isolated from DNA–HnRNA hybrids as described by Hough and Davidson (1973).

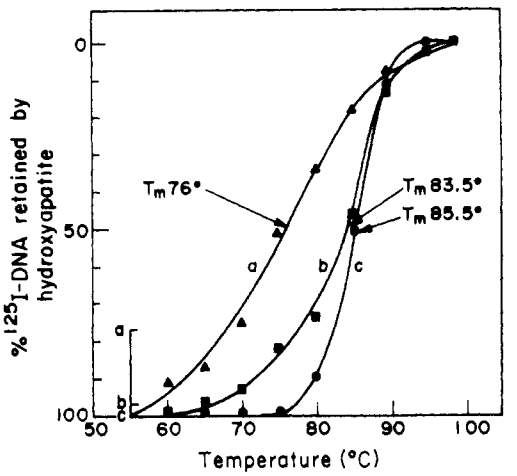

FIGURE 3: Integral melting curve of DNA by thermal elution from hydroxyapatite as described by Britten and Kohne (1967). The DNA was applied in 0.12 M PB to a jacketed hydroxyapatite column equilibrated at 55°. The per cent DNA eluted after exhaustive washing at 55° in 0.12 M PB is shown by the bar at the left. The column temperature was raised in 5° increments, equilibrated at the temperature for 5 min, and then washed with 6 column volumes of 0.12 M PB. The eluent was either precipitated and counted as described in Methods in the case of [^{125}I]DNA or read at A_{260} in the case of native DNA: (●) native DNA; (■) reassociated single copy [^{125}I]DNA; (▲) reassociated middle repetitive [^{125}I]DNA.

average of 350 bases (single-strand length) which is approximately the number average length of the middle repetitive sequences (about 100–300 bases) and below that of the single copy sequences (about 800 bases) of rat DNA (Bonner *et al.*, 1973; Wilkes *et al.*, 1974). Thus, if we assume random shearing of the DNA, each fragment will contain predominantly either repetitive or single copy sequences. The difference between the second-order reaction rate describing the single copy (k = 6.7×10^{-4}) component and that describing the major component of the middle repetitive DNA (k = 1.0) is about three orders of magnitude.

The efficiency with which these components can be isolated is shown in Figure 2. In this experiment the isolated single copy and middle repetitive components have been labeled *in vitro* with ^{125}I and reassociated with excess unlabeled total DNA. The reaction rates describing the major component of the middle repetitive DNA (k = 9.2×10^{-1}) and of the single copy DNA (k = 6.1×10^{-4}) are in good agreement with the reaction rates of these components estimated from the reassociation profile of total DNA (Table I). The second-order reaction curves that have been fitted to the reassociation profile of purified middle repetitive and single copy DNA (see Figure 2) indicate that no gross selection for kinetic components other than the major ones has taken place during the isolation procedure. This is particularly germane with respect to middle repetitive DNA which probably contains minor fractions that reassociate at rates different from that describing the major fraction. Also shown in Figure 2 is the reassociation of DNA isolated from DNA–RNA hybrids. We will describe these data in the next section.

The melting profiles of reassociated purified single copy and middle repetitive DNA are shown in Figure 3. Single copy DNA melts with a T_m about 2° below that of native DNA indicating excellent fidelity of base pairing. Compared to native DNA the melting profile of single copy DNA has a distinct "foot" of material melting between 60 and 75°. This foot can probably be accounted for by a brevity of duplex length resulting from the renaturation of short single copy sequences generated by random shearing. These observations are in accord with those of other workers (Britten and Kohne, 1967; Brown and Church, 1971; Hahn and Laird, 1971; Firtel and Bonner, 1972). The middle repetitive DNA has a broader, less cooperative, melting profile, with a T_m about 10° lower than native DNA. Part of this lowering of T_m might be attributed to the extreme shortness of the repetitive duplex regions (Bonner *et al.*, 1973; Wilkes *et al.*, 1974), as suggested by the findings of Hayes *et al.* (1970). A portion of the lowering of T_m could be due to base-pair mismatch occurring during reassociation as suggested by the sensitivity of repetitive components to manipulation of reaction parameters (Britten and Kohne, 1968; McCarthy and Duerksen, 1970). We do not know the base composition of middle repetitive DNA, a parameter that could also affect the T_m.

Since the reassociation rates of the purified components are close to those predicted from the parameters of Table I, and since the single copy duplexes have a T_m close to that of native DNA, it is unlikely that iodination of these components has had a marked effect on the reassociation rate or stability of the duplexes.

Hybridization of High Molecular Weight HnRNA to Middle Repetitive or Single Copy DNA. Purified single copy or middle repetitive [^{125}I]DNA was allowed to react with excess sheared, high molecular weight HnRNA under conditions allowing the formation of DNA–RNA hybrids. After appropriate incubation times double-stranded material was isolated by chromatography on hydroxylapatite. The double-stranded material includes predominantly RNA–DNA hybrids but also has traces of DNA duplexes (Figure 4 and Table II). DNA duplex formation was measured by extensive ribonuclease treatment of double-stranded material followed by measurement of the surviving DNA duplexes. The estimation of the per cent of DNA hybridized by RNA probably yields a reasonable estimate of complementary DNA. Although some of the DNA

TABLE II: Hybridization of HnRNA to DNA.

	Volume (ml)	RNA Concn (mg/ml)	Hours of Incubation	% DNA in DNA–RNA Hybrids	% DNA in DNA Duplexes
(a)[a]	5	0	0		2.3
	5	0.105	0.2	2.0	2.3
	5	0.105	0.8	3.0	2.3
	5	0.105	1.4	4.3	2.4
	2	0.495	0.6	6.8	2.3
	2	0.495	1.2	8.1	2.5
	2	0.495	2.0	9.4	2.5
(b)	0.5	0	0		0.8
	0.5	0.23	0.18	2.3	2.9
	0.3	0.23	0.4	5.4	1.7
	0.1	0.61	1.3	7.8	1.7
(c)	0.3	0	0		3.2
	0.3	10	9	1.2	2.8
	0.3	10	18	2.8	3.3
	0.3	10	24	3.2	3.4
	0.2	10	36	3.8	3.4
	0.2	10	54.5	4.2	3.6
	0.2	10	96	4.5	3.6
(d)	0.025	0	0		1.2
	0.025	10.1	3.0	2.1	2.1
	0.025	10.1	16.0	5.2	1.0
	0.025	10.1	48.0	5.5	1.8

[a] Letters refer to particular reactions described in legend to Figure 4.

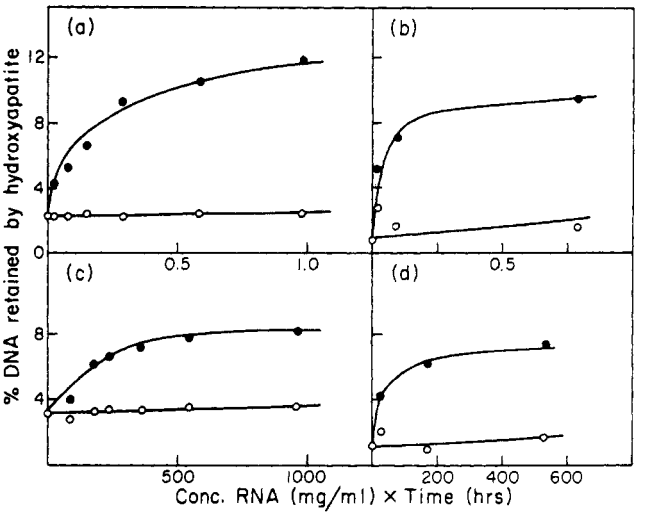

FIGURE 4: Hybridization of HnRNA (sonicated to about 6–8 S in size) to ^{125}I-labeled purified kinetic components of rat nuclear DNA (sheared to a number average of 350 bases single-strand length as judged by electron microscopy). The extent of hybridization and of DNA duplex formation was estimated by chromatography of the reaction mix on hydroxyapatite as described in Methods. Hybridization to middle repetitive DNA was carried out in 0.12 M PB-1 mM EDTA at 62° and to single copy DNA in 0.48 M PB-1 mM EDTA at 69°. (a) Hybridization of sucrose purified HnRNA to middle repetitive [^{125}I]DNA; (●) RNA–DNA hybrids; (○) DNA duplexes. Specific activity of DNA was 1.8×10^5 cpm/μg. RNA to DNA ratio was 4500:1. (b) Hybridization of Me_2SO purified HnRNA to middle repetitive [^{125}I]DNA: (●) RNA–DNA hybrids; (○) DNA duplexes. Specific activity of DNA was 6.8×10^6 cpm/μg. RNA to DNA ratio was 3100:1. (c) Hybridization of sucrose-purified HnRNA to single copy [^{125}I]DNA: (●) RNA–DNA hybrids; (○) DNA duplexes. Specific activity of DNA was 1.7×10^6 cpm/μg. RNA to DNA ratio was 1011:1. (d) Hybridization of Me_2SO purified HnRNA to single copy [^{125}I]DNA: (●) RNA–DNA hybrids; (○) DNA duplexes. Specific activity of DNA was 8.9×10^6 cpm/μg. RNA to DNA ratio was 1960:1. Further conditions are tabulated in Table II.

fragments in hybrid form will be longer than the "true" length of the DNA site complementary to the RNA, random shearing of the DNA will presumably compensate for this effect by the production of DNA fragments that contain complementary RNA sites too short to form hybrids at the criterion of hybridization used.

Hybridization of both sucrose purified HnRNA and Me_2SO purified HnRNA to DNA is shown in Figure 4. During its preparation the sucrose purified HnRNA was not submitted to denaturing conditions and there is apparently aggregation with at least a small amount of GC rich RNA (presumably rRNA precursor) and possibly with HnRNA molecules with a slower sedimentation velocity than bulk high molecular weight HnRNA. On the other hand, high molecular weight HnRNA prepared through Me_2SO is free of such contaminants (Holmes and Bonner, 1973). Thus it is of interest to compare the hybridization properties of the two HnRNA preparations.

The final points of the hybridization curves of Figure 4 show that Me_2SO purified high molecular weight HnRNA hybridizes to at least 7.8% of the middle repetitive DNA (half-reaction at 0.05 mg/ml hr^{-1}) and to at least 4.5% of the single copy DNA (half-reaction at 40 mg/ml hr^{-1}). The equivalent values for sucrose purified HnRNA are 9.4% (half-reaction at 0.12 mg/ml hr^{-1}) and 5.5% (half-reaction at 120 mg/ml hr^{-1}). In all cases DNA duplexes have been subtracted from the values given here. The unnormalized data are shown in Figure 4. It is unlikely that these are true saturation values since the reactions have probably not gone to completion. We consider that there is no gross difference between the per cent hybridization recorded between sucrose purified and Me_2SO purified HnRNA. However, a comparison of half-reaction values indicates that the sucrose purified HnRNA is hybridizing more slowly than the Me_2SO purified HnRNA by a factor of about 2–3. This is consistent with the view that sucrose purified HnRNA contains a contaminant, such as rRNA precursor, that is not contributing significantly to hybrid formation but is effecting the apparent rate of hybridization by contributing nonreacting nucleotides.

The rate of hybridization of sheared high molecular weight HnRNA to middle repetitive and single copy DNA roughly estimates the proportion of repetitive and single copy transcripts. From Figure 4 half-reactions for Me_2SO purified, high molecular weight HnRNA occur at 0.05 mg/ml hr^{-1} (0.5 mol l. sec) and 40 mg/ml hr^{-1} (400 mol l. sec) for middle repetitive and single copy hybrids, respectively, yielding pseudo-first-order rate constants of 1.0 and 1.7×10^{-3}. From the rate of formation of middle repetitive and single copy DNA duplexes it is estimated that the rate constants for the formation of hybrids with these DNA components should be 22 and 1.0×10^{-2}, respectively. From a comparison of these rates it is estimated that about 5% of the RNA consists of middle repetitive transcripts and 17% consists of single copy transcripts. The poly(A) segments of high molecular weight HnRNA account for less than 1% of the nonhybridized material (Frankel *et al.*, 1974). Thus it is not clear why these estimates do not sum to 100%, but parameters such as the viscosity of the reaction mixture due to high concentrations of RNA and variations in the concentrations of different RNA sequences probably contribute to this discrepancy.

Figure 5 shows the melting profiles of single copy and

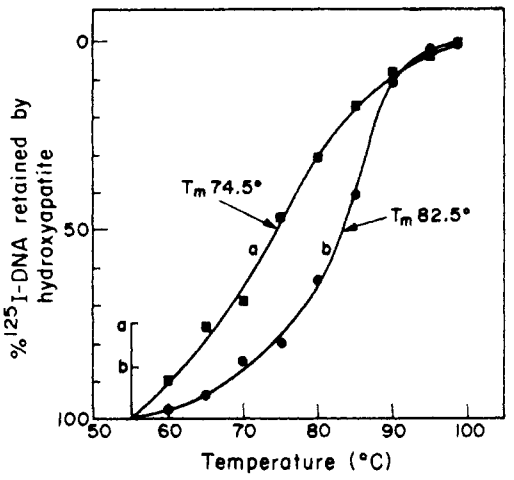

FIGURE 5: Integral melting curve of [^{125}I]DNA–RNA hybrids assayed as described in the legend to Figure 3: (●) single copy [^{125}I]DNA–RNA hybrids; (■) middle repetitive [^{125}I]DNA–RNA hybrids. The hybrids were prepared from reactions such as those presented in the final points of Figure 4a, c.

middle repetitive DNA–RNA hybrids (prepared with sucrose purified HnRNA) as determined by thermal elution from hydroxylapatite. These profiles are similar to the melting profiles of homologous DNA shown in Figure 3. The single copy DNA–RNA hybrids melt with a T_m about 3° below the T_m for native DNA indicating excellent fidelity in base pairing, in agreement with the results of others (Davidson and Hough, 1969; Hahn and Laird, 1971; Brown and Church, 1971; Gelderman *et al.*, 1971; Grouse *et al.*, 1972; McConaughy and McCarthy, 1972; Firtel, 1972). The middle repetitive DNA–RNA hybrids melt with a T_m about 12° below that of native DNA. An estimated 20–30% of the melting profile is contributed by DNA duplexes. It is not known to what extent the reduced T_m is due to base-pair mismatch or to shortness of hybrid duplexes (Hayes *et al.*, 1970).

To test if the observed hybridization takes place with a subfraction of DNA sequences that have relative concentrations different from the major reassociation components of the purified DNA classes, [^{125}I]DNA was isolated from RNA–DNA hybrids of either single copy or middle repetitive input DNA (prepared with sucrose purified HnRNA) and reassociated in the presence of excess unlabeled total DNA as described in a previous section. The results are shown in Figure 2. The DNA from the hybrids reassociates with approximately the same kinetics as the input DNA. These data are consistent with the view that the middle repetitive DNA complementary to HnRNA contains sequences present at approximately the same degree of repetition as the major reassociating component of the input middle repetitive DNA and that the majority of the hybridization of HnRNA to purified single copy DNA is to *bona fide* single copy sequences or to sequences of low reiteration. In the limit each middle repetitive sequence will have one representative in the isolated single copy DNA fraction but the hybridization to these contaminants would only represent about 0.02% at saturation.[2]

Hybridization of High Molecular Weight HnRNA to Nuclear DNA Present in Excess. In the presence of an excess of sheared DNA the rate of hybridization of RNA is predominantly governed by the concentration of complementary DNA sequences (Gelderman *et al.*, 1971; Melli *et al.*, 1971). In the experiment shown in Figure 6 sheared high molecular weight HnRNA, purified through Me_2SO, and rRNA were reacted with an excess of sheared total nuclear DNA.

In qualitative terms the major part (about 38% of the input RNA or 76% of the reacting RNA) of the HnRNA hybridization occurs with kinetics ($C_0t_{1/2}$ = 2.1 × 10^3) similar to those describing the reassociation of the single copy DNA ($C_0t_{1/2}$ = 1.5 × 10^3). A portion of the observed hybridization (about 12% of the input RNA, 24% of the reacting RNA) occurs in the middle section of the reaction. Assuming that this part of the hybridization can be described by a single second-order curve, the $C_0t_{1/2}$ (5.6) of this component is a little greater than that describing the major reassociating component of the middle repetitive DNA ($C_0t_{1/2}$ = 1.0). The observed hybridization to the middle repetitive DNA is probably not due to contamination of the high molecular weight HnRNA with rRNA because the reaction rates of these two classes of RNA under the same experimental conditions differs by a factor of about 6 ($C_0t_{1/2}$ rRNA = 34).

The reason that only 50% of the HnRNA hybridizes even at high C_0t's is unknown but could well be due to the fact that complementary DNA sequences are not in excess over all single copy HnRNA transcripts. This assumption could be tested by increasing the DNA to RNA ratio. Even in model systems such as bacterial rRNA (Melli *et al.*, 1971) only 70–90% of the RNA can be rendered ribonuclease resistant.

The estimates of the $C_0t_{1/2}$ of the kinetic components of high molecular weight HnRNA are only approximations. Several unquantitated parameters such as possible variations in base composition, secondary structure of the RNA, sensitivity of the hybrids to ribonuclease, base-pair mismatch, and differences in the ratio of complementary DNA sequences to RNA across the C_0t curve could all contribute to the determination of the relative proportions and reaction rates of the observed components.

Discussion

The experiments described in this paper strongly argue for the occurrence of both repetitive and single copy transcripts in high molecular weight HnRNA under specific conditions defining duplex stability. Previous reports from other groups have demonstrated the occurrence of both classes of such sequences in whole cell or total nuclear RNA (Davidson and Hough, 1969; Greenberg and Perry, 1971; Gelderman *et al.*, 1971; Hahn and Laird, 1971; Melli *et al.*, 1971; Hough and Davidson, 1973) and it seems reasonable to suppose that this would also be the case with HnRNA of very large size. However, this paper represents the first experimental verification of this supposition, using HnRNA demonstrated to be of high molecular weight under stringent denaturing conditions. The fact that such high molecular weight HnRNA contains both repetitive and single copy sequences raises an additional question, namely: are these sequences present on separate molecules or are they interspersed on the same molecules? This is the subject of a further report (Holmes and Bonner, 1974).

The information content of high molecular weight HnRNA is remarkably large. At least 4–6% of single copy DNA or about 3–4% of total DNA is complementary to high molecular weight HnRNA. Assuming asymmetric transcription, this represents at least 0.8–1.1 × 10^8 base pairs of transcribed DNA.

The quantitative information that can be extracted from experiments utilizing repetitive sequences is less reliable. This

[2] This number is calculated in a manner analogous to that described by Holmes *et al.* (1974).

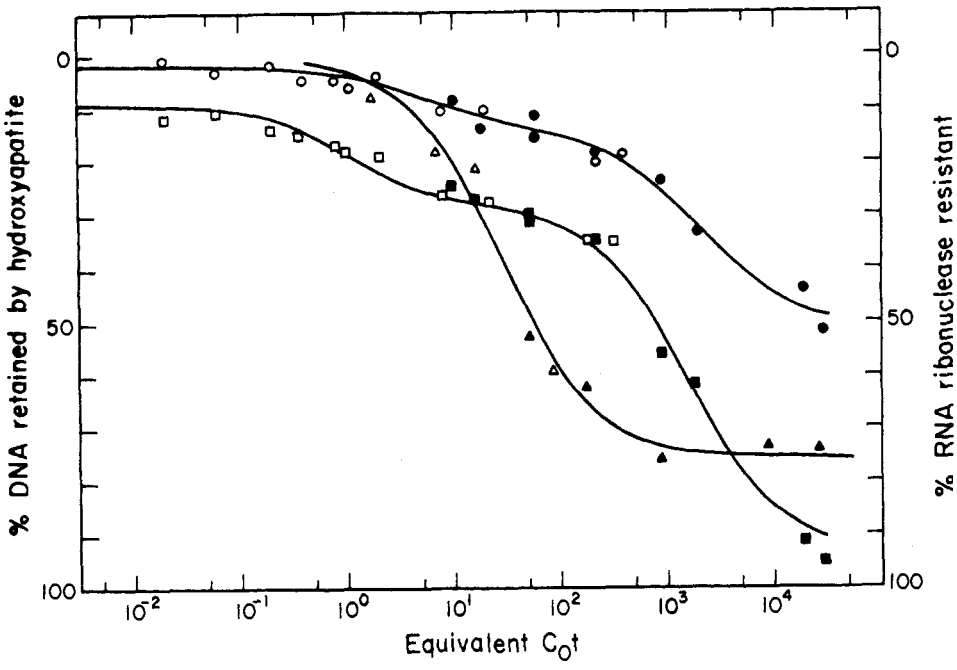

FIGURE 6: Computer analysis of the hybridization of sonicated [^{3}H]HnRNA (prepared through Me_2SO) and [^{3}H]rRNA to nuclear DNA (sheared to a number average of 350 bases single-strand lengths as judged by electron microscopy). The reassociation of DNA has been included for comparison. Squares represent DNA reassociation; circles represent [^{3}H]HnRNA hybridization; triangles represent [^{3}H]rRNA hybridization. The mass ratio of DNA to [^{3}H]HnRNA was about 170,000:1 and that of DNA to [^{3}H]rRNA was about 319,000:1 (ratio of rDNA to rRNA about 90:1). Hybridization was carried out in either 0.12 M PB (open symbols) or 0.48 M PB (closed symbols) at 62 or 69°, respectively. The reassociation of DNA was monitored by passage of an aliquot of the reaction mixture through hydroxylapatite as described by Britten and Kohne (1967). After mild ribonuclease treatment as described in Methods the RNA–DNA hybrids were monitored by the Cl_3CCOOH precipitation method of Melli *et al.* (1971). The rate parameters describing the hybridization of rRNA to DNA in excess as judged by Cl_3CCOOH precipitability can be compared to those determined by hydroxylapatite binding (Strauss and Bonner, 1972). In control solutions in which RNA was incubated without DNA, about 8–16% of the RNA remained ribonuclease resistant at the end of ribonuclease treatment. This has been subtracted as background from each of the points presented in this figure. At a C_0t of 10^4, 5–15% of the input RNA was Cl_3CCOOH soluble. The computer program was supplied by Dr. R. J. Britten. The root mean square of the line describing the HnRNA hybridization reaction is 0.021.

is due to parameters affecting the reaction that cannot at present be evaluated. Thus only rough approximations about the information content of the middle repetitive transcripts can be made. A minimum of 7.8% of the middle repetitive DNA or 1.6% of the total DNA is complementary to high molecular weight HnRNA. Assuming asymmetric transcription this represents about 4.5×10^7 base pairs of DNA of the same or similar sequences. In addition only a rough approximation of the proportion of high molecular weight HnRNA represented by either middle repetitive or single copy transcripts can be obtained. From the rate parameters of the RNA excess hybridization the ratio of middle repetitive to single copy transcripts is about 5:17 (1:3) and from the DNA excess hybridization the ratio is about 12:37 (1:3) of the reacting RNA or about 12:88 (1:7) of the total RNA, assuming that the unreacted RNA consists of only single copy transcripts, which is an attractive possibility.

The function of high molecular weight HnRNA remains unknown. Although most of the nucleotides in high molecular weight HnRNA never reach the cytoplasm several lines of circumstantial evidence indicate that a portion of HnRNA may be precursor to polysomal mRNA (*cf.* Darnell *et al.*, 1973). Therefore it is possible that some of the sequences in high molecular weight HnRNA could be precursor to mRNA transcribed from repetitive DNA (Kedes and Birnstiel, 1971; Weinberg *et al.*, 1972; Delovitch and Baglioni, 1973) or from single copy DNA (Suzuki *et al.*, 1972; Harrison *et al.*, 1972). There is no evidence pertaining to the function of the nucleotides that are turned over in the nucleus, although several possibilities exist. For example, they could represent sequences that are involved in post-transcriptional processing and packaging of HnRNA or that represent the transcriptional product of particular DNA sequences such as control regions (Britten and Davidson, 1969) or RNA polymerase binding sites, etc.

Several reports have discussed the possibility that puffs, produced from the chromomeres of polytene chromosomes in Diptera, might be responsible for the production of discrete HnRNA molecules and that, by implication, such chromomeres might represent the structural manifestations of a unit of transcription (Berendes, 1968; Grossbach, 1969; Daneholt, 1972; Lambert, 1973). Since the molecular weight of a portion of rat ascites HnRNA ($5–10 \times 10^6$ daltons) corresponds to the expected molecular weight of the giant RNA transcripts of Dipteran puffs (Edström and Daneholt, 1967; Daneholt, 1972) it is conceivable that the mammalian genome is at least partially organized into units of transcription homologous to those of Diptera.

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