Sequence Composition of Rat Ascites Chromosomal Ribonucleic Acid†

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ABSTRACT: A population of chromatin-associated low molecular weight RNA molecules (cRNA) prepared from rat Novikoff ascites cells hybridizes to a minimum of 16% of isolated middle repetitive and a minimum of 1% of isolated single copy rat DNA as measured in solution. This sums to about 4.0% hybridization of total DNA. In solution the rate of hybridization of cRNA to isolated middle repetitive DNA is approximately that expected if 80–100% of the cRNA consists of middle repetitive transcripts. In contrast cRNA hybridizes to about 4.7% of total rat DNA immobilized on filters at a rate at least 100 times slower than that predicted from the solution hybridization, suggesting that steric or geometric effects are considerable in this type of assay. In a solution hybridization reaction to total DNA, present in excess, at least 50% of the cRNA hybridizes at an average rate similar to the major component of the middle repetitive DNA. The T_m of cRNA, hybridized to filter-bound DNA, is at least 12° lower than native DNA. The lowering of T_m may be due, in part, to base-pair mismatch and, in part, to the shortness of the hybrids.

The preparation and properties of low molecular weight RNA molecules associated with isolated chromosomes (chromatin) have been described from a variety of tissues (Frenster, 1965; Huang and Bonner, 1965; Benjamin *et al.*, 1966; Bonner and Widholm, 1967; Jacob and Busch, 1967; Wang, 1967; Jacobson and Bonner, 1968; Prestayko and Busch, 1968; Shih and Bonner, 1969; Dahmus and McConnell, 1969; Huang, 1969; DeFilippes, 1970; El-Khatib *et al.*, 1970; Shaw and Huang, 1970; Heyden and Zachau, 1971; Jacobson and Bonner, 1971; Mayfield and Bonner, 1971; Sivolap and Bonner, 1971; Arnold and Young, 1972; Holmes *et al.*, 1972; Kanehira *et al.*, 1972; Marzluff *et al.*, 1972; Mayfield and Bonner, 1972; Montecuccoli *et al.*, 1972; Szeszák and Phil, 1972; Getz and Saunders, 1973; Patel and Holoubek, 1973; Tolstoshev and Wells, 1973).

The low molecular weight RNA components of chromatin differ from one another in size and base composition. No experimental evidence is available that clearly defines a function(s) for any of these RNAs. Previous reports from this laboratory have described the preparation and properties of a particular population of chromatin-associated RNA molecules isolated from rat Novikoff ascites termed chromosomal RNA (cRNA). cRNA from rat ascites can be distinguished from tRNA, rRNA or their breakdown products principally by chromatography on DEAE-cellulose, disc gel electrophoresis, labeling kinetics, base composition, and hybridization competition (*cf.* Holmes *et al.*, 1972). Differences in these properties taken together demonstrate that preparations of rat ascites cRNA are not detectably contaminated with tRNA, rRNA, or their breakdown products.

Rat ascites cRNA hybridizes to a large amount of nuclear DNA (about 4–5%) immobilized on filters (Dahmus and McConnell, 1969; Holmes *et al.*, 1972). The present communication extends these findings by investigating the ability of rat ascites cRNA to hybridize to isolated middle repetitive or single copy rat DNA sequences by solution hybridization techniques.

Experimental Section

Methods. DNA and cRNA were prepared from rat Novikoff ascites chromatin by the method of Dahmus and McConnell (1969). rRNA and tRNA were prepared from rat ascites cells as described by Dahmus and McConnell (1969).

Purified middle repetitive and single copy nuclear DNA were prepared as described elsewhere (Holmes and Bonner, 1974). tRNA, rRNA, and cRNA were labeled *in vitro* with [^{3}H]dimethyl sulfate by a modification of the procedure of Smith *et al.* (1968), as described elsewhere (Holmes and Bonner, 1974). The specific activity of cRNA was 82,000 cpm/μg corresponding to about 3% of the total bases methylated. The specific activity of rRNA was 110,000 cpm/μg (4% bases methylated) and that of tRNA was 180,000 cpm/μg (6% bases methylated).

RNA Hybridization to Purified DNA Components. [^{3}H]-cRNA was hybridized to either purified middle repetitive or single copy nuclear DNA in phosphate buffer (pH 6.8)–1 mM EDTA as described elsewhere (Holmes and Bonner, 1974). The reaction conditions are described in Table I and the legend to Figure 1. Hybridization was assayed by chromatography on Sephadex G-100 as described in the legend to Figure 1.

cRNA–DNA hybrids eluted from Sephadex G-100 were subjected to buoyant density centrifugation in CsCl as described by Hough and Davidson (1973).

RNA Hybridization to Excess Total DNA. [^{3}H]cRNA was hybridized to excess total nuclear DNA (111,000:1 DNA to RNA ratio) sheared to about 350 bases (single-strand length as judged by electron microscopy) as described elsewhere (Holmes and Bonner, 1974). The reaction conditions are described in the legend to Figure 4. Hybridization was monitored by the method of Melli *et al.* (1971).

DNA–RNA Hybridization with DNA Immobilized on Filters. DNA was denatured at 100° for 10 min in 1/100 SSC and applied to nitrocellulose filters (Schleicher and Schuell B-6, 5 mm) in the presence of 6 × SSC, as described by Gil-

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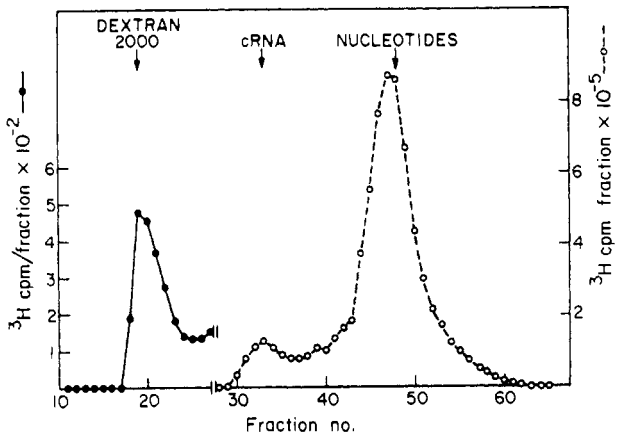

FIGURE 1: Chromatography of cRNA–DNA hybrids on Sephadex G-100. [^{3}H]cRNA was hybridized to unlabeled middle repetitive DNA (46 mg/ml × hr) and treated with ribonuclease as described in the legend to Figure 3. The mixture was then passed over Sephadex G-100. Dextran 2000, isolated cRNA, and a mixture of 2′-3′-ribonucleotides (Calbiochem) were used to standardize the column (2 × 45 cm). The column was eluted with 0.12 M phosphate buffer (pH 6.8)–1 mM EDTA at 4°; 2.5-ml fractions were collected.

lespie and Spiegelman (1965); 12 or 2 μg of DNA containing trace amounts of [^{14}C]DNA were applied to the filters. About 70% of the DNA remained on the filters at the end of the hybridization experiment under conditions given in the legend to Figure 6.

Melting Profile of cRNA–DNA Hybrids. [^{3}H]cRNA was hybridized to filters containing nuclear DNA, treated with RNase, washed, and counted as described in the legend to Figure 6. The filters were removed from the counting vials, dried for several hours at room temperature, and washed on both sides with 1 × SSC. The filters were heated from 55 to 98° at 5° intervals in 1 ml of 0.12 M phosphate buffer (pH 6.8). A separate filter was used for each temperature increment. After heating, the filter was washed on both sides with 0.12 M phosphate buffer (pH 6.8), dried, and counted in a toluene-based scintillation fluid in a Beckman 200-B scintillation system. The results were corrected for a loss of DNA from the filter during heating.

Results

Hybridization of cRNA to Middle Repetitive and Single Copy DNA. This section describes the results of hybridizing cRNA to either isolated middle repetitive or single copy rat DNA in solution. A later section compares these results with the hybridization of cRNA to total rat DNA immobilized on filters.

At the criterion chosen for reassociation [$(Na^+) = 0.18$ at 62°, $T_m - 23°$], single copy DNA ($C_0t_{1/2} = 1.5 \times 10^3$) comprises about 65% of the total DNA and middle repetitive DNA ($C_0t_{1/2} = 1.0$) makes up about 19% of the total DNA (Holmes and Bonner, 1974).

Several investigators have made use of hydroxyapatite to follow the formation of DNA–RNA hybrids (Davidson and Hough, 1969; Gelderman *et al.*, 1971; Brown and Church, 1971; Hahn and Laird, 1971; Firtel, 1972; Grouse *et al.*, 1972; McConaughy and McCarthy, 1972; Holmes and Bonner, 1974). This method cannot be applied to the present work because cRNA–DNA hybrids do not bind quantitatively to hydroxyapatite under standard conditions. The reason for this failure is not known but could be due to the small size of the RNA–DNA duplexes. Hough and Davidson (1973) observed a similar inability of a portion of repetitive DNA–RNA hybrids from *Xenopus* to bind to hydroxyapatite although these hybrids met the criteria of duplex structures by chromatography on Sephadex and by buoyant density in CsCl. Therefore we also made use of these methods, as described below, to follow the formation of cRNA–DNA hybrids.

TABLE I: Hybridization of cRNA to DNA.

Concentration of RNA (mg/ml)	Hours of Incubation	% Hybridization
A. Middle Repetitive DNA[a]		
0.001	0.25	2.7
0.001	0.25	2.5
0.001	0.5	6.1
0.001	1.15	10.4
0.001	2.55	11.5
0.001	2.55	12.3
0.001	11.5	16.3
0.001	11.5	17.3
0.91	9.75	16.6
0.91	9.75	15.4
9.2	5.0	16.2
9.2	5.0	16.8
B. Single Copy DNA[b]		
21	1.8	0.23
21	4.0	0.44
21	7.2	0.6
21	11.0	0.84
21	18.0	1.04

[a] Reaction carried out in 0.12 M phosphate buffer (pH 6.8)–1 mM EDTA at 62°. Ratio of RNA to DNA about 440:1. [b] Reaction carried out in 0.48 M phosphate buffer (pH 6.8)–1 mM EDTA at 69°. Ratio of RNA to DNA about 210:1.

Excess [^{3}H]cRNA was hybridized to either unlabeled middle repetitive or single copy rat DNA. At the end of the reaction the hybrids were subjected to mild ribonuclease treatment and passed over Sephadex G-100 as shown in Figure 1. The extent of hybridization can be estimated from the radioactivity associated with the DNA in the excluded volume of the column. If DNA and [^{3}H]cRNA are mixed, immediately treated with ribonuclease, and passed over the column no radioactivity is found in the excluded volume. [^{3}H]cRNA is associated with the DNA in the excluded volume only after it is allowed to react under conditions permitting the formation of hybrids.

When an aliquot from the pooled, excluded fractions of the Sephadex G-100 column was subjected to buoyant density centrifugation in CsCl, as shown in Figure 2, all the detectable radioactivity was found between a density of 1.67 and 1.75 (density of native rat DNA = 1.694, denatured DNA = approximately 1.7 and free RNA >1.9). This supports the view that the ribonuclease resistant cRNA is associated with DNA.

Figure 3 shows the extent of hybridization of [^{3}H]cRNA to purified middle repetitive and single copy DNA, using as a criterion for hybridization the appearance of ribonuclease resistant [^{3}H]cRNA in the void volume of Sephadex G-100. A minimum of 16% of the middle repetitive and a minimum of 1% of the single copy DNA are hybridized by cRNA. The single copy DNA contains, in the limit, one copy of each

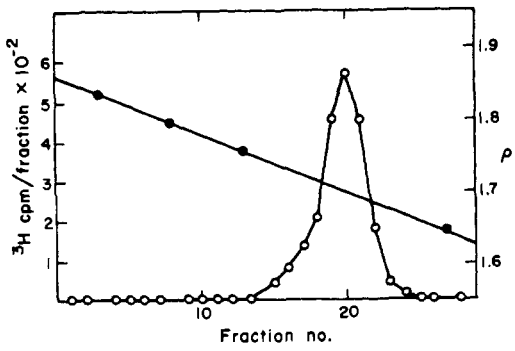

FIGURE 2: Buoyant density centrifugation in CsCl of [^{3}H]cRNA hybrids. The three peak fractions of the excluded volume peak of Sephadex G-100 (see Figure 1) were pooled and 3 ml was added to 4 g of CsCl. The samples were overlaid with oil and centrifuged at 25° for 52 hr at 32,500 rev/min in a Spinco 50.1 swinging bucket rotor. At the end of centrifugation the tubes were dripped onto Whatman 3MM filters, washed with 10% Cl_3CCOOH followed by 60% ethanol, dried, and counted. The refractive index of some of the fractions was measured to monitor the CsCl gradient.

family of the middle repetitive DNA. However, hybridization of cRNA to these repetitive representatives would account, very roughly, for only 0.3% at saturation of the observed hybridization to single copy DNA.

$$\frac{3/1500}{0.65} \times 10^2 \left(\frac{\%\ \text{total DNA hybridized by repetitive transcripts/no. of DNA repetitive families per genome}^1}{\%\ \text{total DNA hybridized by single copy transcripts}} \times 10^2 \right)$$

In a later section it is shown that repetitive cRNA hybrids have significant base-pair mismatch. Thus the hybridization values presented do not necessarily reflect the amount of particular DNA sequences transcribed to yield cRNA but rather reflect the amount of DNA similar to, as well as identical with, cRNA.

A rough estimate of the repetitive transcript content of cRNA can be obtained from Figure 3a. The half-reaction of hybridization of cRNA to middle repetitive DNA occurs at 0.0014 mg/ml hr^{-1} (0.014 mol l. sec^{-1}) yielding a pseudo-first-order rate constant of 49. It is estimated from the rate of formation of middle repetitive DNA duplexes that RNA hybridization to middle repetitive DNA should occur with a rate constant of about 60. Therefore it is estimated that 80–90% of cRNA consists of repetitive transcripts. By a similar calculation it is estimated that only 0.1% of the cRNA sequences represents transcripts of single copy DNA.

Several parameters of this RNA-excess hybridization experiment remain unquantitated such as the effects of viscosity and base-pair mismatch on hybridization rate and the effect of base-pair mismatch on the apparent degree of hybridization. Therefore our values for the per cent DNA hybridized by cRNA and the kinetic composition of cRNA molecules should be viewed only as approximations and not as absolute values.

Hybridization of [^{3}H]cRNA to Total Nuclear DNA Present in Excess. [^{3}H]cRNA was hybridized to excess total nuclear DNA (sheared to about 350 bases, single strand length). The hybridization was carried out as described in the legend to Figure 4. Under the conditions used the rate of hybridization is determined predominantly by the concentration of complementary DNA sequences. The results of this experiment are shown in Figure 4. Assuming a single second-order reaction the $C_0t_{1/2}$ of the observed hybridization is approximately 1.8 (root mean square of fit = 0.05). This is similar to

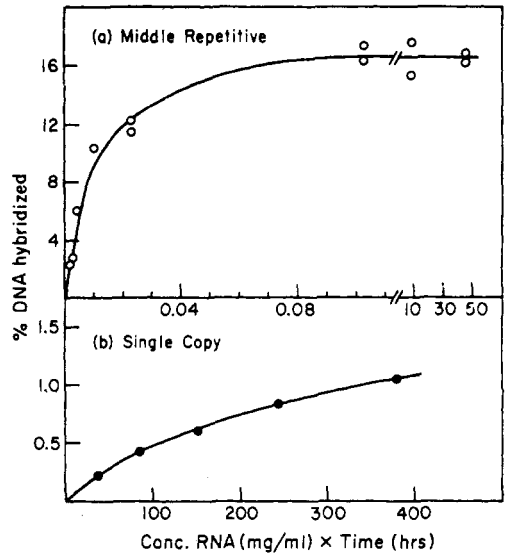

FIGURE 3: Hybridization of dimethyl sulfate labeled [^{3}H]cRNA (82,000 cpm/μg) from rat ascites to purified kinetic components of rat nuclear DNA (sheared to about 350 bases single strand length). After hybridization the reaction mixture was adjusted to 0.24 M phosphate buffer (pH 6.8) and treated with ribonucelase A (50 μg/ml) and ribonuclease T1 (50 units/ml) at 37° for 10 min and chromatographed on Sephadex G-100 (see Figure 1). The [^{3}H]cRNA which eluted in the void volume of Sephadex G-100 was scored as hybrid. See Table I for experimental details.

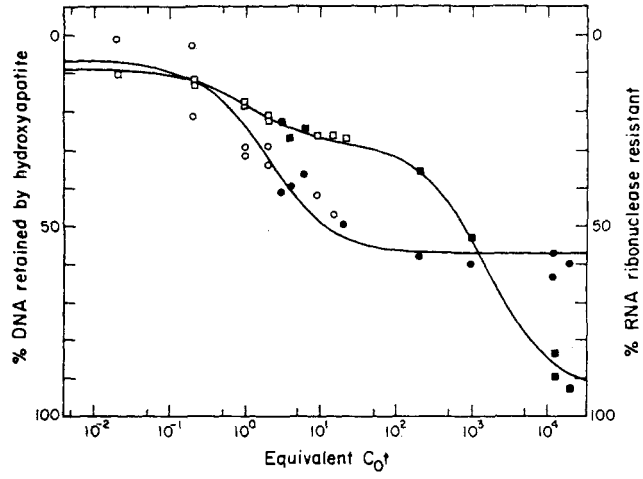

FIGURE 4: Computer analysis of the hybridization of dimethyl sulfate labeled [^{3}H]cRNA (82,000 cpm/μg) from rat ascites in the presence of an excess of rat nuclear DNA (350 nucleotides single strand length). The ratio of [^{3}H]cRNA to DNA was about 1:111,000. The hybridization was carried out in either (open symbols) 0.12 M phosphate buffer (pH 6.8)–1 mM EDTA or (filled symbols) 0.48 M phosphate buffer (pH 6.8)–1 mM EDTA at 62 or 69°, respectively. The reassociation of DNA (squares) was followed by monitoring the A_{260} of an aliquot of the reaction mixture after passage through a hydroxylapatite column (Britten and Kohne, 1967). After mild ribonuclease treatment (20 μg/ml of ribonuclease A and 20 units of ribonuclease T1 in 0.24 M phosphate buffer for 15 min at 37°) the RNA–DNA hybrids (circles) were monitored by the Cl_3COOH precipitation method of Melli *et al.* (1971).

[1] This estimate is obtained by dividing the number of base pairs of repetitive DNA per genome (5.4×10^8, Holmes and Bonner, 1974) by the average reiteration frequency of the repetitive DNA (1.8×10^3, Holmes and Bonner, 1974) times the number of base pairs per average repetitive sequence (200, Bonner *et al.*, 1973; Wilkes *et al.*, 1974).

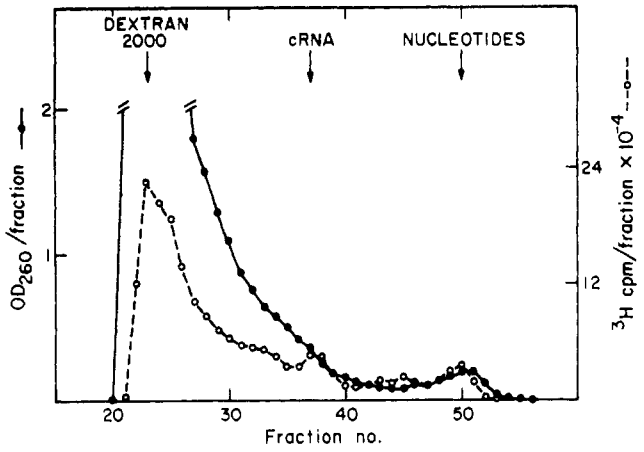

FIGURE 5: Chromatography on Sephadex G-100 of [^{3}H]cRNA–DNA hybrids (incubated to a C_0t of 2×10^2 as described in the legend to Figure 4). The hybrids were not subjected to ribonuclease treatment. Chromatography carried out as described in the legend to Figure 1.

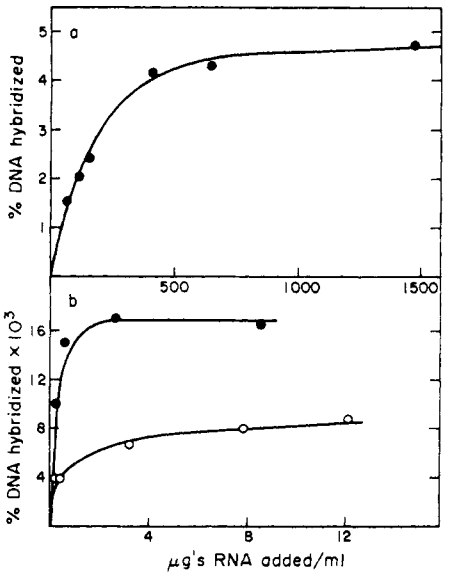

FIGURE 6: Hybridization of [^{3}H]RNA to total rat DNA immobilized on filters. Hybridization was carried out at 37° for (a) 18 hr in the presence of 0.12 M sodium phosphate buffer pH 6.8 and 30% (v/v) formamide or (b) 24 hr in 3.7 × SSC (0.15 M sodium chloride–0.015 M sodium citrate) and 50% (v/v) formamide in a final volume of 0.2 ml. Each vial contained two DNA filters and one blank filter. Before the addition of the filters the hybridization solution containing the RNA was heated to 100° for 5 min and cooled rapidly in ice. Following incubation, filters were rinsed in 2 × SSC, washed by filtration on both sides with 2 × SSC and treated with a mixture of 50 μg/ml of preboiled pancreatic RNase A and 50 units/ml of T1 RNase at 37° for 0.5 hr in 2 × SSC. The filters were again rinsed in 2 × SSC and washed by filtration in 2 × SSC, dried and counted: (a) (●) [^{3}H]cRNA (82,000 cpm/μg), 10 μg input DNA per filter; (b) (○) [^{3}H]tRNA (180,000 cpm/μg), (●) [^{3}H]rRNA (110,000 cpm/μg). 2 μg of input DNA per filter.

the $C_0t_{1/2}$ of the major reassociating component of middle repetitive DNA (1.0) but is different from that of rRNA ($C_0t_{1/2}$ = 39) determined by the same procedure (Holmes and Bonner, 1974). There is sufficient scatter of cRNA hybridization points to render it difficult to determine if a single second-order fit or if a multiple component fit would best describe the data. Thus we cannot determine from these data alone whether cRNA contains mainly one or several kinetic components. However, from the solution hybridization experiment with RNA excess described above it would appear that cRNA consists of predominantly one or a very few kinetic components.

It should be pointed out that the tendency of cRNA to form mismatched hybrids is likely to reduce the apparent rate of hybridization. This reduction may be partly offset by an increase in hybridization rate due to the high G + C content of cRNA [see below (Wetmur and Davidson, 1968)]. Since the relative contribution of these parameters is unknown our estimate of the kinetic complexity of cRNA should be viewed only as an approximation.

Only about 50% of cRNA hybridizes even at high C_0t's. Incomplete reaction may be attributed in part to degradation of RNA resulting from the high temperature and long periods of incubation and in part to the formation of ribonuclease sensitive hybrids as explained below. Using a first-order rate constant of 1.4×10^{-9} (Eigner *et al.*, 1961) it is estimated that the weight-average molecular weight of cRNA is reduced from about 16,000 daltons to 13,000 daltons by thermal scission of phosphodiester bonds during the course of a hybridization reaction lasing 90 hr ($C_0t = 1.2 \times 10^4$). Figure 5 shows the pattern of chromatography on Sephadex G-100 of cRNA reacted with DNA to a C_0t of 2×10^3 without subsequent ribonuclease treatment. About 15% of the radioactivity elutes in a position corresponding to nucleotides and oligonucleotides that are probably too short to form stable hybrids. A further 10% of the radioactivity elutes in a position corresponding to free cRNA. Thus without ribonuclease treatment it is estimated that about 70–80% of the RNA is in hybrid form. An aliquot from the same reaction mixture yields only 55% hybridization (Figure 4) after ribonuclease treatment as judged by the Cl_3CCOOH prescription method. The difference in hybridization estimated by these two methods might be accounted for by the withdrawal of some of the cRNA into duplex structure that are ribonuclease sensitive. The formation of these duplex structures would probably prevent, or reduce the rate of, further hybridization of the RNA in these duplexes but, at the same time, the duplexes would not be scored as hybrids due to their ribonuclease sensitivity.

Hybridization of RNA to DNA Immobilized on Filters. Figure 6 shows the results of hybridizing cRNA to DNA immobilized on filters (Gillespie and Spiegelman, 1965) under conditions that assay essentially only the hybridization of repetitive transcripts. cRNA hybridizes to approximately 4.7% of the total DNA. Since approximately 19% of the total DNA is middle repetitive (Holmes and Bonner, 1974) this value represents an equivalent of about 25% of the middle repetitive DNA. This is somewhat above the value of 16% obtained when the cRNA is hybridized to purified middle repetitive DNA in solution (see Figure 3).

A comparison of the rates of hybridization of cRNA to purified middle repetitive DNA in solution (half-reaction 0.0014 mg hr^{-1}) or to total DNA on filters (half-reaction 1.8 mg hr^{-1} or 0.36 mg hr^{-1} when normalized for the fact that 0.19 of the total DNA is represented by middle repetitive sequences) indicates that the solution reaction proceeds 150–250 times faster than the filter reaction.

The hybridization of tRNA and rRNA to DNA immobilized on filters has been included in Figure 6 for comparison. cRNA hybridizes to about 100 times more DNA than rRNA and tRNA. Assuming a complexity of 2.45×10^6 daltons for rRNA (Loening, 1968), 2.4×10^4 daltons for tRNA (Tissières, 1959), and 1.8×10^{12} daltons for the rat haploid genome (Sober, 1968) it is estimated that there are approximately 130 and 7000 genes for rRNA and tRNA, respectively. These values are in agreement with the findings

of others (Brimacombe and Kirby, 1968; Mohan *et al.*, 1969; Quincey and Wilson, 1969).

The melting profile of cRNA hybridized to DNA on filters is shown in Figure 7. cRNA hybrids have a lower T_m (73.5°) and a broader melting profile than exhibited by native DNA (T_m = 85.5°) as judged by its thermal elution from hydroxyapatite (Holmes and Bonner, 1974). According to an equation derived from the work of Hayes *et al.* (1970), the T_m of short perfectly paired duplexes is significantly lower than that of equivalent duplexes of infinite length

$$T_m(n) = T_m(\infty) - 440/n$$

where (n) = number of base pairs of duplex. The T_m of a perfectly paired hybrid of infinite length of the same base composition as ascites cRNA [55% G + C (Dahmus and McConnell, 1969; Holmes *et al.*, 1972)] is predicted to be 91°. However, using the above equation and assuming that cRNA forms perfectly base-paired hybrids of 50 base pairs (ascites cRNA is about 40–80 nucleotides long, Dahmus and McConnell, 1969) then the T_m of cRNA hybrids is predicted to be 80°. Since the observed T_m is about 7° lower than this theoretical value it is assumed that some base-pair mismatch must be present in cRNA hybrids.

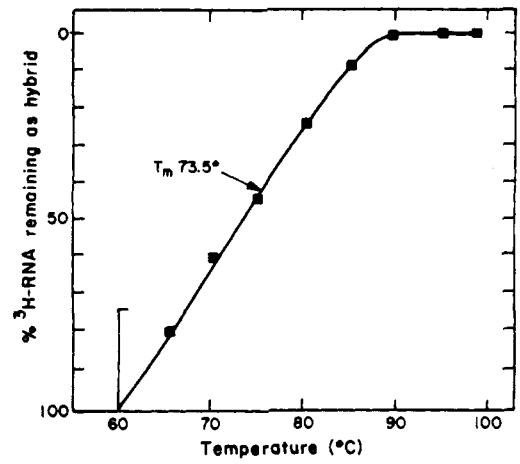

FIGURE 7: Integral melting profile of (■) [³H]cRNA hybrids after hybridization to DNA immobilized on filters (as described in the legend to Figure 6). The melting profile was prepared as described in the Experimental Section using filters similar to the ones which produced the final hybridization point of Figure 6a. The bar at left on the figure indicates [³H]RNA melting at 60°.

Discussion

The principal finding of this paper is that cRNA, prepared from rat ascites, hybridizes extensively to the middle repetitive fraction of rat nuclear DNA and only to a small proportion of single copy DNA. This is demonstrated by hybridizing a large excess of cRNA to either isolated middle repetitive or single copy DNA in solution. Under such conditions cRNA hybridizes to a minimum of 16% of middle repetitive DNA sequences and a minimum of 1% of single copy DNA sequences. From the rate constants of these values it is estimated that approximately 80–90% of cRNA molecules is represented by sequences hybridizing to middle repetitive DNA and about 0.1% by sequences hybridizing to single copy DNA. These values should be viewed only as rough approximations and not as absolute determinations for reasons noted in the appropriate Results section.

As an alternative approach cRNA was hybridized to DNA present in excess. If the complementary DNA sequences are present in sufficient excess then the rate at which the cRNA hybridizes is governed predominantly by the rate of reassociation of complementary DNA (Gelderman *et al.*, 1971; Melli *et al.*, 1971). The results of this experiment indicate that a minimum of 50% of the cRNA hybridizes at an approximate, average rate ($C_0t_{1/2}$ = 1.8) similar to the major reassociating component of the middle repetitive DNA ($C_0t_{1/2}$ = 1.0). The remainder of the cRNA fails to hybridize for reasons that are not certain at present, although it is possible that a portion of cRNA is rendered unavailable for hybridization by thermal degradation during the course of the reaction and by the formation of some spurious RNA–DNA associations that are ribonuclease sensitive, occurring as a result of the vast DNA excess. Even in model systems such as bacterial rRNA (Melli *et al.*, 1971) only 70–90% of the RNA can be rendered ribonuclease resistant.

In the presence of excess DNA, cRNA does not show detectable hybridization to either the highly repetitive or the single copy DNA. However, if a portion of the cRNA was transcribed from foldback or palindrome regions or other portions of highly repetitive DNA that renature with less than second-order kinetics, it would be expected that these DNA components would form duplex structures before hybridization of cRNA to this DNA could occur. However, it is unlikely that cRNA contains a significant (greater than 20%) percentage of transcripts from palindromes because of the kinetic evidence obtained from the RNA excess hybridization experiment.

cRNA prepared from a variety of organisms hybridizes to a large percentage (2–5%) of total DNA immobilized on filters (Bonner and Widholm, 1967; Dahmus and McConnell, 1969; Mayfield and Bonner, 1971, 1972; Holmes *et al.*, 1972). Since the filter hybridization assays essentially only repetitive transcripts this constitutes indirect evidence that cRNA contains repetitive transcripts. Sivolap and Bonner (1971) showed more directly that cRNA contained repetitive transcripts by hybridizing pea bud cRNA to repetitive pea DNA immobilized on filters. These investigators also hybridized pea bud cRNA to single copy pea DNA immobilized on filter. However, due to the low specific activity of the cRNA used their estimate of the percentage hybridization to single copy pea DNA is difficult to evaluate. It may also be difficult to reach a sufficient R_0t (RNA concentration × time) to hybridize cRNA to single copy DNA on filters, especially considering that the filter reaction may be going approximately 100 times slower than the equivalent solution reaction, as demonstrated by our results.

The current experiments confirm the view that ascites cRNA does not consist of tRNA or breakdown products of tRNA or rRNA (Heyden and Zachau, 1971; Artman and Roth, 1971). The main points to be considered are (1) cRNA hybridizes to at least 100 times more DNA immobilized on a filter than do either tRNA or rRNA and it does not compete with tRNA or rRNA for sites on DNA immobilized on filters in reciprocal competition experiments (Dahmus and McConnell, 1969; Holmes *et al.*, 1972); (2) the high value of hybridization of cRNA to DNA immobilized on filters is confirmed by solution hybridization of cRNA to purified DNA components. The kinetics of hybridization of RNA to purified middle repetitive DNA in solution are those predicted for a reasonably uniform population of middle repetitive transcripts; (3) in a DNA excess hybridization a significant proportion of cRNA reassociates with approximately the kinetics of the major kinetic component of the middle

repetitive DNA and, on average, with about an order of magnitude faster than rRNA. Other reasons for considering ascites cRNA distinct from tRNA or rRNA have already been reviewed (Holmes *et al.*, 1972). Preparations of cRNA from other tissues have not been analyzed so extensively and the possibility that they contain tRNA or rRNA breakdown products cannot be eliminated.

The function of cRNA remains unknown. The validity of previous reports (Bekhor *et al.*, 1969; Huang and Huang, 1969) that cRNA is necessary for the sequence specific reconstitution of chromatin can be questioned on the basis of using heterologous RNA polymerase to transcribe chromatin and of the interpretation of experiments that seek to test differences between the transcribing portions of chromatin by hybridization competition between only repetitive RNA transcripts. Circumstantial evidence indicates that cRNA may be positively correlated with gene activity. A different population of sequences of cRNA is present in different tissues of the same organism (Bonner and Widholm, 1967; Mayfield and Bonner, 1971) and new sequences of cRNA are produced during rat liver regeneration (Mayfield and Bonner, 1972). However, caution is required in interpreting these experiments. Different tissues frequently contain several cell types, and a difference in an RNA population, as judged by per cent hybridization to DNA or by hybridization competition, may reflect a modulation in the concentration of particular RNA subpopulations and not necessarily the *de novo* production of new transcripts. Since cRNA is strongly associated with non-histone chromosomal proteins (Jacobson and Bonner, 1968; Huang and Huang, 1969; Huang, 1969; Jacobson and Bonner, 1971; Marzluff *et al.*, 1972; Patel and Holoubek, 1973) it has been speculated that cRNA may be involved in gene regulation.

Serious consideration should be given to the possibility that cRNA is a breakdown product of other classes of rat ascites nuclear RNA such as the large transcripts of rapidly labeled RNA (HnRNA) (Holmes and Bonner, 1973). It is unlikely that cRNA consists of random breakdown products of high molecular weight HnRNA, perhaps generated during isolation, because cRNA consists predominantly of middle repetitive transcripts whereas high molecular weight HnRNA consists mainly of single copy transcripts (Holmes and Bonner, 1974). However, it is possible that cRNA is a product of a portion of the repetitive sequences of HnRNA perhaps representing the residue after post-transcriptional modification of HnRNA. This aspect of the biogenesis of cRNA is currently under investigation in this laboratory.

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Cell-Free Synthesis of Amino-Terminal L-Pyroglutamic Acid†

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ABSTRACT: The cell-free synthesis of amino-terminal L-pyroglutamic acid has been demonstrated using a microsomal protein-synthesizing system prepared from the plasma cell tumor, RPC-20. Glutamic acid is the precursor of the pGlu and the conversion occurs in the RPC-20 microsomal system using RPC-20 or rat liver glutamyl-tRNA. pGlu-containing peptides were isolated by subtilisin digestion of the products of cell-free protein synthesis, and pGlu was released by treatment of these peptides with the bacterial enzyme, pyrrolidonyl peptidase. Two isoaccepting glutamyl-tRNA species were obtained on reverse phase chromatography of RPC-20 tRNA. Both these tRNAs function in cell-free polypeptide synthesis on RPC-20 microsomes and both Glu-tRNAs can participate in the synthesis of pGlu. These results are discussed in terms of previously suggested functions for pGlu in protein biosynthesis.

L-Pyroglutamic acid (pyrrolidonecarboxylic acid) is the amino-terminal amino acid of a number of naturally occurring peptides and proteins. These include the algal peptides eisenine and fastigiatine (Ohira, 1939; Dekker *et al.*, 1949), the hypotensive undecapeptide, eledoisin (Erspamer and Anastasi, 1962), fibrinogen (Blomback *et al.*, 1963), and the heavy and light chains of immunoglobulins (Wilkinson *et al.*, 1966; Apella and Perham, 1968). Although the chemistry of pGlu[1] and pGlu-containing peptides has been studied in some detail, little is known about the biosynthesis of this unusual amino acid. Recently, Twardzik and Peterkofsky (1972) have demonstrated that mouse plasmacytoma cells can incorporate exogenous glutamic acid into N-terminal pGlu. This study marked the first conclusive demonstration of the synthesis of pGlu from glutamic acid. Early studies of pGlu biosynthesis using glutamine (Bernfield and Nestor, 1968) were complicated by the fact that glutamine cyclizes easily to pGlu under midly acidic or basic conditions (Blomback, 1967). With glutamic acid this artifactual cyclization is not a serious problem.

The results of Twardzik and Peterkofsky (1972) identified glutamic acid rather than glutamine as the direct precursor of pGlu, and suggested that pGlu was formed by an enzymatic reaction, but these studies gave no indication of the actual mechanism of cyclization of glutamic acid to pGlu. It has been suggested (Rush and Starr, 1970; Twardzik and Peterkofsky, 1972) that the cyclization occurs after attachment of glutamic acid to a specific glutamyl-tRNA. The pGlu-tRNA thus formed could then participate in the initiation of protein synthesis in certain mammalian systems. To examine this possibility, the synthesis of pGlu has been studied using a cell-free amino acid incorporating system from the mouse plasmacytoma, RPC-20. The specificity of the tumor glutamyl-tRNAs and rat liver tRNA for this synthesis has been studied and the results form the substance of this report.

Material and Methods

Materials. The RPC-20 plasma cell tumor was maintained as previously described (Bridges and Jones, 1973). [^{3}H]Glutamic acid (13.5 Ci/mmol) was supplied by Schwarz/Mann. L-Pyroglutamic acid was purchased from Sigma Chemical Co. and L-pyroglutamyl-L-alanine was supplied by Cyclo Chemical Co. Subtilisin was from Nutritional Biochemicals and ethylenimine was obtained from K & K Chemicals. A sample of dried *Aerobacter cloacea* was generously donated by Dr. Alan Peterkofsky. Pyrrolidonyl peptidase was prepared from *A. cloacea* cells essentially as described by Doolittle and Armentrout (1968) except that the 30,000*g* supernatant was brought to 50% saturation with ammonium sulfate. After Sephadex G-200 chromatography, the enzyme was stored as a slurry in 60% ammonium sulfate and was stable for several months.

Preparation of Components for Cell-Free Protein Synthesis. tRNA, microsomes, and soluble enzymes were prepared from the RPC-20 tumor as previously described (Bridges and Jones, 1973). Rat liver tRNA was prepared by the same procedure as that used for RPC-20 tRNA.

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[1] Abbreviations used are: pGlu, L-pyroglutamic acid; pGlu-Ala, L-pyroglutamyl-L-alanine.