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Dual Labeling in Standard DNA·RNA Hybridization Studies Using ^{125}I-Labeled Nuclear RNA and ^{3}H-Labeled DNA†

Carleton T. Garrett* and Michael E. McNulty

ABSTRACT: Standard DNA-RNA hybridization studies, using nucleic acids isolated from mammalian tissues, are frequently hindered by relatively low levels of radioactivity in pulse-labeled RNA and in an inability to reliably estimate the amount of DNA present in the hybrid. In the method described here nuclear RNA is labeled in vitro with ^{125}I to 400 000–800 000 cpm/μg and DNA is obtained from a rat glial tumor line grown in culture and labeled to specific activities of 42 000–79 000 cpm/μg. DNA-RNA hybridization is conducted in an all solution system at RNA:DNA ratios of 3.5:1 to 18:1. Assay background is controlled by pretreatment of the hybrid and free RNA at the conclusion of the annealing study with RNase, then isolation of the hybrid together with a small fraction of free RNA oligonucleotides on hydroxyapatite. The partially purified hybrids are then trapped on Millipore filters. Assay background is 0.004% of total counts present in the annealing reaction. Comparison of the annealing reactions of pulse-labeled liver nuclear RNA and in vitro ^{125}I-labeled nuclear RNA in saturation, kinetic, and competitive hybridization studies shows them to be essentially the same. Nuclear RNA labeled by either tritium or iodine shows a 10–20-fold greater concentration of the annealing sequences over that found in the microsomal RNA. Minor differences are noted between the nuclear RNAs in the initial rates of reaction and in the magnitude of the decrease in percent hybridization at low levels of unlabeled competitor RNA. This may be due to preferential labeling in pulse-labeled RNA of molecules which are present in lower concentrations or are transcribed from more frequently repeated DNA sequences than the average population of annealing RNA molecules. The technique has application in systems where the amount of tissue for RNA extraction is small or where the system does not permit the obtaining of pulse-labeled RNA, as in experimental rodent skin carcinogenesis or in dealing with RNA from the tissues of large mammals or humans.

Standard competitive hybridization studies measure only a small portion of RNA sequences, e.g., only those sequences transcribed from the reiterated portion of the genome (Holmes and Bonner, 1974a,b). In spite of this limitation, these studies have provided direct evidence for the possible importance of nuclear to cytoplasm transport mechanisms in maintaining the differentiated state in normal tissues and in the pathogenesis of neoplasia (Shearer and Smuckler, 1972; Garrett et al., 1973a–c; Shearer, 1974a,b).

Three factors control the sensitivity of the competition study, after such conditions as the assay incubation temperature, ionic strength, and duration of incubation have been determined. These are the ability to accurately determine the amount of RNA annealed, which is a function of the specific activity of the RNA, a means of accurately determining the DNA present in the hybrid, either chemically or by dual labeled testing, and a favorable net RNA counts annealed to assay background

† From the Department of Pathology, University of South Florida, College of Medicine, Tampa, Florida 33620. *Received March 31, 1975.* Publication No. 75-306. This investigation was supported by a grant from the National Cancer Institute (CA-15647).

ratio. Here assay background refers to counts above machine background detected in samples without DNA or with *Escherichia coli* DNA, and hence represents a measure of the sum of the nonspecific factors which cause interference in accurately determining the amount of RNA in the hybrid.

In studying RNA from mammalian tissues, the above conditions are not easily met. Large amounts of [^{3}H]orotic acid (5+ mCi/200 g body weight) are useful for labeling liver nuclear RNA in the rat (Garrett, et. al., 1973a), but labeling in other organs and in transplantable hepatomas is considerably less efficient (Garrett et. al., 1973b). Moreover, the use of the low energy emitter tritium in the RNA and a higher energy emitter as ^{32}P or ^{14}C in the DNA presents problems in accurately determining RNA, since spillover of ^{32}P or ^{14}C into the ^{3}H channel from the constant amount of DNA present will adversely effect the annealed RNA to assay background ratio.

A possible solution to the problems of dual labeling of RNA and DNA and obtaining high specific activity RNA lies in the use of in vitro labeled ^{125}I-RNA and in vivo labeled ^{3}H-DNA. Since the DNA constitutes a collection of sequences on which RNA molecules partition themselves, its source does not have to be the same tissue or organ from which the RNA is obtained. In practice, however, it must be from the same species as the source of the RNA. Possible sources of DNA capable of being labeled to high specific activity efficiently would be cultures of various tumors, or normal tissues as fibroblasts or amniotic cells. Annealed RNA labeled with ^{125}I can be accurately determined even at small amounts in a γ counter without any interference from the radioactivity in ^{3}H, since the latter is not a γ emitter. The contribution to the tritium channel caused by Compton scattering from the γ rays is not significant if the tritium window is made narrow and the DNA is highly labeled as in the presently described experiments.

The central problem in the use of in vitro labeled ^{125}I-RNA in annealing studies, in which DNA·RNA hybrids are trapped on cellulose acetate filters, is the presence of high assay backgrounds (Getz et al., 1972; Scherberg and Refetoff, 1973). In the present studies background is controlled by first treating the hybrid and unannealed RNA with RNase, then a partial separation of the oligonucleotides from the hybrid on hydroxyapatite, followed by final trapping of the hybrid on Millipore HAWP filters. Banding of ^{125}I-RNA in cesium sulfate (Scherberg and Refetoff, 1974) prior to its use in hybridization studies is also helpful with some preparations of ^{125}I-RNA.

Materials and Methods

DNA and RNA Extraction. Unlabeled rat DNA from rat liver nuclei and unlabeled and 50-min pulse-labeled rat liver nuclear and microsomal RNA were obtained from 175–225-g Sprague–Dawley rats as previously described (Garrett et al., 1973a). Unlabeled rat DNA from rat glial tumor cells and ^{3}H-labeled and ^{14}C-labeled DNA were extracted from pellets of crude nuclei isolated from rat glial tumor cells according to the same procedure as for DNA from rat liver nuclei.

Labeling of Rat Glial DNA. C_6 rat tumor glial cells (Benda et al., 1968) were innoculated at lower density into Ham's F-10 medium (Ham, 1963) containing 10% fetal calf serum, 0.02 M Hepes buffer, pH 7.4, and penicillin and streptomycin at 100 U and 100 μg/ml, respectively, and grown in 250-ml plastic tissue culture flasks to confluency. In addition, the culture media contained either 1 μCi/ml of [^{3}H-methyl]thymidine or 0.05 μCi/ml [^{14}C-methyl]thymidine for obtaining either ^{3}H-labeled or ^{14}C-labeled DNA, respectively. Culture media was changed every 3 days. Monolayers were then washed with ice-cold 0.85% NaCl and trypsinized with 0.5% trypsin in CMF-PBS (CMF-PBS is 0.13 M NaCl, 0.004 M KCl, 5×10^{-4} M Na_2HPO_4, 1×10^{-4} M KH_2PO_4, 0.011 M glucose), and the cells washed three times in cold 0.85% NaCl by centrifugation. The cells from 18 flasks were then suspended in 3 ml of RSB (RSB is 0.01 M Tris, pH 7.6, 0.01 M KCl, 1.5×10^{-3} M magnesium acetate, and 0.006 M mercaptoethanol), swollen for 15 min at 0 °C, homogenized in a Potter-Elvehjem type homogenizer, and centrifuged at 7000*g* for 10 min. The supernatant was discarded and the pellet of crude nuclei was used for isolation of ^{3}H-labeled or ^{14}C-labeled DNA, as described above. Specific activities were obtained in the range of 42 000–79 000 cpm/μg for ^{3}H-labeled and 10 000 cpm/μg for ^{14}C-labeled DNA at counting efficiencies of 35 and 50%, respectively.

^{125}I in Vitro Labeling of RNA. Unlabeled rat liver nuclear RNA was labeled with ^{125}I in the form of potassium iodide, essentially as described by Getz et al. (1972). The initial reaction mixture contained 450 μg of RNA and 1.65 mCi of carrier-free ^{125}I in 0.4 ml consisting of 1.5×10^{-4} M KI and 3.5×10^{-3} M $TlCl_3$. This was incubated at 60 °C for 30 min. Following a second incubation at 60 °C for 20 min in the presence of sodium sulfite and ammonium acetate buffer and elution through Sephadex G-25 with 0.01 M sodium phosphate (pH 6.8), the RNA was absorbed onto a hydroxyapatite column of 1-cm^3 bed volume and washed with 24 ml of 0.05 M sodium phosphate buffer (pH 6.8). It was then eluted with 4 ml of 0.45 M sodium phosphate buffer (pH 6.8). The RNA containing solutions (usually 1–2 ml total volume) were then dialyzed overnight against 1–2 l. of 1/100 SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate). RNA with specific activities of 400 000–800 000 cpm/μg at a counting efficiency of 37% were routinely obtained by this method.

Preparation of Hydroxyapatite. Hydroxyapatite was prepared from 0.50 M Na_2HPO_4 and 0.50 M $CaCl_2$ solution prefiltered through Millipore type HAWP filters exactly as described by Miyazawa and Thomas (1965).

Cesium Sulfate Sedimentation of [^{125}I]RNA. A modification of the method described by Scherberg and Refetoff (Scherberg and Refetoff, 1974) was used. Cesium sulfate (99% pure), purchased from Gallard-Schlesinger Chemical Mfg. Corp., was dissolved in water and filtered through Millipore HAWP filters to remove insoluble materials, and then the solution evaporated to dryness. Step gradients were formed from the purified salt, consisting of 1.5 ml of saturated cesium sulfate solution overlaid with 3.3 ml of cesium sulfate solution of density 1.43–1.45 g/ml containing 200–250 μg of [^{125}I]RNA. The gradients also contained 0.04% *N*-lauroylsarcosine. The gradients were then covered with mineral oil and spun in a Spinco SW41 rotor at 35 700 rpm for 20 h at 25 °C. Following this, the labeled RNA formed a sharp band in the region of 1.66 g/cc. The overlying solution was aspirated and the ^{125}I-RNA was collected and dialyzed against several liters of $^{1}/_{100}$ SSC over a 48-h period to remove all traces of cesium sulfate.

DNA–RNA Hybridization. This technique is a modification of that previously described for filter-bound DNA (Garrett et al., 1973a). All studies here were conducted with both RNA and DNA in solution. In studies using RNA pulse labeled for 50 min with [^{3}H]orotic acid, the DNA was either unlabeled

[1] Abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSC, standard saline citrate; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Tris, tris(hydroxymethyl)aminomethane.

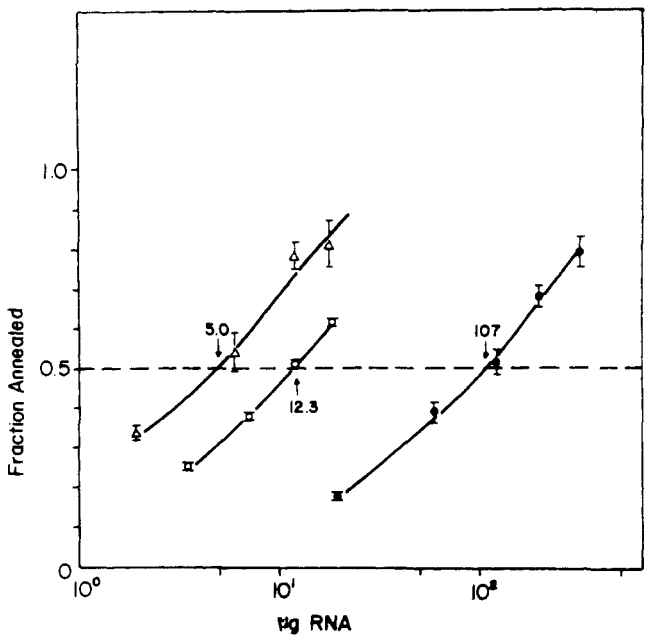

FIGURE 1: Saturation of DNA by ^3H pulse-labeled nuclear and microsomal RNA and ^{125}I-labeled nuclear RNA. One and two micrograms of unlabeled rat DNA and 0.5 μg of ^3H-labeled rat glial DNA were annealed with increasing amounts of 50-min pulse-labeled liver nuclear and microsomal RNA and ^{125}I-labeled nuclear RNA, as described in the text. The values for RNA are per μg of DNA in the reaction. Each point and standard errors are based on triplicate samples. Specific activities and maximum counts bound are: (Δ—Δ) ^3H nuclear RNA, 32 500 cpm/μg, 445 cpm; (○—○) ^{125}I nuclear RNA, 450 000 cpm/μg, 1845 cpm; (●—●) ^3H microsomal RNA, 400 cpm/μg, 115 cpm.

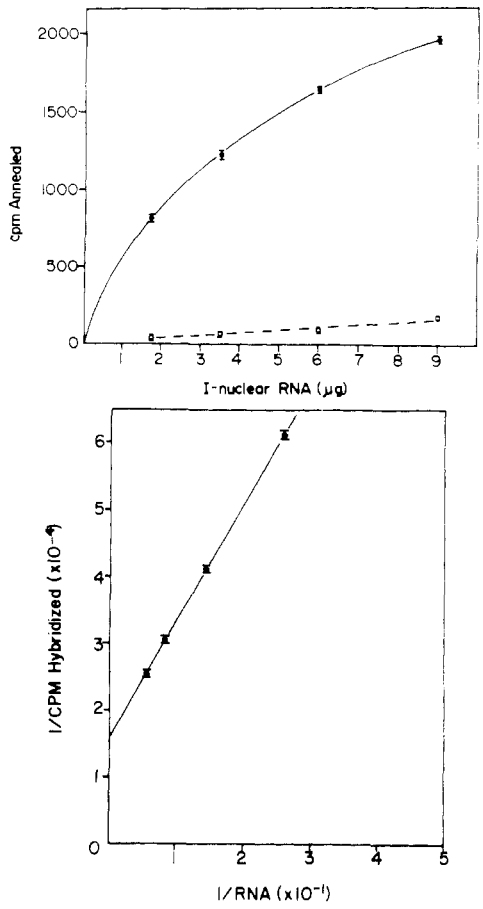

FIGURE 2: Saturation of ^3H glial cell DNA by ^{125}I-labeled nuclear RNA vs. assay background. (A, top) Conditions are specified in Figure 1. ^{125}I counts are normalized to 37.2% recovery of DNA. Average DNA recovered in this study was 32.4%. (●—●) Net normalized ^{125}I cpm above assay background; (○—○) assay background. (B, bottom) Double-reciprocal plot of saturation points in part A adjusted per μg of DNA.

or labeled with ^{14}C. In studies using ^{125}I-labeled RNA the DNA was labeled with ^3H. Following isolation, DNA was denatured, sonicated to a size approximately 250 bases in length, as judged by sucrose gradient centrifugation, and stored frozen at −10 °C. DNA was always denatured again, immediately prior to use, by heating in a boiling water bath for 3–5 min at a concentration of 20 μg/ml in $^1/_{100}$ SSC and, after chilling in an ethanol dry ice bath, was added as the last component to the reaction mixture. The reaction mixture consisted of 0.39 M NaCl, 0.01 M Tes, pH 7.0, and denatured DNA and RNA in the amounts specified in the individual studies. The hybridization reaction was performed in 1- or 2-ml sealed glass ampules at 67 °C for 20 h. At the conclusion of annealing reactions, using 50-min pulse-labeled nuclear or microsomal RNA, the contents of the reaction mixture were drawn up into 4 ml of 6 × SSC and filtered through Millipore type HAWP filters at room temperature. The filter disks were then washed on each side with 50 ml of 6 × SSC and treated at room temperature with 4 ml of 2 × SSC containing 20 μg/ml of pancreatic RNase and 2 units/ml of T1-RNase. They were then rewashed, dried, and counted in glass vials using 15 ml of toluene- PPO-POPOP counting mixture. At the conclusion of the annealing reactions using ^{125}I-labeled RNA and ^3H-labeled DNA, the contents of the reaction mixtures were made up to 1 ml final volume in a solution containing 0.34 M NaCl, 0.04 M KCl, 0.001 M $MgCl_2$, 0.01 M Tris (pH 7.6), and 10 μg/ml of pancreatic RNase and incubated at 37 °C for 1 h. They were then shaken with 1 cm^3 of hydroxyapatite and washed by low-speed centrifugation with 8 ml of 0.01 M, 24 ml of 0.05 M, and 24 ml of 0.1 M sodium phosphate buffer (pH 6.8). All of these buffers contained additional NaCl to a final sodium ion concentration of 0.675 M. The hybrid was then eluted with 8 ml of 0.45 M sodium phosphate buffer, centrifuged at 14 000*g* to remove all traces of hydroxyapatite, chilled to 9–10 °C, and filtered through chilled Millipore HAWP filters. Filters were then washed on each side with 50 ml of 4 × SSC, dried, and counted in plastic scintillation vials using 15 ml of toluene-PPO-POPOP counting mixture to determine ^3H.

For measuring ^3H in annealing studies, using pulse-labeled RNA and unlabeled DNA β counting efficiency was approximately 32%. For dual label studies a narrow window at the lower end of the ^3H energy spectrum was set allowing an 8% counting efficiency of ^3H and a 25% "spillover" of γ counts, or 11–12% spillover of ^{14}C counts into the ^3H channel. In dual-label studies using ^{125}I-RNA and ^3H-DNA, DNA counts were 2000–3500 cpm after subtraction of spillover counts in the range of 200–400 cpm. γ counting efficiency was 37%. ^{14}C counting efficiency was 50% and the window was set to allow no ^3H counts in the carbon channel. Machine backgrounds were 12–15 cpm for γ, 8–10 cpm on the narrow ^3H window, 25–30 cpm on the full ^3H window, and 20–25 for ^{14}C.

Results

Saturation of DNA by ^3H-Labeled Liver Nuclear and Microsomal RNA and by ^{125}I-Labeled Liver Nuclear RNA. In order to provide a basis for evaluating the behavior of ^{125}I in vitro labeled RNA, in DNA·RNA hybridization studies, 50-min pulse-labeled tritiated rat liver nuclear and microsomal RNA were separately annealed in increasing concentrations

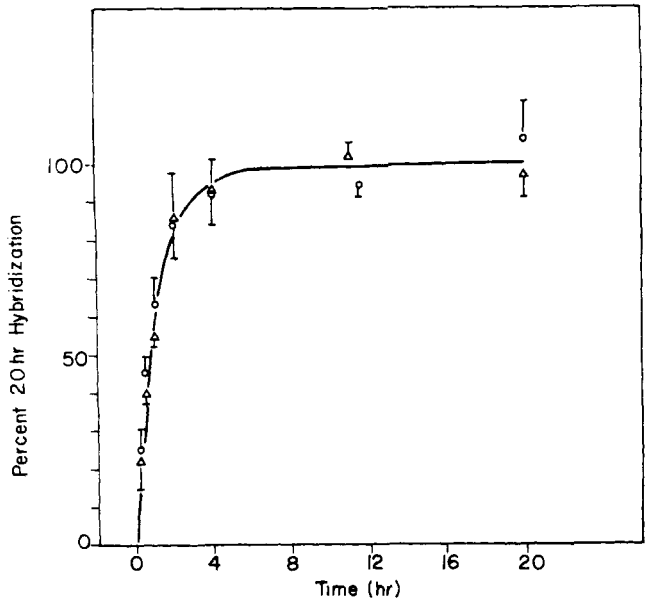

FIGURE 3: Kinetics of annealing of ^{3}H nuclear RNA. Six micrograms of rat liver nuclear RNA pulse labeled for 50 min with 2.5 mCi of [^{3}H]-orotic acid was incubated for increasing periods of time with 0.75 μg of ^{14}C-labeled DNA in 0.3 ml. Δ—Δ and O—O represent separate assays with triplicate samples for each point. Specific activity of RNA, 71 000 cpm/μg; DNA, 850 cpm/μg. Other assay conditions as in Figure 1.

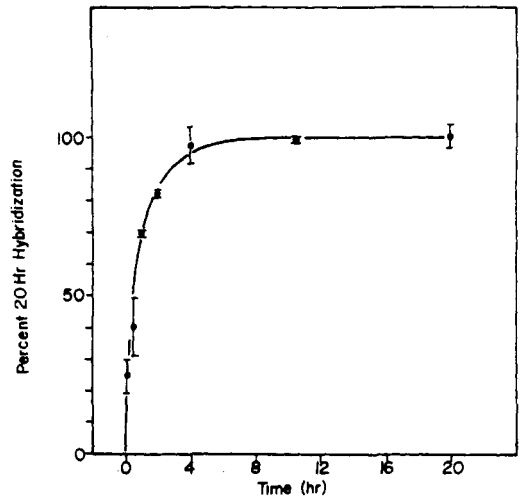

FIGURE 4: Kinetics of annealing of ^{3}H microsomal RNA. Rat liver microsomal RNA (456 μg) pulse labeled for 50 minutes with 2.5 mCi of [^{3}H]orotic acid was incubated for increasing periods of time with 3 μg of labeled rat liver DNA in 1.2 ml. Specific activity of RNA, 1000 cpm/μg; DNA, 115 cpm/μg.

with 1 or 2 μg of rat DNA extracted from the rat liver nuclei. The standard deviations shown are based on the values of triplicate samples for each point. Because of the small quantity of DNA used in these studies, the exact amount trapped on the filters could not be examined directly. However, in subsequent studies performed with ^{14}C-labeled DNA approximately 50–75% of the DNA was retained by the filters. The results of the saturation studies have been plotted on a semilogarithmic scale and are shown in Figure 1. The abscissa represents the RNA/μg of DNA at each point. The 100% saturation value was estimated in each study by the double reciprocal plot method of Bishop (Bishop et al., 1969). An estimate of the concentration of the sequences in the nuclear and microsomal RNA relative to each other may be obtained by comparing the ratio of the amounts of RNA from each required to achieve 50% saturation, since the period of the annealing reaction was constant for all samples (Britton and Kohne, 1968; Birnstiel et al., 1972). The concentration of sequences being measured by this assay appears to be 20 times greater in nuclear RNA than in microsomal RNA, which is consistent with these sequences belonging to the heterogeneous fraction of the RNA (Soeiro et al., 1966, 1968). A second set of studies with pulse-labeled nuclear and microsomal RNA and unlabeled DNA confirmed a difference in annealing efficiency of approximately the same magnitude.

^{125}I-labeled rat liver nuclear RNA was then annealed in a similar fashion with ^{3}H-labeled rat glial DNA. The results are plotted in Figure 1 and replotted on a linear scale in Figure 2A which also includes the value of the assay background for each experimental point. The double-reciprocal plot for these data is shown in Figure 2B. The apparent difference in the half-saturation values for the tritiated and iodinated nuclear RNA's is not significant. A similar saturation study with a different preparation of ^{125}I-RNA and ^{3}H-DNA gave a 50% saturation value of 5.4 μg of RNA. A saturation study with 50-min pulse-labeled RNA and ^{14}C-labeled DNA gave a 50% saturation value of 7.9 μg of RNA. The reason for this apparent onefold variation in the concentration of annealing nuclear RNA species is not known, but could be explained by the contamination of different preparations of nuclei by small but varying amounts of cytoplasmic RNA.

In Figure 2A, the relationship between assay background and net annealed counts is shown. These background counts are 0.12–0.14% of counts passed through the Millipore filters and 0.004% of total RNA counts in the annealing reaction. Backgrounds are 5–10% of the net total annealed counts. Figure 2B shows the double-reciprocal plot of the data in 2A to obtain the 100% saturation value.

Kinetics of Annealing of ^{3}H Pulse-Labeled RNA and ^{125}I-Labeled RNA with DNA. ^{125}I in vitro labeled nuclear RNA and nuclear and microsomal RNA labeled in vivo with [^{3}H]orotic acid as described in Methods were further compared by examining their rate of annealing with DNA. ^{3}H pulse-labeled nuclear and microsomal RNA were annealed with ^{14}C-labeled DNA from glial tumor cells. ^{125}I-labeled RNA was annealed with ^{3}H rat glial tumor DNA. RNA/DNA ratios for these studies were 8:1 for nuclear RNA's and 152:1 for microsomal RNA. All kinetic studies were carried out at a uniform concentration of DNA of 2.5 μg/ml. Because the specific activity of the ^{14}C-labeled DNA would have resulted in a large number of counts relative to those from annealed tritiated RNA in the ^{3}H channel, it was reduced to the desired value by mixing the ^{14}C-labeled rat glial DNA with unlabeled DNA obtained in an identical fashion. The mixture was then denatured and sonicated as described in the Methods. The results for ^{3}H-labeled nuclear and microsomal RNA and ^{125}I-labeled RNA are shown, respectively, in Figures 3, 4, and 5. To make comparison easier the data have been plotted as percent of the 20-h hybridization vs. time. A reproducible difference is noted between the initial rates of reaction of the iodinated and pulse labeled nuclear RNA's. ^{125}I-RNA achieves 50% of its 20-h value in the first 10 min, while pulse-labeled RNA requires between 30 and 60 min to achieve this value. However, in both cases, the annealing reaction appears to be essentially complete by 6 h.

The absence of any decrease in ^{125}I counts detected between 11 and 20 h, as seen in Figure 5, supports the findings of others (Getz et al., 1972; Scherberg and Refetoff, 1973) that the ^{125}I bound to RNA is stable to dissociation from the RNA during

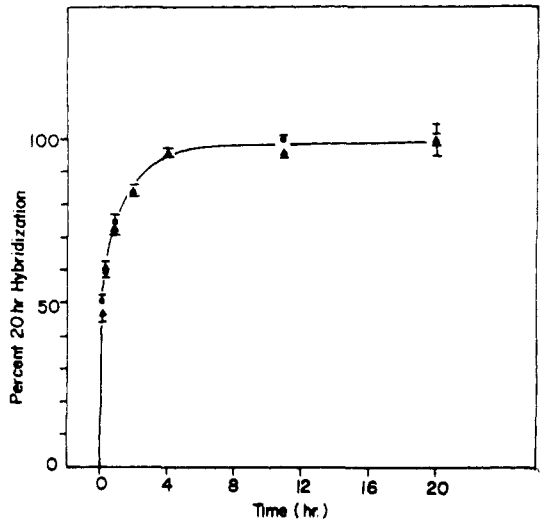

FIGURE 5: Kinetics of annealing of ^{125}I-nuclear RNA. Four micrograms of in vitro labeled ^{125}I-rat liver nuclear RNA was annealed with 0.5 μg of ^{3}H-labeled rat glial tumor DNA in 0.2 ml for increasing periods of time. ^{125}I counts are normalized to 37.2% recovery of DNA. Average DNA recovery in these studies was 33.1%. Specific activities of RNA (●—●), 400 000 cpm; (▲—▲) 800 000. Other conditions are the same as in Figure 1.

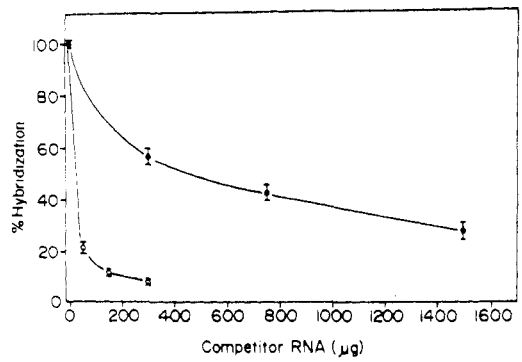

FIGURE 6: Competition of unlabeled nuclear and microsomal RNA against ^{125}I nuclear RNA. 4 μg of ^{125}I rat liver nuclear RNA in 0.2 ml was annealed with 0.5 μg of ^{3}H rat glial tumor DNA in the presence of increasing amounts of either unlabeled rat liver nuclear RNA or microsomal RNA. Counts annealed in the absence of competitor were 1251 ± 13 cpm when adjusted to 37.2% recovery of DNA. Average DNA recovery of this experiment was 23%. (○—○) Unlabeled nuclear RNA; (●—●) unlabeled microsomal RNA.

the annealing study. In separate studies (Garrett and McNulty, unpublished) a decrease of 1% was noted in the average specific activity of triplicate samples of ^{125}I-RNA incubated at 67 °C for 20 h, when compared to identical samples of ^{125}I-RNA which had been kept frozen. This difference was not statistically significant. Assay backgrounds for the studies shown in Figure 5 are again only 5–10% of the net total annealed counts.

Competitive Hybridization of ^{125}I-Labeled and ^{3}H-Labeled Nuclear RNA vs. Unlabeled Nuclear and Microsomal RNA with Rat Glial DNA. The above studies have shown a close quantitative similarity of DNA saturation and kinetics of annealing between ^{3}H pulse-labeled RNA and ^{125}I-labeled RNA. Also of importance, however, is whether iodination of the RNA has resulted in a loss in its base-pairing specificity. One observation which has been frequently demonstrated by competitive hybridization studies using pulse-labeled RNA is that liver nuclear RNA appears to contain sequences not present in the cytoplasm (Shearer and McCarthy, 1967; Garrett et al., 1973b,c; Shearer, 1974a,b). In the present studies ^{125}I in vitro

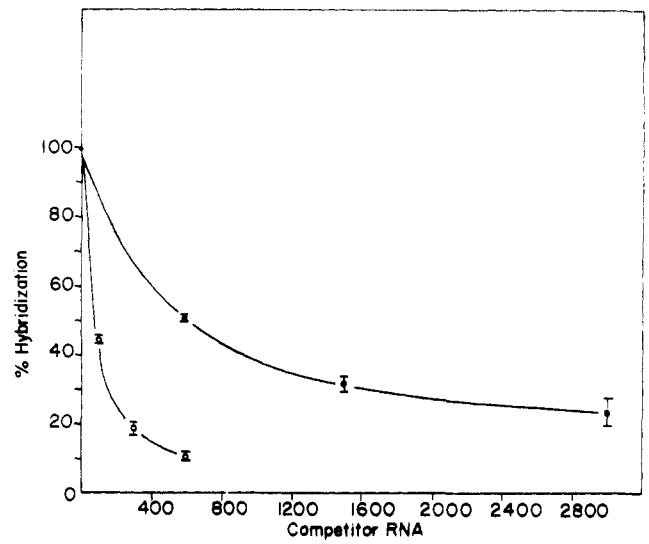

FIGURE 7: Competition of unlabeled nuclear and microsomal RNA against pulse-labeled ^{3}H nuclear RNA. Eight micrograms of ^{3}H rat liver nuclear RNA in 0.4 ml was annealed with 1 μg of ^{14}C rat glial tumor DNA in the presence of increasing amounts of either unlabeled rat liver microsomal or nuclear RNA. Specific activities are as in Figure 3. Assay conditions are as in Figure 6. Counts annealed in the absence of competitor were 450.

labeled rat liver nuclear RNA and ^{3}H pulse-labeled rat liver nuclear RNA were competed separately with unlabeled liver nuclear and microsomal RNA. In the study with ^{125}I-RNA the DNA was labeled with ^{3}H, and in the study with ^{3}H-RNA the DNA was labeled with ^{14}C. Nucleic acid concentrations were identical in both studies, and both studies utilized the same RNase treatment and fractionation of samples on hydroxyapatite.

The results shown in Figure 6 and 7 essentially demonstrate the pattern seen by ourselves and others, indicating a markedly greater competitive efficiency of nuclear vs. microsomal RNA. Both studies do show continued decrease in the percent hybridization with microsomal RNA at the highest competitor point. However, double-reciprocal plots (Bishop et al., 1969; Hansen et al., 1970) of both sets of data show that qualitative differences exist between the nuclear and microsomal RNAs and that the microsomal RNA does not contain some sequences present in the nuclear RNA. One minor difference between the competition studies is that unlabeled nuclear RNA appears to be more efficient as a competitor against ^{125}I-RNA than against ^{3}H-RNA. Possible reasons for this are given in the Discussion. For the study with ^{125}I-RNA, average net counts/min for the 150- and 300-μg nuclear RNA competitor points were 1.5 and 1.2 times assay background, respectively. Assay background in the absence of competitor RNA was 61 cpm and for all points averaged 53 cpm, being lowest in the case of the two highest microsomal RNA competition points.

Discussion

^{125}I-labeled liver nuclear RNA behaves quantitatively similar to 50-min pulse-labeled liver nuclear RNA with regard to saturation of DNA by RNA, rate of annealing of RNA with DNA, and response to unlabeled nuclear and microsomal RNA in competition studies. The percent of the rat genome which anneals with RNA at the theoretical saturation point of ^{125}I-labeled nuclear RNA is 3.4%. This compares favorably with the value of approximately 2% obtained by a different technique for the fraction of the rat genome which anneals with

the middle repetitive sequences from rat ascite tumor high-molecular-weight nuclear RNA (Holmes and Bonner, 1974a). Two minor differences were noted in these studies between pulse-labeled and in vitro labeled RNA. In kinetic studies the initial rate of reaction is slower for pulse-labeled RNA and its hybridization is not as rapidly suppressed by competitor nuclear RNA as is that of in vitro labeled RNA. One possible explanation of these findings, especially for the kinetic studies, would be that ^{125}I-RNA has a larger molecular weight than pulse labeled RNA (Wetmur and Davidson, 1968). However, molecular weight determination on sucrose gradients showed pulse labeled RNA to be approximately 200–215 nucleotides long, while ^{125}I-RNA are 90–100 nucleotides in length. A second hypothesis, which ties the kinetic and competition observations together, is that during pulse labeling isotope tends to accumulate initially in RNA molecules which are present in lower concentrations or are transcribed from more frequently repeated DNA sequences than the average population of annealing RNA molecules. This appears to be the more likely explanation at this time. Aside from these minor differences, kinetic and competition studies with the two labeled RNA's are essentially identical.

In an all-liquid hybridization system, DNA/DNA reannealing must be considered. Incubation of DNA under the present conditions results in an equivalent C_0t value of 1.92 (Britten et al., 1974). DNA renaturation studies performed in this laboratory and by others indicate some reiterated DNA sequences will anneal at this value of C_0t. However, this value is 10–50 times smaller than the value of C_0t which marks completion of annealing of reiterated sequences (Britten and Kohne, 1968; Garrett, unpublished results). The ratio of RNA sequences to complementary DNA sequences is also important and can be estimated on the basis of data presented here and published elsewhere. The present studies indicate that approximately 3.4% of the genome is complementary to repetitive sequence (heterogeneous) nuclear RNA. Studies by others have shown that 10% or more of nuclear RNA is heterogeneous (Soiero et al., 1968), and 30% of heterogeneous nuclear RNA is transcribed from reiterated sequences (Greenberg and Perry, 1971). Under the present conditions, that is, 4 μg of nuclear RNA and 0.5 μg of DNA, this results in an RNA to complementary DNA ratio of 7:1 or greater. Thus, the reaction of DNA with RNA should be favored. If any RNA sequences are present in relatively few copies, however, DNA/DNA reannealing might affect their detection.

The use of in vitro ^{125}I-labeled RNA and in vivo ^{3}H-labeled DNA permits independent determination of the amount of annealed RNA and DNA. The assay background is reduced to acceptable values through pretreatment of the hybrid and free RNA with RNase, then removal of most of the free RNA counts by isolation of the hybrid on hydroxyapatite. Assay backgrounds in these studies represent only about 0.004% of total counts in the annealing mixture. Occasional preparations of ^{125}I-RNA gave ratios of net annealed counts to assay background of only 2–4 following this procedure. If these preparations of RNA are then banded on cesium sulfate density gradients and subsequently annealed with DNA, the ratios were increased to 10 or greater. Our experience with this procedure thus parallels that of Scherberg and Refetoff. Unlike their studies, however, our hybridization system is clearly measuring sequences other than ribosomal RNA.

The hybridization system as performed in these studies has certain theoretical and practical advantages. First, the all-solution hybridization system has a considerably faster rate of reaction than the filter technique (Holmes et al., 1974). Second, the small amount of DNA present in the assay makes the unlabeled competitor more effective. This is because until the total amount of labeled plus unlabeled RNA sequences is sufficient to completely saturate the complementary sites on the DNA both the labeled and unlabeled RNA sequences will anneal and no decrease in the measured counts will occur (Bolle et al., 1968).

Third, the ability in this assay to increase the specific activity of the RNA to very high values, through the use of the relatively inexpensive isotope ^{125}I, reduces the amount of RNA and DNA necessary to successfully accomplish hybridization studies. This could have application in an area such as experimental skin carcinogenesis or in other systems where there is very little tissue with which to work and where the sequences being measured are a small fraction of the sample of RNA.

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Sequence Composition of the Template-Active Fraction of Rat Liver Chromatin†

Joel M. Gottesfeld,‡ György Bagi,§ Beckie Berg, and James Bonner*

ABSTRACT: Rat liver chromatin has been separated into nuclease-sensitive and -resistant fractions after mild digestion with DNAase II. The nuclease-sensitive material is further fractionated into Mg^{2+}-soluble and -insoluble chromatin fractions. The kinetics of production of these chromatin fractions have been investigated. After a brief enzyme treatment (5 min at 10 enzyme units/A_{260} unit of chromatin at pH 6.6), 11% of the input chromatin DNA is found in the Mg^{2+}-soluble fraction. This DNA has a weight-average single-strand length of about 400 nucleotides and, as determined by renaturation kinetics, comprises a subset of nonrepetitive DNA sequences and a subset of families of middle repetitive sequences. This demonstrates the nonrandom distribution of repetitive and single copy sequences in the Mg^{2+}-soluble fraction of chromatin. Previous studies have shown that the Mg^{2+}-soluble fraction is enriched in nonrepeated sequences which are transcribed in vivo (Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F., and Bonner, J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 2193–2197). We now report that the Mg^{2+}-soluble fraction of liver chromatin contains a low proportion of sequences in common with the Mg^{2+}-soluble fraction of brain chromatin. Thus, fractionation does not depend on some general property of chromatin but is specific with regard to the template activity of the tissue from which the chromatin was obtained.

Previous work from this laboratory has shown that a minor fraction of DNA in interphase chromatin is more rapidly attacked by the endonuclease DNAase II than is the bulk of chromatin DNA (Marushige and Bonner, 1971; Billing and Bonner, 1972; Gottesfeld et al., 1974a). The amount of DNA in this fraction is variable depending upon the source of the chromatin, but is approximately equal to the proportion of DNA available in a given chromatin preparation for transcription by exogenous RNA polymerase (Billing and Bonner, 1972). The nuclease-sensitive fraction can be separated from the major portion of chromatin by simple procedures based on the solubility of this fraction in either standard saline–sodium citrate (Marushige and Bonner, 1971) or divalent cations (Billing and Bonner, 1972; Bonner et al., 1973; Gottesfeld et al., 1974a). This fraction differs from either unfractionated chromatin or the nuclease-resistant fractions in many respects: namely, chemical composition, chromosomal protein populations, template activity for support of RNA synthesis with exogenous bacterial polymerase, DNA sequence complexity, and DNA sequence homology with cellular RNA (Gottesfeld et al., 1974a). The nuclease-sensitive fraction appears to have the properties expected for transcriptionally active chromatin: it is enriched in nonhistone chromosomal proteins and depleted in histone protein (Marushige and Bonner, 1971; Gottesfeld et al., 1974a, 1975). Nascent RNA is copurified with the Mg^{2+}-soluble fraction of both ascites and HeLa cell chromatin (Billing and Bonner, 1972; Bonner et al., 1975; Pederson and Bhorjee, 1975; Gottesfeld, in preparation) and, most important, the Mg^{2+}-soluble fraction of liver chromatin is enriched in nonrepetitive DNA sequences complementary to cellular RNA (Gottesfeld et al., 1974a). Thus, many lines of evidence support the notion that the nuclease-sensitive, Mg^{2+}-soluble fraction of chromatin corresponds to transcriptionally active regions of chromatin in vivo.

Although active chromatin has been found to differ from inactive regions of chromatin in many respects, the DNA of the Mg^{2+}-soluble fraction is organized in a manner similar to the bulk of the DNA in chromatin; after limited digestion with DNAase II, agarose gel electrophoresis reveals a repeating unit of about 200 base pairs with DNA of the Mg^{2+}-soluble fraction (Gottesfeld and Bonner, 1976). After prolonged nuclease digestion about half of this DNA is rendered acid soluble; furthermore, nuclease-resistant complexes of DNA, RNA, histone, and nonhistone protein have been isolated from Mg^{2+}-soluble chromatin (Gottesfeld et al., 1975). These results are consistent with the finding that active genes are complexed with protein in nuclease-resistant structures (Axel et al., 1975; Lacy and Axel, 1975).

In this report, we expand upon our earlier investigations. From cross-reassociation experiments with the DNAs of the different chromatin fractions, we conclude that fractionation is highly DNA sequence specific. We find that the nuclease-sensitive fraction contains a subset of single-copy sequences and a subset of families of repetitive sequences. Thus, the

† From the Division of Biology, California Institute of Technology, Pasadena, California 91125. *Received November 6, 1975.* These studies were supported by the United States Public Health Service (Grants GM 86 and GM 13762).
‡ Present address: MRC Laboratory of Molecular Biology, Cambridge, England CB2 2QH.
§ Present address: Biological Research Center, Institute of Plant Physiology, Szeged, Hungary.