Ribonucleic Acid–Deoxyribonucleic Acid Hybridization and Hybridization–Competition Studies of the Rapidly Labeled Ribonucleic Acid from Normal and Chronic Lymphocytic Leukemia Lymphocytes*

Paul E. Neiman and Patrick H. Henry

ABSTRACT: The rapidly labeled ribonucleic acid synthesized *in vitro* by circulating lymphocytes from normal individuals and from patients with chronic lymphocytic leukemia was studied by the techniques of ribonucleic acid–deoxyribonucleic acid hybridization and hybridization–competition. A maximum of 12% of labeled ribonucleic acid from normal lymphocytes and 10% of labeled chronic lymphocytic leukemia–ribonucleic acid formed ribonuclease-resistant hybrids with homologous deoxyribonucleic acid. The ribonucleic acid from both cell types reacted with deoxyribonucleic acids from various animal species in proportion to their known genetic relatedness. At a [^{3}H]ribonucleic acid to deoxyribonucleic acid ratio of 1.5:1 available complementary sites on the deoxyribonucleic acid were saturated sufficiently to give experimental values in hybridization–competition experiments which corresponded closely to a saturation-dependent, theoretical competition curve. Heterologous ribonucleic acid from several species competed in the reaction between chronic lymphocytic leukemia [^{3}H]ribonucleic acid and homologous deoxyribonucleic acid in proportion to the nucleotide sequence relatedness of the deoxyribonucleic acids from which they were transcribed. This specificity of the competition system was strictly dependent upon the conditions of the experiment. Unlabeled ribonucleic acid chronic lymphocytic leukemia lymphocytes competed completely with labeled ribonucleic acid from normal lymphocytes. However, unlabeled normal lymphocyte ribonucleic acid competed incompletely with chronic lymphocytic leukemia [^{3}H]ribonucleic acid and reached a plateau at 74% of the theoretical value for an identical ribonucleic acid. This suggests that the lymphocyte of chronic lymphocytic leukemia synthesizes species of ribonucleic acid that are either absent in the normal lymphocyte or present in very low concentration.

A striking morphologic similarity exists between the normal small lymphocyte and the lymphocyte which appears in the blood of patients with chronic lymphocytic leukemia. Both types of lymphocytes are small (10 μ in diameter), have a high ratio of nucleus to cytoplasm, and an RNA:DNA ratio of 0.3. Despite their similar appearance, chronic lymphocytic leukemia lymphocytes can be functionally distinguished from their normal counterparts. For example, several investigators have demonstrated that the chronic lymphocytic leukemia cell responds poorly to *in vitro* stimulation by phytohemagglutinin or to specific antigens (Oppenheim *et al.*, 1965), whereas the normal lymphocyte will grow and divide *in vitro* under these same conditions (Nowell, 1960). The gradual replacement of normal lymphoid tissue and bone marrow by these leukemic lymphocytes results in the failure of hematopoietic and immunologic functions characteristic of the disease state. It is reasonable to propose that an altered pattern of gene expression underlies these abnormalities and that this alteration would be reflected by changes in the RNA synthesized by the chronic lymphocytic leukemia lymphocytes.

Previously reported studies from this laboratory (Henry *et al.*, 1967; Torelli *et al.*, 1968) have demonstrated that the chronic lymphocytic leukemia and normal small lymphocyte synthesize 28S and 18S rRNA at a slow rate and to a limited extent. The vast majority of the nucleic acid synthesized by both types of cells is a rapidly labeled, high molecular weight nuclear RNA which is heterodisperse (30–60 S) in a sucrose density gradient. RNA–DNA homology studies demonstrated that a portion of this RNA readily formed hybrids with homologous DNA. An RNA with similar properties has been described in a number of types of animal cells, for example, the HeLa cell and the avian erythroblast (Warner *et al.*, 1966; Attardi *et al.*, 1966; Scherrer *et al.*, 1966a).

Despite these gross similarities between the rapidly labeled RNA synthesized by both types of lymphocyte, it remains a possibility that different genetic loci are being transcribed in the chronic lymphocytic leukemia lymphocyte. Therefore, different nucleotide sequences would be present in the RNA of this cell. We utilized the techniques of RNA–DNA hybridization and hybridization–competition to characterize further the rapidly labeled complementary RNAs synthesized *in vitro* by the chronic lymphocytic leukemia and normal lymphocyte.

* From the Medicine Branch, National Cancer Institute, Bethesda, Maryland 20014. *Received May 16, 1968.*

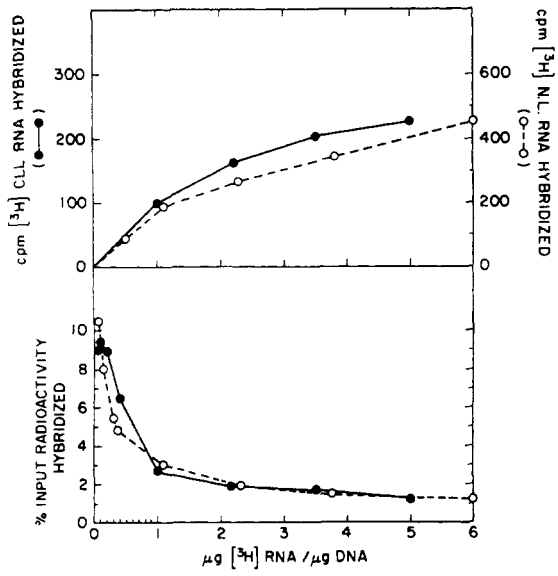

FIGURE 1: The hybridization of RNA from chronic lymphocytic leukemia and normal lymphocytes labeled for 6 hr. The reaction was carried out in sealed-glass ampules at 67° for 16 hr in 0.1 ml of two-times standard saline citrate with a DNA concentration of 20 μg/ml and RNA concentrations as indicated for RNA:DNA ratios above 0.5. For smaller ratios the RNA concentration was held at 1.0 μg/ml and the DNA concentration increased. The points represent the average of duplicate samples. The upper panel indicates the cpm of normal and chronic lymphocytic leukemia lymphocyte RNA bound to DNA at increasing RNA:DNA ratios. The lower panel describes the data in terms of per cent of the input radioactivity retained as an hybrid.

Experimental Section

Materials

Nyon fiber (Leuko-Pak) was purchased from Fenwal Corp. [5-^{3}H]Uridine (22 Ci/mmole), [^{3}H]adenosine (4 Ci/mmole), and [^{3}H]cytidine (25 Ci/mmole) were products of Nuclear-Chicago Corp. Pancreatic ribonuclease and pancreatic deoxyribonuclease I were purchased from Worthington Biochemical Corp. Toluene phosphor (Liquifluor) was obtained from Nuclear-Chicago Corp. Synthetic poly G was purchased from Miles Laboratory. Phytohemagglutinin was purchased from Burroughs Wellcome, Ltd.

Methods

Preparation of Lymphocytes. Leukocyte-rich plasma from normal individuals was prepared from heparinized fresh whole blood by the sedimentation of red cells with one-tenth volume of 6% Dextran in normal saline. Separation of lymphocytes from polymorphonuclear leukocytes was achieved by passage of the white cell rich plasma, diluted with an equal volume of Eagle's minimal essential (Eagle, 1959), through a column of thoroughly washed nylon fiber according to the method of Greenwalt *et al.* (1962; Torelli *et al.*, 1968). The usual yield was 6–8 × 10^8 cells (>99% lymphocytes) from 500 ml of blood.

The patients with chronic lymphocytic leukemia selected for study were untreated inidviduals with white blood cell counts ranging from 50,000 to 200,000 per mm^3. Their lymphocytes were prepared by sedimentation of the blood directly in the heparinized syringe used for venepuncture. The vast predominance of small lymphocytes (>95%) in the blood of these patients made the nylon fiber column separation unnecessary once it was demonstrated that the omission of this step had no effect on subsequent results. The chronic lymphocytic leukemia lymphocytes were simply deposited from the plasma by centrifugation at 1000 rpm for 10 min.

Labeling of RNA. The lymphocytes were suspended in minimal essential medium (10^7 cells/ml) with 20% autologous plasma, glutamine (0.3 g/l.), penicillin, streptomycin, and 10 μCi/ml each of [^{3}H]uridine, [^{3}H]adenosine, and [^{3}H]cytidine and incubated at 37° in 8-oz prescription bottles for periods of 1–6 hr. The cells were harvested by centrifugation at 1200 rpm and washed once with cold normal saline.

Extraction of RNA. The cell pellet was suspended in two volumes of reticulocyte standard buffer[1] and the cells were allowed to swell for 5 min at 4°. Crystalline deoxyribonuclease I (RNase free) was added (final concentration 1 mg/ml) and the cells were disrupted with a tight-fitting Dounce homogenizer for 2 min (the DNase served to liquify the gelatinous mass of DNA which forms following rupture of the cell nuclei, and which may trap considerable quantities of lymphocyte RNA). The RNA was then extracted from the homogenate according to a previously described hot phenol–*m*-cresol–sodium dodecyl sulfate procedure (Torelli *et al.*, 1968). The RNA pellets were dissolved in two-times standard saline citrate with 0.05% sodium dodecyl sulfate to yield a final concentration of about 5 mg/ml. The usual yield was 1.0 mg of RNA from 10^9 lymphocytes. The RNA retained its hybridization characteristics for periods of 1–2 weeks when stored at −20°.

Preparation of DNA. DNA was extracted from human lymphocytes and *Escherichia coli* by the method of Marmur (1961) and from mouse, monkey, hamster, and rat liver by the method of Hiatt (1962).

RNA–DNA Hybridization. All the RNA–DNA hybridization reactions were carried out in 1-ml sealed-glass ampules at 67° for 16 hr with a reaction volume of 0.1 ml (two-times standard saline citrate with 0.05% sodium dodecyl sulfate). The hybrid complexes were trapped on membrane filters (Millipore, HA 47 mm) and treated with pancreatic ribonuclease according to a previously described modification of the method of Nygaard and Hall (1963; Torelli *et al.*, 1968). The dried filters were assayed for radioactivity directly in toluene phosphor in a Packard Tri-Carb liquid scintillation spectrometer. The samples were counted for a sufficient period of time to achieve a statistical accuracy of ±1.0%. Hybridization–competition studies were performed by premixing the labeled RNA with varying amounts of unlabeled RNA from various sources followed by the completion of the hybridization procedure described above.

[1] Abbreviations used: standard saline citrate, 0.015 M sodium citrate–0.5 M NaCl; reticulocyte standard buffer, 0.01 M NaCl–0.01 M Tris–0.0025 M $MgCl_2$ (pH 7.4).

TABLE I: Specificity of Direct Hybridization Reaction.[a]

Source of DNA	% Control Hybridization	
	Normal Lymphocyte [^{3}H]-RNA	Chronic Lymphocytic Leukemia [^{3}H]-RNA
Normal lymphocyte	100	100
Chronic lymphocytic leukemia lymphocyte	100	100
Monkey liver	65	65
Rat liver		12
Mouse liver	20	16
Chicken liver	8	5
E. coli	0	0

[a] The reactions were carried out with 2 μg of [^{3}H]-RNA and 20 μg of DNA in 0.1 ml of two-times standard saline citrate in duplicate. The mean of the counts per minute bound to homologous DNA was set at 100%. The *E. coli* DNA reaction was used as a blank and was 10^{-4} or less of the counts added per vial.

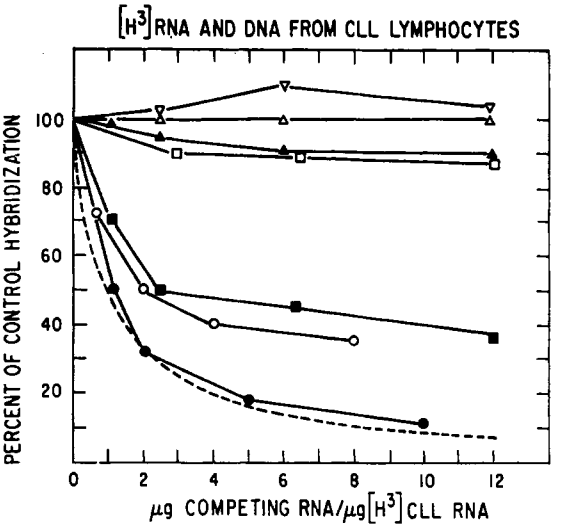

FIGURE 2: The competition of unlabeled RNA from various sources in the hybridization of labeled RNA from chronic lymphocytic leukemia lymphocytes with chronic lymphocytic leukemia DNA at an [^{3}H]RNA:DNA ratio of 1.5. The experiment was carried out with 3.2 μg of DNA and 4.8 μg of chronic lymphocytic leukemia [^{3}H]RNA (3500 cpm/μg) premixed with various quantities of unlabeled RNA from *E. coli* (▽—▽), synthetic poly G (△—△), mouse L 1210-leukemia cells (▲—▲), chicken liver (□—□), monkey kidney cells (■—■), KB cells (human epidermoid carcinoma) (○—○), and chronic lymphocytic leukemia lymphocytes (●—●). The final volume was 0.1 ml of two-times standard saline citrate, reaction time was 16 hr, and each point determined by the average of duplicate samples. The counts per minute bound to DNA in the absence of unlabeled RNA were taken at 100%, and the dashed line represents the theoretical competition curve at saturation for an identical RNA.

Results

Saturation of DNA. Experiments were carried out to explore the nature of the reaction between labeled lymphocyte RNA and its homologous DNA. It has been previously reported that at least 90% of the 6-hr pulse-labeled RNA in both types of lymphocyte is confined to the nucleus and sediments primarily in the 30–60S region of a sucrose gradient (Henry *et al.*, 1967; Torelli *et al.*, 1968; P. E. Neiman and P. H. Henry, unpublished observations). The results of varying the concentration of [^{3}H]RNA per microgram of DNA in the reaction mixture are illustrated in Figure 1. The maximum percentage of the radioactivity in the RNA synthesized in 6 hr that formed a ribonuclease-resistant hybrid was 12% in the normal lymphocyte and 10% in the chronic lymphocytic leukemia cell. This maximum percentage of hybridization occurred in the presence of a tenfold excess of DNA and decreased sharply as the RNA:DNA ratio approached 1.0–1.5. At this point there appeared to be an inflection in the saturation curve although no clear saturation plateau was attained. As the ratio of RNA:DNA was increased beyond 1.5, a nearly constant fraction of 1–2% of the input radioactivity entered into the RNA–DNA hybrid. Since these studies were performed in free solution, which permits both DNA:DNA and RNA:DNA interaction, the possibility remains that the rate of DNA:DNA reassociation among the polynucleotide families would remove a sufficient number of these sequences from the reaction. This might be expected to reduce the maximum percentage of hybridization and yield apparent saturation of the DNA at a spuriously low RNA:DNA ratio. In the course of similar experiments with the DNA immobilized on filters (Gillespie and Speigelman, 1965) we have obtained approximately the same maximum percentage of hybridization although the time required for the reaction was 48 hr rather than 16 hr (P. E. Neiman and P. H. Henry, unpublished observations). This suggests that most of the sequences available for hybridization on filters containing immobilized DNA were also available in the liquid system. Thus, in succeeding experiments a ratio of radioactive RNA:DNA of about 1.5 was considered adequate for partial saturation of the DNA with those species of RNA present in sufficient concentration to react under the specified experimental conditions.

Specificity of the Hybridization Reaction. Experiments were carried out to determine the ability of complementary [^{3}H]RNA from lymphocytes to discriminate between DNAs from various sources. These were performed in the presence of a tenfold excess of DNA to maximize the extent of hybrid formation. The results are described in Table I. The extent of hybridization of chronic lymphocytic leukemia [^{3}H]RNA and normal lymphocyte [^{3}H]RNA with their homologous DNAs was taken as 100% homology. The data demonstrated that human DNA from either normal or leukemic sources

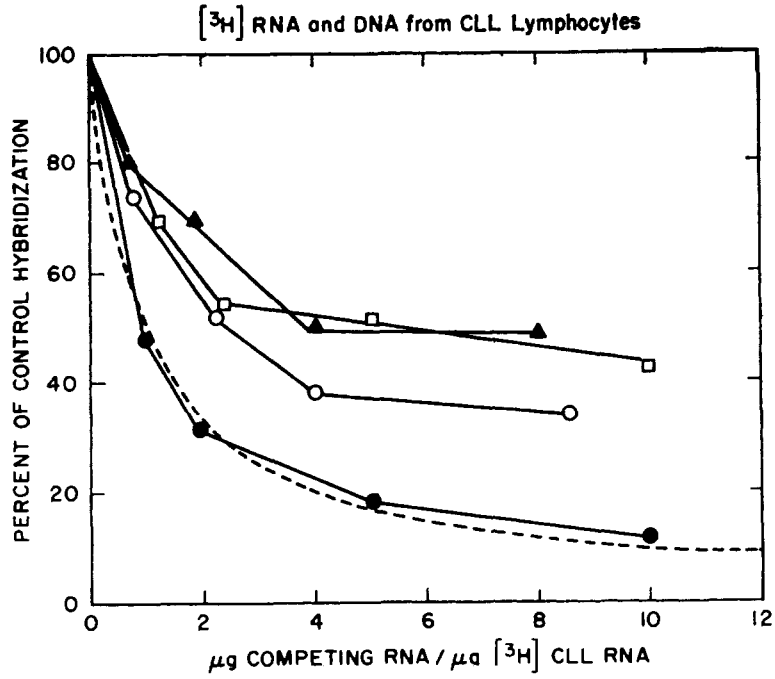

FIGURE 3: The competition of unlabeled RNA from various sources with labeled chronic lymphocytic leukemia RNA at a [³H]RNA:DNA ratio of 4.0. Studies were carried out with 2.0 μg of chronic lymphocytic leukemia DNA and 8 μg of chronic lymphocytic leukemia [³H]RNA premixed with unlabeled RNA from chronic lymphocytic leukemia lymphocytes, KB cells, chicken liver, and mouse L 1210 leukemia cells. The reaction was performed in 0.1 ml of two-times standard saline citrate in sealed-glass 1-ml ampules and incubated for 16 hr at 67°. The symbols are the same as in Figure 2.

was equivalent. Both chronic lymphocytic leukemia and normal lymphocyte RNA reacted with monkey DNA to approximately 65% of the extent of their reaction with homologous DNA. The reaction of chronic lymphocytic leukemia and normal lymphocyte RNA was 16 and 20%, respectively, with mouse DNA and 5 and 8% with chicken DNA. No reaction was observed with *E. coli* DNA.

Hybridization–Competition. The technique of hybridization–competition was used to detect possible differences in the rapidly labeled RNAs from normal and chronic lymphocytic leukemia lymphocytes. Studies were carried out by incubating saturating amounts of chronic lymphotic leukemia [³H]RNA with chronic lymphocytic leukemia DNA in the presence of increasing quantities of unlabeled RNA from various sources. If the types and distribution of RNA species in the unlabeled preparation are the same as those in the radioactive RNA, the addition of the unlabeled material should effectively dilute the specific activity of the [³H]RNA in proportion to the amount of cold RNA added. Thus, the radioactivity of the hybrid product would be reduced in a predictable manner and would permit the construction of a theoretical competition curve for comparison with actual experimental values (Merits *et al.*, 1966; Bolle *et al.*, 1968).

Figure 2 illustrates a competition experiment in which 4.8 μg of [³H]RNA from chronic lymphocytic leukemia lymphocytes was reacted with 3.2 μg of homologous DNA. Unlabeled RNA extracted from the lymphocytes of a second patient with chronic lymphocytic leukemia competed to 95% of the theoretical value at a ratio of unlabeled to [³H]RNA of 10:1. *E. coli* RNA and synthetic poly G were noncompetitive. RNA extracted from mouse lymphocytes (L 1210 leukemia) and chicken liver cells competed to the extent of 10 and 12%, respectively, of the theoretical value for an identical RNA. Unlabeled RNA from monkey kidney cells competed to 65% of the theoretical value. In addition, RNA from a different human cell line, the KB cell, competed to only 70% of the theoretical value and could be clearly distinguished from chronic lymphocytic leukemia lymphocyte RNA. The species specificity of the hybridization–competition reaction was significantly reduced when the ratio of [³H]RNA:DNA was increased to 4. The results of a competition experiment in which 8 μg of chronic lymphocytic leukemia [³H]RNA was reacted with 2 μg of homologous DNA are shown in Figure 3. There was no significant change in the degree of competition by unlabeled chronic lymphocytic leukemia RNA or KB RNA when compared with the previous experiment (Figure 2). However, chicken and mouse cell RNAs interrupted the formation of the radioactive hybrid to 50% of the control reaction. These data demonstrated that one can discriminate between these RNAs on the basis of nucleotide sequence and that the degree of specificity is dependent upon the experimental conditions employed.

The results of a competition experiment in which unlabeled RNA from chronic lymphocytic leukemia and normal lymphocytes was used in the hybridization reaction between 4.8 μg of [³H]RNA from normal lymphocytes and 3.2 μg of lymphocyte DNA are shown in Figure 4. The RNA from both types of lymphocytes

TABLE II: Extent of Competition by Unlabeled Normal Lymphocyte and Chronic Lymphocytic Leukemia RNAs with Chronic Lymphocytic Leukemia [^{3}H]RNA for Lymphocyte DNA.[a]

Competing RNA	% Theoretical Competition[a]	Mean ± Std Dev
Chronic lymphocytic leukemia lymphocyte	94	94 ± 3.9
Chronic lymphocytic leukemia lymphocyte	91	
Chronic lymphocytic leukemia lymphocyte	97	
Chronic lymphocytic leukemia lymphocyte	90	
Chronic lymphocytic leukemia lymphocyte	89	
Chronic lymphocytic leukemia lymphocyte	98	
Normal lymphocyte[b]	75	73 ± 2.4
Normal lymphocyte	75	
Normal lymphocyte	75	
Normal lymphocyte	69	
Normal lymphocyte	72	

[a] The percentages listed are the per cent of the theoretical reduction of the radioactivity of the control hybrid achieved at a tenfold excess of competing unlabeled RNA over [^{3}H]RNA. [b] Stimulated with phytohemagglutinin (0.02 ml/ml) for 72 hr.

competed equally well and closely followed the theoretical curve. Thus, we were not able to detect the synthesis of labeled species of RNA in the normal cell which were not also present in the chronic lymphocytic leukemia lymphocyte. Figure 5 shows the results of the reverse experiment testing the competitive effect of unlabeled RNA from normal and chronic lymphocytic leukemia lymphocytes in the hybridization reaction between [^{3}H]-RNA from chronic lymphocytic leukemia lymphocytes and lymphocyte DNA. The RNA from chronic lymphocytic leukemia cells again competed closely along the theoretical curve. In contrast, the RNA from normal lymphocytes competed incompletely to only 75% of the predicted value as did RNA extracted from normal lymphocytes stimulated with phytohemagglutinin for 72 hr. The results of several such experiments are shown in Table II. The mean value for unlabeled RNA from six patients with chronic lymphotic leukemia (using labeled RNA from three other patients with chronic lymphocytic leukemia) is 94% of

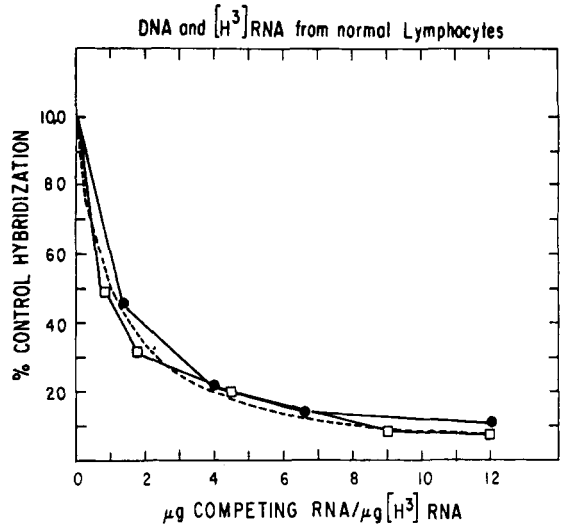

FIGURE 4: The competition of unlabeled RNA from chronic lymphocytic leukemia and normal lymphocytes in the hybridization of [^{3}H]RNA from normal lymphocytes with lymphocyte DNA. The reaction was performed in 0.1 ml of two-times standard saline citrate in 1.0-ml sealed-glass ampules and incubated for 16 hr at 67°. (—●—●—) Competition by unlabeled normal lymphocyte RNA. (—□—□—) Competition by unlabeled chronic lymphocytic leukemia RNA.

the theoretically predicted value for competition by identical RNAs. The extent of competition for unlabeled lymphocyte RNA from ten normal individuals in six experiments is 74% of the theoretical value. This difference is significant at the 1% level by rank analysis.

Discussion

The technique of hybridization–competition is an excellent tool for detecting differences in the products of gene transcription. It has been employed extensively in the studies of the mRNAs for early and late functions in bacterial and animal viruses (Hall *et al.*, 1964; Bautz *et al.*, 1966; Dove, 1966; Pène and Marmur, 1967; Oda and Joklik, 1967). The technique has also been utilized with animal cells to study, for example, the species of RNA synthesized in the differentiating amphibian embryo (Denis, 1964) in the developing embryonic mouse liver (Church and McCarthy, 1967a) and in the regeneration of mouse liver following partial hepatectomy (Church and McCarthy, 1967b). In these studies it was possible to demonstrate differences in the RNAs synthesized by animal cells during periods of major alteration of their structure or function. However, it is important to note that there are certain important limitations in animal nucleic acid homology studies. These stem from the observation that the formation of DNA–DNA duplexes does not require complete focus specificity because there are many similar, but not identical, nucleotide sequences within at least a portion of a given animal DNA (Britten and Kohne, 1966). The extent to which these related families of polynucleotides reassociate is strongly influenced by experimental conditions (Martin and Hoyer, 1966; McCarthy, 1967). Similar considerations also apply to the formation of RNA–

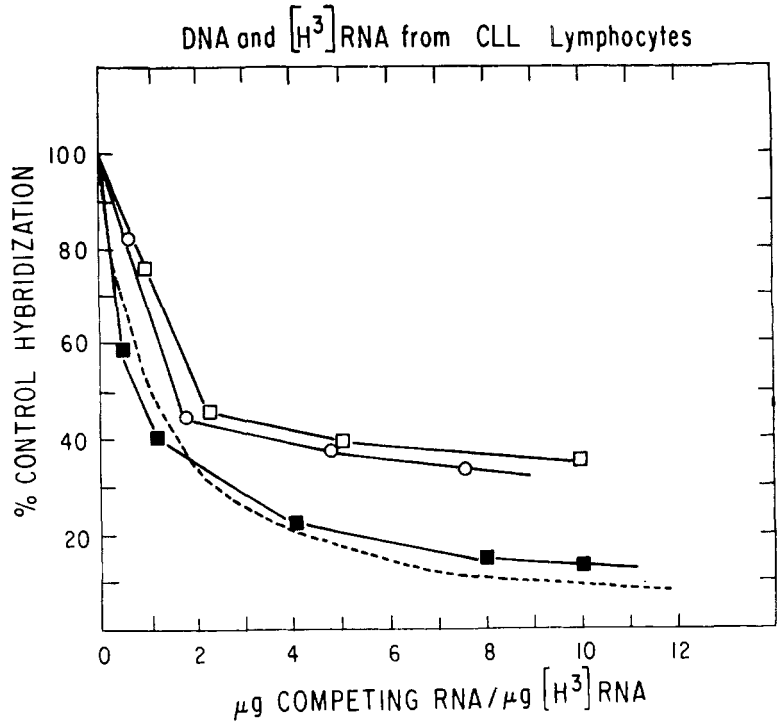

FIGURE 5: The competition of unlabeled RNA from chronic lymphocytic leukemia and normal lymphocytes in the hybridization of [^{3}H]RNA from chronic lymphocytic leukemia lymphocytes with chronic lymphocytic leukemia DNA. Unlabeled RNA from chronic lymphocytic leukemia lymphocytes is indicated by -■-■-, normal lymphocytes by —□—□—, and phytohemagglutinin stimulated lymphocytes by —○—○—. The reaction conditions are the same as listed for Figure 4.

DNA hybrids. Thus, an RNA molecule may react with a genetic focus other than that responsible for its synthesis (Denis, 1966a). This is apparent from the cross hybridization observed between RNA and DNA obtained from related species and further supported by the ability of RNA from a related species to compete in a homologous reaction (Church and McCarthy, 1967a). It has been estimated that these families of repeating sequences in mammalian DNA may comprise as much as 30% or more of the base sequences (Britten and Kohne, 1966, 1968). The remaining DNA sequences are presumably unique and RNA molecules synthesized on these sites will, under standard conditions, enter into DNA–RNA interaction only if their concentrations are sufficiently great. It has, therefore, been suggested that hybridization studies with mammalian nucleic acids are better visualized as a means of discriminating between the products of these families of cistrons rather than the titration of specific genetic loci (Church and McCarthy, 1967c). When differences can be demonstrated between populations of animal cell RNA molecules they probably represent a minimal estimate of the actual differences, since the degree of resolution is limited by the presence of the closely related polynucleotide sequences.

The present studies were performed with two types of human lymphocyte, one normal and the other neoplastic. The cells are virtually identical from the standpoints of morphology and apparent degree of maturation. It was hoped that these similarities would minimize any differences that are dependent upon the stages of cellular maturation or differentiation which are frequently troublesome in biochemical comparisons of normal and neoplastic cells.

When the rapidly labeled RNAs from these two types of lymphocyte were compared by direct hybridization no significant differences were noted. The maximum percentage of the radioactivity in the RNA that formed a ribonuclease-resistant hybrid (RNA:DNA = 0.1) was 12% in the normal lymphocyte and 10% in the chronic lymphocytic leukemia cell. As the RNA:DNA ratio approached 1.5, the percentage of the input radioactivity which formed a hybrid decreased sharply. This was interpreted as representing at least a partial saturation of the DNA and indeed the experimental values obtained in hybridization–competition experiments corresponded closely to a saturation-dependent, theoretical competition curve. The saturation curve did not, however, attain a plateau at RNA:DNA ratios as great as 6. In animal cells an RNA:DNA ratio of 50 or greater has been required for saturation of the homologous DNA (Whitely *et al.*, 1966; Glišin *et al.*, 1966; Denis, 1966b). An exception to this is the duck erythroblast system in which apparent saturation of the DNA occurred at an RNA:DNA ratio of 6.0 to 8.0 (Scherrer *et al.*, 1966b). The duck erythroblast, like the small lymphocyte, has a high ratio of nucleus to cytoplasm. The failure to attain a plateau in our studies may represent a less specific interaction of the nucleic acids at higher RNA concentrations or simply a reflection of the concentration of the reacting species of RNA and the period of incubation. It is known that when mammalin DNA is allowed to react with itself under conditions similar to the ones

used in our studies (two-times standard saline citrate, 67° for 16 hr) the sequences which reassociate are the polynucleotide families that are present in high concentration (Britten and Kohne, 1966). If the same reasoning holds for DNA:RNA interactions, then only those species of RNA that are present in abundance would have formed hybrids in our studies.

The specificity of the hybridization and hybridization–competition reactions was tested by utilizing nucleic acids from several types of animal cells. The species specificity of the direct RNA–DNA hybridization reaction was nearly identical with that reported for DNA–DNA homology studies (Hoyer *et al.*, 1965). The competition reaction was also quite specific as shown by its ability to discriminate between animal cell RNAs in proportion to the degree of base sequence relatedness of the DNAs from which they were transcribed. It should be noted, however, that this degree of interspecies specificity was true only at the low RNA concentrations used in these experiments. When the initial ratio of [^{3}H]RNA to DNA was 4 or greater, we observed that the addition of increasing amounts of unlabeled heterologous animal cell RNAs did interrupt the hybrid formation in excess of the probable sequence similarity of the competing polynucleotides. This type of nonspecific reduction in hybrid formation has been previously reported with animal cell RNA (Merits *et al.*, 1966; Birnboim *et al.*, 1967) and indicates that the partial interruption of DNA–RNA hybrid formation may, under some conditions, greatly overestimate the degree of similarity of the competing RNAs (McCarthy, 1967).

The hybridization–competition experiments demonstrated that the unlabeled normal lymphocyte RNA was an incomplete competitor in the reaction between radioactive chronic lymphocytic leukemia RNA and lymphocyte DNA. This indicates the chronic lymphocytic leukemia cell is synthesizing species of RNA that are either absent in the normal lymphocyte or present only in very low concentration. On the other hand, the unlabeled chronic lymphocytic leukemia RNA competed completely in the reaction between radioactive normal lymphocyte RNA and lymphocyte DNA. Thus, we were unable to detect any species of RNA in the normal small lymphocyte that were not also present in the chronic lymphocytic leukemia cell. However, since perfect matching of nucleotide sequences is probably not required for hybrid formation with mammalian nucleic acids (Denis, 1966a,b; McCarthy, 1967), this finding does not prove that the competing sequences in the chronic lymphocytic leukemia RNA are identical with the labeled normal lymphocyte RNA. The role of the additional species of complementary RNA synthesized by chronic lymphocytic leukemia lymphocytes and the relationship of this RNA to the functional deficiencies noted in this disease are not known. The complementary RNA examined in this study shares with mRNA the properties of rapid synthesis and efficient hybridization with DNA. On the other hand, this class of RNA in the lymphocyte, although synthesized in the nucleus, has not been shown to be a precursor of cytoplasmic mRNA (Henry *et al.*, 1967; Torelli *et al.*, 1968). The efficiency with which this class of nuclear RNA forms hybrids and the description of RNA species with some of the same properties in several animal cell types suggest that it may have contributed to the RNA which forms hybrids in animal cell nucleic acid homology studies (Birnboim *et al.*, 1967). Thus, some of the new information transcribed into the RNA which is associated with major alterations in cellular structure and function may well be carried in this "giant" nuclear RNA. The importance of this RNA in the cellular economy of the normal and malignant lymphocyte is further underlined by the fact that it represents a significant portion of all the RNA synthetic activity of these cells. Nevertheless, until more specific information is available regarding function, we can only speculate that the transcription of new complementary RNA molecules in the chronic lymphocytic leukemia lymphocyte may underlie its functional deficiencies and possibly the disease state itself.

Acknowledgments

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Chemical Studies on Amino Acid Acceptor Ribonucleic Acids. VIII. Degradation of Purified Alanine *Escherichia coli* B Transfer Ribonucleic Acid by Pancreatic Ribonuclease*

Claudio G. Alvino, Lynne Remington, and Vernon M. Ingram

ABSTRACT: The mono- and oligonucleotides in total pancreatic ribonuclease digests of purified *Escherichia coli* B alanine transfer ribonucleic acid peak 1 were separated on DEAE-cellulose columns in 7 M urea. Certain oligonucleotides were further separated by paper chromatography or by DEAE-Sephadex A-25 column chromatography. Their sequences and the properties of a new minor base were studied by alkaline hydrolysis and by digestion with ribonuclease T_1-takadiastase, snake venom diesterase, or micrococcal nuclease.

The nucleotide sequences of several yeast tRNAs are now known through the studies of Holley and his coworkers (1965) on alanine tRNA, of Madison *et al.* (1966) on tyrosine tRNA, of Zachau *et al.* (1966) on the sequences of two serine tRNAs, and of RajBhandary *et al.* (1966, 1967) on phenylalanine tRNA. It would be of interest to compare these nucleotide sequences from one organism, yeast, with those of a tRNA with the same amino acid acceptor activity from a different species. Alanine $tRNA_1$[1] from *Escherichia coli* B was chosen because Goldstein *et al.* (1964) had shown that it could be purified easily by countercurrent distribution and because so much is already known about the structure–function relationships in the corresponding yeast tRNA. The present report describes the study of some of the oligonucleotides produced by complete digestion with pancreatic RNase from *E. coli* B alanine $tRNA_1$.

Materials and Methods

Purification of E. coli B Alanine Acceptor RNA. Mixed amino acid acceptor RNA was purchased from General Biochemicals (Chagrin Falls, Ohio). Countercurrent distribution (Figure 1a,b) was carried out as described by Goldstein *et al.* (1964) in a 200-tube automatic apparatus (E. C. Apparatus Co., Swarthmore, Pa.). The countercurrent distribution runs were performed at a controlled temperature of 24°. Batches of 0.7–1.0 g of mixed acceptor RNA were distributed at one time. The RNA was recovered from the fractions, five tubes together, and the amino acid acceptor activity was determined by the method of Ingram and Pierce (1962). The countercurrent distribution method gives a major (I) and minor peak (II) (Ala-$tRNA_1$ and Ala-$tRNA_2$, respectively). The structural work reported in this paper has all been on the major peak (Ala-$tRNA_1$), corresponding to fractions 14–18 in Figure 1b. The purity of the RNA used was estimated to be about 80% from its acceptor activity.

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[1] Abbreviations used are: $tRNA_1$, transfer ribonucleic acid peak 1; p indicates a phosphate group; OH is used to indicate a free 2 (3′)-hydroxyl end group; P-RNase, pancreatic ribonuclease; T_1 or T_1-RNase, ribonuclease T_1-takadiastase.