Molecular Evidence for the Association of RNA Tumor Viruses with Human Mesenchymal Malignancies

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I. The Strategy of the Search for RNA Tumor Viruses in Human Malignancies

Our overall purpose has been and remains to explore the possible involvement of RNA tumor viruses as etiologic or cofactor agents in human neoplasias and to exploit any leads that emerge that could be of any conceivable use in the prevention, diagnosis, or therapy of human cancer.

The task of identifying the existence and the causative role of the animal RNA tumor viruses was inadvertently made easier by breeding high cancer incidence animal strains. In the process, a homogeneous genetic background was created that was permissive for viral replication. As a consequence, virus particles reached levels that made their detection inevitable. Those who are concerned with human neoplasias are for the most part faced with the same difficulties encountered by the early animal oncologists prior to the availability of inbred strains. It follows that a search for putative human viral agents requires more sensitive devices than those which sufficed to establish their presence in the genetically homogeneous animal systems. In the quest for such tools, we quite naturally turned to molecular hybridization and the other methodologies developed by molecular biologists in the past several decades.

Our investigations evolved through a number of stages that are conveniently identified by the questions we posed for experimental resolution:

1) Do human neoplasias contain RNA molecules possessing detectable homology to the RNA of tumor viruses known to cause similar cancers in other mammals?

2) If a positive outcome is obtained, do the RNA molecules identified in tumors possess the size and physical association with reverse transcriptase that characterize the RNA of the animal oncornaviruses?

3) If such RNA exists in human tumors, is it encapsulated in a particle possessing the density and size of the RNA tumor viruses?

4) Is the RNA of human tumor particles homologous to the RNA of the viruses causing the corresponding disease in animals?

5) The "virogene-oncogene" concept proposes that all animals prone to cancer carry in their germ line a complete copy of the information required to convert a cell from normal to malignant for the production of tumor virus particles. Is this concept valid for randomly bred populations and, in particular, for the human disease?

II. The Animal Models as a Point of Departure

When we began our investigations, there were relatively few animal oncornaviruses available in amounts adequate for the sort of biochemical experiments required. Table I lists these and records certain relevant features that served as a

Table I: Comparsion of Some Representative Oncornaviruses

Virus	Indigenous Host	Homology*					Disease
		AMV	RSV	MuLV	MSV	MMTV	
AMV	Chicken	+	+	−	−	−	Leukemia
RSV (RAV)	Chicken	+	+	−	−	−	Sarcoma
MuLV	Mouse	−	−	+	+	−	Leukemia, lymphoma
MSV (MuLV)	Mouse	−	−	+	+	−	Sarcoma
MMTV	Mouse	−	−	−	−	+	Breast cancer

* The results of molecular hybridizations between [^{3}H]DNA complementary to the various RNAs and the indicated RNAs. The plus sign indicates the hybridizations were positive and the negative sign indicates none could be detected.

guide in these expermients. There are two avian viruses, myeloblastosis virus (AMV) and the Rous sarcoma virus (RSV), that cause mesenchymal malignancies in chickens. In addition, we have the murine leukemia virus (MuLV) and the murine sarcoma virus (MuSV) that induce similar diseases in mice. Finally, we have the murine mammary tumor virus (MMTV), which is the unique etiologic agent for mammary tumors.

When these viruses are examined for sequence homologies amongst their nucleic acids, a rather informative pattern emerges. It will be noted that the two chicken agents have sequences in common, but do not show detectable homology with any of the murine agents. Turning to the murine viruses, we find that the nucleic acids of the leukemia, lymphoma, and sarcoma agents are homologous to one another, but not to either of the two avian agents or to the mammary tumor virus. Finally, the mouse mammary tumor virus has a singular sequence homologous only to itself.

It is important to understand that a plus sign does not indicate identity, but simply sufficient similarity to be detectable by the relaxed hybridization conditions used in these initial studies. Similarly, a negative sign does not imply the complete absence of sequence homology, but rather that none was observable by the procedures used.

If analogous, or similar, virus particles are associated with the corresponding human diseases, certain predictions may be hazarded on the basis of the specificity patterns exhibited in Table I, and they may be listed as follows:

(a) In view of the lack of homology between the avian and murine agents, it is unlikely, from simple evolutionary considerations, that human agents, should they

exist, would show more homology to the avian group than to the murine oncornaviruses.

(b) It follows that the murine tumor viruses would represent the more hopeful source of the molecular probes required to search for similar information in the analogous human cancers.

(c) If particles are found to be associated with human mesenchymal tumors (in leukemias, sarcomas, and lymphomas), their RNAs might show homology to one another and possibly to that of the murine leukemia virus.

(d) If RNA particles are identified in human breast cancer, they should not exhibit homology to the RNA of virus-like particles associated with the human mesenchymal neoplasias or to MuLV RNA, but might exhibit some homology to the RNA of the murine mammary tumor virus.

On the basis of both availability and the specificity considerations outlined above, it is clear why the murine agents were initially chosen for producing the necessary molecular probes to look for corresponding information in the human disease. Furthermore, the desire to monitor the biological consistency of our findings dictated that we examine in parallel the human neoplasias listed. Such a parallel examination would permit us to determine whether our findings in humans mirrored biologically what was known from the animal experimental models. For this purpose, we focused our efforts on the mesenchymal neoplasias and on breast cancer.

III. Molecular Hybridization with Radioactive DNA Probes

The DNA-RNA hybridization procedure we used to answer the question whether human tumors contain viral-related RNAs was one that we had designed (1) some fifteen years ago to answer questions of almost precisely this nature in the case of virus-infected bacteria. The method depends on the ability of any piece of single-stranded DNA to find its complementary RNA and form, under the proper conditions, a double-stranded hybrid structure. The reaction is highly specific and has proved to be of considerable value in molecular biology over the past decade.

The required radioactive DNA was synthesized by supplying detergent-disrupted virus preparations with magnesium and the deoxyriboside triphosphates, with one of them being labeled with tritium. When the synthesis is completed, the protein and the RNA present are eliminated, and the residual radioactive DNA is purified to completion. Each [^{3}H]DNA preparation is then rigorously examined for specific hybridizability to its appropriate template and for its inability to complex with irrelevant RNAs. After satisfying the specificity criteria, the purified viral-specific tritiated DNA is mixed with cytoplasmic RNA prepared from a variety of tumors and annealed under the conditions described in Figure 1. The hybridizations are always carried out with a vast excess of tumor RNA. Since the viral-specific tritiated DNA is small compared with the RNA, any complexes formed between them will behave physically more like RNA than DNA. Such complexes are readily detected by isopynic separation in equilibrium density gradients of cesium sulfate. At the end of the centrifugation, the distribution of the tritiated DNA is examined across the gradient. Any uncomplexed DNA will remain at a density correspond-

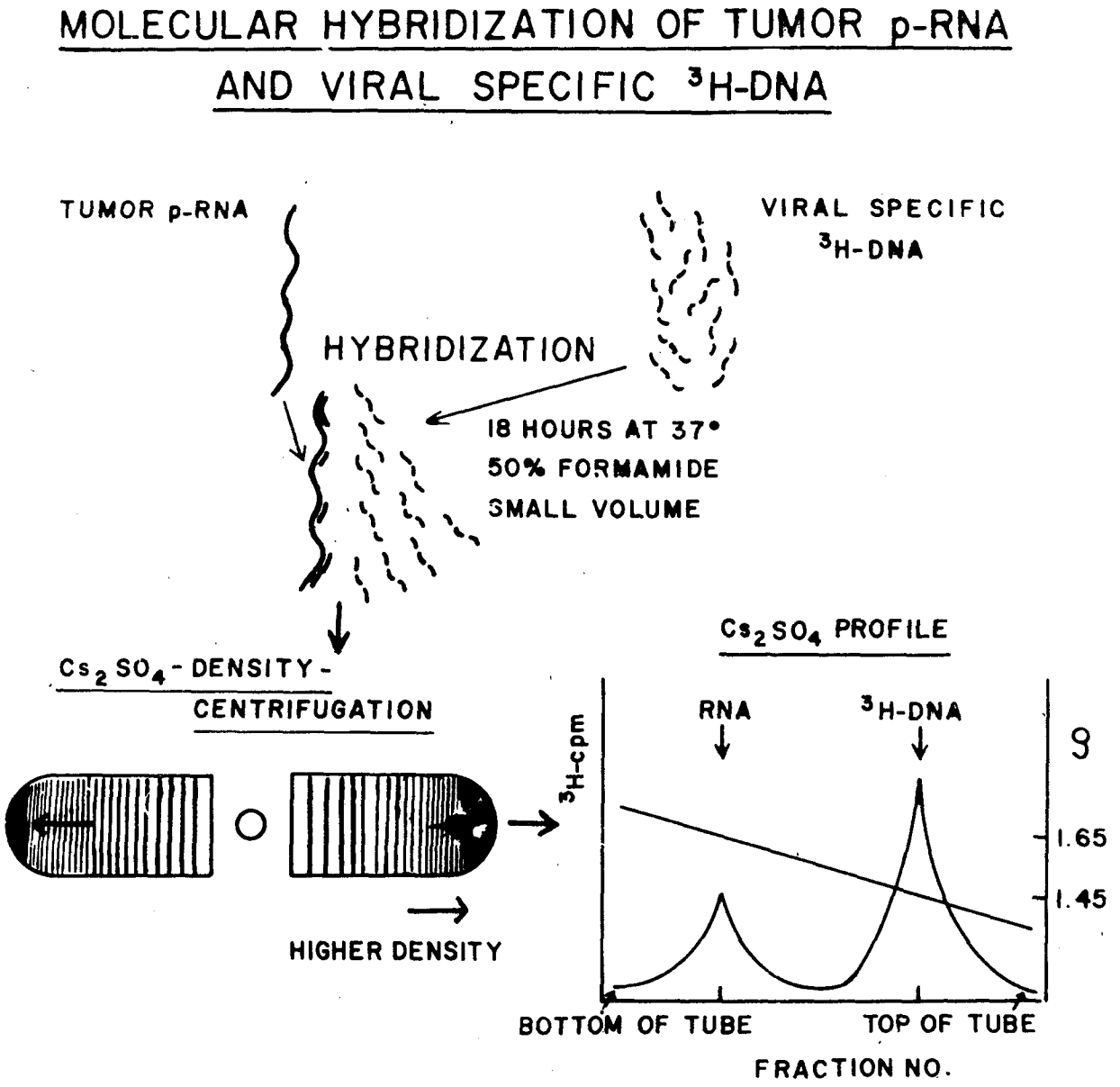

Fig. 1: Molecular hybridization and detection with viral-specific [^{3}H]DNA and tumor RNA (see text for further details).

ing to about 1.45. The DNA molecules that have annealed either partially or completely to RNA will band at or near the density of RNA (ϱ = 1.65). The movement of the tritiated DNA from the DNA density region to the RNA density region is then the signal that the probe used has found complementary sequences in the tumor RNA with which it is being challenged.

The human neoplasias examined included adenocarcinoma of the breast (2–4), the leukemias (5), the sarcomas (6), and the lymphomas (7). The leukemias encompassed both acute and chronic varieties of the lymphatic and myelogenous types. The human sarcomas studied included fibro, osteogenic, and liposarcomas. The lymphoma series contained Hodgkin's disease, Burkitt's tumors, lymphosarcomas, and reticulum cell sarcomas. Control adult and fetal tissues were always examined in parallel, and these were invariably negative. In the case of breast

tissue, the two benign diseases, fibroadenoma and fibrocystic disease, were also included and were found to be negative.

Table II summarizes in diagramatic form the outcome of the survey of human neoplasias with the animal virus probes. The pluses signify that the corresponding

Table II: Homologies among Human Neoplastic RNAs and Animal Tumor Viral RNAs

	Human neoplastic RNAs			
Viral RNAs	Breast Cancer	Leukemia	Sarcoma	Lymphoma
MMTV	+	−	−	−
RLV	−	+	+	+
AMV	−	−	−	−

The results of molecular hybridization between [^{3}H]DNA complementary to the various viral RNAs and pRNA preparations from the indicated neoplastic tissues. The plus sign indicates that hybridizations were positive and the negative sign, that none could be detected (5).

tritiated DNA complexed with the indicated tumor RNAs and the minuses, that no such complexes were detected. The positives in these earlier studies ranged from 67 % for breast cancer to 92 % for the leukemias. What is most noteworthy of the pattern exhibited in Table II is its concordance with predictions deducible from the murine system. Thus, human breast cancer contains RNA homologous only to that of the murine mammary tumor virus. The human leukemias, sarcomas, and lymphomas all contain RNA sharing sufficient homology to that of the Rauscher murine leukemia virus (RLV) to make a stable duplex. These mesenchymal neoplasias contain no RNA homologous to the MMTV RNA. Finally none of the human tumors contains RNA detectably related to that of the avian myeloblastosis virus. The homology of leukemic RNA to that of RLV and the homology of RNA from human breast cancer to that of MMTV have been confirmed (8, 9).

In summary, the specificity pattern of the unique RNA found in the human neoplasias is in complete agreement with what has been described for the corresponding virus-induced malignancies in the mouse.

IV. The Simultaneous Detection Test

The existence of RNA in human tumors having sequence homology to virus particles causing homologous diseases in mice does not of course establish a viral etiology for these diseases in man. The next step requires the performance of experiments designed to answer the second and third questions raised in the introductory paragraphs, i. e., those relating to the size of the RNA being detected and whether it is associated with the reverse transcriptase in a particle possessing other features of complete or incomplete oncornaviruses.

What we sought was a method of detecting the presence of particles similar to the RNA tumor viruses that would be simple, sensitive, and sufficiently dis-

criminating so that a positive outcome could be taken as an acceptable signal of the presence of a viral-like agent. To achieve this goal, we devised a test that depended on the simultaneous detection of two diagnostic features of the animal RNA tumor viruses.

The oncornaviruses exhibit two identifying characteristics. They contain a large (1×10^7 daltons in molecular weight and composed of subunits each of which is 3×10^6 daltons) single-stranded RNA molecule having a sedimentation coefficient of 70S, or 35S if the 70S molecule has broken down into its subunits. They also have reverse transcriptase (10, 11), an enzyme that can use the viral RNA as a template to make a complementary DNA copy.

The possibility of a concomitant test for both the enzyme and its template was suggested by our prior experience with RNA transcriptase in which we found (12) that the growing RNA chain could be detected as a complex with its DNA template on removal of the protein from the reaction mixture. Similar observations were made in examinations of the early reaction intermediate (13, 14) of the reverse transcriptase reaction.

It was on this basis that Schlom and Spiegelman (15) developed the simultaneous detection test that was used to demonstrate (16) the presence in human milk of particles containing 70S RNA and the reverse transcriptase. The test was modified (17) to be applicable to tumor tissue using the mouse mammary tumor as the experimental model.

Figure 2 diagrams the procedure used. Tumor cells are first broken by the use of the Dounce homogenizer and nuclei, mitochondria, and large cell membrane fragments removed by low speed centrifugation. The supernatant is subjected to trypsin digestion to inactivate any nucleolytic enzymes and the trypsin is neutralized by trypsin inhibitor. The supernatant is then centrifuged at 150,000 X g to yield a cytoplasmic pellet containing virus particles, if present. The resulting pellet is then banded isopycnicly in a sucrose density gradient and the fraction between 1.16 and 1.19 g/ml is collected by centrifugation. The recovered pellet is then treated with a nonionic detergent (NP40) to disrupt possible viral particles, and the disrupted preparation is used in a brief endogenous reverse transcriptase reaction. The product of the reaction, with its RNA template, is freed of protein and analyzed in a glycerol velocity gradient to determine the sedimentation coefficient of the tritiated DNA. In addition, the product is subjected to equilibrium centrifugation in a Cs_2SO_4 gradient to determine its density.

The presence of particles encapsulating 70S RNA and a reverse transcriptase will be indicated by the appearance of a peak of newly synthesized DNA traveling at a speed corresponding to either a 70S RNA or a 35S RNA molecule. That the apparently large size of the [^{3}H]DNA is due to its being complexed to an RNA molecule can be readily verified by subjecting the purified nucleic acid to ribonuclease prior to velocity examination. The disappearance of the 70S and 35S [^{3}H]DNA peaks following RNase treatment proves that the [^{3}H]DNA was complexed to large RNA molecules. Similarly, if the reaction is positive, newly synthesized DNA should appear in the RNA and/or hybrid regions of the Cs_2SO_4 gradient, and these peaks should again be eliminated by prior treatment with ribonuclease.

The simultaneous detection test was first applied to human breast cancer (18) in

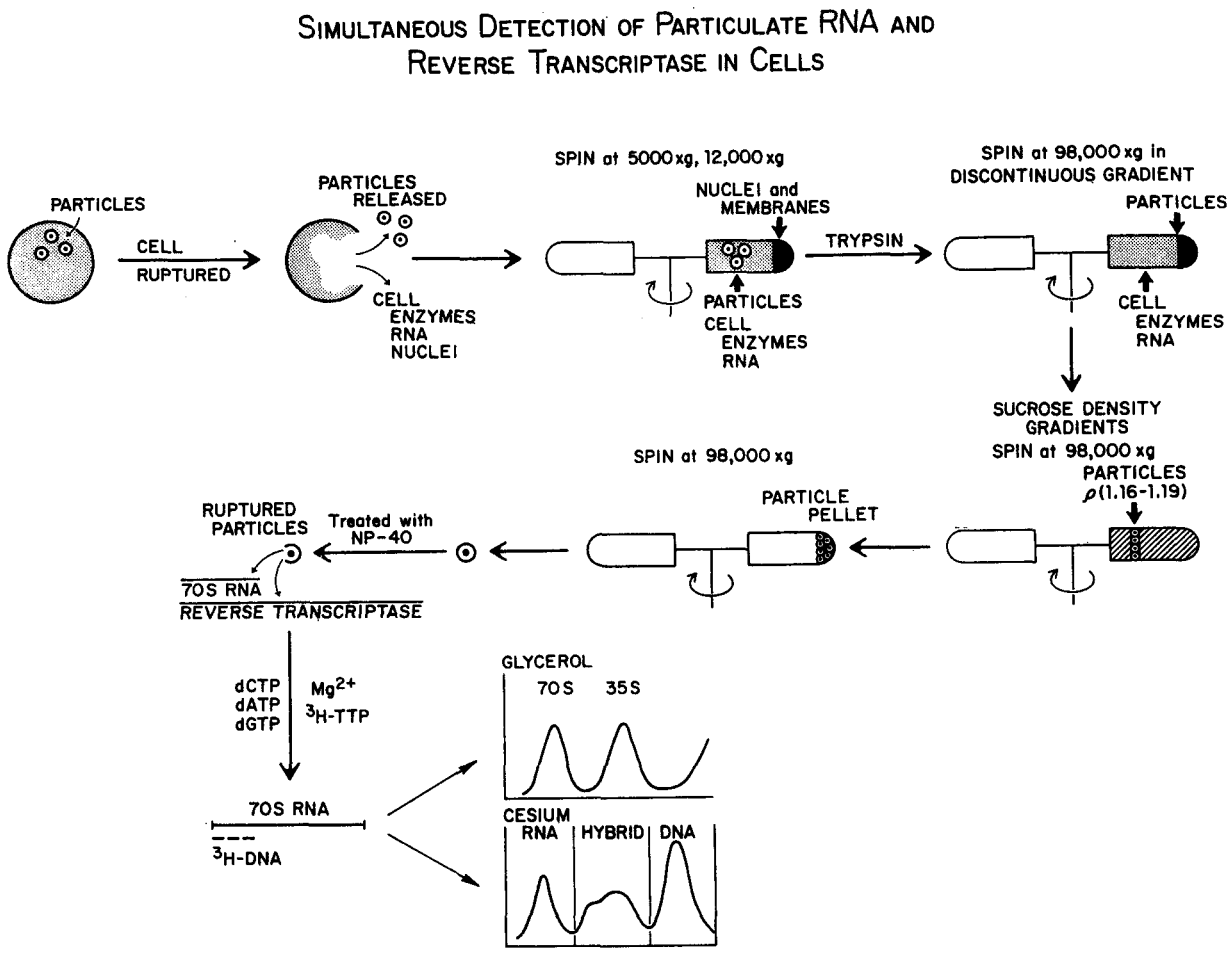

Fig. 2: Simultaneous detection test for 70S RNA and reverse transcriptase in neoplastic tissue (see text for further details).

a series including 38 adenocarcinomas and ten non-malignant controls. It was found that 79 % of the malignant samples were positive for the simultaneous detection reaction and all of the control samples from normal and benign tissue were negative. It was further shown that the particles possessing the reverse transcriptase activity and its 70S RNA template localize at a density between 1.16 and 1.19 g/ml, the density characteristic of the oncogenic viruses.

The data obtained therefore indicate that one can, with a high probability, find in human breast cancers particulate elements of the right density that encapsulate RNA-instructed DNA polymerase and a 70S RNA.

V. Application of the Simultaneous Detection Test to Mesenchymal Tumors

In our initial study of the leukemias (19), peripheral leukocytes were prepared from the buffy coats of both leukemic and nonleukemic control patients. Cells were disrupted and fractionated as described in Figure 2. Representative experiments examining the effects of ribonuclease treatment of the product and omission of one of the deoxytriphosphates during the reaction are shown in Figure 3. We see the telltale 70S peaks of DNA synthesized by the pellet fractions from the leukocytes of patients with acute lymphoblastic and acute myelogenous leukemias. The elimination of the complex by prior treatment with ribonuclease (Figure 3A) shows

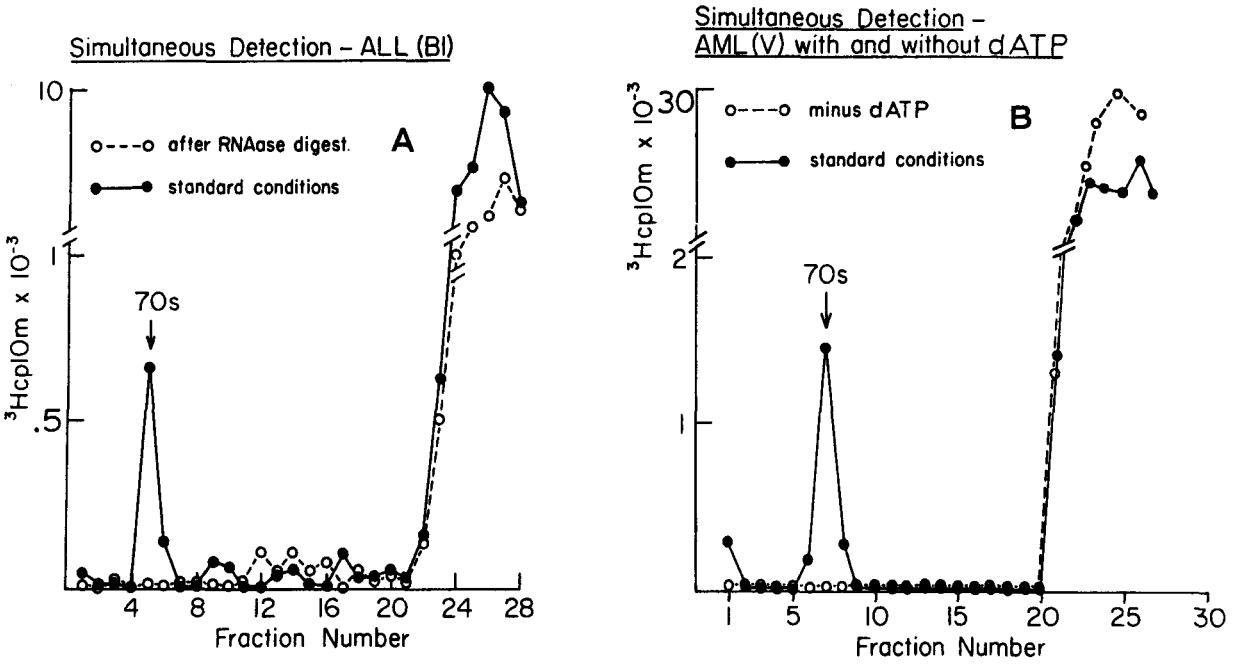

Fig. 3: Detection of 70S RNA [^{3}H]DNA complex in human leukemic cells. 1 gm of leukemic WBC was washed in 5 ml of 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4, resuspended in 4 ml of 5 per cent sucrose, 0.005 M EDTA, 0.01 M Tris-HCl, pH 8.3, and ruptured with three strokes of a Dounce homogenizer. The nuclei were removed by low speed centrifugation (2,000 g, 5 min, 2°). The supernatant was brought to a final concentration of 1 mg/ml trypsin (Worthington) and incubated at 37° for 30 min. A tenfold excess of lima bean trypsin inhibitor (Worthington) was added (final concentration 3 mg/ml) and the solution again centrifuged at 2,000 g for 5 min at 2°. The supernatant was then centrifuged at 45,000 rpm for 60 min at 2°. The resulting cytoplasmic pellet was resuspended in 0.5 ml of 0.01 M Tris-HCl, pH 8.3, brought to 0.1 per cent Nonidet P-40 (Shell Chemical Co.) and incubated at 0° für 15 min. DNA was synthesized in a typical reverse transcriptase reaction mixture (final vol 1 ml) containing: 50 µmol of Tris-HCl, pH 8.3, 20 µmol NaCl, 6 µmol $MgCl_2$, 100 µmol each of dATP, dGTP, dCTP, and 50 µmol-[^{3}H]dTTP (Schwarz Biochemical, 800 cpm per pmol). 50 µg/ml actinomycin D were added to inhibit DNA-instructed DNA synthesis. After incubation at 37° for 15 min, the reaction was adjusted to 0.2 M NaCl and 1 per cent SDS, and deproteinized by phenol-cresol extraction. The aqueous phase was layered on a 10 to 30 per cent gradient of glycerol in TNE buffer (0.01 M Tris-HCl, pH 8.3, 0.1 M NaCl, 0.003 M EDTA) and centrifuged in a SW-41 rotor Spinco at 40,000 rpm for 180 min at 2°. Fractions were collected from below and assayed for TCA-precipitable radioactivity. In this, as in all sedimentation analysis, 70S RNA of the avian myeloblastosis virus was used as a marker.

(A) One aliquot of product was run on the gradient as a control and the other was pretreated with 20 µg of RNase 1 (Worthington) for 15 min at 37° prior to sedimentation analysis. (B) Reactions with and without dATP (19).

that the tritiated DNA is indeed complexed to a 70S RNA molecule. Further, the omission of dATP (Figure 3B) leads to a failure to form the 70S complex, a result expected if the reaction is in fact leading to the synthesis of a proper heteropolymer. In similar experiments, it was shown that omission of either dCTP or dGTP also resulted in the absence of the 70S RNA-[^{3}H]DNA complex, all of which argues against nontemplated end addition reactions.

In some cases leukemic cells were obtained in amounts adequate to permit a more complete characterization of the product. Hybridization of the human product to the appropriate viral RNAs provides the most revealing information since

it tests sequence relatedness to known oncogenic agents. We summarize in Table III the results of examining the peripheral leukocytes of 23 patients, all in the active phases of their disease, including both acute and chronic leukemias. Of the 23 leukemic patients examined, 22 showed clear evidence that their peripheral leukocytes contained particles mediating a reaction leading to the appearance of endogenously synthesized DNA in the 70S region of a glycerol gradient. Nine of these were tested for ribonuclease sensitivity and in all cases the complexes were destroyed. In nine others, the DNA was recovered from the complex and annealed to RLV RNA and

Table III: Simultaneous Detection of 70S RNA and Reverse Transcriptase in Leukemic Cells (19)

Leukemias	Simultaneous detection cpm	RNase sensitivity	Hybridization to RLV-RNA	Hybridization to AMV-RNA or MMTV-RNA
Acute Lymphatic				
1	400	+	NT	NT
2	95	+	NT	NT
Acute Lymphatic/ Lymphosarcoma				
3	805	+	+	–
4	200	NT	+	–
5	185	+	NT	NT
6	105	+	NT	NT
Acute Myelogenous				
7	170	+	NT	NT
8	985	+	+	–
9	305	+	NT	NT
10	1295	+	+	–
11	1010	NT	+	–
12	115	NT	+	–
13	415	NT	+	–
14	400	NT	+	–
15	605	+	NT	NT
16	215	+	+	–
17	285	+	NT	NT
18	0	NT	NT	NT
19	1400	NT	+	–
Chronic Lymphatic				
20	200	+	NT	NT
Chronic Myelogenous				
21	405	NT	+	–
22	390	NT	+	–
23	600	NT	+	–

NT = Not Tested

to either MMTV RNA or AMV RNA. In all nine, hybridizations occurred with RLV RNA and not to either of the unrelated MMTV RNA or AMV RNA. In four patients, enough DNA complex was formed to permit a complete characterization of the product. In all four, the DNA complexes were destroyed by ribonuclease and the purified DNA hybridized uniquely to RLV RNA.

In addition to this initial group, we subsequently examined 85 leukemic patients and 38 patients with lymphomas (20, 21), including Hodgkin's disease, African Burkitt's lymphoma, lymphosarcoma, and reticulum cell sarcoma. The results of the simultaneous detection tests on these and corresponding control tissues are summarized in Table IV. It is noteworthy that positive outcomes were observed

Table IV: Simultaneous Detection on Mesenchymal Tissues

The simultaneous detection test was carried out as described in Figure 2. Peripheral white blood cells (WBC) were used in the leukemias, acute myelogenous (AML), chronic myelogenous (CML), acute lymphocytic (ALL) and chronic lymphocytic (CLL).

Tissue	Positives No. (Avg. cpm)	Negatives No. (Avg. cpm)	% Positives
Malignant			
AML	58 (985)	1 (10)	98
CML	18 (520)	0	100
ALL	25 (790)	0	100
CLL	4 (350)	0	100
Hodgkin's Disease (spleens)	22 (379)	6 (14)	79
Burkitt's Lymphoma	9 (369)	2 (14)	82
Other Lymphomas	7 (347)	1 (24)	88
Normal or Benign			
WBC	0	48 (12)	0
Spleens	0	34 (15)	0

in more than 99 % of the leukemic patients, whether they were acute or chronic, lymphocytic, or myelogenous. Thus despite their disparate clinical pictures and differing cellular pathologies, these various types of leukemias are associated with virus-like particles containing RNA with similar, though probably not identical, viral-related information. In the leukemias, we always dealt with peripheral white blood cells from patients with active disease, and this may account for almost total lack of negative responses. In the lymphomas, we were confined to examining spleens and lymphomatous tumor material where control over the content of malignant cells is more difficult to exercise. However, even here the proportion of positives is high, ranging from 79 % to 88 %. In contrast with these results are those obtained with the control series of 48 white blood cell samples and 34 spleens. The non-neoplastic samples included some with elevated white blood cell counts (in the range of 25,000/mm^3) due to a variety of disorders. None of the 82 samples

from cancer-free patients exhibited any evidence of positive reactions. The difference in average cpm of positives and negatives is such that a diagnostic decision is unambiguous.

VI. Implications of Simultaneous Detection Tests on Human Breast Cancer and the Mesenchymal Tumors

The experiments we have just summarized on human breast cancer and the leukemias were designed to probe further the etiological significance of our exploratory investigations (2, 5), which identified in these neoplasias RNA homologous to those of the corresponding murine oncornaviruses. The data obtained with the simultaneous detection test established that at least a portion of the tumor-specific virus-related RNA we were detecting was a 70S RNA template physically associaed with a reverse transcriptase in a particle possessing a density between 1.16 and 1.19 g/ml, three of the diagnostic features of the animal RNA tumor viruses. Further, the DNA synthesized in the particles from both classes of neoplasias hybridized uniquely to the RNA of the corresponding oncornavirus. Note that this last result is complementary to and completes the logic of our experimental approach. We started out by using animal tumor viruses to generate [^{3}H]DNA probes that were used to find related RNA in human neoplastic tissue. We concluded by using analogous human particles to generate [^{3}H]DNA probes, which were then used to determine sequence relatedness to the RNA of the relevant oncornaviruses. None of the human probes hybridized to the avian viral RNA. The probe generated by the particles from human breast cancer was homologous only to the RNA of mouse mammary tumor virus, whereas the human leukemic probe was related in sequence only to RLV RNA, the murine leukemic agent. The biologically logical consistency of these results adds further weight to their probable relevance to the human disease.

VII. On the Problem of Germ-line Transmission of Viral Information

We now come to grips with the fifth question raised in the introductory paragraphs, the virogene-oncogene concept (22), which derives from animal experiments and argues that all animals prone to cancer contain in their germ line at least one complete copy of the information necessary and sufficient to convert a cell from normal to malignant and produce the corresponding tumor virus. This hypothesis presumes that the malignant segment normally remains silent and that its activation by intrinsic or extrinsic factors leads to the appearance of virus and the onset of cancer.

There are various ways of testing the validity of the virogene-oncogene hypothesis, but the pathways differ in the technical complexities entailed. One approach commonly used attempts to answer the question: Does every normal cell contain at least one complete copy of the required viral-related malignant information? The methodologies used included the techniques of genetics, chemical viral induction, and molecular hybridizations. However, for a variety of reasons, none of these gave, or could give, globally conclusive answers. Genetic experiments do not readily distinguish between susceptibility genes and actual viral information. Further, even if genetic data succeeded in identifying some structural viral genes, it

would still be necessary to establish that *all* the viral genes are represented in the genome. Attempts to settle the question by demonstrating that *every* cell of an animal can be chemically induced to produce viruses have thus far, for obvious reasons, not been tried. The best that has been achieved along these lines is to show that *cloned* cells do respond positively. However, the proportion of clonable cells is small and *clonability may well be a signal for prior infection with a tumor virus.*

Finally, the quantitative limitations of molecular hybridization make it almost impossible to provide definitive proof that each cell contains one complete viral copy in its DNA. Although it is not very difficult to show that 90 % of the information is present, it is ~~the~~ last 10 % that constitutes the insurmountable barrier and 10 % of 3×10^6 daltons amounts to a far from trivial 3×10^5 daltons, the equivalent of about one gene.

A useful way to obviate these technical difficulties is to invert the problem. Instead of asking whether one complete copy exists in normal cells, the question can be phrased in the following terms: Does the DNA of a malignant cell contain viral-related sequences that are *not* found in the DNA of its normal counterpart? Phrasing the issue in this manner leads to the design of experiments that avoid the uncertainties generated by the demonstrated fact that many indigenous RNA tumor viruses share, completely or partially, *some* sequences with the normal DNA of their natural hosts (23). The crucial point is of course whether *all* of the viral sequences are to be found in normal DNA. The approach we adopted requires removal of those viral sequences that are contained in non-neoplastic DNA by exhaustive hybridization of the viral probe to normal DNA in vast excess. Any unhybridized residue can then be used to determine whether malignant DNA contains viral-related sequences not detectable in normal tissue.

We first investigated this question in the case of the human leukemias (24) and the strategy, as diagrammed in Fig. 4 (a and b), may be outlined in the following steps:

a) Isolate from leukemic cells the fraction enriched for the particles encapsulating the 70S RNA and RNA-directed DNA polymerase;

b) Use this fraction to generate [^{3}H]DNA endogenously synthesized in the presence of high concentrations of actinomycin D to inhibit host and viral DNA-directed DNA synthesis;

c) Purify the [^{3}H]DNA by hydroxyapatite and Sephadex chromatography with care being exercised to remove by self-annealing and column chromatography all self-complementary material in the tritiated probe;

d) Use the resultant [^{3}H]DNA to detect complementary sequences in normal and leukemic leukocyte DNA;

e) If viral-related sequences are detected in *both*, remove those found in normal leukocytes by exhaustive hybridization to normal DNA; and

f) Test the residue for specific hybridizability to leukemic DNA.

In carrying out the recycling and test hybridizations, it is imperative that conditions be chosen to account for the possibility that the leukemia-specific sequences are present in only one copy per genome, a possibility which is in fact realized (24). To this purpose, the concentration in moles per liter (C_0) of DNA and the time (t in seconds) of annealing is adjusted to C_0t values of 10,000, which are adequate to locate unique sequences.

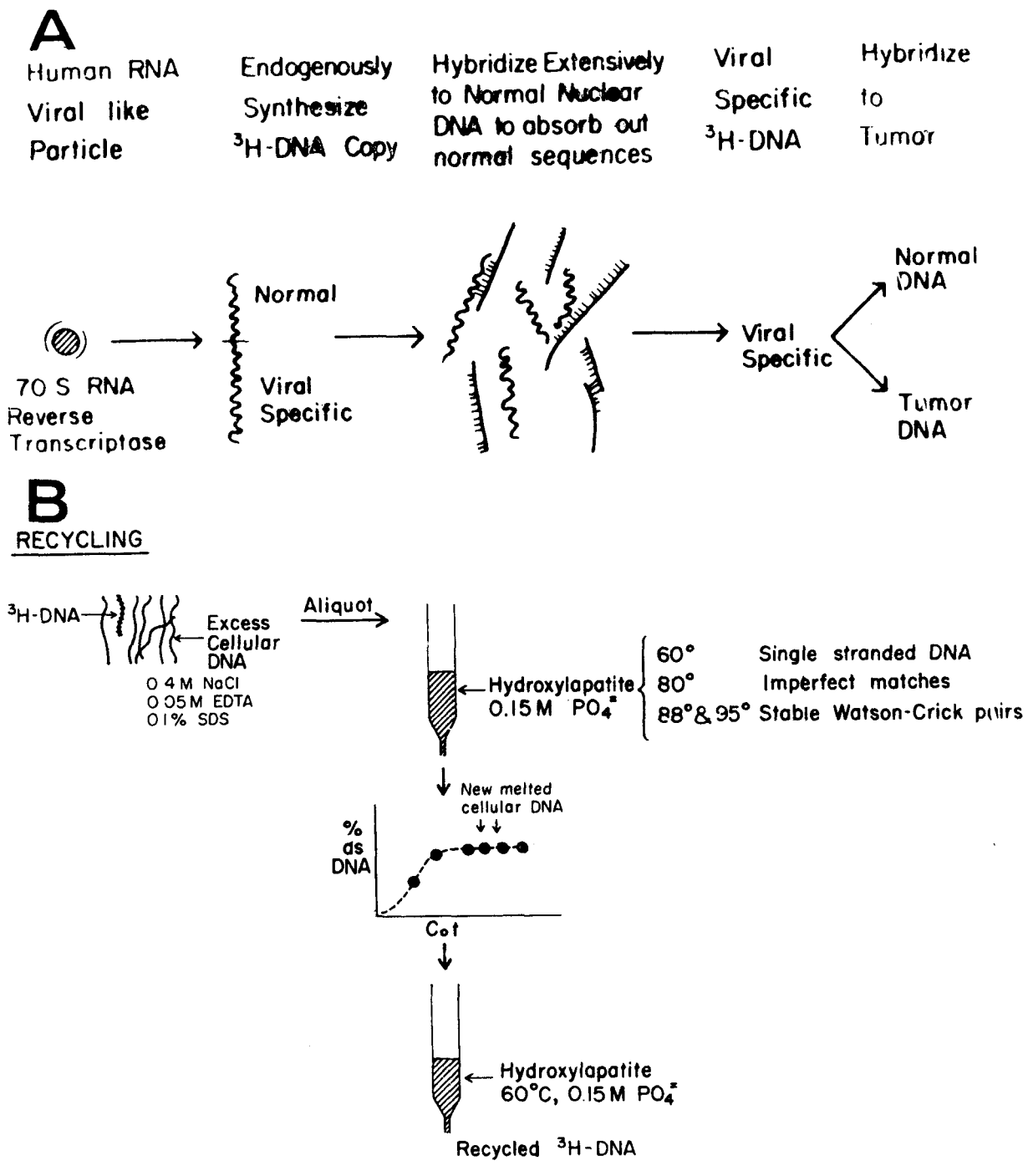

Fig. 4: (A) Generation of [^{3}H]DNA by human leukemic particles and hybridization of sequences shared with normal DNA. (B) Separation of leukemia-specific sequences by hydroxyapatite chromatography. See text for further details.

A typical outcome of hybridizing such recycled tritiated DNA to normal and leukemic DNA is shown in Fig. 5. It is evident that no complexes stable at temperatures above 88° are formed with normal DNA. On the other hand, 57 % of the recycled [^{3}H]DNA probe forms well-paired duplexes with leukemic DNA. A series of such experiments was performed with particle-generated [^{3}H]DNA and nuclear DNA obtained from 8 untreated patients with either acute or chronic myelogenous leukemia. In every case (Table V), the [^{3}H]DNA, after being sub-

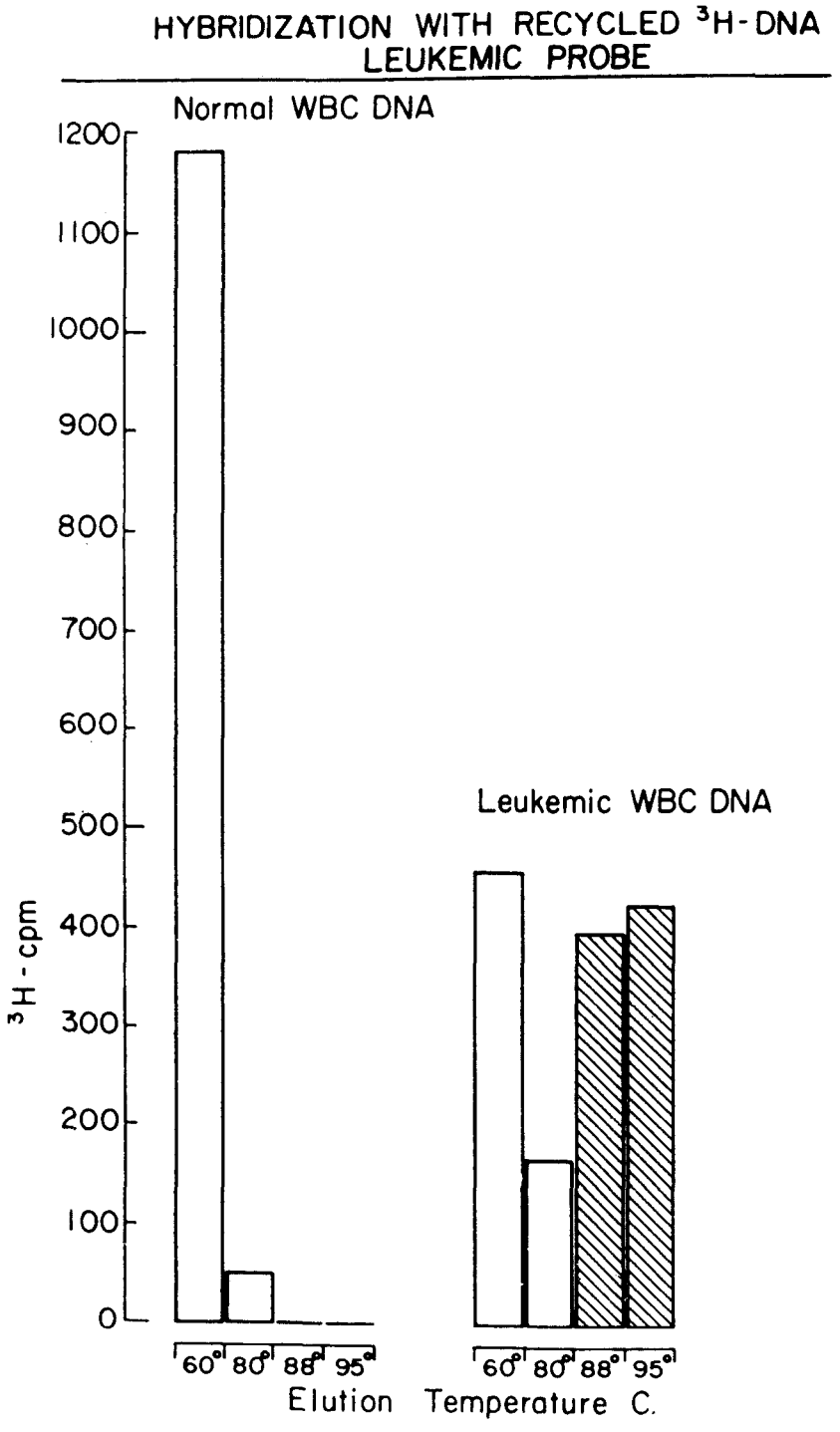

Fig. 5: Hydroxyapatite elution profile of a hybridization reaction of recycled leukemic [^{3}H]DNA to nuclear DNA from normal leukocytes and from leukemic leukocytes of the patient from which the [^{3}H]DNA was derived.

jected to exhaustive annealing to normal DNA, yielded a residue that forms stable duplexes only with leukemic DNA, in agreement with the experiment of Fig. 5.

In estimating the implication of these results, it must be recalled that the leukemia-specific sequences found (24) in leukemic cells are present as nonreiterated copies per genome. This was established by the C_0t values (concentration of nucleotides X time) required to detect them. The sensitivity used to examine

Table V: Exhaustive Hybridization of [^{3}H]DNA Probe Synthesized by Leukemic Particles with Normal-leukocyte Nuclear DNA, Followed by Hybridization of the Nonhybridizing Recycled Leukemic [^{3}H]DNA Probe to Normal DNA and to Leukocyte Nuclear DNA from the same Leukemic Patient (24).

		Leukemic [^{3}H]DNA hybridized to normal-leukocyte DNA		Recycled leukemic [^{3}H]DNA hybridized to leukocyte DNA			
				Leukemic		Normal	
		cpm	% Hybridization	cpm	% Hybridization	cpm	% Hybridization
1	(AML)	3020	61	523	56	0	0
2	(CML)	1350	40	1020	51	0	0
3	(CML)	2580	51	431	35	3	0
4	(CML)	510	45	101	36	0	0
5	(AML)	1100	43	303	48	4	0
6	(AML)	4520	49	1130	46	1	0
7	(AML)	390	42	45	69	0	0
8	(AML)	1450	49	510	52	0	0

Background was 30 cpm and all counts recorded represent cpm above background. CML = Chronic myelogenous leukemia. AML = Acute myelogenous leukemia.

normal cells for the leukemia-specific sequences was such that 1/50th of an equivalent of that found in leukemic cells would have been readily detected. Consequently, one may conclude that the vast majority of normal cells do not contain this particular stretch of malignant-associated information and it cannot therefore be represented in the germ line of nonleukemic individuals.

VIII. Unique Sequences in Hodgkin's and Burkitt's Lymphomas and their Relatedness

We have already noted that, like the leukemias, Hodgkin's and Burkitt's lymphomas have particles containing reverse transcriptase and a 70S RNA template related in sequence to that of RLV. It was of obvious interest to determine whether the lymphomas also parallel the leukemias in possessing a unique sequence not detectable in normal tissue. If they do, one can in addition ascertain whether the sequences found in Hodgkin's and Burkitt's lymphomas are related to each other. The outcome has evident significance for the possible relevance of the sequence to malignancy.

[^{3}H]DNA probes were synthesized with particles isolated from four Burkitt's tumors and three Hodgkin's disease specimens. Sequences shared with normal DNA (between 35 % and 40 %) were then removed as described for the leukemias (24) to yield the recycled [^{3}H]DNA probes (25).

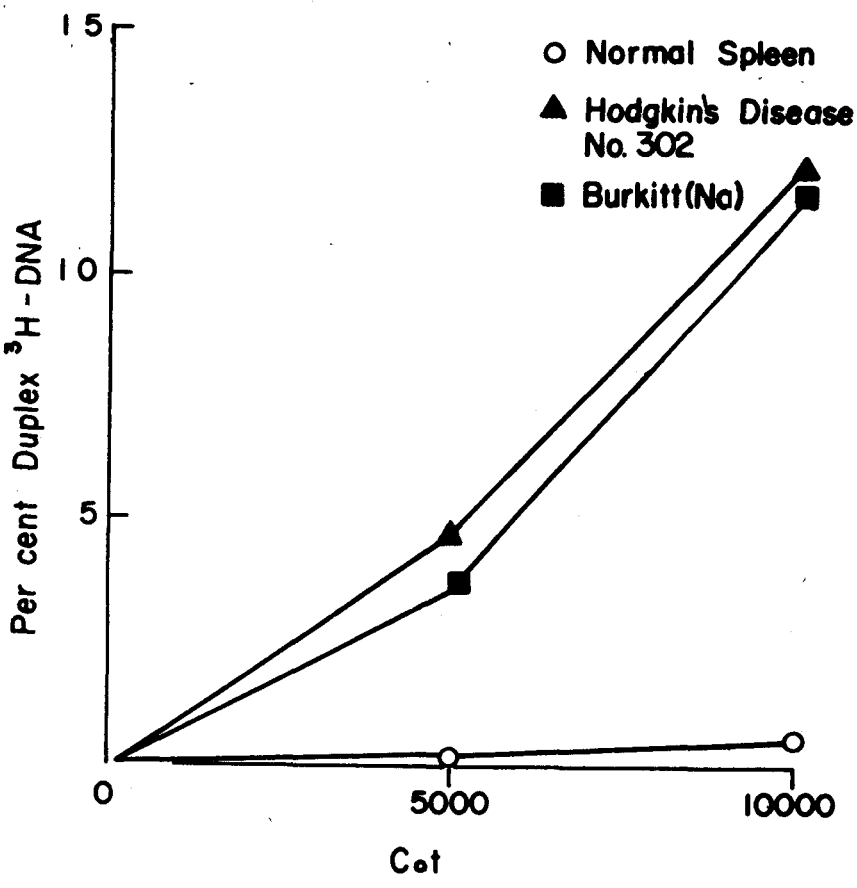

Fig. 6: Hybridization of recycled Hodgkin's disease #302 [³H]DNA to nuclear DNA isolated from normal spleen (0–0), Hodgkin's disease #302 (▲ – ▲), and Burkitt's lymphoma (NA) (■ – ■). Equal aliquots were removed from the hybridization vessel at each Cot value and hybrid formation was analyzed by hydroxyapatite chromatography. The input counts for each point were 1,500–2,000 cpm and only those duplexes eluting at 88° and above are counted as stably hybridized.

Figure 6 shows the outcome of challenging recycled Hodgkin's disease [³H]DNA with nuclear DNAs from normal spleen, Hodgkin's disease spleen, and from Burkitt's lymphoma. Few, if any, stable duplexes are formed with normal DNA. Note, however, that although the probe was made with Hodgkin's disease particles, the [³H]DNA hybridized to Burkitt's lymphoma nuclear DNA virtually as well as it complexed to DNA from Hodgkin's spleen. The converse is also true, as may be seen from Table VI, which summarizes the results of our findings in the recycled [³H]DNA challenged with nuclear DNA from normal and malignant tissues (25). Again, normal DNA is unable to form significant amounts of stable complexes (elution at 88° and above) with the [³H]DNA probes. In all instances, the lymphoma [³H]DNAs hybridized in stable complexes to the nuclear DNA of the original types from which the particles were obtained and used to generate the labeled DNA. Further, with only one exception, all of the Burkitt's and Hodgkin's disease [³H]DNAs cross hybridize with each other's DNA.

In summary, several features emerged from this study of the lymphomas. The particle-related sequences found in Burkitt's and Hodgkin's lymphomas possess sequences in common, an observation in accord with our earlier findings (7, 20,

Table VI: Hybridization of Recycled [^{3}H]DNA Probes Synthesized with Human Lymphoma Particles with Nuclear DNA from Normal and Tumor Tissues

Origin of [^{3}H]DNA recycled probe	Nuclear DNA	No. tested positive total	Percent positives
Burkitt's lymphomas	Burkitt's	7/7	100
	Hodgkin's	2/2	100
	Normal (spleen)	0/4	0
	IM* (spleen)	0/1	0
	IM* (cell)	0/1	0
Hodgkin's disease	Hodgkin's	3/3	100
	Burkitt's	2/3	67
	Normal (spleen)	0/3	0

* IM = infectious mononucleosis

21, 26), that Hodgkin's and Burkitt's particles both share sequences with the Rauscher murine leukemia agent. Further, in view of the previous association of the Epstein-Barr virus with Burkitt's lymphoma (27, 28) and the non-neoplastic infectious mononucleosis (29, 30), it is revealing to note from Table VI that the leukocyte DNA of patients with infectious mononucleosis was devoid of the Burkitt's sequences detected by the recycled [^{3}H]DNA lymphoma probe, indicating that these latter sequences are specific for neoplastic tissues. The fact that the particle-related sequences in Hodgkin's and Burkitt's tumors are related to each other adds further weight to this conclusion. Finally, the observation that cells carrying multiple copies of the DNA of the Epstein-Barr virus do not complex with recycled [^{3}H]DNA probes from either Hodgkin's or Burkitt's particles proves that these particle sequences have no detectable relation to the DNA of the Epstein-Barr virus.

IX. Evidence from Studies of Identical Twins

Although the comparison of leukemic patients with normal suggests that healthy individuals do not contain the leukemia-specific sequences, the data do not rule out the possibility that those who do come down with the disease do so because they in fact inherit the required information in their germ line. One way to resolve this issue is to study the situation in identical twins. Since identical twins are monozygous, i. e., derive their genomes from the same fertilized egg, any chromosomally transmitted information must be present in both. It had already been shown by Goh and his colleagues (31, 32) in the case of chronic myelogenous leukemia that only the leukemic member of each of two identical twin pairs contained the marker Philadelphia chromosome. It was of obvious interest to examine this situation for the leukemia-specific sequences. If the leukemic member of the pair contains the particle-related DNA sequences, and does so because he inherited them through his germ line, then these same sequences must be found in

the leukocyte DNA of his healthy sibling. To perform the experiment, it was necessary to locate identical twins with completely convincing evidence for monozygosity and where only one of them was leukemic. Further, the twins had to be of adult age since at least a unit of whole blood is required to provide enough leukocyte DNA to carry out the required hybridization.

Two sets of identical twins satisfying all these requirements were found and an experiment similar to the one outlined above was performed with each pair (33). In each instance, particles containing the reverse transcriptase and 70S RNA were again isolated from the leukocytes of the leukemic members and used to generate the [^{3}H]DNA endogenously. The [^{3}H]DNA was purified and sequences shared with normal DNA removed by exhaustive hybridization in the presence of a vast excess of normal DNA from random healthy blood donors. This was then followed by hydroxyapatite chromatography to separate paired from unpaired [^{3}H]DNA. It is important to emphasize that in the recycling step, the normal DNA used came from the leukocytes of healthy, random blood donors and not from the normal twin. To have used the latter would have obviously confused the issue. The residue of the tritiated DNA that did not pair with the normal DNA was then used to test for the presence of a sequence in the leukocyte DNA of the patient and that of his healthy sibling.

The results obtained with the two sets of twins are described in Fig. 7, and it is evident that the same situation holds between the members of the twin pairs as was observed in the comparison of unrelated leukemic patients and random normals (Fig. 5 and Table V). The leukemic twin contains particle-related sequences that cannot be detected in the leukocytes of his healthy sibling.

The fact that we could establish a sequence difference between identical twins implies that the additional information found in the DNA of the leukemic members was inserted after zygote formation. This finding argues against the applicability of the virogene hypothesis to this disease since it would demand that the leukemia-specific sequences found in the DNA of the individual with the disease must surely also exist in the genome of his identical twin. These results are also inconsistent with the possibility that individuals who succumb to leukemia do so because they inherit the complete viral genome.

X. Implications of the Unique DNA Sequences in Leukemias and Lymphomas

The data we have summarized on the existence of viral lateral-related sequences unique to the DNA of human malignant cells imply that they are inserted in somatic DNA, a process known to occur with RNA tumor viruses in animal cells in tissue cultures (34) and in whole animals (35). The fact that these viral sequences can be incorporated into somatic DNA suggests that this could also occur in early embryogenesis and thus involve a cell destined to differentiate into the germ line. An event of this nature would be selected for in any attempts at developing inbred strains characterized by high frequency of cancer.

Indeed this seems to have occurred in the course of producing the AKR mouse, a strain in which spontaneous leukemia occurs with virual certainty. It has been shown (36) that the DNA of the AKR mouse contains murine leukemia virus sequences that are not present in the DNA of the NIH Swiss mouse. These se-

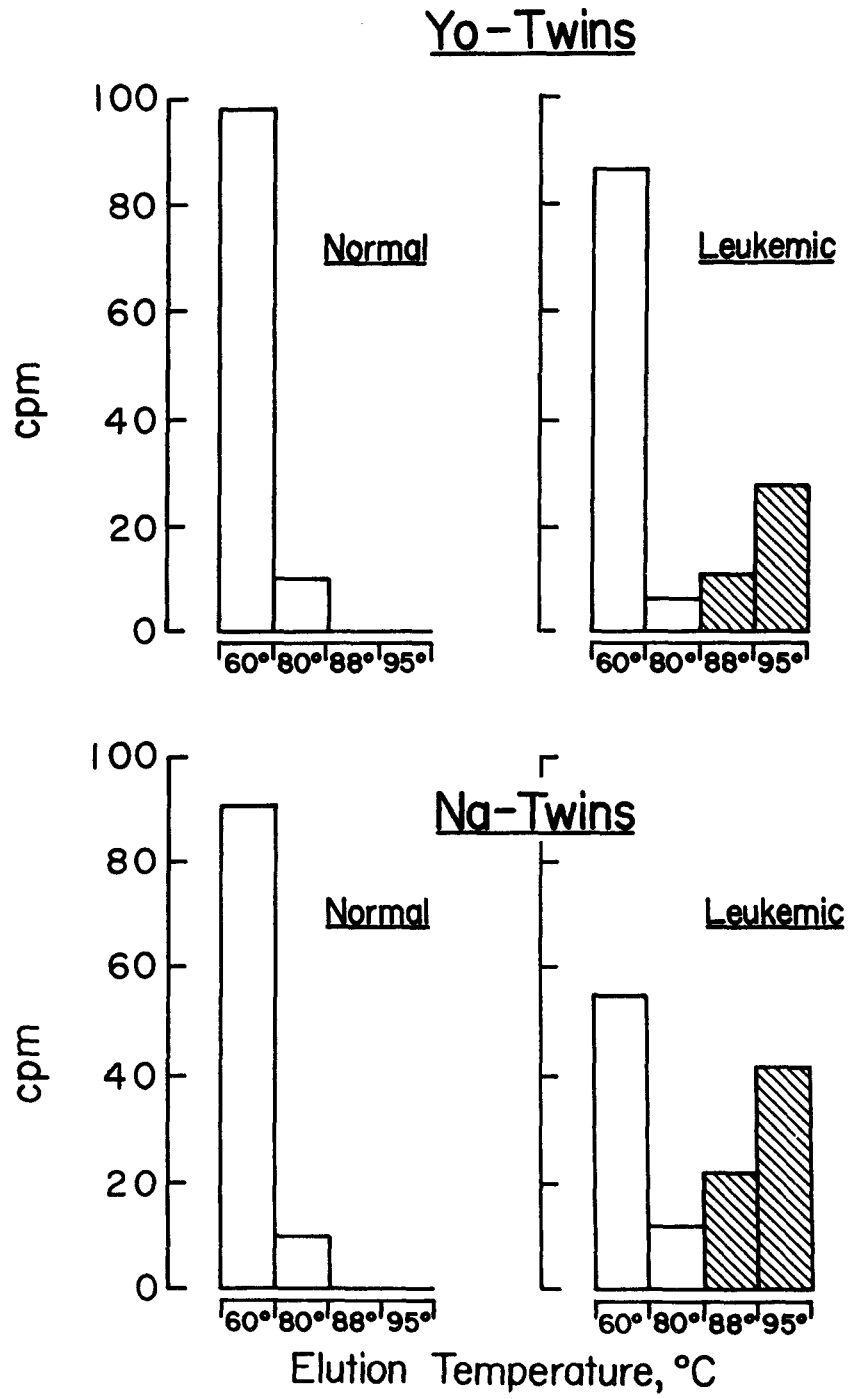

Fig. 7: Hydroxyapatite elution profile of a hybridization reaction of the recycled leukemic twin [^{3}H]DNA probe to nuclear DNA from normal leukocytes, normal twin leukocytes, and leukocytes from the same leukemic twin. The annealing reaction mixtures contained 20 A_{260} units of cellular DNA, 0.004 pmol of [^{3}H]DNA, and 15 μmol NaH_2PO_4 (pH 7.2) in a final vol of 0.01 ml. The reaction was brought to 98° for 60 sec and 0.04 mmol of NaCl was added. The reaction mixture was then incubated at 60° X 50 hr. The reaction was stopped by the addition of 1 ml of 0.05 M $NaHPO_4$ (pH 6.8). The sample was then passed over a column of hydroxyapatite of 20-ml-bed vol at 60°. The column was washed with 40 ml of 0.15 M $NaHPO_4$ (pH 6.8) at 60°, 80°, 88°, and 95°. Fractions of 4 ml were collected, the A_{260} of each fraction was read, and the DNA was precipitated with 2 μg/ml of carrier yeast RNA and 10 per cent trichloroacetic acid. The precipitate was collected on Millipore filters, which were dried and counted. In all cases, greater than 80 per cent of the nuclear DNA reannealed. A background count of 8 cpm was subracted in all instances (33).

quences were localized by genetic and molecular hybridization, and it was found that they are either identical to or closely linked to the Akv-1 locus.

The case of the AKR mouse was very likely an inadvertent result of its selection. An even more remarkable instance is the deliberate insertion of viral sequences into the germ line (37). This was accomplished by infection of preimplantation mouse embryos at the 4–8 cell stage with the murine leukemia virus (MuLV) followed by reimplantation in the uteri of surrogate mothers. Of 15 such animals born, one developed lymphatic leukemia at 8 weeks of age. Molecular hybridizations revealed leukemia-specific viral sequences in the DNA of all eight different organs examined, whether they were of mesenchymal origin or not. In agreement with our earlier findings (35), these sequences were not found in normal mesenchymal tissue nor were they detected in the DNA of non-target tissues in animals made leukemic by injection of virus after birth.

In summary, except for strains deliberately inbred for high spontaneous occurrence of disease, the mesenchymal neoplasias of mice and men would appear to have a similar underlying mechanism. In both instances new viral-related sequences are found in the DNA of the malignant cells and these are not found in the DNA of uninvolved tissues. They are therefore not germinal unless one wishes to invoke a rather unlikely specific elimination in the course of the differentiation of every cell but the malignant one. Despite its implausibility, this possibility should be exploited by testing for the leukemic-specific sequences in the germ line DNA (sperm) of leukemic individuals.

It must be emphasized that conclusions as to the validity of the virogene-oncogene hypothesis are only relevant to the particular instances examined and cannot be generalized to any other viral-related cancers even in the same animal, let alone to other species. In any event, it is evident that our findings with respect to the human mesenchymal tumors suggest more optimistic pathways for the control of these diseases than would be available if the total information were already in the genome. The data imply that we may not be forced to master the control of our own genes in order to cope with these neoplasias.

The fact that the human particles possess sequences homologous to those found in viral agents known to cause the corresponding neoplasias in mice encourages the hope that they are relevant to human disease. However, despite the considerable progress that can be recorded, no definitive proof exists at the present writing that the virus-like particles found in the human neoplasias are either viruses or etiologic agents of the cancers in which they are found. Proof will ultimately come when it proves possible to produce the relevant malignancy in a susceptible animal by injection of the particles purified from human tumors. It should, however, be noted that we have known of the mouse mammary tumor virus for more than 35 years and no one has yet succeeded in producing mammary tumors with this agent in any animal other than the mouse.

Under the circumstances, it would seem prudent not to wait for the definitive experiment with the human particles, but rather to proceed with attempts at further exploration of their significance and possible clinical usefulness.

We should like to list a few areas of possible exploitation and then turn our attention to a brief description of what has been accomplished along these lines.

1. The existence of the leukemia-specific sequences can provide the clinician with

a hitherto unsuspected parameter that could potentially be a useful adjunct in monitoring therapy.

2. One could attempt to grow the human particles in tissue culture in order to provide a more accessible source of these particles for further biochemical characterization with the ultimate hope of generating useful reagents for diagnostic, therapeutic or monitoring purposes.

3. Another, less ambitious approach is to purify one of the protein subcomponents from the human particles for further characterization. This could then be used for the production of a monospecific antiserum that might be clinically useful.

XI. Particulate Reverse Transcriptase in the Leukocytes of Leukemic Patients in Remission

We have already noted (Table V) that positive simultaneous detection tests, indicating the presence of particles containing reverse transcriptase and the 70S RNA template, were obtained in more than 99 % of the leukemic patients examined. It was of obvious interest to see whether these particles could be detected in the leukocytes of leukemic patients who are in good clinical remission.

Peripheral blood leukocytes were obtained from patients at the Baltimore Cancer Research Center and from the M. D. Anderson Hospital. The leukocytes from some of the leukemic patients were obtained by leukophoresis and immediately stored at $-7°$ until used. The clinical statuses of the patients at the time of leukophoresis are summarized in Table VII. A complete remission was defined as the absence of symptoms related to the disease, normal results on physical examination, a hemoglobin of greater than 10 g/100 ml, leukocyte count greater than 3000/mm^3, platelet count greater than 100,000/mm^3, no blasts in the peripheral blood smear, and less than 5 % blasts in the bone marrow.

Table VIII summarizes the results of simultaneous detection assays for high molecular weight RNA and reverse transcriptase in the leukocytes from the patients examined. Outcomes are designated as positive only when the peaks of tritiated DNA found in the 70S and 35S regions were eliminated by prior treatment with ribonuclease, a feature establishing that the [^{3}H]DNA is complexed to a large RNA molecule. If the peaks are not removed subsequent to RNase digestion, the reaction is scored as a negative outcome. In the present study two untreated leukemic patients were available for testing prior to remission induction and both were positive at that time. Three of the nine patients in complete remission demonstrated a 70S or 35S peak of acid-precipitable radioactivity that was abolished by RNase treatment. The "negatives" were subjected to a simultaneous detection assay via a cesium sulphate gradient, a procedure that obviates the problem generated by fragmentations of the RNA template during manipulation.

It will be noted from Table IX that samples from the untreated patients were all positive. The [^{3}H]DNA-RNA hybrids were detected in nine out of eleven patients in complete remission. In this group, six of seven AML patients and two of three acute lymphocytic leukemia (ALL) patients demonstrated a positive reaction. In five of the patients, the simultaneous detection tests were negative by velocity sedimentation analysis but were positive when analyzed in the cesium

Table VII:

Patient	Age	Sex	Diagnosis+	Clinical* status	Remission duration at time of sample (days)	Total remission duration (days)
1-A	62	F	AML	UN	–	–
1-B	"	"	"	CR	7	64
2-A	42	M	AML	UN	–	–
2-B	"	"	"	CR	45	96
2-C	"	"	"	REL	–	–
3-A	40	F	AML	UN	–	–
3-B	"	"	"	CR	7	32
4-A	54	F	AML	UN	–	–
4-B	"	"	"	CR	7	218+
5	34	F	AML	CR	42	109
6	53	M	AML	CR	7	87
7	53	F	AML	CR	100	112
8	40	M	ALL	CR	1214	1600+
9	42	M	ALL	CR	1062	1662+
10	35	M	ALL	CR	1149	1735+
11	42	M	AML	CR	1051	1590+

+ AML = acute myelogenous leukemia; ALL = acute lymphocytic leukemia
* UN = untreated; CR = complete remission; REL = relapse
Clinical status of leukemic patients when leukophoresis was performed for enzyme studies.

Table VIII:

Patient	Clinical status	CPM		Reaction
		70S	35S	
1B	CR	0		–
2A	UN	400		+
2B	CR	224		+
2C	REL		475	+
4A	UN	320		+
4B	CR	0		–
6	CR	975		+
7	CR	0		–
8	CR	0		–
9	CR	0		–
10	CR	0		–
11	CR	120		+

Test for 70S and 35S RNA-[^{3}H]DNA in leukocytes from leukemic patients. CPM in 70S or 35S represents acid-precipitable radioactivity that was removed from the 70S and 35S by prior treatment with ribonuclease A and T_1.

Table IX:

Patient	Clinical Status	RNA Region		Reaction
		CPM	% Total	
1A	UN	459	26	+
1B	CR	293	43	+
2A	UN	869	39	+
2B	CR	346	31	+
2C	REL	829	90	+
3A	UN	129	33	+
3B	CR	210	30	+
3C	CR	216	40	+
5	CR	248	30	+
6	CR	236	32	+
7	CR	187	29	+
8	CR	296	12	+
9	CR	0	0	−
10	CR	503	61	+
11	CR	52	32	−

Cesium sulfate analysis of RNA[^{3}H]DNA in leukocytes from leukemic patients. The CPM in the RNA or RNA-DNA hybrid region and the percent of the total CPM applied to the gradient are enumerated. A positive reaction represents heat and ribonuclease-sensitive acid-precipitable radioactivity in the RNA or hybrid region of the gradient as described in the text. The [^{3}H]DNA found in the hybrid density regions is hybridized to smaller DNA-RNA complexes, which would result from fragmentation of the larger 70S and 35S RNA molecules.

sulphate gradients. Hybrids of small molecular size would not be identified as 70S with 35S complexes, but can be detected as complexes in the hybrid region of the cesium sulphate gradient. Only one of the patients (#9) was negative by both glycerol and cesium sulphate gradient analyses. This patient did not appear to differ clinically at the time of the examination from the other remission patients exhibiting positive reactions. In the course of these studies we also examined by cesium sulphate analysis two pooled, normal white blood cell samples and five non-neoplastic spleens for the presence of particles capable of yielding RNA-tritiated DNA hybrids in an endogenous reaction and all were negative, as had been true in our previous studies (Table V).

It is obvious that finding the leukemia characteristic particles in the white blood cells of patients in remission is disappointing and does not accord with the generally accepted assumption that there is a normal and a leukemic population of leukocytes in acute leukemia (38). The goal of contemporary chemotherapy and immunotherapy is to reduce the size of the leukemic component (to zero if possible) to allow the bone marrow and the peripheral blood to repopulate with non-neoplastic cells. A number of clinical observations suggest that remission leukocytes are in fact normal cells. First, the prolongation of life is directly proportional to the duration of the remission. Secondly, a small but increasing number of patients

with acute lymphoblastic leukemia in long-term remission appear to go on to cure, indicating a permanent extinction of the leukemic cell population (39).

The morphology and functional properties of remission leukocytes have been studied by a number of techniques. These include karyotype analysis (40–45), ability to form colonies in agar (46–49), and the detection of leukemia-related antigens (50, 51). In general, these studies have supported the concept that remission leukocytes represent the return of a population of normal cells. Also, relapses are usually heralded by the detection of the abnormality associated with leukemic cells, and a number of these techniques have been suggested as ancillary tools in following the response of patients to chemotherapy. However, there have been instances in the above reports of patients in well-consolidated remissions whose peripheral leukocytes or bone marrow cells demonstrated persistence of aneuploidy, leukemia-related antigens, or abnormal colony formations in agar; these abnormalities appear unrelated to the effects of maintenance of chemotherapy. In this connection, mention should be made of Killmann's deductions (52) based on the demonstrated capacity of leukemic cells to differentiate; on these grounds he questions whether the normal-looking cells observed in the bone marrow of AML patients in remission are in fact derived from non-leukemic ancestor cells.

The data presented indicate that with regard to certain biochemical markers, which may be virus-related, remission leukocytes may more closely resemble the leukemic cells than normal cells. Quite surprisingly, two of the three ALL patients in long-term remission still had evidence of particles in their peripheral leukocytes. Further, the enzyme was detected in seven of eight patients with AML in remission. We are unable to determine if all or a fraction of the peripheral white cells studied possessed the leukemic characteristics. Thus we cannot directly answer the question whether one or two white cell populations are present.

Mak and his colleagues (53, 54) have described particulate activity in the supernatants of short-term cultures derived from bone marrow of leukemic patients in remission. The activity of the cultures from remission patients equaled, and in some instances exceeded, that detected in the cultures derived from patients in relapse.

There are a number of plausible explanations for the persistence of the particulate enzyme and its associated template in the remission leukocytes. The normal cell found in remission could be infected with a non-oncogenic C-type virus or conversely the remission leukocyte could have acquired resistance to transformation whereas susceptibility to infection was unaltered. Second, as a result of chemotherapy, a portion of the leukemic clone could have evolved into a non-neoplastic clone still capable of expressing some viral function. There are a number of *in vitro* models for the latter phenomenon. Thus, it has been shown that cells transformed with a murine sarcoma virus can spontaneously, or after exposure to antimetabolites, revert to a normal morphology. Certain clones of these morphological revertants behave in a non-malignant manner, yet some viral functions are expressed or can be induced (55).

A more direct method for examining such questions is to use the molecular hybridization to answer the following questions: 1) Do remission cells have leukemia-specific DNA nucleotide sequences? 2) If present, are some leukemia-specific DNA sequences not expressed or are critical DNA sequences deleted? These areas are presently under investigation.

XII. Attempts to Produce Human RNA Tumor Particles in Cell Cultures

All would agree that an important advance would result from the establishment of particle-producing cells in short, or preferably long-term culture. An alternative but equally useful outcome could be obtained by the successful infection of established cell lines with the human virus-like particles. Although not yet achieved, a number of recent reports suggest that this desirable situation may eventually be obtained. Thus, McGrath *et al.* (56) describe a human breast carcinoma cell line that may ultimately be converted into a source of breast cancer particles. Kotler *et al.* (57) have succeeded in using arginine starvation to induce the release of virus-like particles from human leukemic cells. We have already noted that short-term cultures of leukemia bone marrow aspirates in a conditioned medium has led to the production of particles recoverable from the culture supernatants (53, 54).

Probably the most interesting recent announcement along these lines came from Gallo and his colleagues (58, 59, 60) who reported the isolation of a C-type virus (HL23V) from cultured peripheral white blood cells derived from a patient with acute myelogenous leukemia. The reverse transcriptase of this putative human oncornavirus was found to be antigenically related to the reverse transcriptase of the simian sarcoma virus type-1 (SSV-1) and to the gibbon ape lymphoma virus (GALV). The spontaneously released viruses from the human leukemia cells were successfully transmitted to A204, a human rhabdomyosarcoma cell line. The infected A204 (HL23V) culture was an excellent producer, yielding virus in sufficient quantities to permit biochemical and immunological characterization.

The potential implications of these observations made it mandatory to undertake the task of identifying the nature of the virus particles released. We will here briefly summarize our efforts along these lines.

The A204 (HL23V) culture produced high titers of particles that were found by [^{3}H]-uridine labeling to possess the characteristic buoyant density (1.16 g/ml) of oncornavirus. Simultaneous detection assays (15) of the culture supernatants demonstrated that the particles encapsulated 70S RNA and reverse transcriptase.

The reverse transcriptase from A204 (HL23V) culture supernatants was examined for relatedness to the SSV enzyme. Figure 8 shows that the antiserum prepared against the SSV reverse transcriptase was capable of inactivating the reverse transcriptase activity of HL23V particles only to about 60 %. The partial inhibition of enzyme activities suggested the possible presence of a second virus containing an antigenically unrelated enzyme.

To identify the unknown component in the HL23V particles, a search was instituted amongst known oncornaviruses using immunologic and molecular hybridization techniques. Probable candidates were quickly narrowed down to the RD-114/CCC baboon endogenous virus group. Figures 9A and 9B show hydroxyapatite temperature elution profiles of viral (SSV and BV-M7) cDNA annealed to the total RNA from HL23V particles. The extent of the hybridization and the thermal stability indicate that the HL23V particles contain the complete information of both the simian sarcoma virus (SSV) and the baboon endogenous virus (BV-M7).

To determine whether all of the genetic information of HL23V can be accounted for by these two viruses, the reciprocal hybridization was performed. In these

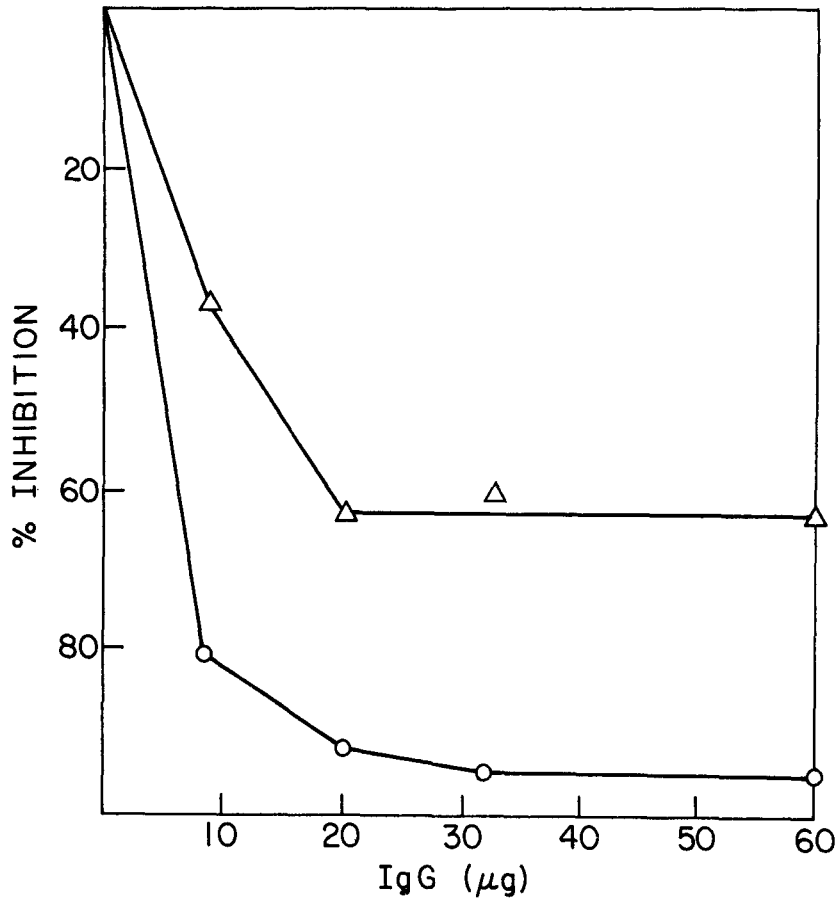

Fig. 8: Effects of anti-SSV reverse transcriptase IgG on HL23 reverse transcriptase activity. Increasing amounts of IgG purified from normal goat serum and from goat antiserum directed against SSV reverse transcriptase were mixed with NP-40-disrupted HL23, incubated for 15 min at 37° and then assayed for reverse transcriptase. SSV was similarly treated and assayed. Both HL23 and SSV input were standardized to incorporate 10 pmoles of [^{3}H]-TP in a synthetic template assay. The reverse transcriptase reactions (100 μl) contained the following in μmoles: Tris-HCl (pH 8.0), 5; $MnCl_2$, 0.02; KCl, 4; dithiothreitol, 0.04; 0.02 each of dGTP and [^{3}H]-dGTP (500 cpm/pmole) and oligo dG_{12}: poly Cm at 4 μg/ml. After incubation at 37° for 30 min, the reactions were terminated and assayed for acid-precipitable radioactivity. Using incorporations at identical inputs of normal IgG as control, the percent inhibition of the SSV and HL23 polymerase activity by the increasing levels of immune IgG were computed. 0 = V; △ HL23 virus.
the increasing levels of immune IgG were computed. 0 = SSV; △ = HL23 virus.

experiments, cDNA probe synthesized endogenously with HL23V particles, was annealed to RNAs from SSV-1 or BV-M7 or both. Figure 10 shows that the HL23V-cDNA hybridized 37 % and 44 % to the RNAs of BV-M7 and SSV-1, respectively. These individual hybridizations were additive as demonstrated by the complete complexing of HL23V-cDNA to a mixture of BV-M7 and SSV-1 RNAs. These data indicate that the genetic information of HL23V virions is completely accounted for, within the limits of the sensitivity of the molecular hybridization technique used, by the complete genomes of both SSV-1 and BV-M7.

To supplement and confirm these findings by an independent method, competition molecular hybridizations were performed using cDNA synthesized from SSV. Viral RNA from SSV was labeled with ^{125}I. SSV-cDNA can protect this ^{125}I-SSV-RNA more than 79 % from ribonuclease digestion at molar ratios of 5:1 (cDNA:RNA). Table X shows that when unlabeled viral RNAs were added in vast excess to compete this homologous reaction, both SSV and HL23V RNA

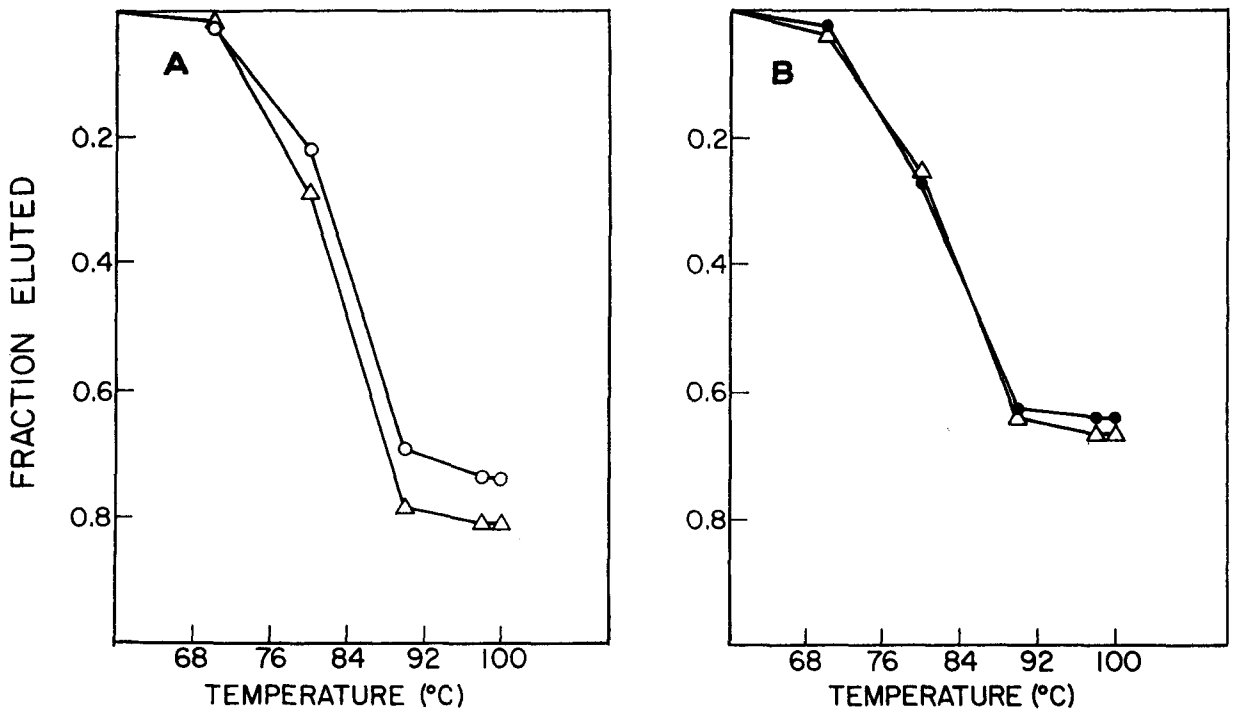

Fig. 9: Hybridization of [^{3}H]-DNA transcripts of (A) SSV-1 and (B) BV (M7 isolate) to HL 23 viral RNA. SSV-1 and BV-M7 were obtained from Pfizer, Inc. (Maywood, N. J.) and were derived from sucrose density gradient-banded tissue-culture supernatants of a chronically infected human lymphoblastoid cell line (NC-37) and baboon kidney-canine thymus coculture (BKCT), respectively. HL23 virus was prepared from high-speed pellets of 2-day old media from HL23 virus-infected human rhabdomyosarcoma cell cultures. [^{3}H]-DNA probes for each of the viruses were isolated from the 60–70S RNA:DNA hybrids of standard large-scale simultaneous detection assays. Hybridization reactions were set up between the various probes (500–1000 cpm/assay) and viral RNAs (0.2–1.0 µg) in 20 µl volumes in sealed siliconized glass tubes in 0.8 M phosphate buffer, pH 6.8, 0.1 % SDS and 10 mM EDTA. After heating at 100 °C for 1 min, the reactions were incubated at 68 °C for 20 h (Cot $\geqq$ 2). The reactions were anlyzed by thermal elution hydroxyapatite chromatography. Fractions of the total radioactivity eluted above 60 °C were plotted as a function of temperature. 0 = SSV-1 RNA, ● = BV-M7 RNA, △ = HL23V RNA.

Table X: Analysis of HL23V for SSV Genomic Content by Competition Hybridization

Competing RNA	*Competing RNA* ^{125}I-SSV RNA	% Resistance of ^{125}I-SSV RNA
None	–	72
SSV-1	1500	3
GALV	1200	15
MuLV-R	1800	75
Mouse 18S + 20S	450	74
HL23V	2300	6

Hybridization reactions (5.5 µl) were performed as described in Fig. 9 and contained 0.11 ng ^{125}I-SSV RNA (1.2 X 10^8 cpm/µg), 0.68 ng [^{3}H]-SSV cDNA (2 X 10^3 cpm/µg) and 0.5–0.25 µg of the indicated competing RNA. Following incubation at 68° for 48 h, the reactions were diluted with 0.01 M Tris-HCl, pH 8.0, 0.4 M NaCl, 0.01 M EDTA and divided into four equal aliquots. Ribonuclease A (25 units/ml) and ribonuclease T_1 (5 units/

ml) were added to two aliquots and the samples incubated at 37° for 1 h. Nuclease resistance was the ratio of acid-precipitable ^{125}I in the samples with and without ribonuclease. Recovery of input acid-precipitable ^{125}I was greater than 90 %. SSV RNA was isolated from virions by disruption with SDS, Pronase treatment, rate sedimentation in a sucrose-SDS gradient and equilibrium density gradient centrifugation in potassium iodide. Viral RNAs from GALV-1 and MuLV-R were isolated by similar procedures excepting the KI gradient. HL23V RNA was total RNA from purified virus and the mouse 18S and 20S RNAs were extracted from purified ribosomal subunits from NIH/3T3 tissue culture cells. [^{3}H]-SSV cDNA was synthesized from SSV RNA and oligo dT with AMV DNA polymerase in the presence of 0.1 mg/µl actinomyc in D and 0.5 mg/ml distamycin A. The reaction contained TTP, dATP and dGTP at 1 mM each and [^{3}H]-dCTP (25 Ci/mMole) at 0.05 mM. This SSV cDNA protected ^{125}I-SSV RNA 33 %, 75 % and 87 % from ribonuclease digestion at molar input ratios of 0.4, 3 and 15, respectively (cDNA:RNA).

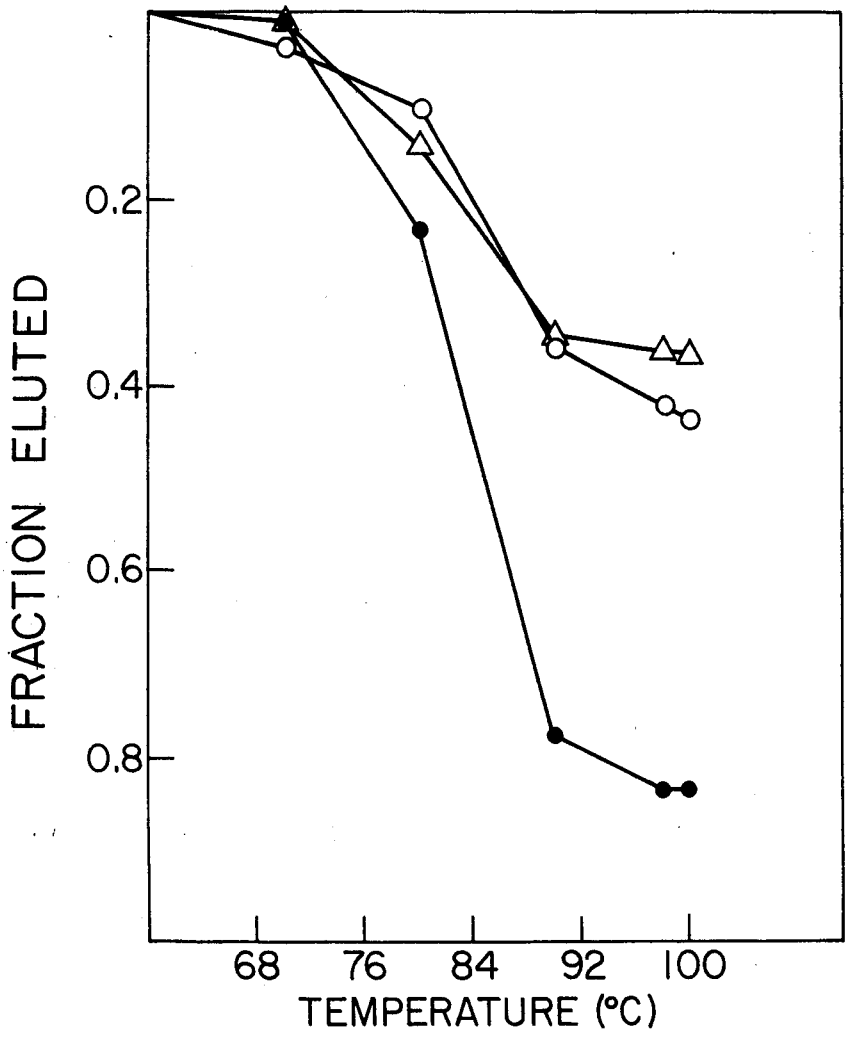

Fig. 10: Reconstruction hybridizations. HL23V [^{3}H]-DNA probe was hybridized to SSV and BV-M7 RNAs individually and in combination.
0 = SSV RNA, △ = BV-M7 RNA,
● = SSV and BV-M7 RNAs.

rendered the ^{125}I-SSV completely digestible. The GALV competed less successfully, illustrating the sensitivity of this technique for detecting the small sequence differences known to exist between GALV and SSV.

HL23V was further tested for its immunological similarity to SSV and BV-M7. The virus was concentrated by ultracentrifugation from culture supernatants of A204 (HL23V) and used as competing antigens in radioimmune assays for the p30 and gp45 of SSV grown in NC-37. As shown in fig. 11, the extent of competition of HL23V in both of these radioimmune assays was indistinguishable from SSV. Further, an immunodiffusion analysis of HL23V was made with antisera

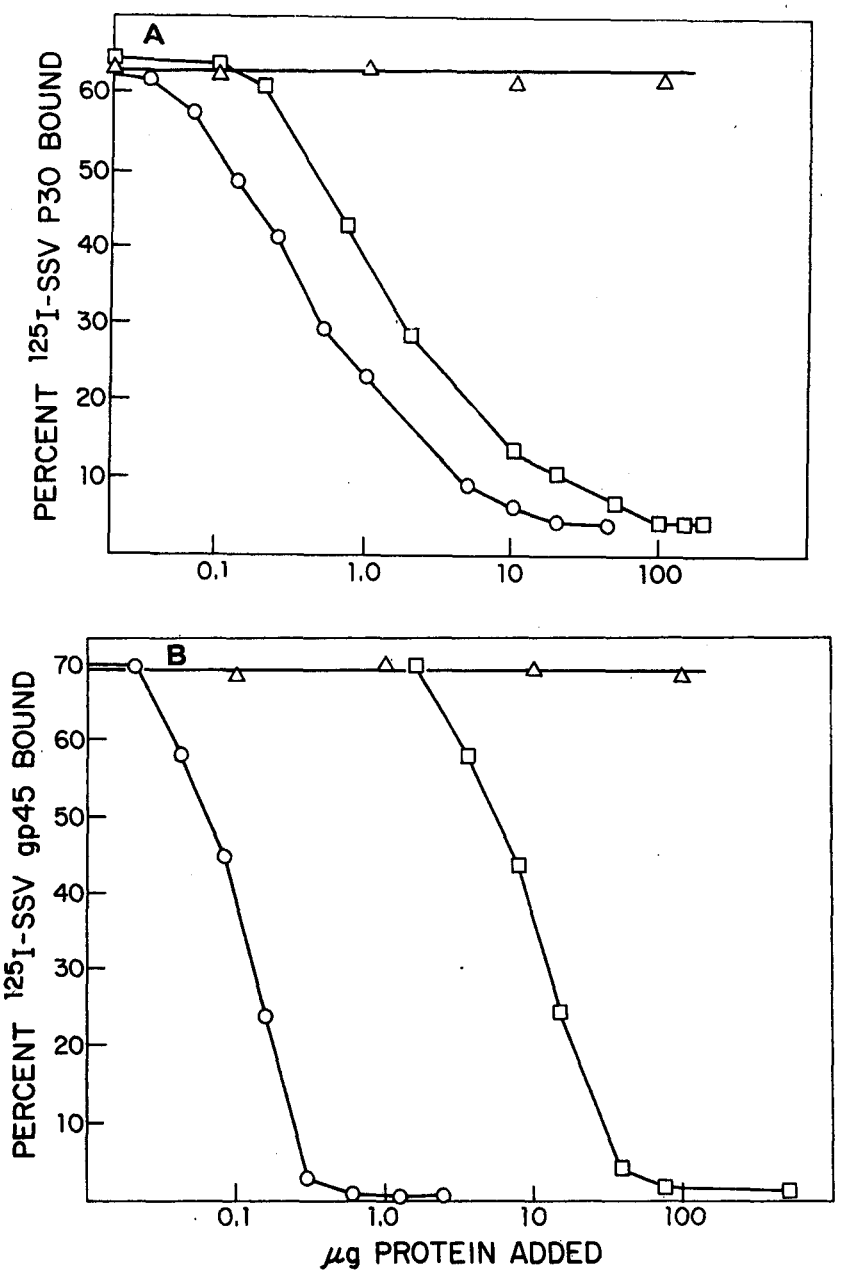

Fig. 11: Competition radioimmunoassays. HL23V was assayed for the presence of proteins antigenically related to the p30 (A) and gp45 (B) of NC-37 grown SSV-1. Antisera used for the studies with SSV-p30 were prepared by immunizing rabbits with the purified p30. An NC-37 absorbed rabbit antiserum prepared by inoculation of disrupted virions and used for the studies with SSV-gp45 was kindly supplied by Dr. D. Larson (Pfizer, Maywood, N. J.). The p30 protein of SSV-1 was purified from sonicated virus, followed by phosphocellulose and Sephadex G75 column chromatography and isoelectric focusing. It was iodinated and repurified and the iodinated p30 antigen preparations obtained had specific activities of 6 X 10^6 cpm/μg. The gp45 protein of NC-37 grown SSV was purified from sonic-disrupted virus by column chromatography on agarose 5M in guanidium hydrochloride. Analysis of the preparations by 5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing indicated homogeneity of the 45,000-dalton antigen. Succinimidyl-3-(4-hydroxphenyl) propionate (ICN Pharmaceuticals, Inc.) was first labeled with ^{125}I and purified. The purified gp45 was then labeled by conjugation with the ^{125}I-labeled ester and chromatographed on a Sephadex G-25 column. Titrations of the antisera were performed in 200 μl reactions that included 0.2 % bovine serum albumin (BSA) in saline, 5000 cpm of ^{125}I-antigen and dilutions of antibody. After 1 h incubation at 37 °C,

100 μg of normal rabbit carrier IgG and a titered amount of goat anti-rabbit IgG were added. The reaction was incubated for 15 h at 4 °C. The samples were centrifuged and both precipitates and supernatants were counted in a Searle Autogamma counter model 1185. The results are expressed as percent cpm precipitated. Greater than 80 % of the labeled antigen could be bound by specific antisera. Competition assays were performed in a similar manner, except that unlabeled competing antigen was added to the original incubation mixture. The unlabeled competing antigens were SSV = (0), HL23V = (□), SSV-gp45 = (●) and MPMV = (△).

prepared against the major and internal structure of proteins of the woolly monkey (p30) and of the baboon virus (p28). The lines of identity obtained with BV-M7 and SSV indicate that, by these criteria, aga in HL23V cannot be differentiated from a mixture of these two agents.

In summary, immunologic and hybridization analyses indicate that the particles produced by A204 (HL23V) consist of a mixture of two viruses that are indistinguishable immunologically and by nucleotide sequence from two known non-human primate viruses, the baboon endogenous virus M-7 (61) and the woolly monkey virus SSV-1 (62). It will be noted that our conclusions and results (63) are in complete agreement with those of Gilden and his collaborators (64) whose experiments, complementary to ours, involved hybridizations to the cytoplasmic RNA of various cell lines infected with HL23V and antigenic analysis of the type-specific p12 and p15 antigens.

Any attempts to establish productive long-term cultures are always exposed to the all pervasive danger of laboratory contamination with animal oncornaviruses. Because of this, any evidence that agents produced in tissue cultures are either identical or even similar to a known animal oncornavirus has been accepted as sufficient evidence to condemn the culture and its particles as irrelevant to the human disease. It is important to recognize, however, that this is not a logically compelling argument. For example, it could well be true that some animal viruses originated from a human source. It is even less certain to conclude that an agent is human if it cannot be identified either by base sequence or by antigenic properties with a known animal virus. This line of reasoning makes the untenable assumption that our catalogue of all tumor viruses is complete.

The clinically relevant question for any putative human candidate particle is not necessarily its origin but its relation to the human disease. Are there at present any criteria that can be used usefully to decide whether a given tissue culture virus (e. g., HL23V) is in fact relevant to human neoplasia? A possible resolution can be achieved by answering the following two questions: (1) Can one provide evidence at the level of protein and/or nucleotide sequence for the presence of the putative agent in the *original tumor material* from which the tissue culture was established? (2) Can one provide evidence at the level of protein and/or nucleotide sequence for the persence of the putative agent in the malignant cells of other patients with the same disease?

A positive answer to the first question in the form of evidence for their presence in the original tumor cells would serve to eliminate the trivial explanation that the particles arose in the culture by laboratory contamination. The answer to the second question will decide the general relevance of the observation to human leukemia. Unless a positive response is obtained in a major portion of the patients examined, no basis exists for identifying the HL23V particles as clinically signifi-

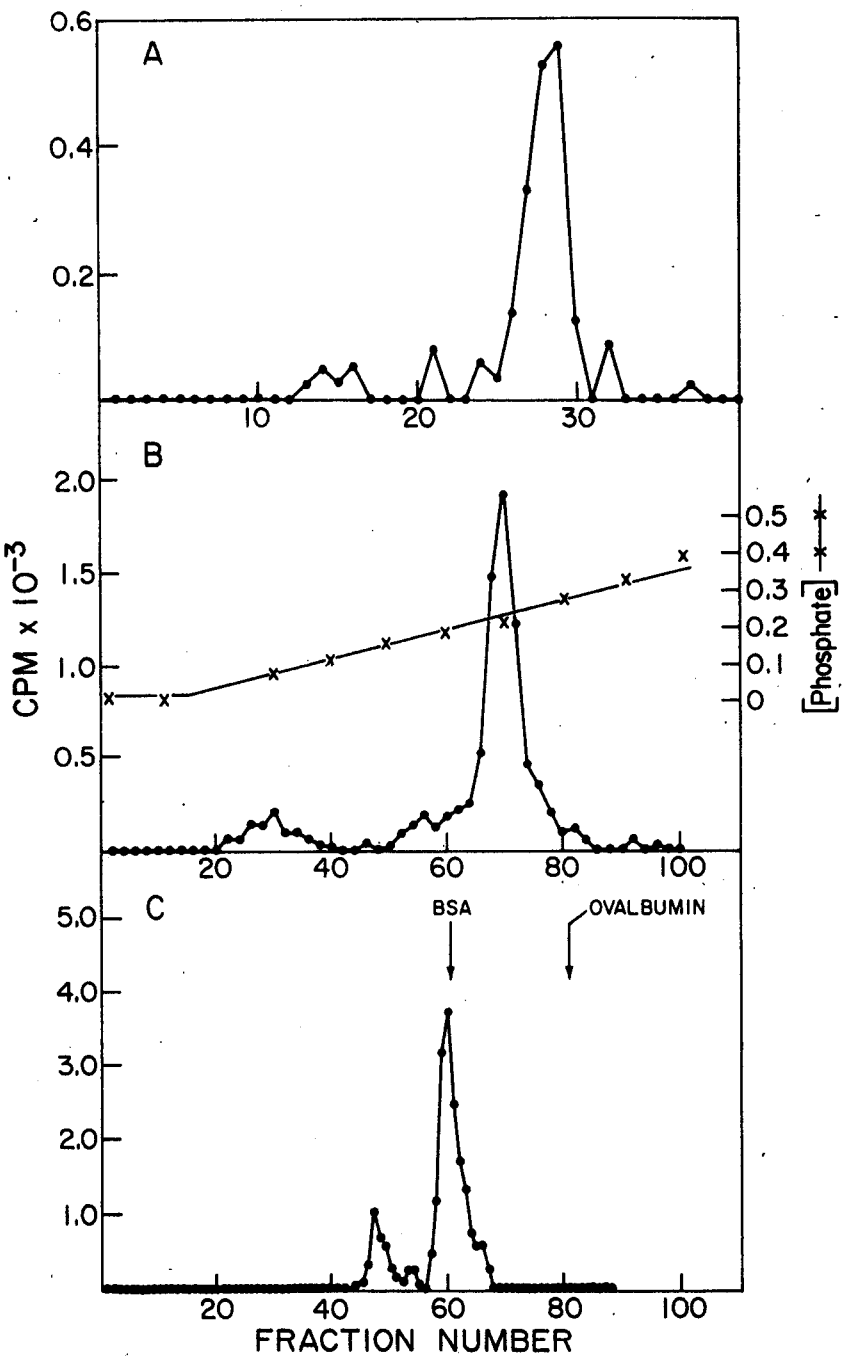

Fig. 12: (A) DEAE-cellulose chromatography of leukemic spleen enzyme. Core-like particles isolated from a leukemic spleen were treated with 1 % Nonidet P-40 and 0.7 M KCl, and the solubilized enzyme activity was chromatographed on a 16.5 cm X 2.5 cm column. Elution was with 0.4 potassium phosphate. 10 µl of each fraction (3.2 ml) were assayed using on oligo dT-poly rA template.

(B) Phosphocellulose chromatography of leukemic spleen enzyme. The fractions from the pooled DEAE-cellulose peak activity were diluted and chromatographed on a 17 cm X 1.5 cm column. Elution was with 160 ml of an 0.01 M-0.05 M potassium phosphate gradient; 1.6 ml fractions were collected and 10 µl aliquots assayed for oligo dT-poly rA-templated activity.

(C) Agarose gel filtration of leukemic spleen enzyme. The peak of activity eluted from phosphocellulose was subjected to gel filtration on a 50 cm X 0.9 cm agarose column. The elution rate was 4 ml/hr and 0.4 ml fractions were collected. Aliquots of 4 µl were assayed for enzyme activity with an oligo dT-poly rA template.

The concentrated phosphocellulose enzyme is then passed through a 0.5 M agarose column on which one routinely observes two peaks of activity (fig. 12C). If the first peak of activity is rechromatographed on the same type of column, a shift of most of the activity to the position of the second peak occurs. Thus, the first peak would appear to be an aggregate (possibly a dimer) of the enzyme. A summary of the column fractionations in terms of recoveries and specific activities is recorded in Table XI.

Table XI: Purification of RNA-dependent DNA Polymerase from a Viral Core Fraction of Human Leukemic Spleen

Fraction	Total Protein (mg)	Total Activity (pmoles)	Specific Activity (pmoles/mg)
1. Viral core region	3.90	2011	5.0×10^2
2. DEAE-cellulose pool	0.20	1949	9.7×10^3
3. Phosphocellulose pool	0.05	1400	2.8×10^4

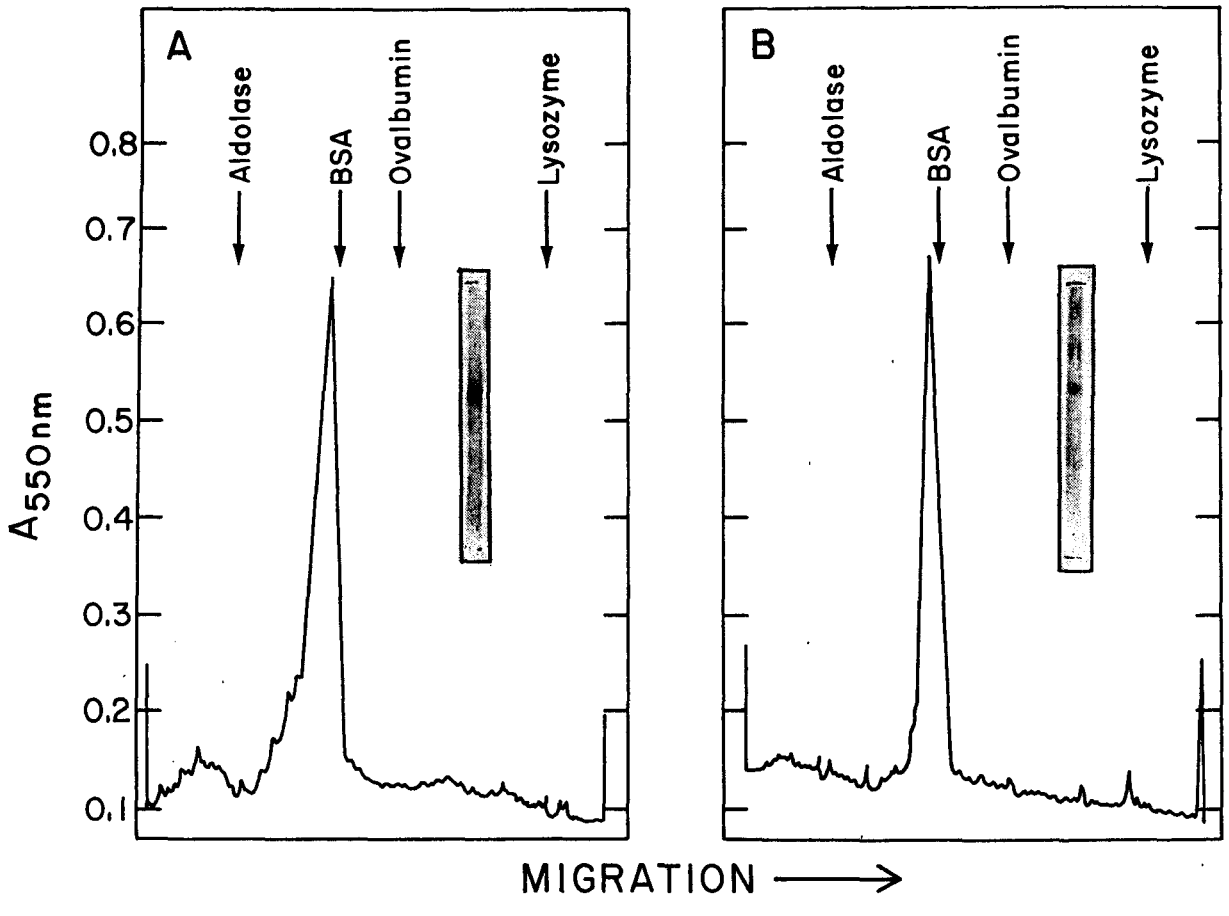

Fig. 13: Sodium dodecyl sulfate-polyacrylamide gel agarose enzymes. Fractions 48(A) and 60(B) of Fig. 12C were subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gels. After staining with Coomassie blue, the gels were scanned in a Gilford Model 2400 spectrophotometer. The stained gel of each fraction is also shown.

To examine the purity of the final enzyme preparation, portions of the two agarose peak fractions of Fig. 12C were electrophoresed on 5 % acrylamide gels in the presence of 0.1 % sodium dodecyl sulfate. Molecular weights of the separated polypeptides were determined using lysozyme, ovalbumin, bovine serum albumin and aldolase as molecular weight markers. As shown by the gel photographs and the scans of figures 12A and 12B, both agarose peaks yielded one major band corresponding to a molecular weight of 70,000 daltons, supporting the conclusion that the first agarose peak was in fact an aggregate of the second. A pool of the two agarose peaks constituted the final enzyme preparation.

Several parameters of the reverse transcriptase were examined. The reaction was found to proceed best at 37° and over a broad pH range from 7.0 to 8.2. The requirement for a divalent cation could be satisfied best by Mg^{++} (6 mM) although Mn^{++} was also effective over a very narrow range (0.5 to 1.2 mM), a feature also observed with the murine leukemic reverse transcriptases. In common with these, the purified leukemic spleen enzyme could utilize DNA but preferred RNA as a template. Further, the RNA templated activity was completely dependent on the presence of all four deoxyriboside triphosphates and exogenously added RNA (Table XII).

Table XII: Deoxynucleoside Triphosphate and RNA Requirements of Leukemic RNA-dependent DNA Polymerase

Reaction*	pmoles [^{3}H]dCMP polymerized**
1. Complete	4.6
2. -dATP	<0.024
3. -dGTP	0.076
4. -dTTP	<0.024
5. -dATP, dGTP, dTTP	<0.024
6. -RNA	0.032

* Incubation was for 20 min at 37° with 1.4 μg avian myeloblastosis virus RNA as template.
** [^{3}H]-dCTP was present at 1.04 X 10^4 cpm/pmole.

The critical diagnostic criterion of a putative reverse transcriptase is the ability to transcribe heteropolymeric RNA into a DNA complement. To test this, a reaction was run with the purified enzyme employing isolated AMV-RNA as the template. The product synthesized was purified, alkali-treated and hybridized either to AMV or RLV-RNA. An analysis on cesium sulfate gradient showed clearly that the tritiated product was complementary only to AMV-RNA. Thus, the enzyme purified from the human leukemic particles satisfied this operational definition of a reverse transcriptase.

The results (69) we have just described represent the first instance of a human reverse transcriptase isolated to the purity required for complete characterization. In addition, the procedure provides enough protein to generate monospecific antisera for ultimate use as detecting devices.

XIV. Present Status and Future Prospects

The application of nucleic acid hybridization and the other techniques of molecular biology have permitted us to illuminate some of the issues of human cancer noted in the questions listed in the introductory paragraphs. The answers obtained may be summarized in the following statements:

(1) Human neoplasias do contain RNA molecules possessing detectable homologies to the RNA of tumor viruses known to cause similar cancers in animal systems.

(2) The RNA molecules indentified in the human tumors possess the size and physical association with reverse transcriptase that characterizes the RNA of the animal oncornaviruses.

(3) Further, the tumor-specific RNA is encapsulated in a particle possessing the size and density of the RNA tumor viruses.

(4) The RNA of the human tumor particles possess homology to the RNA of the viruses causing the corresponding diseases in animals.

(5) Viral-related sequences are unique to the DNA of tumor cells, are inserted postzygotically, and are therefore not resident in the germ line.

The availability of the mesenchymal and mammary tumor animal model systems provided us with the viral agents that generated the radioactive DNA probes used to search for and find the homologous sequences in the particulate fractions of the corresponding human neoplasias. Our invention and perfection of the "simultaneous detection test" enabled us to characterize the size and particulate nature of the RNA detected in the human tumors. Beyond this, it evolved into an important technical advance since it permitted us to bypass the restriction of being forced to start with a known animal viral agent. Thus, we were able to extend our investigations beyond breast cancer and the mesenchymal tumors to other clinically important human neoplasias. The use of the simultaneous detection test established that a variety of human carcinomas contained particles having the diagnostic criteria associated with the oncornavirus-like particles. The neoplasias examined included a variety of cancers of brain (70), the gastrointestinal tract and lung (71), and skin (72).

Having found these particles in the human tumors, where does one go from there? An obvious need is to characterize them more fully biochemically and immunologically so that they can be compared with the similar agents found in the animal systems. However, here we are faced with a logistical problem that has thus far interposed serious obstacles. The amount of tumor material available and its particle content are such that it is difficult to isolate particles in sufficient quantity and purity to perform the desired biochemical examinations. Adequate characterizations will not be readily feasible until these particles are obtainable in suitable yield from established cell lines. To date this desideratum has not been achieved. Nevertheless, it may still prove possible to exploit some of the implications derivable from certain of their features. In particular, at least a portion of the RNA sequences found in the particles associated with each primary tumor site appears to be unique. As we have already noted, the particles found in human breast cancer share no sequences in common with those found in the mesenchymal tumors. These differences appear to extend to the particles found in other sorts or

primary tumors. Thus, we have been able to distinguish by cross hybridization the sequences found in particles from stomach cancers from those found in the colon. Similarly, the sequences found in lung cancer particles were easily differentiated from those found in other tumor sites tested. It would appear from our survey that the sequences found in these particles are histogenically specific.

These results suggest the possibility of developing a novel pathway for specific tumor detection. From the outset, it was evident that the nucleic acid hybridization technology we had developed and used to provide fundamental information of the molecular basis of the cancer cell was not likely to be useful in a clinical setting. In the first place, it has thus far been successfully applied only to tumor cells, and there is little likelihood that there would be enough circulating tumor-specific nucleic acids to be useful for diagnostic purposes. Further, the hybridization procedure is too sophisticated, too laborious, and too expensive to be introduced into the clinical pathology laboratory.

It seems clear that one must find a way of translating the sequence differences of the tumor particles into a parameter that would be more amenable to detection by the devices more commonly used in the clinical laboratory. One approach would depend upon the plausible expectation that the sequence differences observed in the different particles would be reflected in proteins that might be distinguishable antigenically. Were this realized, one could immediately hope to use the very sensitive and less restrictive methods of immunology. These are not only very sensitive, but they are in routine use in clinical laboratories.

To exploit this approach and obviate the logistic and other difficulties attending attempts to study the whole particles, one might focus rather on isolating and characterizing individual protein components. Of these, one of the most amenable is the reverse transcriptase since it can be followed during fractionation by means of its enzyme activity. As we have shown here, this approach has led to the successful isolation and purification of the reverse transcriptase of the particles found in leukemic spleens. These same methodologies can be and have been applied to other human neoplastic tissue. With these specific proteins available, monospecific antisera can be generated that could hopefully be used for detection in the body fluids of tumor-bearing individuals. The potential value of providing a useable specific assay for the presence of tumor cells has been even further enhanced by the recent advances in adjuvant chemotherapy. Efforts along these lines could convert what has thus far been an exercise in molecular biology into a potentially powerful clinical tool.

Acknowledgments

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